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TITLE OF THESIS... C^{14}-phenylalanine Incorporation..
by Wheat-seedling cytoplasmic
Ribosomes.....

UNIVERSITY.... of... Alberta..... Edmonton.....

DEGREE FOR WHICH THESIS WAS PRESENTED... M.Sc.....

YEAR THIS DEGREE GRANTED..... 1972.....

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DATED. July....19.....1972

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THE UNIVERSITY OF ALBERTA

^{14}C-PHENYLALANINE INCORPORATION BY WHEAT-SEEDLING CYTOPLASMIC RIBOSOMES

by

© EDWARD. B. L. TUCKER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

FALL, 1972

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled, "^{14}C-phenylalanine Incorporation by Wheat-Seedling Cytoplasmic Ribosomes", submitted by Edward B. L. Tucker, in partial fulfilment of the requirements for the degree of Master of Science.

Saul Rabkin
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Date July 18, 1972

ABSTRACT

^{14}C-phenylalanine incorporation was induced by homogeneous preparations of wheat-seedling cytoplasmic and chloroplast ribosomes which had been isolated by zonal centrifugation. Wheat embryo synthetase enzymes in the presence of stripped yeast tRNA and ^{14}C-phenylalanine produced ^{14}C-phenylalanyl tRNA which was used in the incorporation system. The incorporation by cytoplasmic 79S ribosomes required high concentrations of ribosomes, high concentrations of magnesium, low concentrations of tris-HCl buffer pH 7.6, and was complete after 45 minutes incubation at 37^{o}C. This transfer of ^{14}C-phenylalanine from ^{14}C-phenylalanyl tRNA into protein was not dependent upon added energy factors. Incorporation by both species of ribosomes was inhibited by puromycin, only the chloroplast ribosomes were inhibited by chloramphenicol, and neither species of wheat-seedling ribosomes was inhibited by cycloheximide. The cytoplasmic ribosomes dissociated into subunits which would reassociate to form the parent species. Puromycin pretreatment of ribosomes resulted in complete dissociation. The reformed parent species would not induce polyphenylalanine synthesis. The results led to the conclusion that the homogenization and separation procedures had cleaved the polysomes into monosomes which contained not only mRNA fragments, but nascent protein as well.

ACKNOWLEDGMENT

The research described in this thesis was performed under the supervision of Dr. Saul Zalik. His guidance, wisdom and assistance was given willingly throughout the study and in the preparation of this manuscript.

I wish to acknowledge my colleagues for helpful criticism and direction during the research; and Dr. Sara Zalik (Department of Zoology) for the use of equipment.

Appreciation is given to my wife, Lindis, for her encouragement and helpful discussion.

The financial assistance was provided by the University of Alberta and the National Research Council of Canada.

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INTRODUCTION

Protein synthesis in eukaryotes is thought to occur by a system generally similar to that of prokaryotes. Dissimilarities in their ribosomes and in the initiation of protein synthesis by them have been reported. Leaves of higher plants contain abundant amounts of the two classes of ribosomes in the same cell. Therefore, despite the difficulties associated with sorting out homogeneous ribosome classes, they afford a unique opportunity for comparative studies.

The ribosomes of the plant cytoplasm are of the eukaryotic class, while the ribosomes found in the chloroplast and mitochondria have characteristics similar to those of prokaryotes. These three types of ribosomes are apparently capable of synthesizing proteins. Lately, a great deal of research evidence has confirmed that the ribosomes found in the cytoplasm are different from those found in the chloroplasts or mitochondria. Various methods used to obtain relatively-homogeneous preparations of ribosomes have included organelle isolation, plant etiolation and the use of seed germ. A different approach in studying protein synthesis of the individual types of ribosomes involves the use of specific inhibitors of protein synthesis. In the present study the approach was to separate the chloroplast and cytoplasmic ribosomes from an heterogeneous mixture by means of zonal centrifugation. Using this method relatively-large quantities of chloroplast and cytoplasmic wheat leaf ribosomes were obtained and the intactness of these was demonstrated by their ability to incorporate ^{14}C-phenylalanine.

This study was undertaken to characterize the general features of an incorporation system containing these ribosomes.

Experiments on the factors required for incorporation, the effect of protein synthesis inhibitors, and ribosomal dissociation and reassociation were performed.

LITERATURE REVIEW

I. Mechanism of Protein Synthesis

A. Introduction

Although the procedure involved in the organization and formation of a protein is a dynamic process, it is often divided into three steps--initiation, elongation, and termination--for ease in comprehension. This section of the literature review is similarly divided; however, Figure 1 attempts to show protein synthesis in a more operational state.

Boulter (1970) states that "most of our knowledge of the mechanism of protein synthesis is derived from experiments using 70S microbial ribosomes, particularly those of E. coli". This portion of the literature review, therefore describes the process with data obtained from bacteria rather than from plants. As is discussed later in this review, protein synthesis in eukaryotes is thought to occur by a mechanism similar to that for prokaryotes. However, a statement made by Mahler and Cordes (1966) seems relevant here. "The very fact that a relatively simple, unified, and self-consistent model appears to have emerged from these efforts might suggest that perhaps some of the generalizations are overly facile and that once the problems are rexamined in search for quantitative rather than qualitative agreement, inconsistencies may emerge that may gnaw at the very foundation of the magnificient edifice".

Figure 1. The mechanism of protein synthesis.

F_1, F_2, F_3 ----initiation factors

A, D ----respectively acceptor and donor site on the ribosome

T_s, T_u ----respectively the unstable and stable transfer factors

G ----GTP dependent elongation factor

UAA, UAG, UGA ----termination codons

R_1, R_2 ----release factors which respectively respond to UAA, UAG and UAA, UGA

S ----a factor which stimulates the recognition of the release factors with their respective termination codons

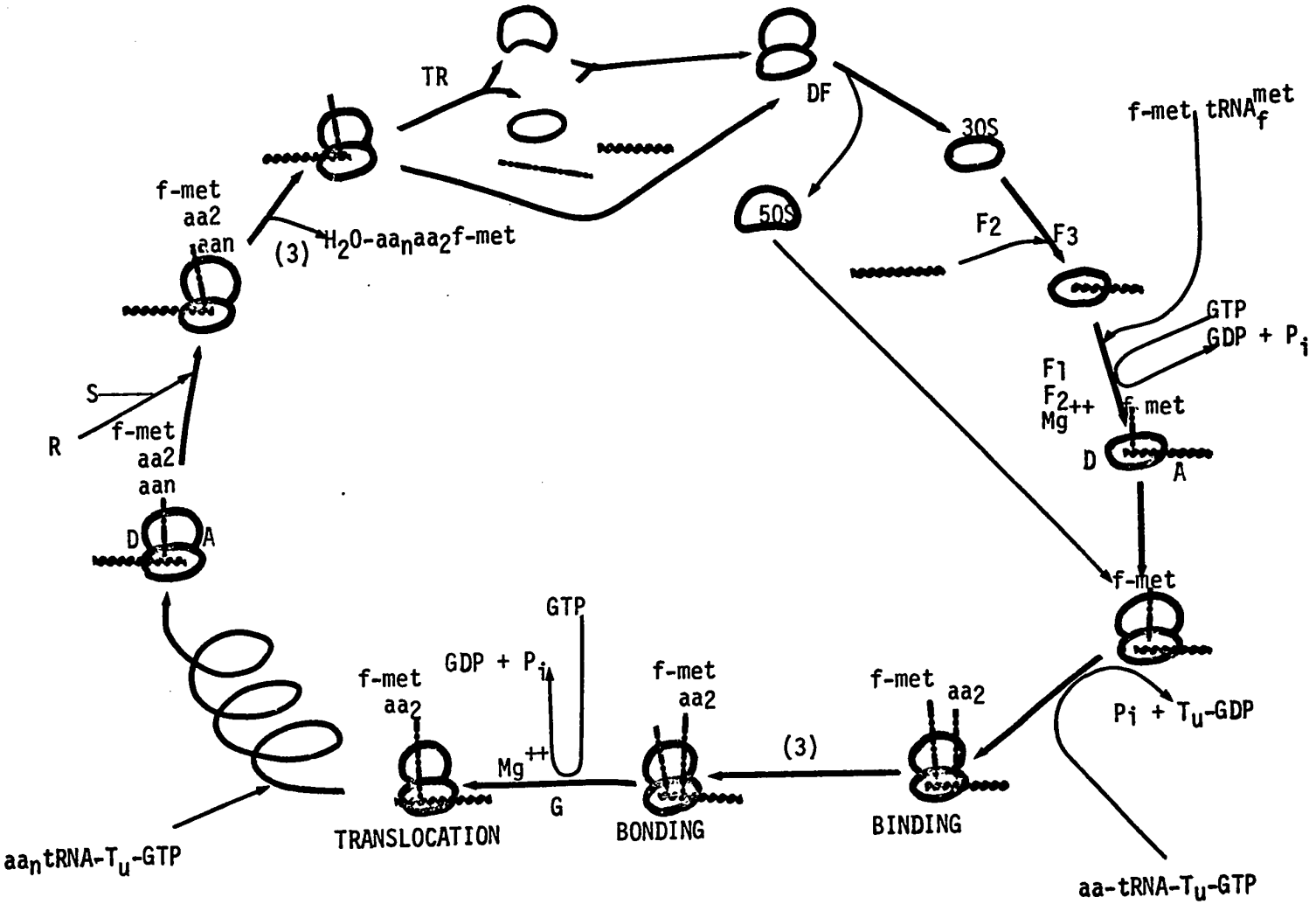
TR ----a release factor which releases tRNA from ribosomes when no translocation is involved

DF ----dissociation factor which dissociates monosomes into subunits

3 ----the enzyme peptidyl transferase

~~~~~~~~----the messenger RNA

————----the transfer RNA
~~~~~~~~


TR
DF
30S
50S
f-met tRNA$_f^{met}$
f-met
aa2
aan
(3) H_2O-aa$_n$aa$_2$f-met
F2
F3
GTP
GDP + P_i
F1
F_2
Mg^{++}
f-met
D
A
S
R
f-met
aa2
aan
D
A
f-met
GTP
GDP + P_i
f-met
aa2
Mg^{++}
G
f-met
aa2
(3)
f-met
aa2
P_i + T_u-GDP
TRANSLOCATION
BONDING
BINDING
aa$_n$tRNA-T_u-GTP
aa-tRNA-T_u-GTP

B. Initiation

The mechanism of protein synthesis has been comprehensively described in reviews by Boulter (1970), Kaji (1970), Lucas-Lenard and Lipman (1971) and Boulter, Ellis and Yarwood (1972). It is generally believed that methionyl tRNA is the "universal initiator" in that both eukaryotic and prokaryotic cells contain two species of met tRNA, one of which is used exclusively for initiation of protein synthesis while the other is used exclusively in the elongation step of protein synthesis. However, in the prokaryotic cells, formylated met tRNA is required for initiation while in the eukaryotic cells, the initiator met tRNA is not formylated. As Boulter et al. (1972) have summarized: " (a) micro-organisms contain a transformylase and a formylatable initiating $tRNA^{met}$; (b) animals and yeast do not contain a transformylase, but the initiating $tRNA^{met}$ is formylatable; and (c) although in plants the initiating $tRNA^{met}$ is not formylatable, the supernatant enzyme fraction appears to contain a transformylase which might, however, originate in the cell organelles". The amino terminal methionine is usually removed by a methionine specific amino peptidase, after the removal of the formyl group by a deformylase enzyme from the amino acid. The methionine on the $tRNA_m^{met}$ is placed into the internal position of the growing peptide chain.

Leis and Keller (1970) found two types of methionine tRNA's in chloroplasts of wheat leaf tissue and two types of methionine tRNA's in the surrounding cytoplasm. The met tRNA's of chloroplast acted in a similar manner to those of E. coli in that one could be formylated and was the initiator of protein synthesis, while the other could not

be formylated and donated its methionine to an internal position of a peptide. Neither of the met tRNA's of the surrounding cytoplasm could be formylated, but one acted as the initiator met tRNA while the other placed its methionine at an internal position of a peptide. Likewise, Yarwood, Boulter, and Yarwood (1971) isolated two major met tRNA species from cotyledons of Vicia faba (L), only one of which was the initiator. They also isolated a minor species which could be exchanged for the initiator met tRNA of E. coli and this was explained as possibly of cell organelle origin. Monasterio et al. (1971) have performed experiments to compare the ability of the two types of met tRNA's in wheat embryo, rat liver and E. coli, to bind ribosomes. In all cases the initiator met tRNA bound more efficiently that did the other met tRNA species.

Thus, results suggest that during evolution of prokaryotes to eukaryotes, methionine was conserved for the initiation of protein synthesis. The sequence of formation of initiator f met $tRNA_f^{met}$, as given in a review by Lucas-Lenard and Lipmann (1971), is shown as follows:

$$\text{ATP} + \text{methionine} \xrightarrow{1} \underset{\text{AMP}}{\overset{\text{enzyme}}{\diagdown\!\diagup}}\!\!-\text{met} + PP_i$$

$$\text{AMP}\!-\!\underset{\text{enzyme}}{\diagup\!\diagdown}\!-\text{met} + \begin{cases} tRNA_m^{met} \longrightarrow \text{met-}tRNA_m^{met} + \text{AMP (internal)} \\ tRNA_f^{met} \longrightarrow \text{met-}tRNA_f^{met} + \text{AMP} \end{cases}$$

$$\text{met-}tRNA_f^{met} + N^{10}\text{-formyltetrahydrofolate} \xrightarrow{2} \text{f-met-}tRNA_f^{met}$$

1) methionyl tRNA synthetase

2) methionyl tRNA transformylase

Initiation of protein synthesis requires the presence of three factors: F1, F2, and F3. All three factors are loosely attached to the 30S subunit and they can be removed by washing the ribosomes with a concentrated salt solution. Sabol et al. (1970) isolated the three initiation factors from the 1.0 M NH_4Cl wash of E. coli ribosomes. These factors have been extensively purified by Wahba et al. (1969) and some of their physical properties have been reviewed by Lucas-Lenard and Lipmann (1971). Figure 1 shows their sequence and depicts their function, as suggested by this review.

Initiation probably takes place in two steps: binding of the 30S subunit to mRNA and binding of f-met tRNA to the 30S ribosomal subunit-mRNA complex. The first step requires F2, and if natural mRNA is the template, F3 is required (Revel et al., 1969). The second step requires both F1 and F2 as well as GTP and magnesium. The F2 factor appears to possess GTPase activity and it is likely that the GTP binds to this factor. With the binding of the 50S ribosomal subunit to this 30S ribosomal subunit-mRNA-f-met-tRNA complex, factor 1 is released (Hershey et al., 1969) and elongation commences.

C. Elongation

Peptide-chain elongation can be described in three steps: binding of charged tRNA to the ribosome, formation of a peptide bond between the incoming amino acid and its predecessor, and translocation of both the mRNA and newly-synthesized peptidyl tRNA with the release of the antecedent tRNA. Two factors (T_u and T_s) have been described by Miller and Weissbach (1970) for the binding reaction and their part

in the reaction is summarized below. Unlike initiation factors, translocation factors are found in the soluble fraction of the cell.

$$T_u\text{-GDP} + T_s \longrightarrow T_s\text{-}T_u + GDP$$

$$T_u\text{-}T_s + GTP + \text{aa-tRNA} \longrightarrow \text{aa-tRNA-}T_u\text{-GTP} + T_s$$

This aa-tRNA-T_u-GTP complex donates the aminoacyl tRNA for its codon-directed binding to a ribosomal site (acceptor site) neighbouring the donor site which is occupied by the initiator or previously-formed peptidyl tRNA, as shown in Figure 1. Peptidyltransferase described by Maden et al. (1968) as an integral part of the 50S subunit, now catalyzes the peptidyl transfer to the newly-bound aa-tRNA from f-met-tRNA or from the previously-formed peptidyl tRNA. The succeeding step of elongation requires a third elongation factor (factor G) plus GTP and magnesium, and is a translocation of both the mRNA and the newly-synthesized peptidyl tRNA from the acceptor to the donor site. At the same time there is a release of the predecessor tRNA. A new aminoacyl tRNA may now enter the unoccupied acceptor site and the steps are recycled until the correct peptide is constructed and the signal for termination occurs.

The two translational factors isolated by Legocki and Marcus (1970) are discussed in the section, "Amino acid incorporation by wheat 80S ribosomes".

D. Termination

Genetic experiments have shown that cleaving of the bond between the formed peptide and the last tRNA that had been added (termination), is effected when the nonsense codes UAA, UAG or UGA are

read (Brenner et al., 1967). Two release factors R_1 (which causes peptide release in response to UAA and UAG) and R_2 (which causes peptide release in response to UAA and UGA) have been isolated and purified by Scolnick et al. (1968). A third termination factor (factor S) has been isolated by Goldstein et al. (1970) which stimulated the recognition of the release factors with their respective nonsense codons. Termination commences when the proper R factor complexes with the ribosome. When the peptidyltransferase catalyzes the transfer of the peptide from its tRNA in the donor site to the acceptor site, which contains the nonsense codon, the peptide is released leaving its tRNA attacted to the ribosome. There are various theories on how the ribosome with bound tRNA dissociates into subunits which may be used in initiation. It is possible that a factor as described by Kaji (1970), the TR factor, could remove the tRNA, leaving the monosome intact or in its subunits. Work by Kaempfer (1970) described the subunits as coming directly from polysomes, but indicated that they could reassociate to form 65S monomers. Miall et al. (1970) has described a dissociation factor (factor DF) which is probably initiation factor 1 and which causes the dissociation of monosomes into subunits ready to begin initiation. Davis (1971) proposed that this DF is present in limiting amounts and therefore strictly governs the number of subunits.

II. Differences between Chloroplast and Cytoplasmic Protein Synthesizing Complex

It is thought that protein synthesis in chloroplasts and in the surrounding cytoplasm occurs by a mechanism similar to that

described for E. coli. It has been established that the protein-synthesizing complex of the chloroplasts is equivalent to that in bacteria (Lyttleton, 1962; Ellis, 1969; Loening and Ingle, 1967; Sissakian et al., 1965), but that it is different from the complex of the surrounding cytoplasm which resembles that in animals (Marcus and Feeley, 1965; Allende and Bravo, 1966). Boardman et al. (1966) working with tobacco leaves, stated that ultracentrifuge analysis showed that the ribosomes of the chloroplast had a sedimentation coefficient of 70S, whereas the ribosomes from the surrounding cytoplasm had a sedimentation coefficient of 80S. This phenomenon has been observed many times now and is accepted as common knowledge. Hadziyev et al. (1968) using ion exchange chromatography showed chloroplast ribosomal RNA (rRNA) to have a higher cytidylic and a lower adenylic acid content that the leaf cytoplasmic rRNA, whereas the guanylic and uridylic acid content of all preparations was the same. Mehta et al. (1968) found wheat chloroplast ribosomes contained 16S and 23S RNA while the cytoplasmic ribosomes contained 17S and 25S RNA. The proteins of chloroplast and cytoplasmic ribosomes have also been found to be different. Odintsova and Yurina (1969) using disc gel electrophoresis found 25 bands for cytoplasmic particles, but only 22 bands for chloroplasts. Gualerzii and Cammarano (1969) calculated approximately 90 proteins in cytoplasmic ribosomes, and 60 proteins in chloroplast ribosomes. Jones, Nagabhushan, Gulyas, and Zalik (1972) reported 75 proteins in the 70S ribosomes and 85 proteins in the 80S ribosomes from wheat leaf. It was concluded that the proteins in cytoplasmic ribosomes are not at all like those found in the chloroplast. Payne

and Dyer (1971) have isolated a 5S rRNA component from the larger subunit of both the cytoplasmic and chloroplast ribosomes of broad bean leaves. Electrophoretic mobilities indicated 118 and 122 nucleotide residues respectively for the cytoplasmic and chloroplast 5S rRNA. More recently Payne and Dyer (1972) have reported yet another 5.8S RNA component, which was hydrogen-bonded to the 25S rRNA of the 80S broad bean ribosome. This RNA could not be found on the rRNA of the broad bean chloroplast ribosomes and gave further evidence that the chloroplast and cytoplasmic ribosomes were different. Comparative electron microscopic studies of chloroplast amd cytoplasmic ribosomes showed their general shape to be the same; however, some of the chloroplast ribosomes showed a cleft which was not seen on the cytoplasmic ribosomes (Bruskov and Odintsova, 1968). Dimensions given for cytoplasmic and chloroplast ribosomes in pea and bean are as follows:

Cytoplasmic	Chloroplast
260±10 A long (pea and bean)	220±10 A long (pea and bean)
190±10 A wide (pea)	170±10 A wide (pea and bean)
220±10 A wide (bean)	

Probably the most important difference between cytoplasmic and chloroplast ribosomes is that the latter have been reported to carry on protein synthesis at a faster rate. Boardman et al. (1966) found the 70S tobacco ribosomes were 10 to 20 fold more active in protein synthesis than the 80S ribosomes. Working with wheat, Hadziyev and Zalik (1970) reached similar conclusions. Boardman et al. (1966) found that optimum activity of 70S ribosomes was obtained at

11 to 15 mM Mg^{+2}, whereas optimum activity of 80S was at 5 mM Mg^{+2}, and furthermore at low Mg^{+2} concentrations the 70S ribosome would dissociate while the 80S would only dissociate after dialysis. The chloroplast 70S ribosome can be dissociated to give subunits of approximately 30S and 50S, whereas the 80S cytoplasmic ribosomes can be dissociated to give subunits of around 40S and 60S.

Protein synthesis by 80S plant ribosomes is inhibited by antibiotics which affect animal ribosomes (eg. cycloheximide), while antibiotics which affect bacterial ribosomes (chloramphenicol, lincomycin) inhibit protein synthesis by plant 70S ribosomes and not the 80S ribosomes (Boulter, 1970; Ellis, 1969, 1970; Ellis and Hartley, 1971).

III. Amino Acid Incorporation by Plant Systems

A. Amino acid incorporation by wheat 80S ribosomes

Most of the work on cytoplasmic amino acid incorporation in wheat has been done on wheat germ, because of the problem of obtaining 80S cytoplasmic ribosomes free of 70S chloroplast ribosomes. The work of Leis and Keller (1971), however, shows that the initiator f met-tRNA for the chloroplast system is present in the wheat germ, which does indicate the presence of the chloroplast system in wheat germ. In addition to this, there is the ever-present problem of a mitochondrial system being present.

The fact that wheat germ ribosomes are free of the active messenger RNA, but protein synthetic activities could be stimulated by poly U addition or by allowing the seeds to imbibe water, has been reported a number of times (Marcus and Feeley, 1965; Allende and Bravo

1966). Incorporating systems used to examine protein synthesis of wheat germ and other plants usually contain: Tris-HCl buffer, pH 7.5 to 7.8; $MgCl_2$, 4 to 10 mM; KCl, 20 to 50 mM; GTP; ATP; an energy regenerating system (creatine phosphate and creatine phosphokinase or phosphoenolpyruvic acid and pyruvate kinase); ribosomes 2 to 10 E_{260} units; 2-mercaptoethanol and supernatant protein. If a poly U system is used, then poly U is added plus ^{14}C-phenylalanine. However, if another radioactive amino acid is used, then a natural messenger or an artificial messenger such as TMV-RNA is needed and the 19 other amino acids are added.

A complete system refers to a system where the radioactive amino acid is added unattached to the tRNA, and in this system, besides the radioactive amino acid added, tRNA plus the synthetase enzymes must be added. If the tRNA is charged with the radioactive amino acid, then the synthetase enzymes need not be included and the system is referred to as the transfer system.

Using a complete system, Marcus and Feeley (1965) reported protein synthetic activity of imbibed or poly U treated wheat embryos. Allende and Bravo (1966) found similar results using both a complete system and a transfer system. For both systems they found activity dependendent upon poly U, supernatant fraction, magnesium, potassium, GTP, and an energy regenerating system. Maximum activity was found at 60 minutes with a magnesium concentration of 7.5 mM and 25 mM KCl. The samples were counted in a gas flow counter with efficiency of 39%. The highest activity that they reported for the transfer system was 294 cpm and the counts decreased, which they felt demonstrated

dependence of factors. Mehta et al. (1969) reported incorporation of ^{14}C-phenylalanine in a system containing cytoplasmic polysomes, which were obtained from the supernatant after pelleting the wheat-leaf chloroplast fragments. Chloroplast polysomes have been reported mainly on membranes (Filippovich et al., 1970), and thus the method of pelleting the chloroplast fragments to obtain a supernatant containing almost entirely cytoplasmic ribosomes is effective. Mehta et al. used 10 mM magnesium and stated that at lower concentrations they isolated ribosome pellets containing less polysomes. In addition they found more cytoplasmic polysomes at 4 days than at 7 days, and this phenomenon was re-emphasized by the fact that poly U stimulated 7-day seedlings, but not 4-day seedlings.

In 1970 Legocki and Marcus described the isolation of two translational factors which are needed for in vitro poly U phenylalanine synthesis. They used a transfer system containing 6.5 mM $MgAc_2$ and 71 mM KCl, which was incubated for 10 minutes at 30°C. The two translocational factors were isolated from the wheat germ supernatant and were purified by DEAE-cellulose chromatography, $(NH_4)_2SO_4$ fractionation, and hydroxylapatite chromatography. The ribosomes they used had been washed twice with 20 mM KCl - 1 mM $MgAc_2$ - 3 mM 2-mercaptoethanol buffer containing 1% desoxycholate. In the system with added translocational factors, 11.8 pmoles of the added 20 pmoles of the charged phenylalanyl tRNA were incorporated. If these factors were omitted there was negligible incorporation of the phenylalanyl tRNA. With binding studies, Legocki and Marcus demonstrated that factor I stimulated the binding of amino-acyl-tRNA to ribosomes in a reaction

dependent upon poly U and GTP. Factor II acted as a translocase rather than a binding factor. Either factor was active in catalyzing ribosome dependent hydrolysis of GTP.

Marcus (1970) described a tobacco mosaic virus RNA-wheat germ ribosome system which incorporated amino acids into peptides. A 5 to 8-minute lag which appeared at the beginning of the protein synthesis reaction was shown to be a rate-limiting ribosome messenger attachment reaction. This reaction was found to require ATP and two soluble factors (initiation factors) and did not occur in systems where the ribosomes were already bound to messenger RNA. Again supernatant factors were isolated by DEAE chromatography. Marcus makes the statement that the possibility does exist that one or more of the factors found to be soluble in extracts of dry embryo may subsequently become attached to the ribosome early in germination. It should be realized that the method used for the isolation of these factors would not exclude RNase; however, as is mentioned later in this thesis, RNase has been reported absent in wheat germ supernatant. To demonstrate that the rate-limiting step which he observed was actually the formation of this initiation complex, Marcus showed that when radioactive TMV-RNA was incubated with the wheat germ ribosome system, depleted of tRNA, there was formed a ribosome-TMV-RNA "initiation" complex which sedimented on a sucrose gradient slightly faster than the ribosome. Puromycin or cycloheximide, which inhibit later steps of protein synthesis, had no effect on this system. When tRNA was added and the reaction extended for 5 minutes, a peak which represents polysome formation appeared in front of the 'initiation' complex peak. This

step was inhibited by the addition of cycloheximide or puromycin. When the incubation time was further increased, the radioactivity was found in a more dense region. Marcus, Bewley and Weeks (1970) used aurintricarboxylic acid (ATA), a compound which specifically inhibits the attachment of messenger RNA to the ribosome, to inhibit the formation of wheat germ initiation complex. If ATA was added after the initiation complex had formed (to stop further formation of this complex) then the peptides could be synthesized following the addition of the two translational factors to the system. However, the initiation complex formed only if both wheat germ initiation factors were added to the system prior to the addition of ATA. The formation of this complex required ATP instead of GTP, but did not require the participation of f-met tRNA nor any other tRNA. This system, described by Marcus, differs from the bacterial system where f-met tRNA is required and where GTP is required instead of ATP.

However, Weeks et al. (1972), from the same laboratory, have since reported that the initiation complex was a 40S ribosomal subunit-mRNA-met-tRNA complex. In the presence of TMV-RNA, the initiator type of methionine tRNA bound only to the 40S subunit. Further, with the addition of ATA (to stop initiation complex formation), the 60S subunit was required before amino acids were incorporated into peptides.

Leis and Keller (1970) have separated, by BD-cellulose chromatography, two chain initiating methionine tRNA's from wheat germ. The major initiating $tRNA^{met}$ was not formylated by wheat germ extracts, and functioned in chain initiation without formylation.

The other initiating $tRNA^{met}$ could be formylated by a wheat germ cytoplasmic transformylase. They demonstrated that the unformylated initiator met-tRNA was bound to the initiator site of wheat germ 80S ribosomes in the presence of AUG by showing formation of methionyl-puromycin after the addition of puromycin to the initiation system.

Marcus, Weeks, Leis and Keller (1970) studied protein chain initiation in the wheat germ system which contained TMV-RNA as messenger. Upon amino terminal analysis of these in vitro products, they showed that methionine was found in the N-terminal of the peptides and thus suggest that methionine was the initiating amino acid. At low magnesium concentrations (1.3 mM) unformylated met-tRNA binds to ribosomes in a reaction requiring viral RNA, ATP, GTP, and supernatant factors.

Schultz et al. (1972) found endogenous messenger RNA on ribosomes from ungerminated wheat germ and suggested that the messenger RNA was preserved in a complex form with the ribosomes. They observed seven fractions of RNA when total RNA was electrophoresed. Fraction I and II were 24S and 17S RNA respectively, while fraction VII was 5S RNA containing 50% tRNA. Fraction V stimulated amino acid incorporation and was believed to be mRNA. Endogenous activity was not found on fractions of the 74S monosome peak, but was found on fractions sedimenting at 90S and 45S. This suggested that messenger RNA was preserved in a complex form with the monomer.

B. Amino acid incorporation in other higher plants

App and Gerosa (1966) reported a rice embryo transfer

poly U system which demonstrated an absolute requirement for poly U, Mg^{+2}, K^{+} or NH_4^{+}, GTP, and supernatant factors. They found that the incorporation decreased when the ribosomes were washed with desoxycholate and that incorporation was almost completely restored when supernatant factors were added. They noted that the transfer reaction of unwashed ribosomes was enhanced by washing and by the addition of supernatant factor. Later, App (1969) purified two factors obtained from the rice embryo supernatant, which were also found on the ribosomes. Factor I on the ribosome was easily washed off with desoxycholate; however, factor II was released only after further washing with 0.5 M KCl. He isolated factor I from the supernatant while factor II was isolated from the ribosome, and both were purified with $Ca_3(PO_4)_2$ followed by gel electrophoresis. It was noted during these experiments that the magnesium concentration for optimum polyphenylalanine synthesis varied with the character of the ribosome and supernatant used. Analytical ultracentrifugation showed KCl-washed ribosomes to be dissociated in 1 mM Mg^{+2}, whereas the desoxycholate-washed ribosomes were not. Increasing the magnesium concentration resulted in the disappearance of subunits and appearance of material on the leading edge of the monosome peak, an occurrence which, according to App, represents polysome formation. When these two "transferases" as App named them, were added back to washed ribosomes, incorporation was stimulated. App noted that activity was found on a large number of fractions in polyacrylamide gel and not just in one band. App et al. (1971) reported that rice embryo ribosomes could more easily be dissociated in 0.5 M KCl if the seeds had been imbibed. This ease of

dissociation, they stated, parallels activation of protein synthesis *in vitro*. The ribosomal subunits from either imbibed or non-imbibed embryos could be separated and reassociated to re-form active ribosomes. Their transfer polymerization system contained 10.5 mM $MgAc_2$, 60 mM KCl, and crude supernatant. All the density gradient centrifugation was done in a resuspension medium containing 20 mM tris, 15 mM KCl and 5 mM 2-mercaptoethanol, in spite of the fact that reassociation incorporation studies were done with ribosomes dissociated with 500 mM KCl. The density gradient profile of dissociated ribosomes from imbibed rice embryos contained a shoulder between the 40S and 60S peak. Dissociation profiles based on analytical ultracentrifugation were obtained by addition of 500 mM KCl and the 40S peak was then the same size as the 60S peak. They found both subunits to be required for active polyphenylalanine synthesis, and that dry embryo subunits could be replaced by imbibed subunits.

Parthier (1971) described a poly U transfer system used with pea seedlings. When 0.4 mg (6 E_{260}) units ribosomes, 1.0 mg of rat liver supernatant protein, 100 µg poly U, 0.75 mg of tRNA and 13 mM $MgCl_2$ were incubated at 34°C, polyphenylalanine synthesis was complete after 5 minutes. Gulyas and Parthier (1971) found that the pea seedling ribosomes could be further activated for poly U directed polyphenylalanine synthesis by washing with high concentrations of NH_4Cl, KCl, detergent or by Sephadex gel filtration. They stated that pea seedling supernatant would not stimulate incorporation because of its high content of nucleases. A single wash with 0.5 M NH_4Cl or 1 M KCl enhanced poly U incorporation two to three times, and successive washing with 0.5 NH_4Cl, up to 4 washings, increased incorporation four

times. It was also demonstrated that washing the ribosomes with the detergents desoxycholate, dodecylsulphate or triton X-100 increased ribosome activity. It was noted that by the addition of pea supernatant protein, formation of polyphenylalanine decreased and tests showed the pea supernatant to affect only poly U. The "nuclease" was heat resistant, and dialysable. They stated that this substance is only present in etiolated seedlings, particularly those of leguminous plants.

Marei et al. (1972) have isolated ribosomes from fig fruit which in a complete system, were dependent upon GTP, ATP, and magnesium for ^{14}C-phenylalanine incorporation. This incorporation was only slightly dependent upon poly U and an examination of the ribosome preparation on a sucrose density gradient revealed many polysomes which proved to be responsible for the observed incorporation.

C. Amino acid incorporation by chloroplasts and chloroplast ribosomes.

1. Amino acid incorporation by chloroplasts

Chloroplasts have all the components necessary for protein synthesis. They contain 70S ribosomes, tRNA's, aminoacyl-tRNA synthetases and soluble enzymes for the transfer of amino acids into peptidyl material, and they can synthesize at least some amino acids (Boulter, 1970). Amino acid incorporation in chloroplasts of the following plants have been reported: wheat (Bamji and Jagendorf, 1966), Ranalletti et al., 1969); tomato (Davies and Cocking, 1967), (Hall and Cocking, 1966); spinach (Heber, 1962), (Spencer, 1965); tobacco (Spencer and Wildman, 1964), Hampton et al., 1966), (Chen and Wildman,

1970); bean (Margulies and Parenti, 1968), (Ranalletti et al., 1969); pea (Filippovich et al., 1970), (Lozano and Griffiths, 1970); and sunflower (Ranalletti et al., 1969).

When doing amino acid incorporation with chloroplasts, the research worker must obtain chloroplasts free of bacteria and free of other subcellular organelles. Boulter (1970) suggests a limit of 10^4 bacteria per ml incorporating system can be tolerated. By using freshly-distilled water Bamji and Jagendorf (1966), could decrease the bacterial counts of agar plates incubated 24 hours, to 10^4 per 0.5 ml reaction mixture. They stated that 10^5 bacteria per 0.5 ml would cause serious problems with their results. Parenti and Margulies (1967) observed 10^3 to $5x10^4$ bacteria per ml chloroplast suspension when they used sterile water, chilled the tissue rapidly and washed the tissue with a solution of calcium hypochlorite. Gnanon et al. (1969) stated that "the incorporation of amino acids by chloroplasts isolated from higher plants by ordinary biochemical techniques (using hypotonic buffers without protective colloids) could be ascribed largely if not completly to contaminating bacteria". They studied protein synthesis in chloroplasts without interference from contamination by isolating chloroplasts with the Honda medium, and by adding respiratory inhibitors to the reaction. Lozano and Griffiths (1970) using aseptic techniques obtained chloroplasts containing only 10^2 to 10^4 bacteria per ml chloroplast suspension. Nuclei, mitochondria, bacteria and entire cells may contaminate the chloroplast fraction; however, relatively-intact, clean chloroplasts were obtained by isolating in a medium containing the protective colloids, Dextran and Ficoll, and by successive washing of the chloroplasts. With the addition of a detergent

(triton X-100) to the system, chloroplast membranes were selectively ruptured leaving the contaminants unaffected and the incorporation was taken to indicate the percent contamination. Parenti and Margulies (1967) estimated 2 to 10% of their incorporation was due to contamination, while Lozano and Griffiths (1970) reported 5%.

As pointed out by Lozano and Griffiths (1970) different requirements for chloroplast protein synthesis have been reported; this is probably due to differences in intactness and purity of the isolated chloroplasts. For chloroplasts isolated from young pea leaves, they obtained optimum conditions of 12 mM $MgCl_2$, 100 mM KCl, 16 mM 2-mercaptoethanol, 2.75 mM ATP, 8.3 mM PEP, 10 ug/0.3 ml pyruvate kinase, to which they needed to add a mixture of GTP, UTP and CTP.

Age appears to have an effect upon activity of chloroplasts as noted by Bamji and Jagendorf (1966) who found chloroplast preparations from 4 or 5 day-old wheat seedlings to be more active than chloroplast preparations from 7 and 8 day-old plants.

2. Amino acid incorporation by chloroplast ribosomes

Sissakian *et al.* (1965) obtained amino acid incorporation by ribosomes isolated from pea seedling chloroplasts. Neither ATP nor amino acids stimulated incorporation, and dialysis of the ribosomes lowered activity which could not be regained. Hadziyev and Zalik (1970) reported chloroplasts of 4-day-old wheat seedlings contained a greater amount of polysomes than chloroplasts of 7-day-old plants. The incorporation was decreased if the polysomes were broken into single ribosomes by RNase treatment, and could not be regained by the addition of poly U. The presence of two types of polysomes in chloroplasts--

free and membrane bound--was noted by Payne and Boulter (1969). They suggested that each type may synthesize a different group of proteins. After Chen and Wildman (1970) incubated intact tobacco chloroplasts with ^{14}C-valine, they observed half of the incorporated amino acids to be in nascent protein on ribosomes in the soluble phase, while the rest was in nascent protein on ribosomes attached to membranes. When they isolated the membrane portion of chloroplast and added ^{14}C-amino acids and supernatant, the ribosomes bound to these membranes synthesized protein. Work by Filippovich et al. (1970) indicated that protein synthesis in chloroplasts of pea seedlings takes place mainly on lamellar membranes. The free and membrane-bound ribosomes had been incubated in a medium containing all the factors for a complete system.

IV. Dissociation of Eukaryote Ribosomes

A. Dissociation of ribosomes by high salt concentrations.

Martin et al. (1971) have reported a method for the dissociation and reassociation of cytoplasmic ribosomes from animals, plants, fungi and protozoa. Large quantities of subunits could be isolated by zonal centrifugation through a 10 to 30% sucrose gradient containing 0.88 M KCl in the buffer. They reported inactivation of plant ribosomes if they were separated at a higher temperature (28^{o}C) and if they were stored frozen at -20^{o}C. However, isolation of the subunits at cooler temperatures (4^{o}C) caused aggregation of subunits and thus contamination of the 60S fraction with the dimerized or compacted 40S subunit. Aggregates of 60S subunits had an S value of 90S and 105S. Pretreatment of ribosomes with puromycin was required for complete dissociation;

however, this treatment may have caused a modification of the subunits which exhibited an increased tendency to aggregate and a different magnesium dependency of polyphenylalanine synthesis.

Subunits mixed in a ratio of 2.5 : 1 (E_{260} units) would reassociate to form active, poly U dependent 75S monosomes. Martin et al. reported pea and wheat to reassociate only fifty per cent. No activity was reported in the 40S subunit and the 20% activity of the 60S subunit was attributed to 40S contamination. Undissociated 75S particles were active in incorporation and were unresponsive to poly U.

Peterman (1971) reported dissociation of rat liver ribosomes by the addition of urea to ribosomes pretreated with 0.6 M KCl and dialysed. Treatment with 2.7 M urea gave 60S subunits free of 40S subunits, while treatment with 2 M urea gave 40S subunits free of 60S ones. When these two subunits were isolated and recombined, poly U directed incorporation was only 25% of control undissociated ribosomes.

Staehelin and Falvey (1971) have isolated from mouse and rat liver, ribosomal subunits which reassociate 100% to form active, poly U dependent 80S ribosomes. When purified polysomes were incubated with all the components for protein synthesis, "run-off" monosomes were formed which, when treated with 0.5 M KCl and centrifuged through a buffer containing 0.3 MKCl and 3 mM $MgAc_2$, dissociated 80 to 90% into 60S and 40S subunits. These workers precipitated the subunits by adding 0.7 volume of 95% cold ethanol. They also noted that when run-off ribosomes were spun through a sucrose gradient containing the incorporation buffer, the majority of ribosomes sedimented in a sharp 70S peak clearly separated from the 80S complexes still carrying peptidyl-tRNA.

Analysis of the subunit RNA demonstrated the 40S to be 100% pure, but the 60S was contaminated with 40S to about 10%.

Battaner and Vazquez (1971) found subunits were produced when yeast ribosomes, which had been washed with 0.5 M NH_4Cl, were dialyzed for two hours against 1000-fold excess of buffer containing 0.2 mM $MgAc_2$. Separation of these subunits was performed by placing them on top of a 7 to 35% sucrose gradient in a buffer containing 50 mM NH_4Cl and 5 mM $MgAc_2$. At the end of the centrifugation, the top 90% of the supernatant was discarded and the remainder of the supernatant (containing only the 40S subunit) was collected, while the pellet (enriched in 60S subunit) was resuspended, recentrifuged and then collected. Reassociating the subunits restored 80 to 100% activity, the 40S did not incorporate at all, and the incorporation by the 60S was explained on the basis of 4 to 8% contamination by 40S.

Wolfe and Kay (1967) dissociated wheat germ ribosomes by dialysis against a buffer containing 0.05 M KCl, 0.025 M tris-HCl (pH 7.5), 0.0003 M $MgCl_2$, and 0.006 M 2-mercaptoethanol. They obtained 60S, 40S and 26S subunits, which could not be reassociated by increasing magnesium concentration to 0.005 M. Jones, Nagabhushan, and Zalik (1972) reported dissociation of wheat cytoplasmic ribosomes into 42S, 49S, and 61S particles after centrifugation through a 7 to 35% convex sucrose gradient in a buffer containing 20 mM tricine (pH 7.5), 10 mM $MgCl_2$, 400 mM KCl, and 5 mM 2-mercaptoethanol. The 42S and 61S recombined to form the 80S monomers upon returning the magnesium concentration to 5 mM. Wheat chloroplast ribosomes could easily be dissociated by the same method; however, the subunits did not readily

recombine to form the parent 70S species.

Nolan and Arnstein (1969) could reassociate subunits of rabbit reticulocyte free ribosomes to form the parent 80S species, but these were not active in a poly U incorporating system. By dissociating rat liver ribosomes in 1 M KCl, Terao and Ogata (1970) obtained subunits which would easily recombine to give parent 80S species active in poly U directed incorporation. Gravela (1971) reported dissociation and reassociation into active particles of Yashida hepatoma ribosomes. These ribosomes dissociated with a comparatively-mild treatment of KCl or EDTA.

B. Dissociation of ribosomes by dissociation factors.

Subramanian et al. (1968) discovered in E. coli a protein factor found only on native 30S subunits which promoted the almost complete dissociation of 70S ribosomes prepared from starved E. coli cells. The DF-I was extracted from native subunits, which had been separated by 10 to 30% sucrose gradients, by storing them on ice in 1 M NH_4Cl. The supernatant from a high-speed centrifugation of this material was dialyzed, and the fraction precipating between 30 - 70% saturated $(NH_4)_2SO_4$ after being dialyzed was collected as the DF-I. They found that all the ribosomes would not dissociate and stated that their preparation might contain a small number of nondissociable ribosomes (presubably fragmented polysomes). These authors observed that increasing the magnesium concentration, reduced the rate of dissociation. Their results support the cycle which hypothesizes that after termination, free 70S ribosomes are produced which must be dissociated for initiation.

Garcia-Patrone et al. (1971) established that antibiotics which affect the 30S subunit modify the DF stimulated dissociation, whereas antibiotics which affect the 50S subunit do not affect dissociation. This agrees with Subramanian et al. (1968) who state that the goal of the DF is the smaller subunit.

To show an analogy between DF and initiation factor, Subramanian and Davis (1970) tested each initiation factor for its ability to dissociate. They concluded that only initiation factor F3 would cause dissociation. Albrecht et al. (1970) fractionated the three initiation factors by DEAE column chromatography and agreed with Subramanian and Davis that F3 and DF were in the same protein fraction. They observed GTP to have no effect on the 100% dissociation they obtained.

By DEAE-cellulose, phosphocellulose and Sephadex G-25 or G-100 chromatography, Miall et al. (1970) purified the DF to yield a single band on gel electrophoresis which had an apparent M.W. of 8,350 to 9,200. GTP had no effect on the ability of this protein to dissociate 80 to 90% of the 70S E. coli ribosomes. Because of similarities in purification (the protein was not adsorbed on DEAE cellulose, but was adsorbed on phosphocellulose) and molecular weight, Miall et al. concluded that DF was equivalent to F1 and not F3 as reported by Subramanian et al.

Petre (1970) isolated a specific protein factor which would dissociate yeast ribosomes. Yeast "native" subunits were separated by differential centrifugation and a protein fraction was removed by washing these subunits with ammonium sulphate. The precipitate from

35 to 75% saturation was collected, dialyzed and heated at 40°C for 30 minutes. After being cleared by slow centrifugation this crude protein fraction from "native" subunits would cause a temperature-dependent dissociation of yeast ribosomes. Because this dissociation factor would promote only 40 to 50% dissociation, Petre suggested the presence of two types of ribosomes. Experiments done to observe the hydrolysis rate of isolated yeast ribosomal RNA indicated that the amount of RNase present in the dissociation factor protein was much lower than would be required for the observed dissociation.

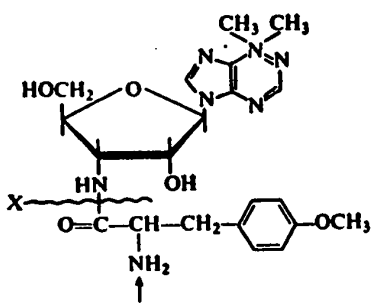
Lawford et al. (1971) extracted from ribosomes enriched with native subunits of rat liver, a protein mixture which they partially purified by $(NH_4)_2SO_4$ fractionation. This partially-purified protein stimulated only a small amount of dissociation when ribosomes reformed from subunits were used as substrate. The DF was active only if the ribosomes had been freed of both their messenger RNA and nascent protein.

C. Puromycin used to stimulate dissociation.

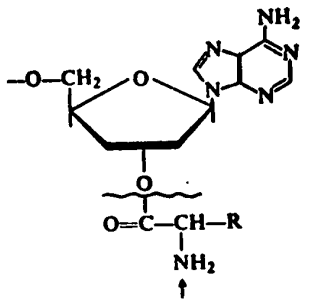
As stated above, runoff ribosomes will accumulate if protein synthesis is blocked by puromycin. Figure 2 indicates the obvious structural relationship of puromycin to an amino acylated tRNA. As described by Mahler and Cordes (1966), puromycin terminates protein synthesis by acting as a substitute for aminoacyl tRNA, thus causing the formation of a peptidly puromycin which is incapable of further chain growth and which is thus released. As stated by Boulter (1970), puromycin inhibits amino acid incorporation by cytoplasmic ribosomes, chloroplast ribosomes, mitochondria and nuclei, and furthermore it is

specific to only protein synthesis.

Figure 2



Puromycin



Terminal adenosine of an amino acyl- (or peptidyl-)tRNA

Puromycin has been used by a number of workers to produce free ribosomes which could be dissociated more easily to give more active subunits. Blobel and Sabatini (1971) held rat liver polysomes on ice in a buffer containing a high concentration of KCl and treated them with puromycin. After 15 minutes most of the nascent protein was released, but the ribosomes remained as 80S particles; however, when the mixture was thenincubated at 37^{o}C for 10 minutes, the runoff ribosomes completely dissociated into subunits. These subunits did not contain tRNA, and would readily reassociate to form monomers which were active in poly U-directed incorporation. This method has also been used by Lawford et al. (1971) to obtain subunits which combine to form 80S rat liver ribosomes active in poly U-dependent incorporation. Stahl et al. (1968), Lawford (1969), and van der Decken et al. (1970) used a method which involves the incubation of polyribosomes under conditions for protein synthesis by using untreated cell sap in the absence of ^{14}C-labelled amino acids, but in the presence of puromycin. Both rat liver and skeletal muscle active subunits were prepared.

MATERIALS AND METHODS

I. Chemicals

Disodium·$4H_2O$ ATP, trilithium GTP, disodium·$4H_2O$ creatine phosphate, creatine phosphokinase (specific activity 81 I.U./mg), a salt-free lyophilized powder obtained from rabbit skeletal muscle, and the potassium salt of polyuridylic acid were Calbiochem A grade (Los Angeles, California, USA). The stripped tRNA obtained from General Biochemicals (Chagrin Falls, Ohio, USA) was soluble RNA (sodium salt) isolated from fresh yeast. The uniformly labelled L-^{14}C-phenylalanine (specific activity 405 mCi/mmole) which was in a sterilized aqueous solution containing 2% ethanol, POPOP, and PPO were purchased from the Radiochemical Centre (Amersham, Searle). The other chemicals were analytical grade: KCl, $MgCl_2$, THAM[tris(hydroxymethyl)aminomethane], bentonite, sucrose, $(NH_4)_2SO_4$, Trichloracetic acid and scintanalyzed toluene were from Fisher Chemicals (Edmonton, Alberta, Canada); 2-mercaptoethanol from Eastman Organic Chemicals, (Rochester N.Y., USA); tricine[N-tris(hydroxymethyl)methyl-glycine] from Calbiochem; triton X-100, $MgAc_2$, phenol and NH_4Cl from Baker Chemical Co. (Phillipsburg N.J., USA); sodium desoxycholate from K&K Laboratories, INC. (Plainview N.Y., California, USA); and diethyl pyrocarbonate from Research Chemical Corp. (Sun Valley, California, USA). The puromycin hydrochloride was obtained from Nutritional Biochemicals (Cleveland, Ohio, USA); chloramphenicol U.S.P. from Parke, Davis and Co. Ltd. (Brockville, Ontario, Canada), cycloheximide from Sigma Chemicals (St. Louis, Missouri, USA); and the nutrient agar was obtained from Difco Laboratories (Detroit, Michigan, USA).

II. Equipment

Analytical ultracentrifugation analysis was done on a Spinco ultracentrifuge model E with schlieren optics. High speed preparative centrifugation was done in a Beckman L2-65B ultracentrifuge equipped for zonal centrifugation. The rotors used were Ti 60, SW 27, and Ti 14. Low-speed preparative centrifugation was done in an IEC (International Refrigerated Vacuum centrifuge), model BD-2, using rotor model 970. Fractionation of column effluents was performed by an ISCO ultraviolet analyzer model UA-2 in a refrigerated cabinet, while all other spectrophotometry was performed with a Beckman Recording Spectrophotometer model DK. Radioactivity was measured in a Nuclear Chicago Mark I liquid scintillation counter.

III. Checking the Chemicals

A. ATP and GTP

These two nucleotides were checked as suggested in circular OR-10 1969 Pabst Laboratories. The absorption spectra of ATP at pH 2 and 7 matched those shown for ATP in the publication and the GTP spectra at pH 1.7 and 11 matched those for GTP. Paper chromatography in an isobutyric acid: concentrated NH_4OH:H_2O (66:1:33) showed only minute amounts of ADP and AMP in the ATP, while no evidence of contaminants was shown in the GTP. The ratios of 250/260, 280/260 were as stated by the publication.

B. Creatine phosphokinase

Creatine phosphokinase was assayed by the method described

in the Worthington Manual [Worthington Biochemical, Corporation (Freehold, New Jersey, USA)]. The formation of phosphocreatine was followed by measuring the inorganic phosphorus liberated in a perchloric acid-molybdate digestion. Using K_2HPO_4 as a standard, an average rate of 0.029 µmoles phosphorus produced per minute was calculated, and evaluated to give an average rate of 34 E.U. per mg protein. Calbiochem stated the specific activity as being between 10 and 18 E.U. per mg with creatine as substrate, so it was concluded that the higher amount of activity could be due to a different lot than was specified in the catalogue. The lot was specified as having 81 I.U. activity.

C. tRNA check

Yeast-stripped tRNA (0.002 gm) was dissolved in 1 ml of 0.01 M KAc buffer pH 5.0 and 0.8 ml was centrifuged on the analytical ultracentrifuge. Only one peak was observed. When 0.001 gm of bovine pancreatic RNase was added to 1 ml of the above solution and the solution incubated for 1 hour, the previously-noted peak had disappeared.

IV. Chemical Preparation

A. Preparation of bentonite

One hundred grams of bentonite was stirred overnight with 50 volumes of water. This suspension was then centrifuged at 1500 xg for 15 minutes and the upper gelatinous part of the pellet was scooped off and collected. This pellet was suspended in 2 l of 0.05 M EDTA pH 7.2 solution and stirred overnight. After centrifugation the pellet was resuspended in distilled, deionized water and centrifuged. This

washing procedure was repeated three times and the pellet from the last wash was weighed, dried in a vacuum oven at 85°C overnight, and reweighed. A factor of 0.078 was calculated which when multiplied by the total wet weight of the pellet gave the actual dry weight of bentonite, and from this a 4% W/V suspension of bentonite was calculated and made.

wet weight of pellet x 0.078 = dry weight

dry weight x 25 = final volume of solution.

B. Plate counting

To dissolve the nutrient agar, 23 gm was suspended in 1000 ml of distilled, deionized water and heated in a boiling water bath. One hundred ml aliquotes were sterilized for 15 minutes at 120°C, 16 psi and stored at room temperature under sterile conditions until used. Dilutions of the ribosome solution were made in a ¼ strength ringer solution pH 7.0 containing in gm/4 l: NaCl, 2.25; KCl, 0.105; $CaCl_2$, 0.12; and $NaHCO_3$, 0.05. Sterile pipettes were used to place 1 ml of sample into a disposable petri dish. A layer of agar at 45°C was poured onto and mixed with this sample. Incubation was for 48 hours at 37°C.

C. Redistillation of phenol

Phenol was placed into a round bottom flask and heated by a heating mantle. The first 3/4 of the condensed vapor was collected and the colorless crystals were stored in a dark-colored, tightly-closed storage bottle, in the dark. When distilled phenol was required, the crystals were melted by placing the bottle in a hot water bath.

D. Preparation of chromatographic materials.

1. Cellex-D

Cellex-D (DEAE cellulose) obtained from Bio: Rad Laboratories (Richmond, California, USA) was regenerated by consecutive washing with the following solutions, and filtering through Whatman No. 1 filter paper in a Buchner funnel: 1.5 l of 0.1 N NaOH; 1.5 l of double distilled water (to neutral); 1.5 l of 0.1 N HCl; 1.5 l of double distilled water (to neutral). The regenerated cellulose was placed in the eluting buffer and packed into the column.

2. Sephadex G-25

Sephadex G-25 (fine) obtained from Pharmacia (Uppsala, Sweden) was sterilized in its respective eluting buffer by autoclaving it for 40 minutes at 120^{o}C and 16 psi.

E. Treatment of sucrose with diethyl pyrocarbonate (DEP)

Six ml of DEP was added to 1.0 l of 40% sucrose solution in the buffer II. The solution was heated in a boiling water bath for 20 minutes and allowed to return to room temperature before being stored overnight at 4^{o}C. Aliquotes of this solution were appropriately diluted with buffer and were used to make the zonal gradient.

F. Buffers

The composition of the buffers used routinely in this study are listed below according to the numbers by which they are designated.

Buffer I 0.7 M sucrose, 100 mM tricine pH 7.5, 5 mM $MgCl_2$, 50 mM KCl 5 mM 2-mercaptoethanol

Buffer II 10 mM tricine pH 7.5, 10 mM $MgCl_2$, 4 mM 2-mercaptoethanol

Buffer III 10 mM tricine pH 7.5, 1 mM $MgCl_2$, 100 mM KCl, 5 mM 2-mercaptoethanol, 10% triton X-100, 0.4 M sucrose

Buffer IV 20 mM tricine pH 7.5, 400 mM KCl, 10 mM $MgCl_2$, 4 mM 2-mercaptoethanol

V. Preparation and Procedures with Ribosomes

A. Plant material

Seeds of Triticum aestivum variety Manitou were placed touching each other on 2 inches of sterilized 3:2:1 soil mix (soil: peat:sand) and covered with 1 inch of vermiculite. After being soaked from the bottom, the flats were placed in a Fleming-Pedlar Coldstream growth cabinet and grown for 4.5 days at a temperature of 70 to 72°F, 24 hours illumination of 1200 to 1400 ft. candles, relative humidity of 45 to 50%, and watered daily with tap water. Prior to harvest for ribosome extraction, the plants were chilled in a cold room at 4°C.

B. Isolation of ribosomes

1. Ribosomes for zonal separation

In a cold room (4°C) 100 gm of wheat leaves were cut with scissors into 1/8 to 1/4 inch pieces and were placed into a glass container kept at -9°C by a $CaCl_2$ salt-ice bath. Two hundred ml of

sterile Buffer I containing 10% triton X-100, 5 ml of 4% bentonite and 0.3 mg sodium desoxycholate was added. The mix was homogenized three times intermittently with a Polytron homogenizer type PT 200D. The homogenate was clarified by passing through an Acme Supreme Juicerator lined with Whatman No. 1 filter paper. In later experiments to obtain higher yields of ribosomes, an additional 100 gm of chopped wheat leaves was added to the clarified supernatant and the homogenization and clarification steps were repeated. The heavy material was then sedimented by spinning 20 minutes at 30,000 xg and the supernatant obtained was layered on top of 10 ml of sterile buffer II containing 1 M sucrose, and was spun for 2 hours at 340,000 xg. The ribosomal pellets were resuspended in 8 ml of Buffer II.

2. Ribosomes without zonal separation

The method of isolation was identical except that the leaves were ground in Buffer III. When the ribosomes were washed with NH_4Cl, the 10 ml Buffer II underlay contained 0.5 M NH_4Cl. The pellet from this high-speed centrifugation was resuspended in Buffer II and an equal volume of 1 M NH_4Cl was added. The mixture was held on ice for 15 minutes before being recentrifuged through the Buffer II containing 1 M sucrose.

C. Separation of ribosomes by zonal centrifugation

The above-mentioned ribosomes were brought to a final volume of 10 ml containing 3% sucrose and were layered on top of a

convex gradient ranging from 7 to 38% sucrose in the sterile Buffer II. The centrifugation in a Beckman Ti 14 zonal rotor spinning at 47,000 RPM was carried out for 3 hours at 25°C or for 7 hours at 4°C, after which the gradient was analyzed via a flow cell in a Beckman model DK Recording Spectrophotometer at 290 nm. The large quantities of ribosomal material used in the zonal separation made absorption at 290 nm a requisite even though maximum absorption was at 260 nm. The peaks corresponding to the 70S and 80S ribosomes were collected and pelleted by centrifugation at 340,000 xg for 3 hours. These pellets were resuspended in Buffer II and were stored less than 24 hours at 4°C or for longer periods under liquid nitrogen (-196°C) before being used in the incorporation experiments. Ribosomal concentration was determined by measuring the absorption at 260 nm.

D. Dissociation of ribosomes and separation of subunits

Ribosomes were suspended in Buffer II and centrifuged through Buffer IV which contained the sucrose gradient as described above. The peak corresponding to the subunits was collected and pelleted by centrifuging at 340,000 xg for 3.5 hours. Subunits were used immediately for recombination or incorporation studies.

E. Swinging bucket centrifugation

Three to five E_{260} units of ribosomal material was applied in a 0.1 to 0.2 ml sample on top of a 5 to 20% linear sucrose gradient in the appropriate buffer. The two sucrose solutions were mixed in a Universal Density Gradient Mixer, obtained from Buchler Instruments

(Fort Lee, New Jersey, USA) and the gradients were formed in nitrocellulose tubes obtained from Beckman Instruments (Palo Alto, California, USA). After spinning for 4.25 hours at 85,000 xg in a Beckman SW 27 swinging bucket rotor, the nitrocellulose tube was punctured at the bottom and the gradient was pumped by a peristaltic pump (LKB-produkter AB, Stockholm-Bromma 1, Sweden) through a flow cell at a rate of 1 ml per minute and the absorbance at 260 nm was recorded.

F. Puromycin treatment of ribosomes

1. Preparation of ribosomes for dissociation in swinging buckets

The method used was similar to that described by Blobel and Sabatini (1971). A ribosome suspension which contained 100 E_{260} units/ml and which had been obtained either by zonal separation or by the method using Buffer III was chilled on ice; 0.2 ml of this solution was added to a solution containing 0.25 ml ice-cold, double-concentration Buffer IV and 0.05 ml of 0.01 M puromycin pH 7.0. This solution was held on ice for 15 minutes, transferred to a 37°C water bath for 10 minutes, and then 0.2 ml aliquotes of it were layered on top of sucrose gradients in the appropriate buffer.

2. Preparation of ribosomes for zonal dissociation

Four ml of a ribosome solution which contained 750 E_{260} units/ml was added to a solution containing double-concentration Buffer IV plus 0.5 ml 0.01 M puromycin and was incubated 15 minutes at 0°C, followed by 10 minutes at 37°C, before being placed on top of

the zonal dissociation gradient.

G. Estimation of RNase in prepared ribosomes

The method described by Sodek and Wright (1969) was followed, except that wheat germ soluble RNA and ribosomes were used as substrate instead of purified RNA and enzyme. The assay mixture consisted of 1 ml of the substrate solution, enzyme (up to 0.4 ml), and 0.1 M NaAc buffer, pH 6.0, to give a final volume of 2 ml. The assay mixture was incubated at 37°C, and 0.5 ml samples withdrawn 10, 40, and 70 minutes after the addition of the enzyme and pipetted into a 0.5 ml 5%perchloric acid containing 0.25% uranyl acetate. The tubes were chilled and the precipitates spun down. A 0.5 ml sample of the supernatant was made up to 5.0 ml with water, and the absorption at 260 nm measured. One unit is equivalent to the production of 0.1 μmole of acid-soluble nucleotides per hour, assuming an average E_{260} of 10 for a μmole/ml mixture of nucleotides.

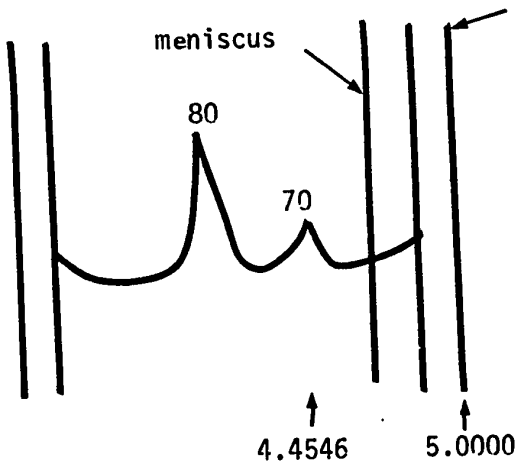
H. Sedimentation values

Sedimentation velocity runs were made at 20°C of the Spinco Model E ultracentrifuge equipped with schlieren optics. Pictures were taken at 0, 4, 8, 12, and 20 minutes after a speed of 39,460 rev/min was reached.

1. Reading the plates

The plates were read backwards from 5.0000 starting at the inside reference hole (see diagram below) on a Gaertner micro-comparator.

The center of each peak was read for every time interval and these values were substracted from 5.0000 to obtain the distance the peak had moved. This value was divided by the Magnification Factor (1.9435 obtained by Dr. Jones) to obtain the distance the ribosome species had moved in the cell, and this distance was added to 5.71 (the distance of the cell from the center of rotation). This final addition gave the distance of the species from the center of rotation at the particular time.



Sample calculation:

5.0000 - 4.4546 = 0.5454

$\frac{0.5454}{1.9435}$ + 5.71 = 5.9906 = the distance the particle is from the center of rotation.

2. Calculating sedimentation values

Sedimentation values were calculated using the equation

$S = \frac{\frac{dx}{dt}}{w^2 x}$ which upon integration gives $S = \frac{2.303}{w^2} \frac{(d \log x)}{dt}$

w = radians/sec. Since 1 revolution = 2 radians, the speed used (39,460 rev/min) gives 4134 radians/second. If log x (the log distance the particle has moved from the center of rotation) is plotted against

t (the time (sec) for the particle to move that distance), then $\frac{d \log x}{dt}$ equals the slope of the line. Because a number of peak distances were measured, these points are plotted on a graph from which a best fit line can be determined and the slope can be calculated. Svedberg units are in 10^{-13} sec.

$$S = \frac{2.303}{(4.134 \times 10^{3})^2} \times \text{slope}$$

The calculations for the distance the particle had moved from the center of rotation (x) and calculation of S value from the log values of x and time in minutes was programmed on an Olivetti-Underwood desk computer for efficient calculation.

VI. Procedure for Incorporation

A. Preparation of synthetase enzymes

Twenty-five g of commercial wheat germ was ground with a mortar and pestle with 100 ml of the sterile homogenizing buffer (0.45 M sucrose, 5 mM $MgCl_2$, 0.05 M tris pH 7.5, and 5 mM 2.mercaptoethanol) used by App (1969) for rice embryos. The supernatant derived after the homogenate had been spun for 15 minutes at 11,000 xg--to remove large debris--was spun at 340,000 xg for 2 hours. Twenty ml of the resulting supernatant was chromatographed on a 30.0 x 3.0 cm column of fine Sephadex G-25 equilibrated with the sterile eluting buffer (0.01 M tris, pH 7.6; 6 mM 2-mercaptoethanol). The portion corresponding to the first fraction absorbing at 280 nm was collected and 14.3 ml of it was immediately used for the aminoacyl coupling reaction. All

glassware used was soaked for 24 hours in chromic acid before being washed, to help deplete the amount of nuclease present. Plastic gloves were worn and other precautions against these nucleases were taken.

B. Preparation of ribosomes from wheat germ

Multiples of the homogenization mixture described above for the preparation of synthetase enzymes gave the yields of wheat germ ribosomes required. The supernatant from the 11,000 xg centrifugation was recentrifuged at 30,000 xg for 20 minutes, and this supernatant was underlayered with 10 ml of Buffer II containing 1 M sucrose. Further steps were the same as described for the purification and dissociation of ribosomes prepared in Buffer III.

C. Preparation of ^{14}C-phenylalanyl-tRNA

The incubation mixture, as described by App (1969), contained in a final volume of 20.0 ml the following: 100 mg creatine phosphate, 20 mg ATP, 800 µg creatine phosphokinase, 8.0 mM $MgAc_2$, 25 µCi ^{14}C-phenylalanine, 0.19 M tris pH 7.6, 27.6 mg stripped yeast tRNA and 14.3 ml wheat germ aminoacyl tRNA synthetase as prepared above. The reaction took place in 15 minutes at 36^{o}C, after which 0.1 volume of 20% (W/V) KAc buffer pH 5.0 was added and the mixture was extracted with an equal volume of 88% (V/V) redistilled phenol. After centrifugation at 11,000 xg for 15 minutes, the aqueous top layer was carefully removed and the RNA was precipitated by adding 1 ml of 20% NaCl and 2.5 volumes of ice-cold ethanol. After standing overnight at

-20°C, the RNA was pelleted by centrifugation, and then redissolved in 20 ml of 0.01 M KAc buffer pH 5.0. The precipitation and centrifugation was redone, and this time the pellet was suspended in 4.0 ml of the buffer. Any traces of phenol were removed by chromatographing the 4 ml of suspended RNA through a sterile 28 cm by 2.5 cm column of Sephadex G-25 equilibrated with 0.01 M KAc buffer pH 5.0. The first fraction absorbing at 280 nm was collected and the RNA was reprecipitated as above. The pellet was resuspended in 4 to 10 ml of the 0.01 M KAc buffer and stored at -20°C. Again precautions as mentioned above were taken against nucleases.

D. Incorporation reaction

Incorporation reaction mixtures containing 8-9000 cpm of ^{14}C-phenylalanyl-tRNA, 25 mM KCl, 16 mM $MgCl_2$, 12 mM 2-mercaptoethanol, 10 mM tris-HCl buffer pH 7.5, 1 mM ATP, 0.5 mM GTP, 5 mM creatine phosphate, 10 µg creatine phosphokinase, and 80 µg of poly U were prepared. To this were added 45 E_{260} units of ribosomes. After 60 minutes incorporation at 37°C, 0.5 ml of 0.1 M cold phenylalanine and 0.5 ml of 20% trichloroacetic acid (TCA) were added. The samples were kept at 80°C for 30 minutes and filtered through a 0.45 µ millipore filter. The filters were washed 10 times with 1.5 ml aliquots of 20% (W/V) TCA and finally with 25 ml of water. The filter discs were dried under a stream of hot air before being placed in 15 ml of scintillation fluid (3 g PPO and 100 mg POPOP/1 toluene) in polyethylene vials. The channels ratio method was used to calculate disintigrations per minute (dpm) from counts per minute (cpm). As a precaution against

bacterial contamination, sterilized glassware and water were used throughout. Aliquots of reagents for single experiments were frozen in acid-washed, sterilized tubes until used and were discarded after once being opened. Reaction mixtures were prepared inside a fiberglass tissue culture hood under a germicidal tube.

E. Preparation of T factor

The method used for the isolation of T factor was described in detail by Legocki and Marcus (1970). Wheat germ, 30 g was blended at low speed for 50 seconds (five blendings each of 10 seconds duration) in 240 ml of 1 mM $MgAc_2$-2 mM $CaCl_2$-50 mM KCl. After this was centrifuged for 10 minutes at 24,000 xg, 0.01 volume of 0.1 M $MgAc_2$, and 0.025 volume of 1 M tris, pH 7.6, were added. Before centrifuging at 340,000 xg for 2 hours, the heavy material in the mixture was removed by a centrifugation for 20 minutes at 30,000 xg. Fifty ml of germ supernatant fraction were dialyzed overnight against two 500 ml changes of medium I (2 mM tris, pH 7.6; 5 mM 2-mercaptoethanol) containing 2 mM $MgAc_2$ and 50 mM KCl. This protein solution was chromatographed on a DEAE-cellulose column, 1.8 x 19 cm, equilibrated with medium I plus 50 mM KCl. Because high purity and separation of T factors was not required, the method of Legocki and Marcus (1970) was modified at this point. Medium I plus 0.3 M KCl was applied to the column and a void volume of 24 ml was discarded before 100 ml of effluent was collected. The protein precipitate resulting from the addition of $(NH_4)_2SO_4$ to 65% saturation was pelleted by centrifugation at 30,000 xg for 10 minutes and was dissolved in 5 ml of medium I.

This crude T factor suspension was dialyzed overnight in two 500 ml changes of medium I before the protein concentration was estimated by using the Folin reagent.

RESULTS AND DISCUSSION

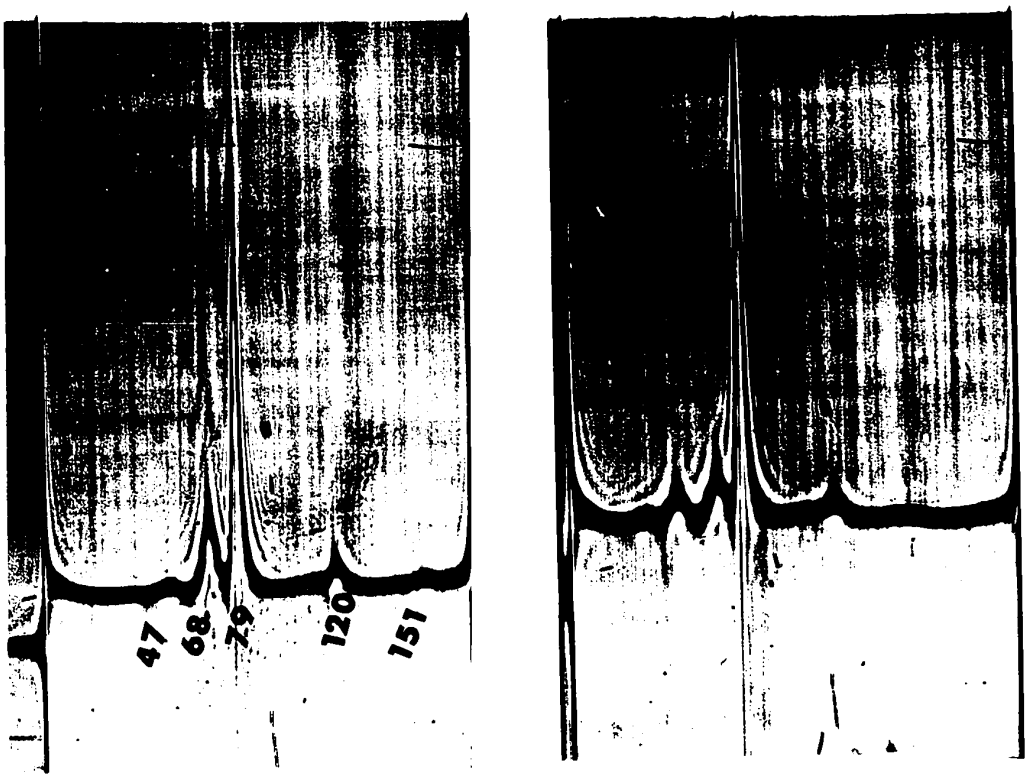
I. Isolation of Ribosomes

A. Characteristics of zonal separated ribosomes

Ribosomes, prepared for zonal separation from the leaves of 4.5 day old wheat seedlings, contained five peaks in the analytical ultracentrifuge (Figure 3A). The sedimentation values measured at 20°C were 47, 68, 79, 120, and 151S. It was assumed that the 79S peak was ribosomes of cytoplasmic origin, while the 68S peak was ribosomes of chloroplast origin. Other evidence bearing on this will be given later. The 47S peak was believed to contain ribosomal degradation products, while the 120S and 151S were believed to be respectively dimers and trimers of the 79S material. Ribosomes prepared for zonal separation from leaves of 5.5 day-old wheat plants contained the same five peaks in the analytical ultracentrifuge (Figure 3B); however, the percentage of 70S peak had decreased in area while the 47S degradation peak had increased.

During tissue preparation it was observed that the older plants were tougher and more difficult to grind, and it was concluded that the increase of degradation product in the 5.5 day-old plant preparations was caused by the more rigorous grinding needed. Therefore, 4.5 day-old instead of 5.5 day-old wheat plants were used in further studies.

The monosomes of the 70S and 80S were separated from each other by zonal centrifugation for either 7 hours at 4°C or 4 hours at 25°C. Both methods gave essentially the same separation. Besides



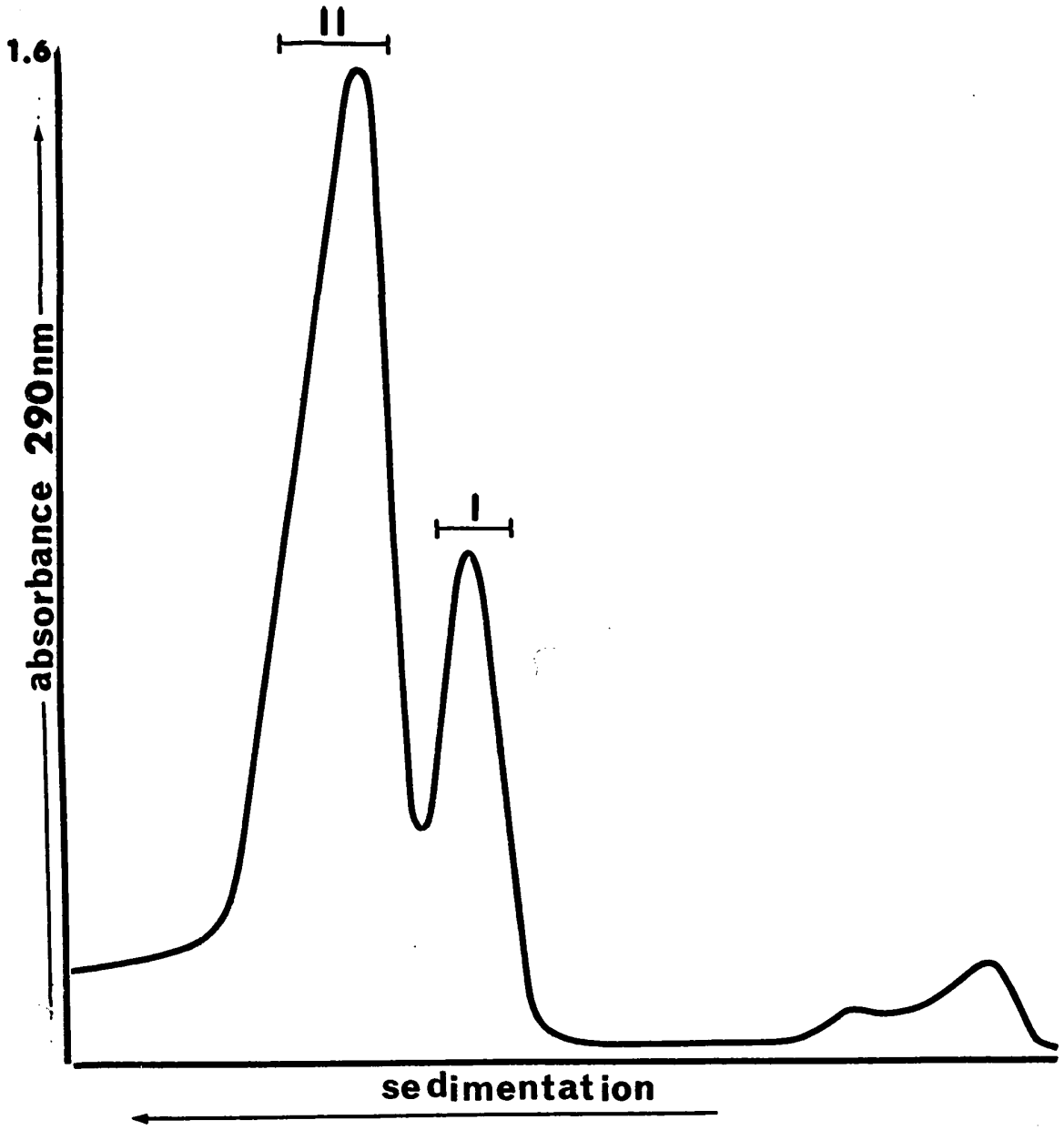
A

Figure 3. Analytical ultracentrifuge pattern of leaf ribosomes from Manitou wheat. A. 4.5 day-old seedlings; B. 5.5 day-old seedlings. Wheat leaves were homogenized in Buffer I (plus 10% triton X-100, 5 ml of 4% bentonite, 0.3 mg Na desocycholate). The ribosomal pellet obtained was resuspended in 20 ml Buffer II, clarified by centrifuging at 30,000 xg for 15 min., and was layered on top of 1 M sucrose in Buffer II before being centrifuged for 2 hours at 340,000 xg. The pellet was suspended in Buffer II and was analyzed. These pictures were taken 8 min. after a speed of 39,460 rev/min was reached and sedimentation values were calculated as is described in the "Methods".

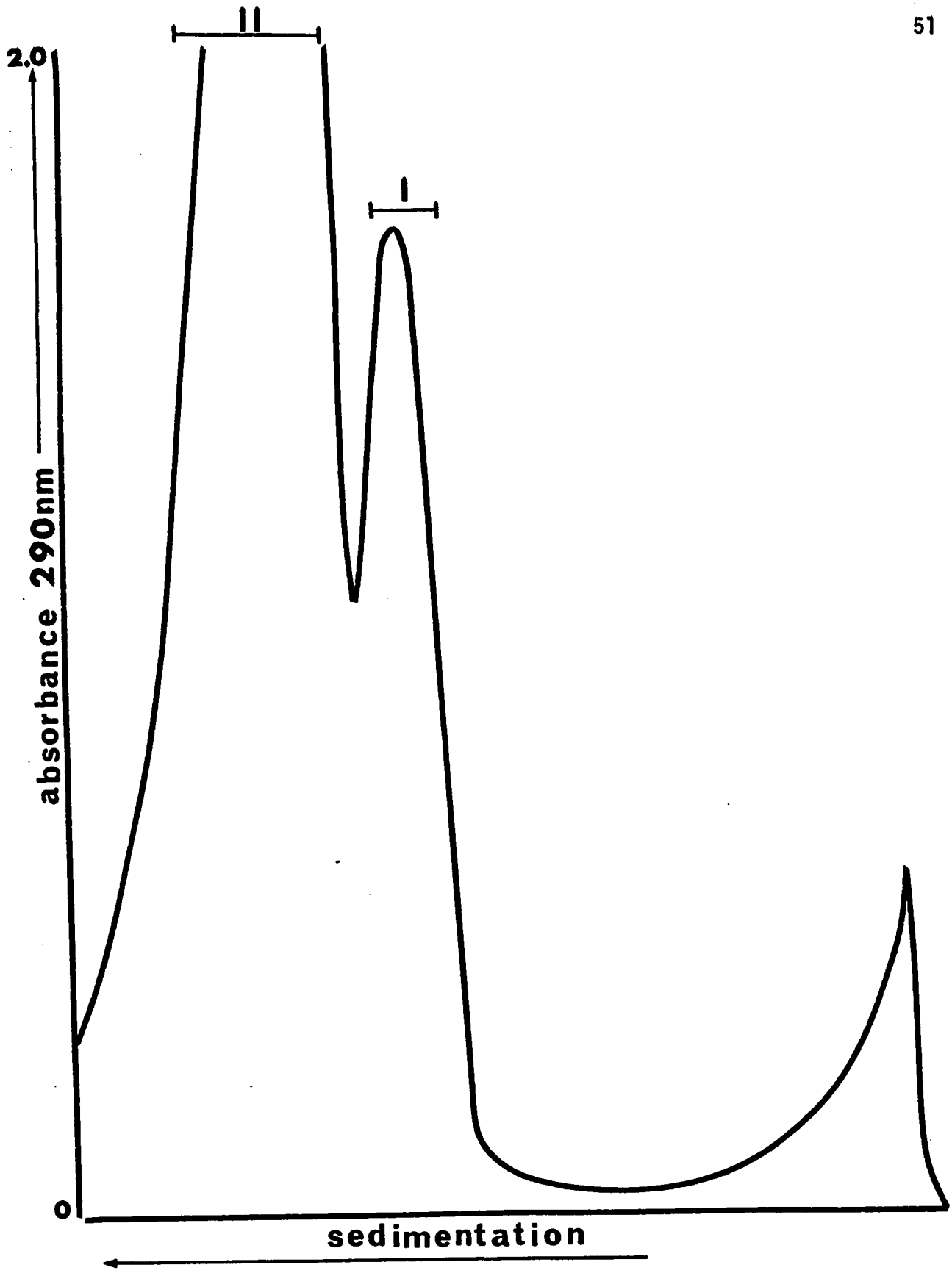
requiring a longer running time, the centrifugation at 4°C was formidable because of condensation in the centrifuge tub and temperature fluctuations of buffer solutions during the loading operation. However, centrifugation was done at 4°C to help retain biological activity. Figure 4A illustrates the separation attained when 1500 E_{260} units were placed on the zonal gradient. Using the triangulation method for calculating the areas of the two peaks, one may infer that the preparation contained 20 to 25% 70S ribosomal material, and 75 to 80% 80S ribosomal material. Figure 4B illustrates that as much as 3000 E_{260} units of ribosomes could be placed on the gradient and still attain sufficient separation. However, higher concentrations overloaded the gradient and one large fraction resulted. It should be noted that ribosomes of 4B have sedimented further down the gradient than the ribosomes of 4A. This phenomenon of non-reproducibility occurred often and was thought to be caused by slight differences in the temperatures of the buffer. The density of the sucrose buffer will change with temperature, thus causing ribosomes on cooler gradients to sediment slower. Because this method was used only for routine separation of the ribosomes, this problem of non-reproducibility was not considered serious.

The 80S ribosome fraction and the 70S ribosome fraction were isolated by centrifuging the collected fraction indicated on Figure 4B. The ribosomes obtained were assessed for purity on the analytical ultracentrifuge, and as is seen in Figure 5A and 5B, the 80S ribosomal fraction was almost devoid of 70S contaminantion and the 70S fraction was almost devoid of 80S contamination.

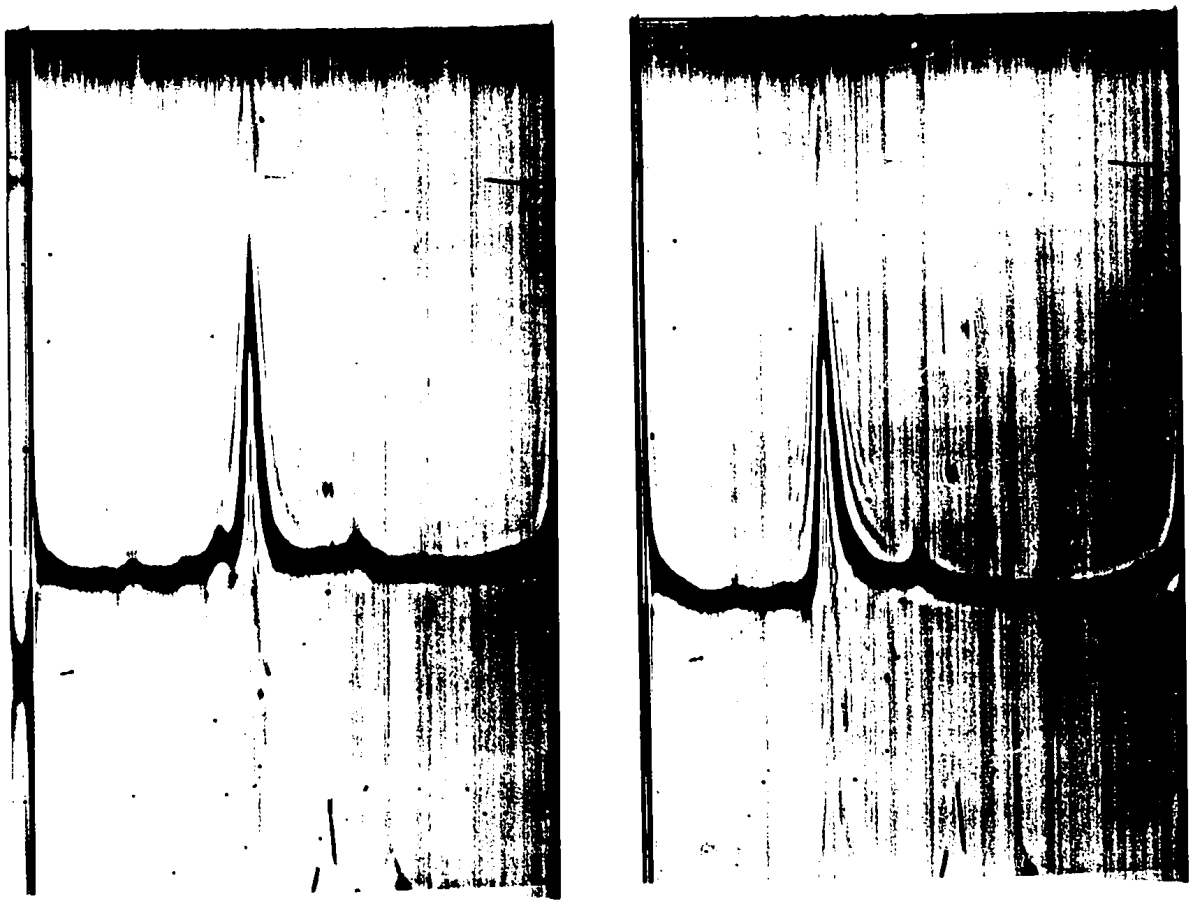
Figure 4. Zonal separation of wheat leaf ribosomes. Ten ml of wheat leaf ribosomes suspended in Buffer II containing 3% sucrose were placed on top of a convex gradient ranging from 7 to 35% sucrose in Buffer II. After centrifuging for 7 hours at $4^{o}C$, the gradient was analyzed via a flow cell at 290 nm and fractions I and II which were respectively 70S and 80S monomers were collected. A. 1500 E_{260} units; B. 3000 E_{260} units.



A



B



A B

Figure 5. Analytical ultracentrifugation pattern of zonal separated 80S and 70S ribosomes from wheat leaves. A. 80S; B, 70S. Ribosomes were zonal separated as is described in the "Methods" suspended in Buffer II and analyzed. These pictures were taken 8 minutes after a speed of 39,460 rev/min was reached. Sedimentation is from left to right.

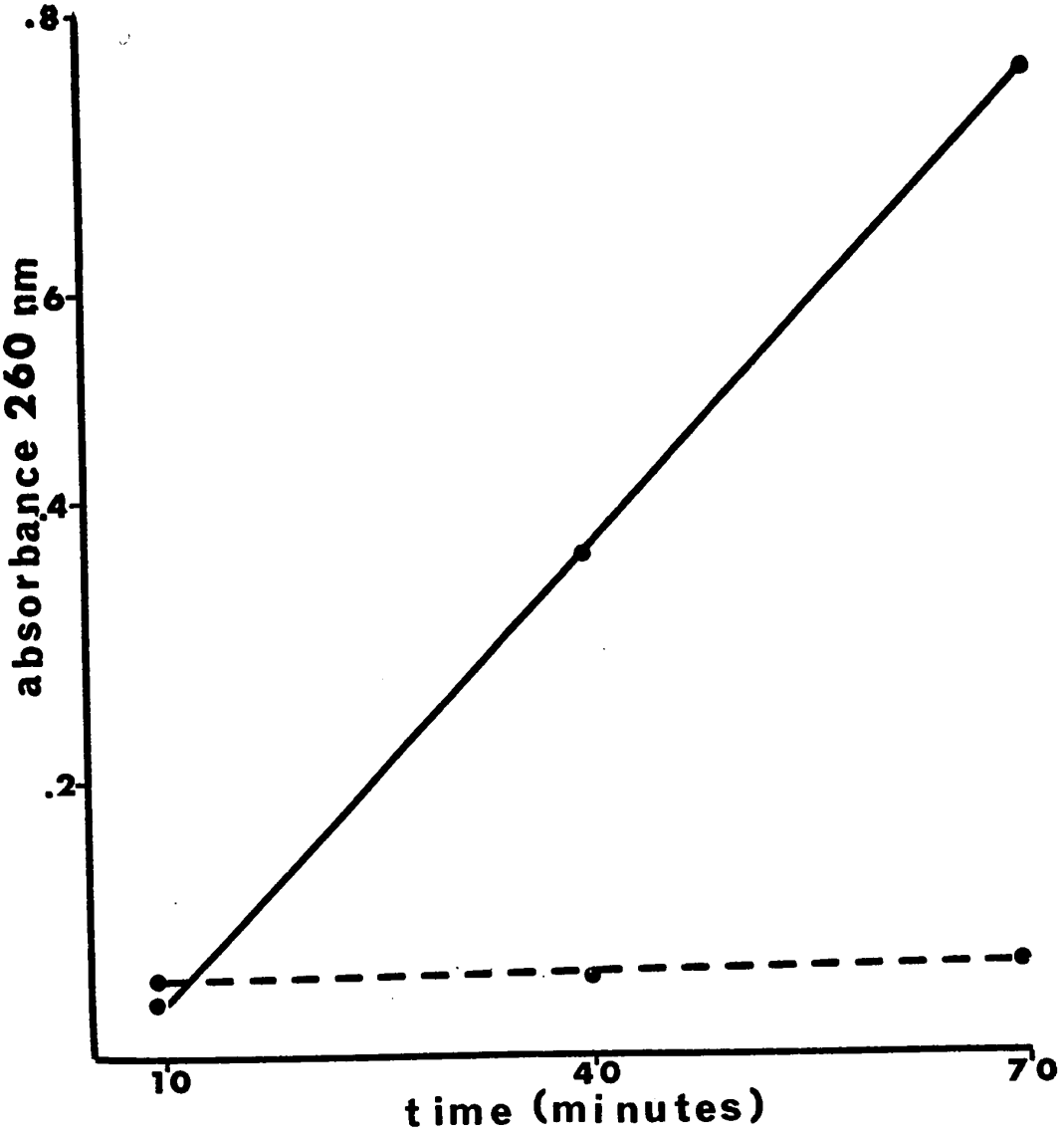
B. Ribonucleases in zonal separated ribosomes

Ribonucleases have been reported in wheat. Matsushita (1959) reported RNase activity in the endosperm of seeds, the cytoplasmic solution, and the cytoplasmic particles (microsomes) of leaves and roots of wheat. He found the RNase on the microsomes to have a pH optimum of 6.0, to be activated by divalent cation, to be situated on the surface of microsomes because it was solubilized by adding taurocholate or BuOH, and to be activated by organic bases such as tris (hydroxymethyl amino methane) and basic amino acids. Hadziyev et al. (1969) reported higher RNase activity in wheat chloroplasts than on the ribosomes. They reported that the RNase degraded mRNA's as well as the ribosomal types of RNA, and that this degradation could be inhibited 50% by the addition of bentonite to the grinding medium. Igarashi (1969) demonstrated the presence of nucleases in E. coli B ribosomes which degraded poly U before in vitro protein synthesis could begin.

The incorporation experiments required ribosomes which were active in protein synthesis and which would not contaminate the incorporation reaction mixture with nucleases that would degrade the poly U and charged tRNA. It was therefore pertinent to examine the ribosomes for RNase activity. From Figure 6 it was calculated that 100 E_{260} units of ribosomes contained 1 unit of enzyme, taken that one unit is equivalent to the production of 0.1 μmole of acid-soluble nucleotide per hour, assuming an average A_{260}^{1cm} of 10 for 1 μmole per ml mixture of nucleotides.

Wyen and Farkas (1971) studied in detail the ribosome-bound nuclease in Avena leaf. They concluded that the ribosomes had

Figure 6. Assay for RNase on wheat leaf ribosomes. Wheat leaf ribosomes were prepared in Buffer I (plus 10% triton X-100, 5 ml bentonite, 0.3 mg Na desoxycholate) and were pelleted by centrifuging 340,000 xg for 2 hours. This pellet was resuspended in 20 ml Buffer II and after clarification by centrifuging 15 minutes at 30,000 xg was layered on top of 1 M sucrose in Buffer II and spun 2 hours at 340,000 xg. The RNase assay mixture contained: 1 ml substrate solution (4.8 mg wheat germ tRNA/ml), 0.4ml ribosome solution (490 E_{260} units/ml), 0.1 M NaAc buffer pH 6.0 to give a final volume of 2 ml. seedling 80S ribosome ——; germ 80S ribosome— — - .


.8
.6
.4
.2
absorbance 260 nm
10
40
70
time (minutes)

bound substantial amounts of the cytoplasmic RNase during isolation and that only part of this RNase could be removed by washing--the rest being tenaciously bound to the ribosomes. Igarashi (1969) treated the ribosomes with a modified high salt washing procedure to remove the nucleases from E. coli ribosomes, but the salt washing concomitantly removed a poly-U directed binding factor which had to be isolated and added back before the ribosomes would regain their activity. Personal communications with Igarashi indicated the isolation of this factor would be impracticable. Cohen (1970) reported that when polyribosomes of rabbit reticulocytes were washed with 0.6 M KCl, they remained intact, but they lost their ability to incorporate amino acids into peptides. Huvos et al. (1970) treated E. coli polysomes with 0.13 µg pancreatic RNase, and found that even though the 28S rRNA had degraded 75%, incorporation had decreased only 50%. Although they stated that the enzyme had been purchased from Reanal, Budapest, they did not state the specific activity of the enzyme. However, 0.13 µg of a comparable pancreatic RNase purchased from Worthington enzymes would be equivalent to 3 enzyme units. From the RNase determination of wheat leaf ribosomes, 0.5 enzyme units were added per 45 E_{260} units of ribosomes (per incubation) and it was therefore relevant to note that some decrease in incorporation could be expected due to the presence of RNase. It was decided that the RNase activity in the ribosomes themselves would be ignored for these first experiments to assure incorporating activity, but precautions against intruding nucleases (from the apparatus, hands, etc.) were taken.

Lanzani and Lanzani (1968) demonstrated RNase activity

on wheat germ microsomes only after the microsomes had been degraded with urea. There was no nuclease activity present in undegraded microsomes nor in the high speed supernatant. The wheat germ ribosomes prepared as described in "Methods" were assayed for RNase activity, and the results as shown in Figure 6 indicated no enzyme activity. Studies in this laboratory on the isolation and characterization of RNA by Dr.s Jones and Nagabhushan demonstrated that the ribosomal RNA of wheat germ was not degraded, while that of the wheat leaves was. These results confirmed the opinion that nucleases are not present in the supernatant of wheat embryo preparations, and thus, in the reaction whereby tRNA was charged with ^{14}C-phenylalanine, the supernatant enzymes were always obtained from wheat germ.

The sucrose used in the zonal separation of ribosomes of cytoplasmic and chloroplast origin was probably contaminated with nucleases which might damage the ribosomes directly or be retained on them. Solymosy et al. (1968) described a method for removing these enzymes from sucrose. Diethylpyrocarbonate (DEP) was used as described in "Methods" in an attempt to produce nuclease-free sucrose, and Figure 7 indicates that results obtained from the zonal centrifugation were not typical and suggested that the ribosomes had agglomerated. Because the characteristic 'apple' odor of DEP was noticed in the prepared sucrose, and because the same separation results were obtained by Dr. Nagabhushan when she attempted to increase ribosomal yield by adding DEP to the isolation medium, it was concluded that the DEP had not been completely eliminated during the heating process. Wolf et al. (1970) described the action of DEP to be non-specific for proteins and

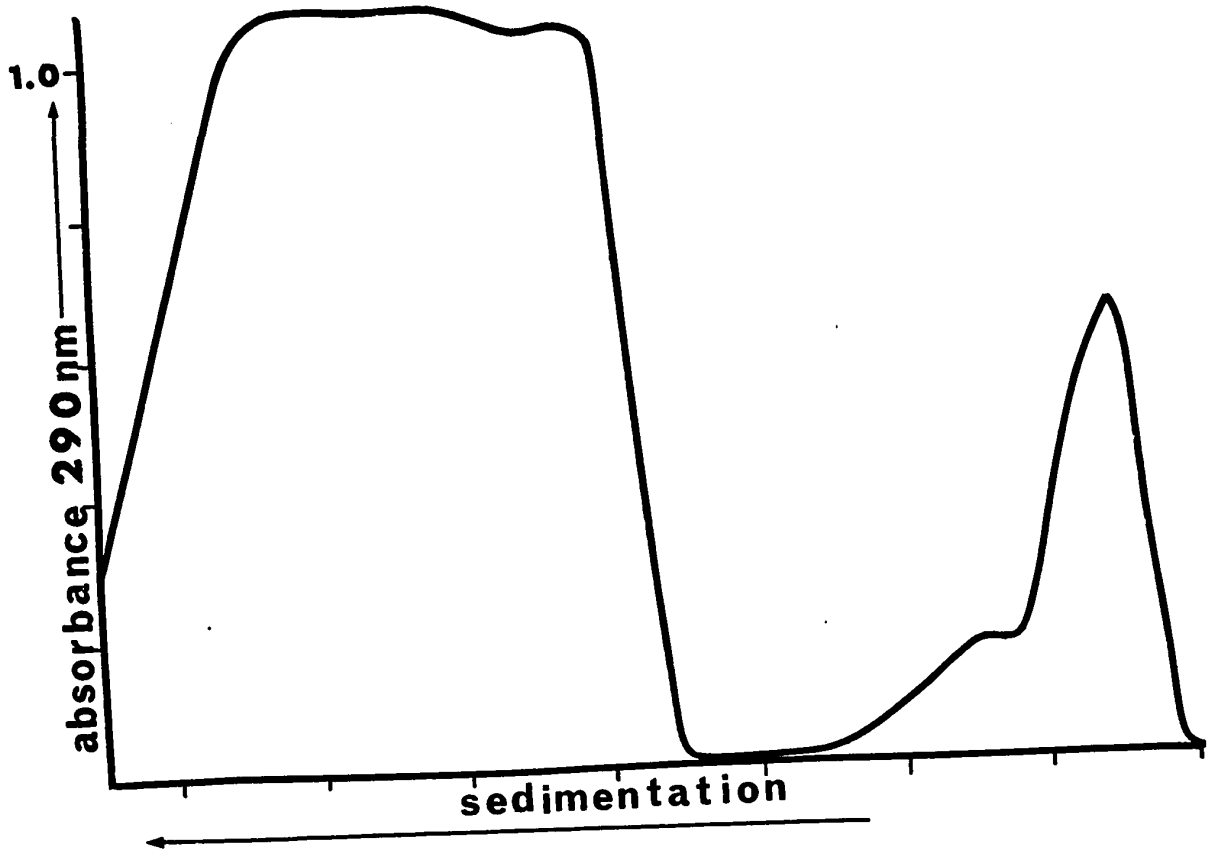


Figure 7. Zonal separation of wheat leaf ribosomes through a DEP treated buffer. Six ml diethyl pyrocarbonate was added to 1,0 l of a 40% sucrose solution in Buffer II. The solution was heated in a boiling water bath for 20 minutes and allowed to return to room temperature before being stored overnight at 4°C. 2500 E_{260} units of ribosomes were centrifuged (47,000 rpm for 7 hours at 4°C) through a 7 to 38% gradient prepared from appropriately diluted aliquots of this DEP treated sucrose solution. The gradient was analyzed via a flow cell at 290 nm.

"that the general feature of this reaction is a formation of inter-molecular peptide-like bonds involving amino acids and carboxylic groups which yield bizzare polymetric proteins". "The intramolecular formation in these bonds results in inactive conformations". Anderson and Key (1970) reported the use of DEP to isolate intact polyribosomes in the presence of pancreatic RNase; however, the DEP concentration needed concomitantly caused the polyribosomes to completely lose their incorporating ability. Weeks and Marcus (1969) reported DEP to protect polyribosomes isolated from imbibed wheat embryos. These polysomes had lost much of their ability for _in vitro_ amino acid incorporation.

It was concluded that although DEP has been used successfully for the isolation of undegraded nucleic acids (Solymosy _et al._, 1968), even small traces of it were enough to alter the protein structure of the ribosomes. However, further experiments aimed at obtaining complete hydrolysis of DEP in the sucrose, might be tried as an inexpensive source of nuclease-free sucrose would be an asset to this work. Nuclease-free sucrose may be purchased from General Biochemical (Chagrin Falls, Ohio, USA) but because large quantities were needed for zonal separation, the price made its use prohibitive. Therefore, the nucleases, if they were present in the sucrose, were tolerated.

II. Phenylalanine Incorporation by Zonal Separated Ribosomes Using a Transfer System

A. Ribosome concentration

Figure 8 indicates the dependency of ^{14}C-phenylalanine

incorporation upon the concentration of 80S ribosomes. The radioactivity of the washed protein-precipitate continued to increase as the concentration of ribosomes increased. Concentrations of ribosomes greater that 70 E_{260} units were found to cause unmanagable amounts of precipitated protein to be formed when the reaction was stopped by the addition of trichloroacetic acid. Due to low yields of zonal separated wheat leaf ribosomes, it was not feasible to use concentrations greater that 45 E_{260} units in each 1 ml assay.

Using the conversion factors suggested by Parthier (1971)--1 mg ribosomal RNA = 25 E_{260} units and 1 mg ribosome = 13 E_{260} units--it was calculated that amino acid incorporations, which had been reported in wheat systems, contained the following concentrations of ribosomes per ml incorporation mixture: Allende and Bravo (1966), 4 E_{260} units; Mehta et al. (1969), 11 E_{260} units; Legocki and Marcus (1970), 10 E_{260} units; Schultz et al. (1972), 2 E_{260} units. App (1969) used 8 E_{260} units of rice embryo ribosomes per ml assay and Parthier (1971) found maximum activity of pea seedling ribosomes system to contain 21 E_{260} units per ml. Beevers and Poulson (1972) reported pea ribosome concentration as milligrams of protein. They added 1 mg protein (the added ribosomes) per 0.5 ml incubation mixture, which may be calculated as 50 E_{260} units of ribosomes per ml incubation mixture.

To attain levels of activity in the described wheat leaf system high enough to draw reliable conclusions, the higher amount of ribosomes (45 E_{260} units) was used.

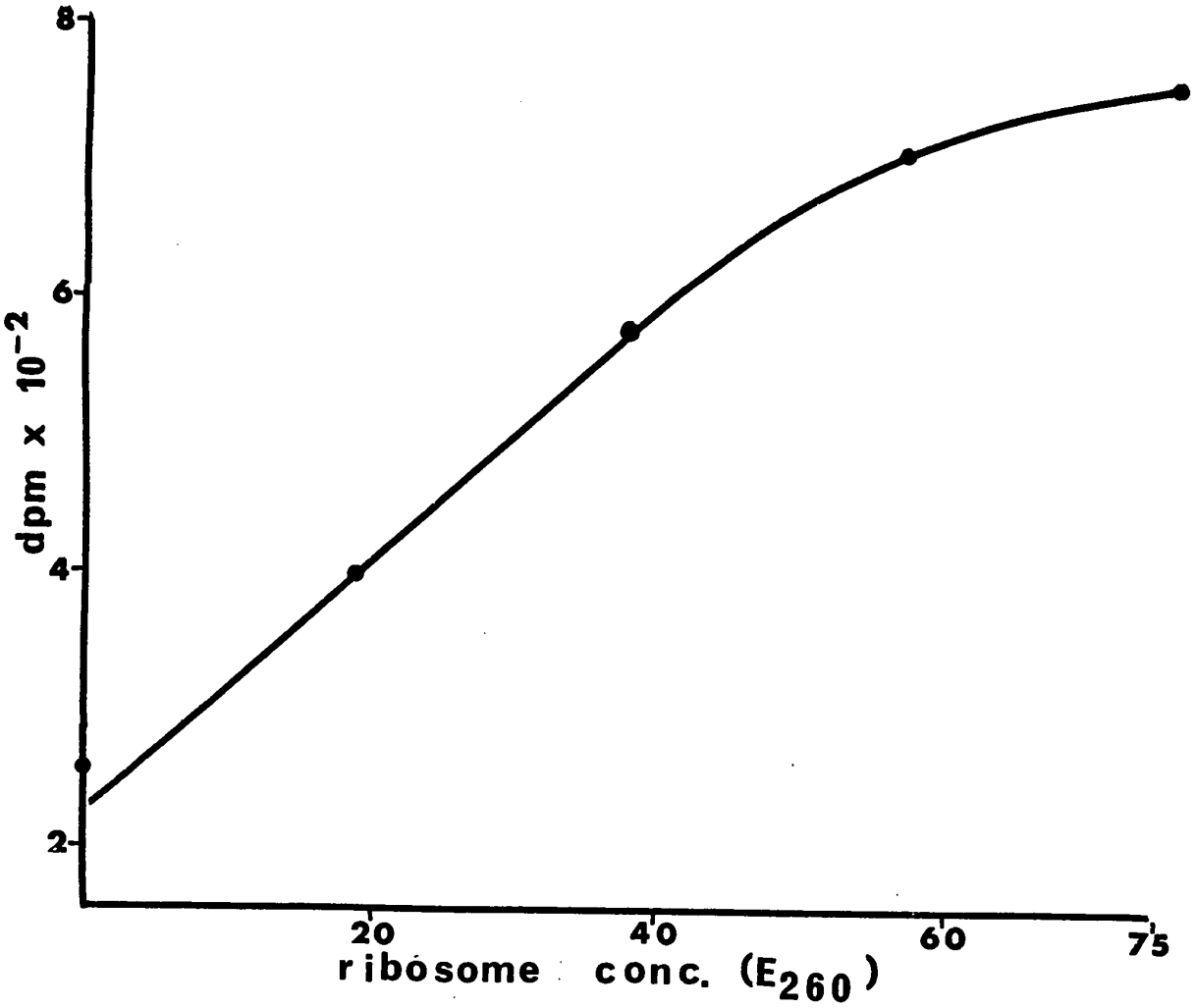


Figure 8. Activity versus concentration of 80S wheat leaf ribosomes. Zonal separated 80S ribosomes were suspended and appropriately diluted with Buffer II before being added to the prepared incorporation media. Tubes were incubated at 37°C for 60 minutes before the reaction was stopped. Activity was measured in a scintillation counter, and dpm was calculated from cpm by the channels ratio method.

B. Incubation time for incorporation

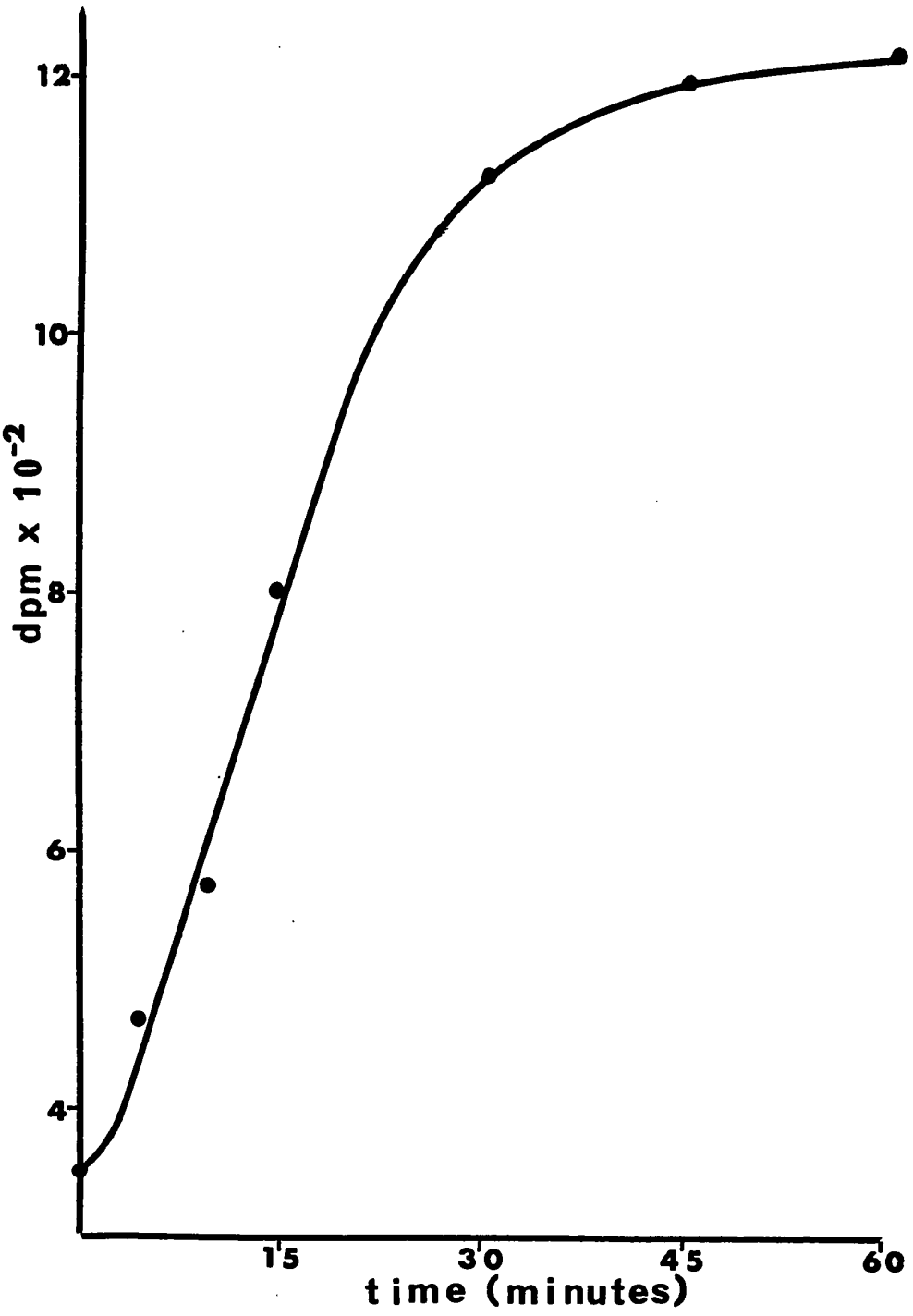
Figure 9 indicates the dependency of ^{14}C-phenylalanine incorporation upon time. Incorporation increased rapidly with time for the first 30 minutes, and then leveled off to reach a plateau at 45 minutes. Although maximum incorporation was acquired at 30 to 45 minutes, it did not decrease if the incubation time was extended. To assure that optimum activity had been attained, the incorporation mixture was incubated for 60 minutes.

C. Buffer effect on incorporation

Figure 10 indicates the effect that the concentration of the tris-HCl buffer pH 7.5, had upon ^{14}C-phenylalanine incorporation. It was noted that the lower concentrations of tris--5, 10 and 20 mM--stimulated higher amounts of incorporation. Likewise, Bewley and Marcus (1970) reported similar observations in a wheat embryo amino acid incorporating system and they stated that the tris buffer acted as a cation in the system, not as an inactive controller of pH. When they used an incorporating mixture containing 5 mM tris-HCl pH 8.1, optimum KCl concentration was at 50 to 60 mM KCl. As is indicated in Figure 10 when the wheat leaf system was used (containing 25 mM KCl and pH 7.6) the optimum tris concentration was 10 mM. It must be remembered that the wheat leaf ribosomes, used in the incorporation system, had been resuspended in a buffer containing 10 mM tricine pH 7.6, 10 mM $MgCl_2$ and 5 mM 2-mercaptoethanol.

As described in "Methods", the ribosomes were prepared in tricine buffer pH 7.6, but the incorporation reaction mixture

Figure 9. ^{14}C-phenylalanine incorporation by cytoplasmic wheat leaf ribosomes versus time. From the incorporation mixture containing zonal separated 80S ribosomes, 1 ml aliquots were pipetted into TCA at the various times. The TCA precipitated samples were incubated at 80^{o} for 30 minutes before being filtered and counted.


12
10
8
6
4
dpm x 10^{-2}
1'5
3'0
4'5
60
time (minutes)

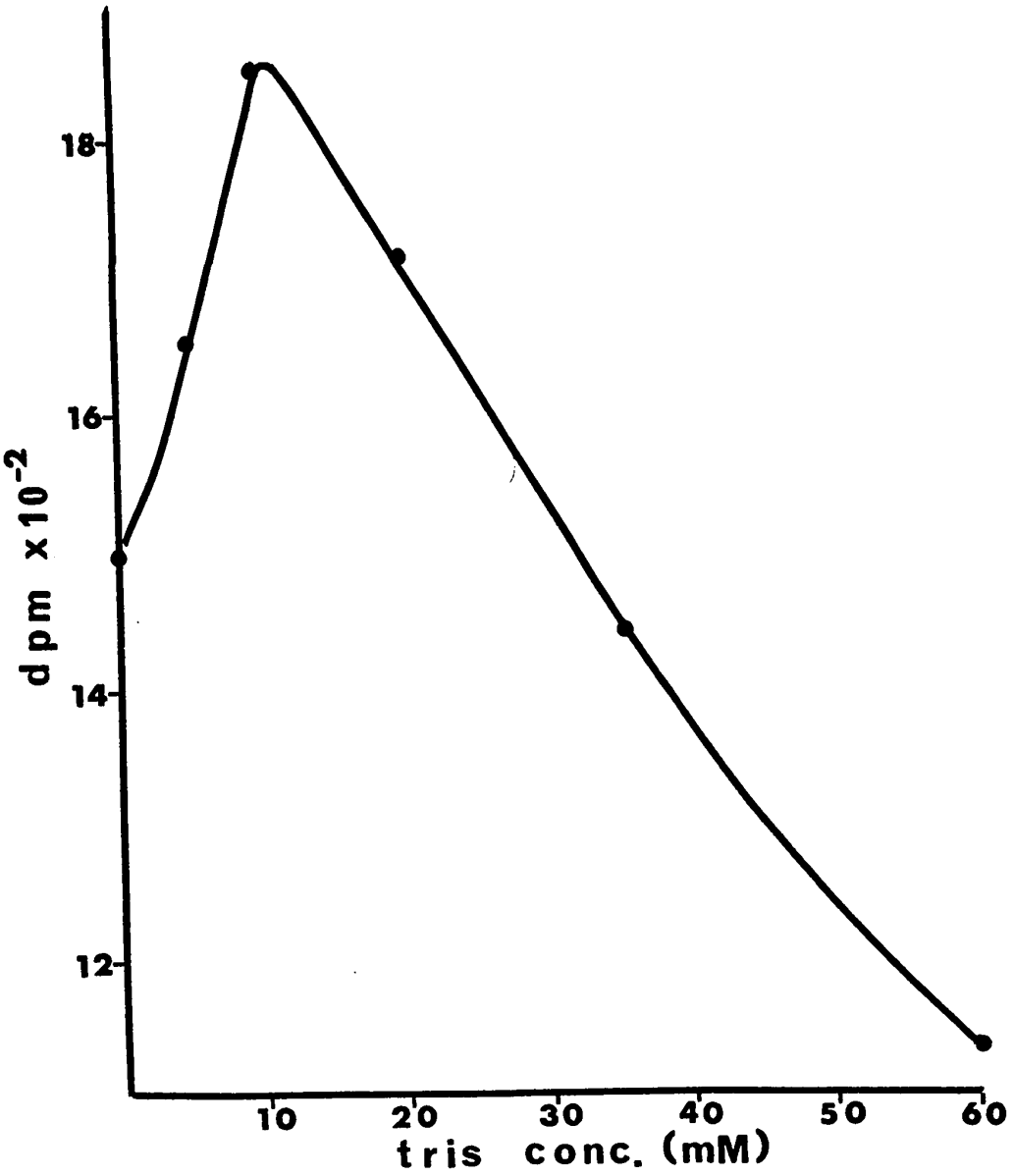


Figure 10. Effect of tris concentration on the phenylalanine incorporation by cytoplasmic wheat leaf ribosomes. The zonal separated 80S ribosomes were resuspended in Buffer II before they were added to the samples containing the various concentrations of tris-HCl buffer pH 7.5. THAM[tris(hydroxymethyl)aminoethane].

contained tris buffer pH 7.6. Personal communications with Dr. Jones indicated ribosome preparations in tricine buffer, unlike those is tris buffer, provided ribosomes which when analyzed on sucrose gradients acted in a way typical of those reported in the literature. An incorporation experiment was performed to compare the incorporating ability of ribosomes prepared in tricine, by incubating them in three different buffer reaction mixtures. All three contained buffer concentrations of 10 mM. As noted in Table 1, there was no difference between the three types of buffers, and it was concluded that tricine could be used to prepare the ribosomes which would be assayed for their activity in a tris incorporation reaction mixture.

Table 1. Effect of buffer on incorporation of ^{14}C-phenylalanine by cytoplasmic wheat leaf ribosomes. Ribosomes, prepared in tricine buffer, were added to the transfer system and made up in 10 mM of the respective buffers. In this and all subsequent tables, incorporation is expressed in dpm calculated from cpm of the hot TCA insoluble protein.

	Buffer		
	tris	tricine	hepes
dpm	2419	2189	2025

D. Dependency of incorporation upon added energy factors.

Table 2 indicates that the incorporation activity was decreased only slightly with the removal of the individual energy factors from the incorporation reaction mixture.

Table 2. Requirement of energy factors for incorporation by cytoplasmic wheat leaf ribosomes. Ribosomes were added to the prepared incorporation mixture minus the energy factor. Two controls were used: incorporation mixture minus ribosomes, and complete incorporation mixture removed at zero time.

System	dpm	% incorp'n
Complete	2045	100
-ATP	1783	87
-GTP	1982	97
-creatine phosphate and cre'n phosphokin.	2051	100
-ribosomes	146	7
+ribosomes at zero time	142	7

This small requirement of energy was not entirely unexpected as Boulter (1970) in his review, had stated that the ATP generating system was not required with the transfer system. The tRNA is already charged, and thus the only energy requirement in the wheat ribosome system would be in the binding and translocation step of protein synthesis. It was concluded that the small amount of energy factor needed for this transfer system had been carried into the incorporation system on the ribosomes.

E. Bacterial contamination

As seen by Table 2, one of the controls (incorporation mixture minus the ribosomes) proved that none of the incorporation

observed by the treatment was due to the added aliquots of the dissolved ingredients. This indicated no bacterial contamination of the aliquots; however, this control does not assay for possible bacterial contamination of the ribosomes. The other control (complete incorporation mixture withdrawn at zero time) indicated that the trichloroacetic acid precipitation step does not trap ^{14}C-phenylalanine and that the activity observed was due to incorporation.

Bacterial contamination of ribosomes was checked regularly by plate counts, and results established that less that 10^4 bacteria per ml ribosomal solution were obtained if the solution of suspended ribosomes was clarified by a centrifugation of 30,000 xg for 15 minutes before plating.

F. Rate of incorporation as a function of magnesium concentration

Spirin and Gavrilova (1969, pp29-33) have reviewed the role of the divalent cation magnesium in the structural stability of the ribosomes. With the gradual removal of magnesium, the monosomes will dissociate into its subunits; and with further removal of magnesium, the structure of the subunits will irreversibly break down. Values calculated for magnesium concentration indicated 1600 to 1800 atoms of magnesium per 80S ribosome, and 2500 atoms of magnesium per 70S ribosome. It was assumed that the ribosomal RNA is in the form of magnesium salt. After reaching the "first critical level of magnesium content in ribosomal particles" as Spirin and Gavrilova called it, the ribosome dissociates. Because the 70S ribosome reached this critical level at a higher concentration of magnesium, it is said to be less

stable than the 80S ribosome. At the second critical level of magnesium, where unfolding occurs, the 70S is again more unstable.

Initiation in a template polynucleotide containing an initiation codon, takes place as was described in the "Literature review". However, if this initiation codon is not present, as in the situation where poly U is required, initiation is stimulated by magnesium concentrations much higher than is required when natural messenger is present. This phenomenon is called non-specific binding and does not require initiation factors. Spirin and Gavrilova (1969, pp112-114) described a model for non-specific initiation, in their review.

In the present study a series of magnesium concentrations was used in the reaction mixture in order to determine its effect on incorporation. As is observed from Figure 11, there was a lag in incorporation at magnesium concentrations below 4 mM. This was followed by a steep increase up to about 14 to 16 mM and the level of incorporation continued to increase up to a concentration of 30 mM, but at a lower rate.

To compare the chloroplast ribosomal rate of incorporation as a function of magnesium concentration with that of the surrounding cytoplasmic ribosomes, it was imperative to extract and use the two species of monosomes from the same preparation. The incorporation mixture (minus the ribosomes and magnesium) was prepared as one batch and divided into two parts. The identical appropriate concentration of the respective ribosomal species was added to each half before the aliquots were pipetted into the tubes containing the range of concentrations of magnesium. Even though the centrifuged ribosomes

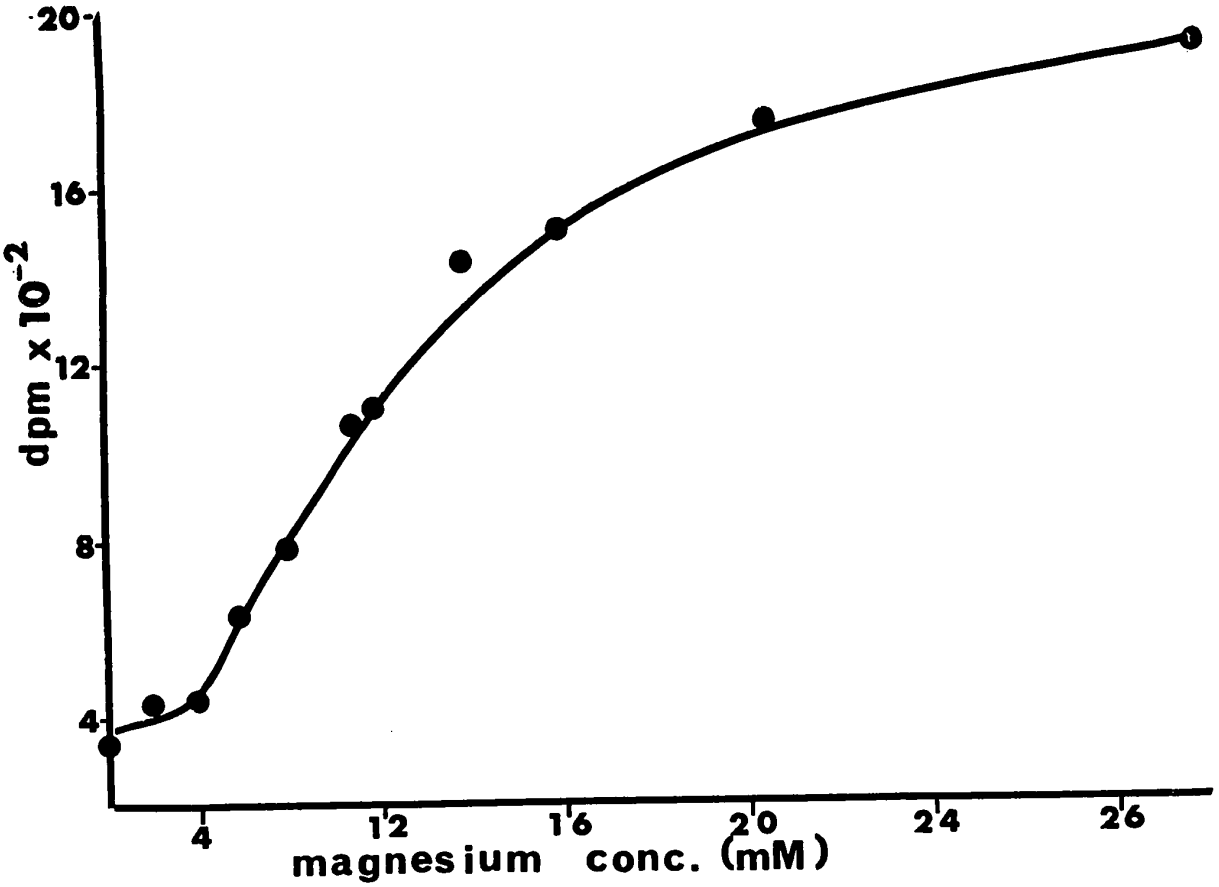


Figure 11. Effect of magnesium concentration on ^{14}C-phenylalanine incorporation by the 80S wheat leaf ribosomes. The reaction mixture contained 45 E_{260} units of ribosomes, 8-9000 cpm ^{14}C-phenylalanyl tRNA, the indicated concentration of magnesium as $MgCl_2$, 80 µg poly U and the other constituents in mM were KCl, 25; 2-mercaptoethanol, 12; tris-HCl pH 7.5, 10; ATP, 1; GTP, 0.5; creatine phosphate, 5.0; and creatine phosphokinase, 5 µg. The incubation was at 37°C for 60 minutes.

were resuspended in 10 mM tris-HCl pH 7.5--containing no magnesium--for these experiments, it can be assumed that the ribosomes will have retained some of their endogenous magnesium.

During this study, the yield of 70S was only sufficient to perform the experiment at five levels of magnesium. As is shown in Figure 12 it was discovered that there was little difference between the chloroplast and cytoplasmic ribosomes in their response to magnesium. Because each incorporation sample had the same concentration (E_{260} units) of ribosomal preparation, it was further concluded that there was not a notable difference between the rates of protein synthesis of the chloroplast and cytoplasmic ribosomes. Boardman et al. (1966) compared the incorporation of ^{14}C-valine into protein, by the supernatant of a 17,000 xg centrifugation of chloroplasts with the supernatant of a 17,000 xg centrifugation of a cell-free homogenate. They concluded that the chloroplast ribosomes were 10 to 20 times more active than the cytoplasmic ribosomes. However, if the chloroplast had been disrupted by homogenization so that the 70S ribosomes bacame mixed with the cytoplasm, or if the chloroplast ribosomes had been pelleted, then their incorporating ability was markedly reduced. In a concluding statement Boardman et al. (1966) remarked, "the present results do not permit conclusions to be drawn about the contributions of the different classes of leaf ribosomes to protein synthesis in the intact leaf". Hadziyev and Zalik (1970) using a method similar to Boardman, reported chloroplast polysomes to be 20 times more active than cytoplasmic polysomes. However, recent attempts in this laboratory to isolate wheat leaf chloroplasts which could be used to prepare the required

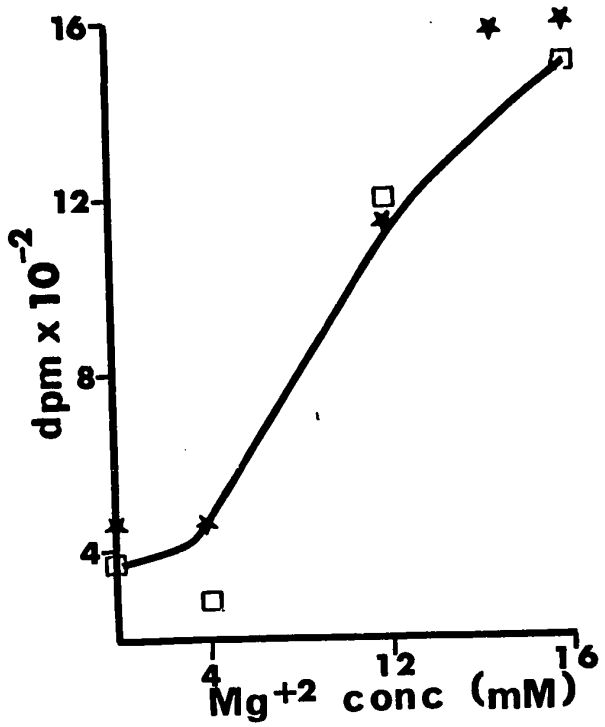


Figure 12. Comparison of the chloroplast ribosomal rate of incorporation as a function of magnesium concentration with that of the surrounding cytoplasmic ribosomes of wheat. Ribosomes were resuspended in 10 mM tris-HCl buffer, pH 7.5 and were added to the incorporation mixture as described under Figure 11. □-ribosomes of cytoplasmic origin; ★-ribosomes of chloroplast origin.

uncontaminated 70S ribosomes were unsuccessful, as were the attempts to isolate uncontaminated 80S ribosomes by sedimenting out the chloroplasts. Therefore, it was concluded that the homogeneity of the ribosome preparations employed by them was not adequate to make reliable comparisons between the two classes of ribosomes.

An incorporation lag at low concentrations of magnesium is evident from the studies by: App (1969) on rice embryo; Igarashi (1969) on E. coli; Nicholls et al. (1970) for rat kidney; Murty and Tamaoki (1972) for mouse lymphoma. However, the data of Legocki and Marcus (1970) using wheat embryo, and Parthier (1971) using pea seedling did not show a pronounced lag. Curiously, in the present study, the wheat leaf ribosomes often exhibited an unexplainable decrease of incorporation at 2 to 4 mM magnesium rather than a lag.

In his review, Boulter (1970) states: "In order to establish that the observed amino acid incorporation is due to the ribosomes themselves you should demonstrate incorporation showing a sharp magnesium optimum, the position of which will depend upon the particular system being investigated". As indicated in the "Literature review", optimum magnesium concentrations for polyphenylalanine synthesis ranged from 7 to 13 mM. However, Nicholls et al. (1970), when studying ^{14}C-leucine incorporation by rat kidney ribosomes observed a plateau rather than a sharp optimum concentration of magnesium. When Murty and Tamaoki (1972) plotted the effect of magnesium on polyphenylalanine synthesis by ribosomes from mouse cancer cells, the curve indicated slight continued stimulation of incorporation past the highest concentration reported (12 mM).

Similarly, in the present study of wheat leaf ribosomes, there was not a sharp magnesium optimum concentration in the range tested. It was thought that high concentrations of magnesium may cause unwanted side reactions, and thus incorporation studies were performed in a buffer containing 16 mM magnesium.

G. Effect of inhibitors of protein synthesis on incorporation

The response of chloroplast and cytoplasmic ribosomes to certain inhibitors has been used as a criterion to their origin. Whereas chloramphenicol inhibits chloroplast ribosomes about 70%, it has been reported to cause only slight inhibition of cytoplasmic ribosomes (Ellis, 1969). Three inhibitors , chloramphenicol, cycloheximide and puromycin were used in this study. It may be seen from Table 3 that the 80S ribosomes were not signigicantly inhibited by chloramphenicol and cycloheximide, but they were inhibited by puromycin. This is in agreement with the results reported by Parisi and Ciferri (1966) who used Castor bean seedling ribosomes. The 70S ribosomes were not inhibited by cycloheximide but they were inhibited by chloramphenicol and puromycin. These findings confirmed that the 70S were different from the 80S, and was taken as an indication that the 70S ribosomes were of chloroplast origin.

Table 3. Effects of chloramphenicol, cycloheximide and puromycin on incorporation by 70S and 80S ribosomes from wheat leaves. Ribosomes were incubated in the presence of the following inhibitors: 23 µg chloramphenicol, 10 µg cycloheximide or 250 µg puromycin.

Ribosome	Inhibitor added and percentage inhibition							
	None		Chloramphenicol		Cycloheximide		Puromycin	
	dpm	%	dpm	%	dpm	%	dpm	%
80S	1288	0	1173	9	1134	12	918	30
70S	1072	0	523	51	1228	0	589	46

As will be further discussed in Section IV (C) "Dissociation and incorporation of ribosomes from plants of differing ages", protein synthesis in the cytoplasm of eukaryotes is known to take place on both free and membrane-bound polyribosomes and workers have provided evidence that separate functions exist for each. Glazer and Sartorelli (1972) have recently reported that the membrane-bound polyribosomes of rat liver were more sensitive to inhibitors of protein synthesis than were the free polyribosomes. Cycloheximide inhibited *in vivo* protein synthesis of membrane-bound polyribosomes 40 to 60% but it had no effect on free polyribosomes. Puromycin inhibited protein synthesis by bound polyribosomes 60% and free polyribosomes by 80%. These experiments have indicated the need for caution in the interpretation of results obtained by inhibitors of protein synthesis and have pointed out that the noted inhibition by puromycin and not by cycloheximide in this wheat cytoplasmic system could be due to the presence of a high amount

of free polyribosomes. Triton X-100 was used to release ribosomes in all cases.

Marcus and Feeley (1966) reported that the formation of an activation complex, in the wheat embryo system, required the presence of three cellular fractions in addition to ATP and magnesium, and was inhibited by cycloheximide. Once this activation complex had formed, the addition of cycloheximide to the protein synthetic medium did not inhibit incorporation. These results obtained by Marcus and Feeley suggested that the 80S ribosomes used for the present study were in an active complex because cycloheximide would not inhibit ^{14}C-phenylalanine incorporation.

H. Species specificity of tRNA and synthetase enzyme

As mentioned in the review by Boulter, Ellis and Yarwood (1972) species specificity in the synthetases has been reported to be negligible by some tRNA's, while completely specific by others. Erokhima et al. (1965), studying pea, yeast and algae, found little specificity for the $tRNA^{phe}$ and synthetases; however, the $tRNA^{met}$ and enzyme from pea and algae, though themselves completely interchangeable, could not replace the yeast component. Tarrago et al. (1970) reported the $tRNA^{met}$ used in elongation of protein synthesis in wheat germ is specifically charged by the homologous enzyme, while the $tRNA^{met}$ used in initiation of protein synthesis, can be charged by the E. coli enzyme. In the present study, wheat germ aminoacyl tRNA synthetases were used to charge stripped yeast tRNA with ^{14}C-phenylalanine. The purification steps of the charged tRNA involved deproteination with

phenol, 3 washings by the ethanol precipation steps, and chromatography through Sephadex G-25; and thus would not allow free ^{14}C-phenylalanine to be carried through into the charged tRNA fraction. The tRNA was indeed charged, for it carried radioactive phenylalanine which would be incorporated into the peptides which were precipitated by trichloroacetic acid.

III. Dissociation Characteristics and Incorporation with Zonal Separated Ribosomes

A. High salt

1. Dissociation

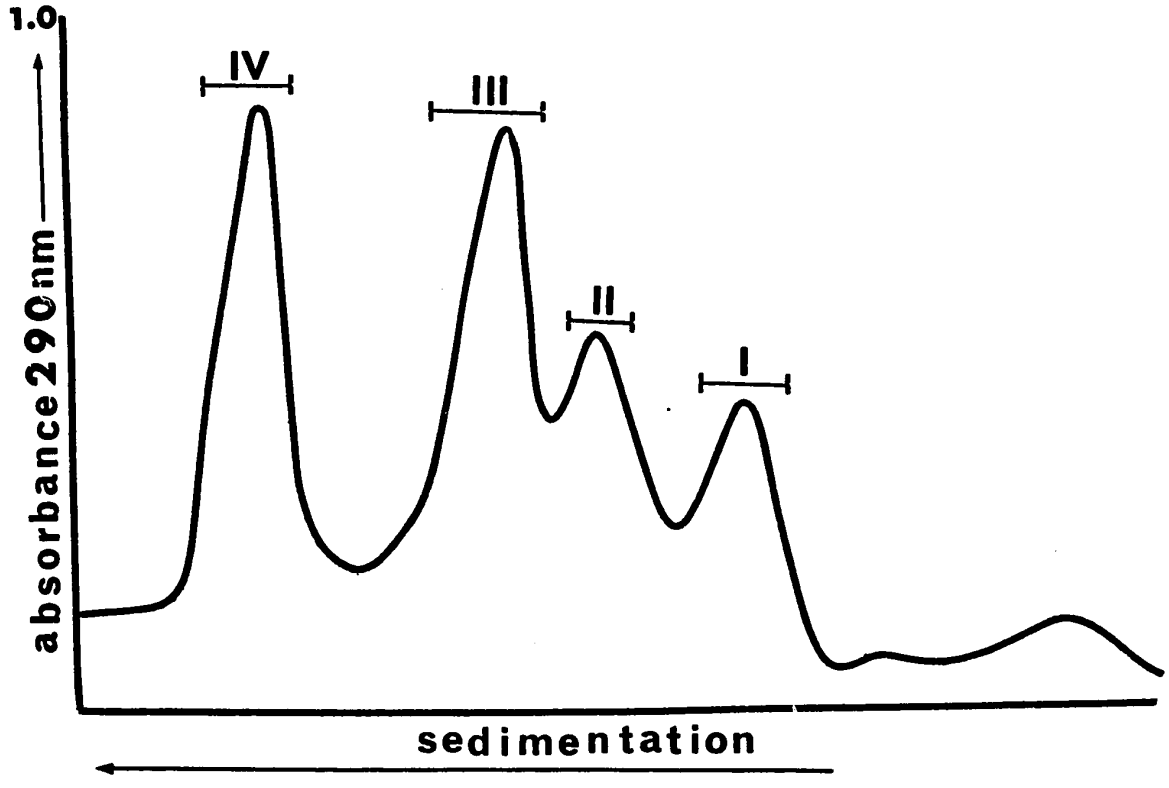
When the zonal separated 80S ribosomes were sedimented through and resuspended in Buffer IV, dissociation was observed on the analytical ultracentrifuge (Figure 13). The sedimentation values calculated for the observed peaks were 76S, 56S, 46S, and 37S. Only about 50% of the 80S ribosomes had dissociated and the 46S peak which had appeared was unexpected. Similar results were observed when the isolated 80S ribosomes were centrifuged through a zonal gradient containing Buffer IV (Figure 14).

2. Incorporation with recombined monomers.

When the subunit fractions were isolated and mixed in a 60:40 ratio of 2:1 (E_{260} units) they readily recombined to form the parent species when the magnesium concentration was increased (Jones, Nagabhushan and Zalik, 1972). As noted in table 4 only the 40S subunit was used because Jones et al. (1972) had described the 40S and 50S

Figure 13. Analytical ultracentrifugation pattern of 80S wheat leaf ribosomes suspended in Buffer IV. Zonal separated 80S ribosomes were resuspended in Buffer IV and were then analyzed. This picture was taken 12 minutes after a speed of 39,460 rev/min was reached.

Figure 14. High salt dissociation of 80S wheat leaf ribosomes. Zonal separated 80S ribosomes were centrifuged through a 7 to 38% sucrose gradient in Buffer IV. After centrifuging for 7 hours at $4^{o}C$, the gradient was analyzed via a flow cell at 290 nm and fractions I, II, III, and IV were collected and were assumed to be respectively 40S, 50S, 60S, and 80S particles.


1.0
absorbance 290nm
IV
III
II
I
sedimentation

material to be two forms--differing only in secondary or tertiary structure--of the same subunit. They had reported that when the two peaks were combined and centrifuged through 10 mM tricine, pH 7.5 (a buffer devoid of $MgCl_2$, KCl, and 2-mercaptoethanol) only one peak corresponding to the 40S peak appeared. And further they found that both subunit forms contained virtually identical proteins and RNA. In the present study, when the subunits were recombined by mixing them in the 16 mM $MgCl_2$ incorporation buffer, they did not support phenylalanine incorporation above that of the subunits, as shown in Table 4.

Table 4. Incorporation by subunits of high salt dissociated 80 ribosomes. The 40S and 60S subunits were isolated from zonal fractions. The ribosomes which would not dissociate by high salt in the zonal separation were isolated and used as an undissociated 80S control. All samples had a final concentration of 45 E_{260} units.

	Ribosomal Material				
	Undissoc't 80S	40S	60S	40S+60S	H_2O
dpm	4364	1175	772	1164	148
%	100	27	18	27	3

B. Puromycin

1. Dissociation

As is shown in Figure 15 A, B, and C; puromycin proved to be useful in the dissociation of the 80S particles. When the 80S ribosomes were preincubated with puromycin at a concentration of

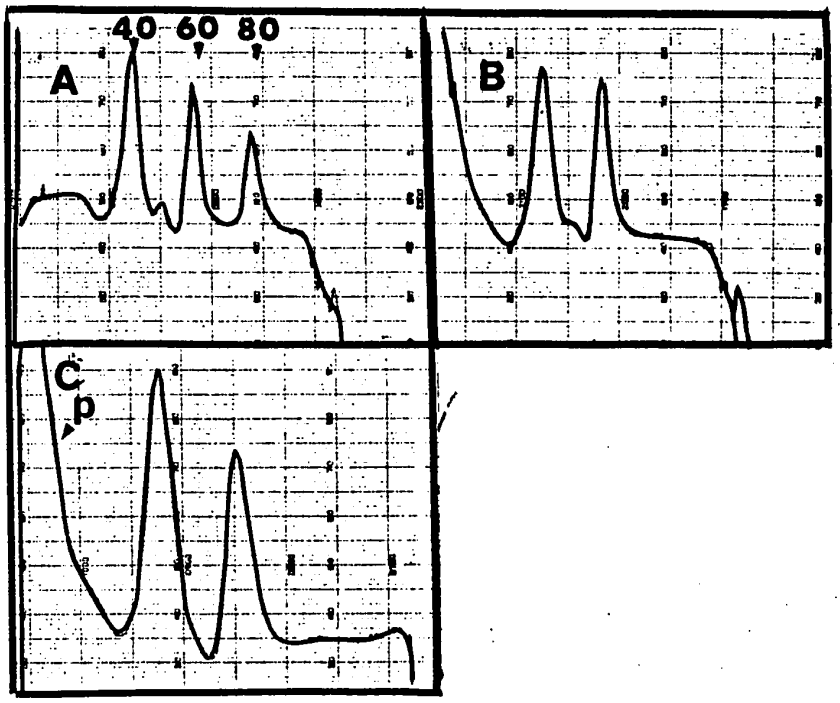


Figure 15. Puromycin stimulated dissociation of wheat leaf cytoplasmic ribosomes. 20 E_{260} units (A and B) or 80 E_{260} units (C) of zonal separated ribosomes in a volume of 0.2 ml, was added to 0.25 ml ice cold double concentration Buffer IV. 0.05 ml of 0.01 M puromycin pH 7.0 (B and C) or 0.05 ml H_2O (A) was added and this solution was held on ice for 15 min, transfered to a 37°C water bath for 10 minutes, and then aliquots containing 5 E_{260} units of ribosomal material was centrifuged 4¼ hours at 85,000 xg at 25°C through a 5 to 20% linear sucrose gradient in Buffer IV. The bottom of the centrifuge tube was punctured and the gradient was pumped at a rate of 1 ml/min through a flow cell and the absorbance at 260 nm was recorded. (P) indicates UV absorbing puromycin.

0.25 μmole puromycin/E_{260} unit ribosome, the monomer peak (seen in Figure 15A) disappeared (Figure 15B). Further studies indicated that when the concentration of ribosomes was 4 times relative to that concentration of puromycin, dissociation was still complete (Figure 15C) and thus when large concentrations of ribosomes needed to be dissociated --as for zonal dissociation--the concentration of puromycin in the preincubation was only 0.016 μmoles/E_{260} unit. In all cases the peak representing the 40S subunits was much larger than was expected, as other research workers had indicated that the peak area of the 40S fraction should be approximately half that of the 60S fraction. In this study it was thought that besides the smaller subunit, the 40S peak contained partially-degraded or unfolded ribosomal material which had been formed when the high KCl (400 mM) was used in the dissociation. In order that this could be examined, an experiment in which various concentrations of KCl were used in dissociation and separation was performed. It may be seen from Figure 16 A to E that measurable dissociation did not commence until the buffer contained a minimum of 200 mM KCl; and at this concentration of KCl, the 50S band was relatively large. However, at KCl concentrations of 400 mM or higher, this peak transformed into the 40S peak which attained an area as great or greater than the 60S peak.

2. Incorporation with recombined monomers.

A zonal separation of 80S ribosomes pretreated with puromycin in the buffer containing 400 mM KCl (Figure 17) indicated that the 80S species almost completely dissociated into 40, 50, and 60S subunits.

Figure 16. Effect of various concentrations of KCl on the dissociation of puromycin treated 80S wheat leaf ribosomes. The puromycin pretreatment buffer as well as the 5 to 20% linear sucrose gradients contained the various concentrations of KCl in Buffer IV. The samples were centrifuged 4¼ hours at 85,000 xg at 25°C and the gradient absorbance at 260 nm was recorded. The buffer concentrations of KCl were: A, 0; B, 100 mM KCl; C, 200 mM KCl; D, 400 mM KCl; E, 500 mM KCl. (P) indicates U.V. absorbing puromycin.

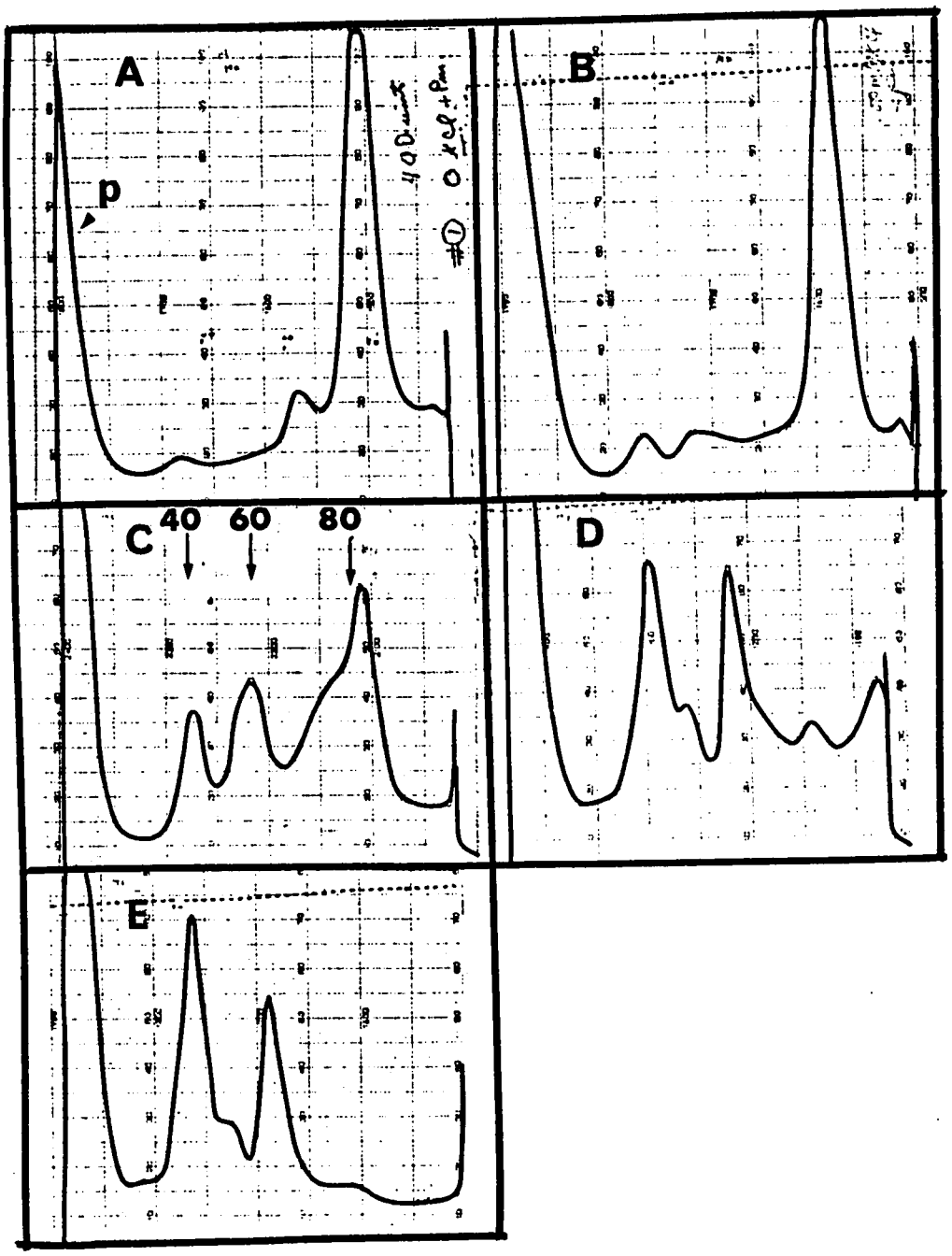
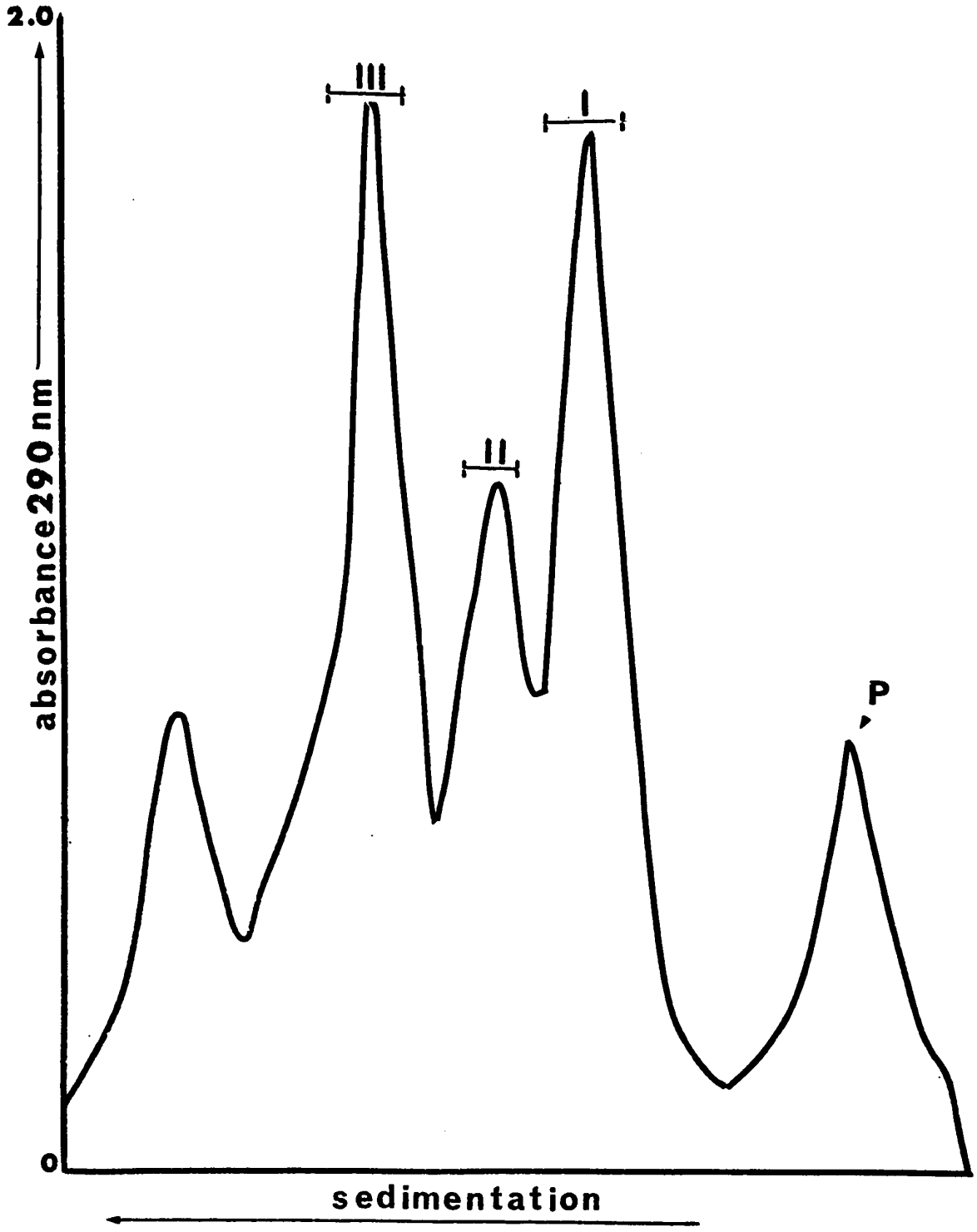

A
p
B
C
40
60
80
D
E

Figure 17. Zonal dissociation and separation of 80S wheat leaf ribosomes pretreated with puromycin. As is described in the "Methods", 3000 E_{260} units of zonal separated 80S ribosomes suspended in Buffer II, were pretreated with puromycin and Buffer IV before being layered on top of the Buffer IV zonal gradient. After centrifuging for 7 hours at 4°C, the gradient was analyzed at 290 nm and Fraction I, II, and III were collected and assumed to be respectively 40S, 50S, and 60S. Peak IV was assumed to be undissociated 80S while "P" indicated the U.V. absorbing puromycin.


2.0
absorbance 290 nm
0
III
II
I
P
sedimentation

The sedimented subunits would completely reassociate to form the parent species when they were centrifuged through a buffer containing the same concentration of tris-HCl, pH 7.5, $MgCl_2$ and KCl as the incorporation mixture (Figure 18), but as is indicated by Table 5, the 40S and 60S reassociated species would not support polyphenylalanine synthesis. It was also noted from Figure 18 that the high concentration of magnesium had caused the sedimentation value of the subunits to change. This shift was again noted in subunits from NH_4Cl treated ribosomes and will be discussed in the next section of the thesis.

Table 5. Incorporation by subunits of high salt dissociated 80S wheat leaf ribosomes which had been pretreated with puromycin. The 40S and 60S subunits were isolated from zonal fractions but the undissociated 80S control was taken directly from the ribosome suspension and was untreated with puromycin or high salt. The respective samples contained in E_{260} units: 80S, 45; 40S, 15; 60S, 30; 40 + 60S, 15 + 30.

	Ribosomal Material				
	Undissoc't	40S	60S	40S+60S	H_2O
dpm	2423	396	334	327	43
%	100	16	14	14	2

IV. NH_4Cl washed Ribosomes

A. Required factors for incorporation

Because of damage to the zonal rotor which would prevent its use for some time, other methods of pursuing the ribosomal dissociation

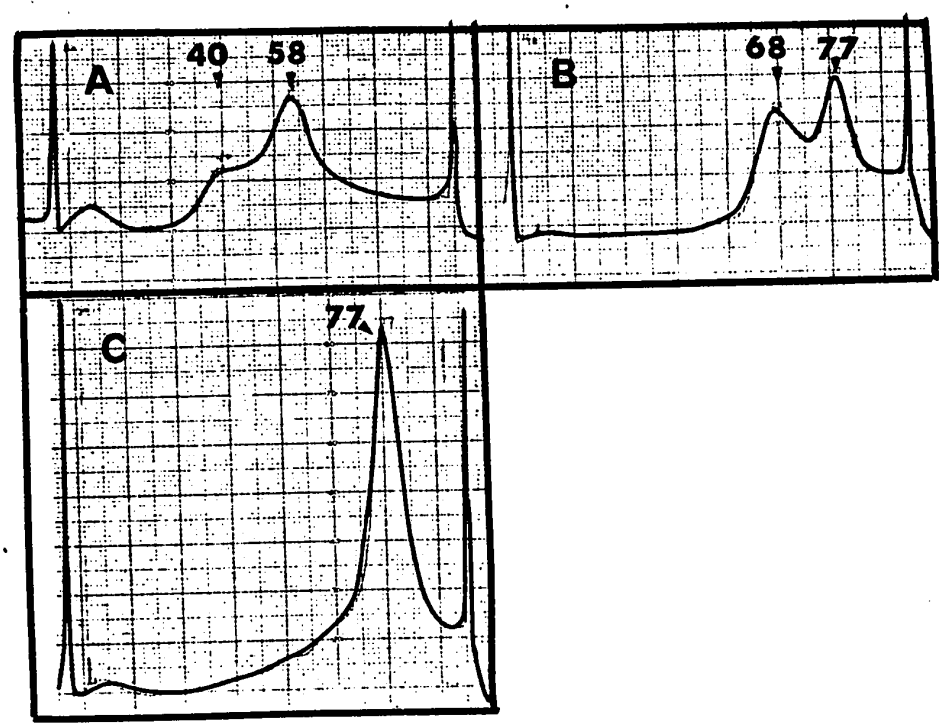
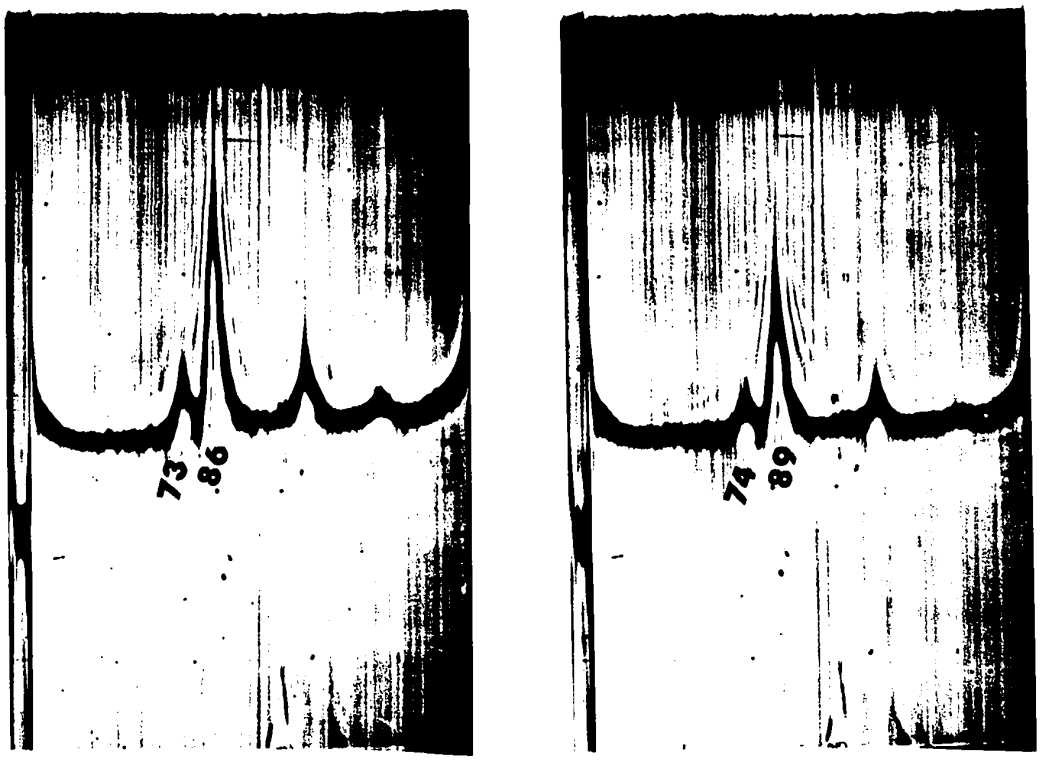


Figure 18. Reassociation of subunits of wheat leaf cytoplasmic ribosomes. Two E_{260} units of zonal separated 40S and 60S subunits suspended in Buffer II were layered separately A, 40S; B, 60S and together C, 40 + 60S, on top of a 5 to 20% linear sucrose gradient buffer containing 10 mM tris-HCl pH 7.5, 16 mM $MgCl_2$, and 25 mM KCl. After centrifugating 4¼ hours at 85,000 xg at 25°C, the absorbance at 260 nm was recorded.

studies had to be used. Research performed in this laboratory had indicated that Buffer III, a buffer containing 100 mM KCl, would selectively dissociate and disrupt the 70S ribosomes while leaving the 80S ribosomes intact. By employing this procedure the time and rigorous treatment of ribosomes, needed for zonal separation, would be decreased and it was thought that the ribosomes might remain more biologically active. Since the objective of this part of the research was to measure incorporating activity of recombined cytoplasmic ribosomal subunits, the small contamination of chloroplast ribosomes was tolerated.

Gulyas and Parthier (1971) had washed etiolated pea seedling ribosomes with 0.5 M NH_4Cl to remove nucleases which decreased polyphenylalanine synthesis. In the present study wheat leaf ribosomes were prepared with Buffer III before being washed with 0.5 M NH_4Cl. Analytical ultracentrifugation indicated that the NH_4Cl washed ribosomes did not differ in sedimentation characteristics from the unwashed preparations, and that these preparations were not devoid of 70S ribosomes (Figure 19 A and B--73S, 74S peaks). An experiment was performed to determine which of the salt and energy factors were required for phenylalanine incorporation in this system and the results demonstrated that only $MgCl_2$ had a significant effect (Table 6). The results observed in Table 6 were derived from two separate experiments, so a comparison of the effect that the removal of each individual factor had on the rate of incorporation between the washed and unwashed ribosomes was not possible. However, comparisons of each system with its control demonstrated that except for the small dependency on poly U by washed ribosomes, the washing with NH_4Cl did not result in the requirement of any factor other than magnesium. In fact, when the incorporation system contained



A B

Figure 19. Sedimentation values of ribosomes prepared by homogenizing wheat leaves in Buffer II. After homogenization, the ribosomes were centrifuged through Buffer II containing 1M sucrose. Part of the ribosome preparation was suspended in 0.5 M NH_4Cl, pH 7.0 before being recentrifuged through Buffer II with 1 M sucrose. Both NH_4Cl washed (B) and unwashed (A) ribosomes were suspended in Buffer II for analysis. These pictures were taken 8 minutes after a speed of 39,460 rev/min was reached and sedimentation values were calculated as is described in the "Methods".

only tris, $MgCl_2$, NH_4Cl washed ribosomes, and phenylalanyl tRNA, at least 80% activity was still obtained.

Table 6. Factors required for incorporation by washed wheat leaf ribosomes. Ribosomes prepared with Buffer III were not washed or were washed with 0.5 M NH_4Cl before they were added to the prepared incorporation mixture minus the factor.

	Incorporating Factors							
	Complete	-KCl	-$MgCl_2$	-2 ETSH	-ATP,CP, CPK	-GTP	-ATP,CP, GTP, CPK	-polyU
-wash dpm	3530	3177	328	3460	3442	3247	3461	3439
%	100	90	9	98	98	92	98	97
+wash dpm	2739	2731	216		2573	2445		1987
%	100	100	8		94	90		73

Yarwood, Payne, Yarwood and Boulter (1971) reported the requirement of elongation factors for poly U-directed enzymatic binding of phenylalanyl tRNA to bean ribosomes. Unlike the transfer factors reported for the wheat germ system by Legocki and Marcus (1970), these factors were very unstable if they had been highly purified. An experiment to examine a transfer factor requirement in the wheat leaf system indicated that washing the ribosomes with 0.5 M NH_4Cl decreased incorporation which could not be regained by the addition of semi-purified transfer factors (Table 7). The fact that addition of the factor preparation decreased incorporation rather than increased it, and that nucleases had been reported absent in wheat germ supernatant,

indicated that the added protein was passively diluting out and interfering with the binding of phenylalanyl tRNA to the ribosomes.

Table 7. Effect of addition of partially purified supernatant factor on the incorporation by NH_4Cl washed wheat leaf ribosomes. Ribosomes were prepared in Buffer III and were not washed or were washed with 0.5 M NH_4Cl. Aliquots of each ribosome type were added to the incorporation mixture containing various quantities (given as µg protein) crude transfer factor.

	Incorporating System					
	No wash	No wash + 250 µg	No wash + 1000 µg	Wash	Wash + 250 µg	Wash + 1000 µg
dpm	4710	4579	3954	3443	3078	2991
%	100	97	84	73	65	64

Tests performed to estimate the RNase activity associated with the ribosomes, gave evidence that all the RNase had been removed after two washings through 0.5 M NH_4Cl.

It was concluded that further incorporation studies with 0.5 M NH_4Cl washed whole ribosomes would be conducted in an incorporation medium containing only tris-HCl, pH 7.5, $MgCl_2$, phenylalanyl tRNA, and ribosomes.

B. Incubation time for NH_4Cl washed ribosomes.

Figure 20 indicates the dependency of ^{14}C-phenylalanine incorporation upon time. Similar to unwashed zonal-separated ribosomes (Figure 9), incorporation increased for the first 30 minutes, and then

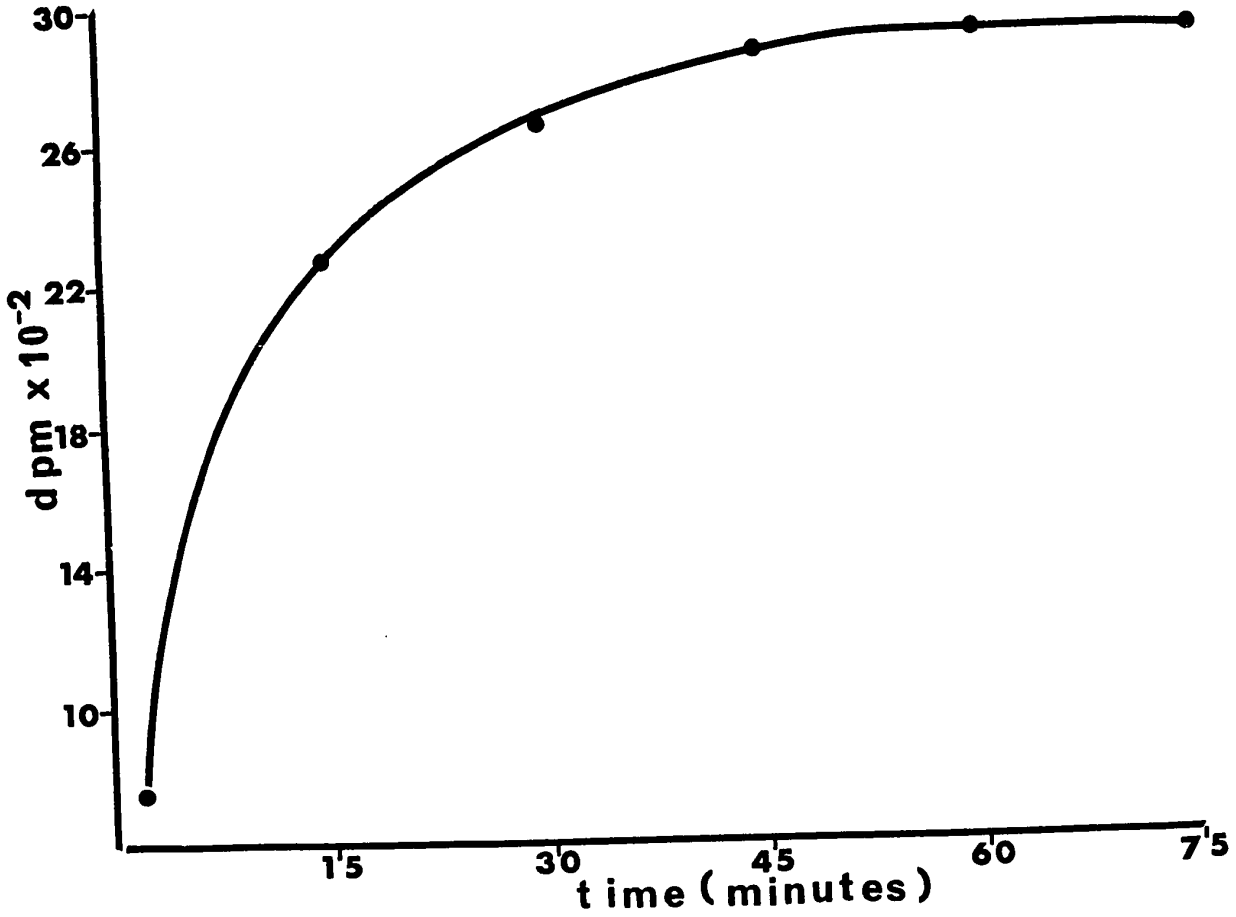


Figure 20. ^{14}C-phenylalanine incorporation by washed wheat leaf ribosomes versus time. The incorporation mixture contained 10 mM tris-HCl pH 7.5, 16 mM $MgCl_2$, ^{14}C-phenylalanyl tRNA (10,000 cpm), 0.5 M NH_4Cl washed ribosomes (45 E_{260} units per ml). From the incorporation mixture, 1 ml aliquotes were pipetted into TCA at the various times. The TCA precipitated samples were incubated at 80°C for 30 minutes before being filtered and counted.

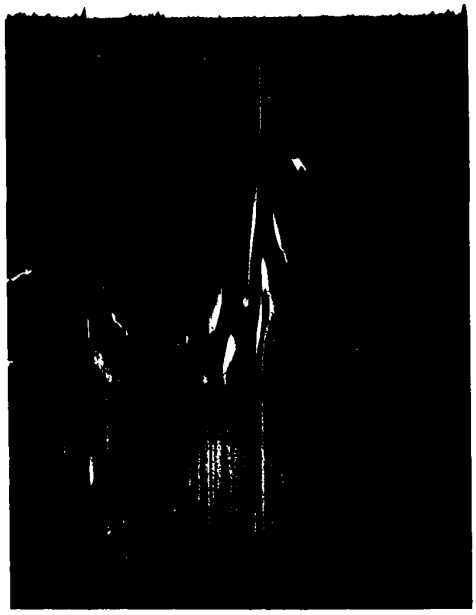
leveled off to reach a plateau at 45 minutes.

C. Dissociation and incorporation of ribosomes from plants of differing ages.

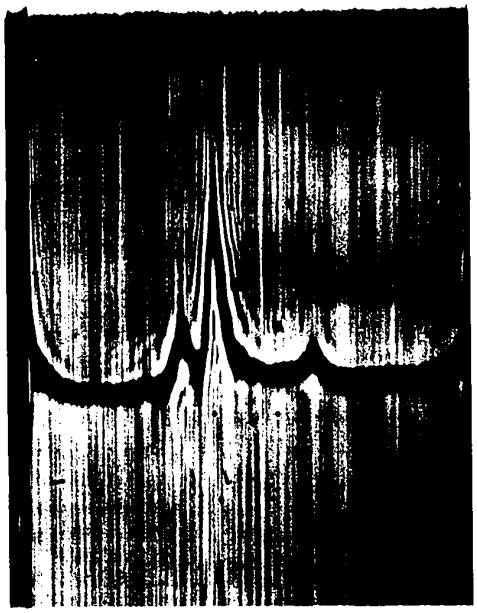
Earlier experiments had shown that ribosomes obtained from wheat germ, would easily dissociate into the reported amounts of 40S and 60S subunits. Hadziyev and Zalik (1970) had reported that chloroplast ribosomes from leaves of 4 to 5 day-old wheat plants were very active in protein synthesis and these were assumed to be mostly in the form of polysomes. It was therefore hypothesized that the fraction of monomers obtained in the present study, was actually fragmented polysomes and thus containing mRNA and nascent protein which were not allowing the ribosomes to dissociate readily. The finding by App et al. (1971) that monosomes prepared from polysomes would not dissociate under conditions which allowed complete dissociation of free monosomes supports this view.

Experiments were performed to compare the ribosomes from wheat seedlings of different ages for their dissociation and incorporation abilities. When an equal weight of leaves was homogenized, the yield of ribosomes from the four ages of wheat plants, varied. The tough yellowing leaves of the 5.5 and 6.5 day plants gave the lowest yield of ribosomes, while the large number of tender young plants required to give the 25 gm of 3.5 day plant leaves gave the highest yield. As seen on the analytical ultracentrifuge (Figure 21 A) the younger plants did not appear to have more polysomes, and it was concluded that the method of preparation had cleaved any polysomes

Figure 21. Analytical ultracentrifugation comparison of ribosomes prepared from wheat leaves of different ages. The leaves of the 6.5 (A), 4.5 (B), and 3.5 day-old wheat plants (C) were homogenized in Buffer III before they were washed with 0.5 M NH_4Cl and before they were centrifuged twice through Buffer II with 1 M sucrose. The ribosomes were suspended in Buffer II for analytical ultracentrifugation. These pictures were taken 8 minutes after a speed of 39,460 rev/min was reached.



A



B

C

into 80S particles. Figure 22 A, B, C, and D illustrates the dissociation of these ribosomes (prepared with Buffer III, washed with 0.5 M NH_4Cl, not pretreated with puromycin) when centrifuged through dissociation buffer containing 400 mM KCl. The ribosomes of the plants of all four ages dissociated into 40S, 50S, and 60S; however, only in the 3.5 day plants was the 40S peak area smaller than that of the 60S peak. When the ribosomes were compared for their ability to incorporate phenylalanine, maximum activity was observed for the ribosomes extracted from the 4.5 day-old plants (Table 8).

Table 8. Incorporation by wheat leaf ribosomes from seedlings of varying ages. Flats of wheat were planted on consecutive days so that the ribosomes could be isolated on the same day. Ribosomes were isolated with Buffer III and were washed twice with 0.5 M NH_4Cl before being added to the prepared incorporation system which contained only $MgCl_2$, tris, and ^{14}C-phenylalanyl tRNA.

	Age of Plant (days)			
	3.5	4.5	5.5	6.5
dpm	4071	5194	3807	4514
%	78	100	73	87

Plant ribosomes have been classed as two types--membrane-bound or free--which specifically synthesize different types of protein (review by Boulter et al., 1972). Radioactive tracer experiments on the developing bean seed by Payne and Boulter (1969) demonstrated that the increase of membrane-bound ribosomes during synthesis of storage

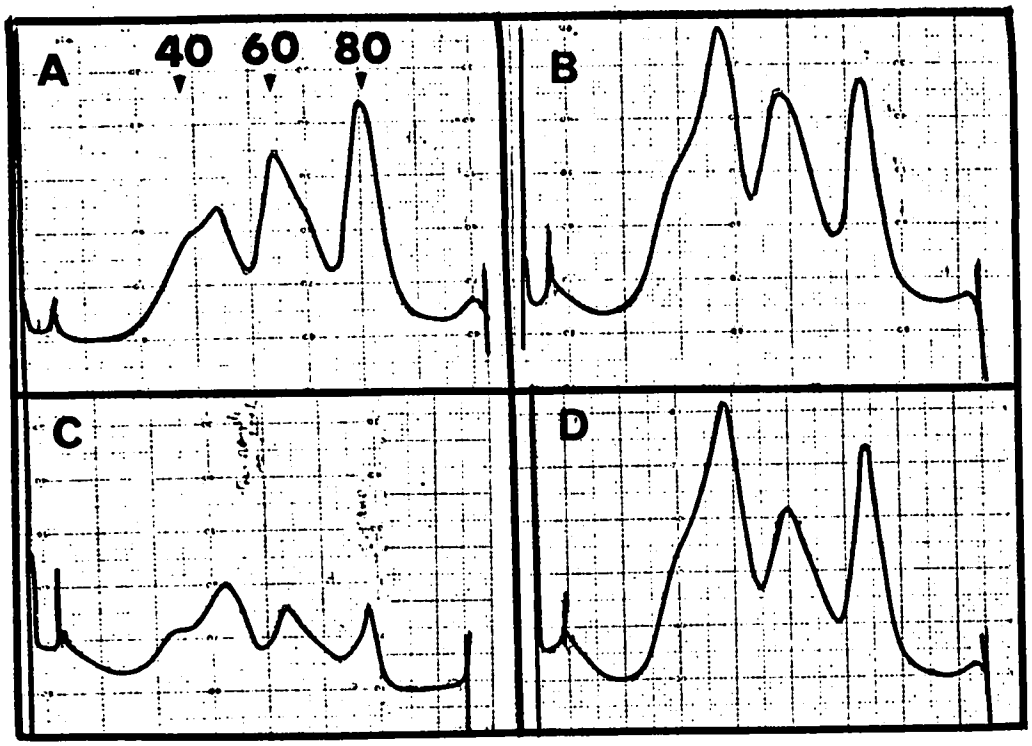


Figure 22. A comparison of the ability of ribosomes, from different ages of wheat seedlings, to dissociate in high salt. Five E_{260} units of ribosomes (prepared with Buffer III, washed with 0.5 M NH_4Cl and suspended in Buffer II) were layered on top of a 5 to 20% linear sucrose gradient in Buffer IV. After centrifuging 4¼ hours at 85,000 xg at 25°C, the absorbance at 260 nm was recorded. A, 3.5; B, 4.5; C, 5.5; and D, 6.5 day-old seedlings.

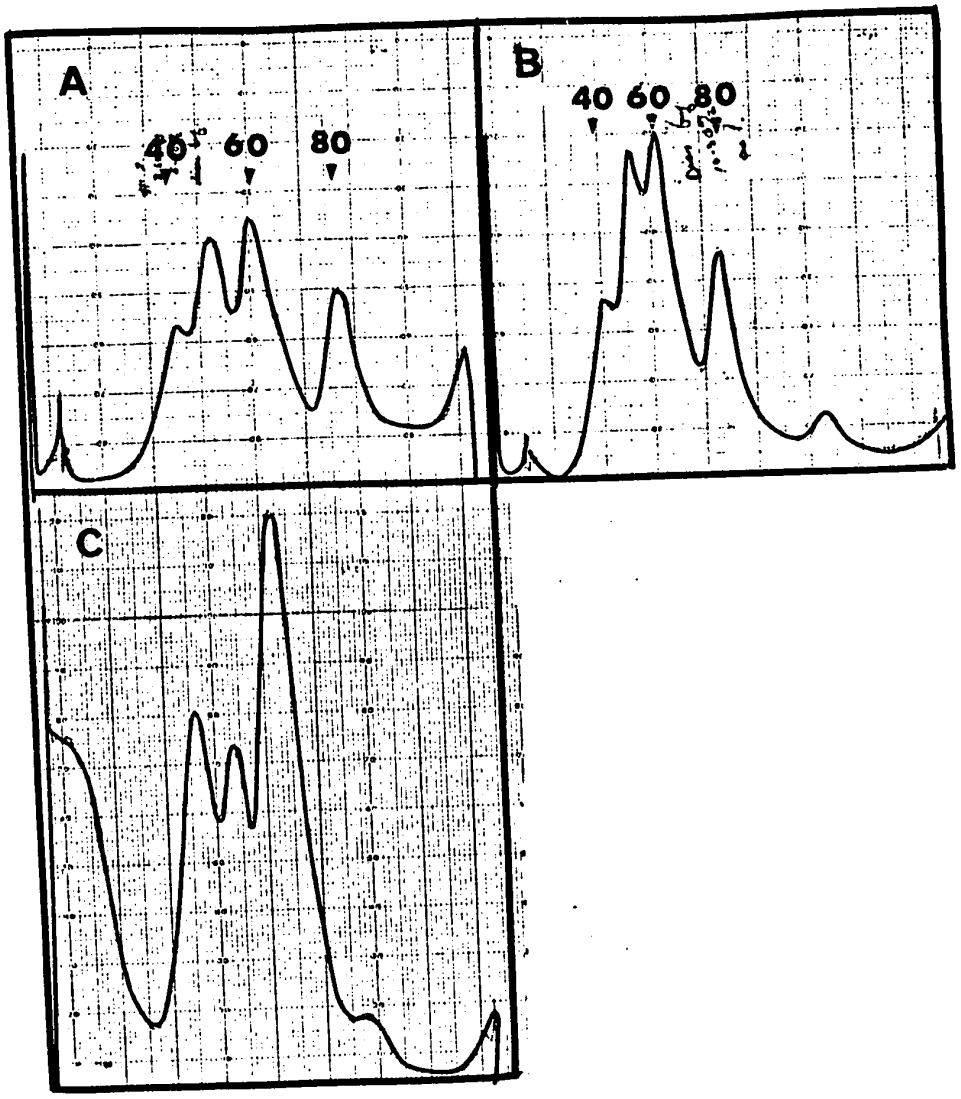
protein was due to the synthesis of new membrane-bound ribosomes rather than attachment of the previously-free ribosomes. As the seeds reached dormancy, the membrane-bound ribosomes detached and were found free in the cytoplasm. The acceptable dissociation of ribosomes from younger leaves (3.5 day-old) and wheat germ, when compared to the dissociation of ribosomes from older seedlings, could be explained by different types of ribosomes--respectively free and membrane bound.

As seen by Figure 23 A, B, and C pretreatment of the ribosomes from 3.5 day-old plants with puromycin aided dissociation. If the ribosomes were spun through a 10 to 30% sucrose gradient containing the dissociation buffer, dimers were revealed (Figure 23B) which otherwise sedimented to the bottom. However, these gradients could not be used for preparation of subunits because the subunits were not as widely separated as when 5 to 20% sucrose gradient had been used. It was concluded that the 3.5 day-old wheat plants could be used to render a yield of ribosomes which would incorporate phenylalanine and which would dissociate into the acceptable ratio of subunits.

D. Conditions for dissociation of ribosomes from 3.5 day-old plants

The 3.5 day-old plants had given promising results and a set of experiments was conducted to establish the best conditions for the required dissociation. Sedimentation of ribosomes from 3.5 day-old plants through dissociation buffers indicated that pretreatment with puromycin and centrifugation through a buffer containing 300 mM KCl would give the optimum desired dissociation (Figure 24 A to D). With the removal of nascent protein by the puromycin pretreatment, the

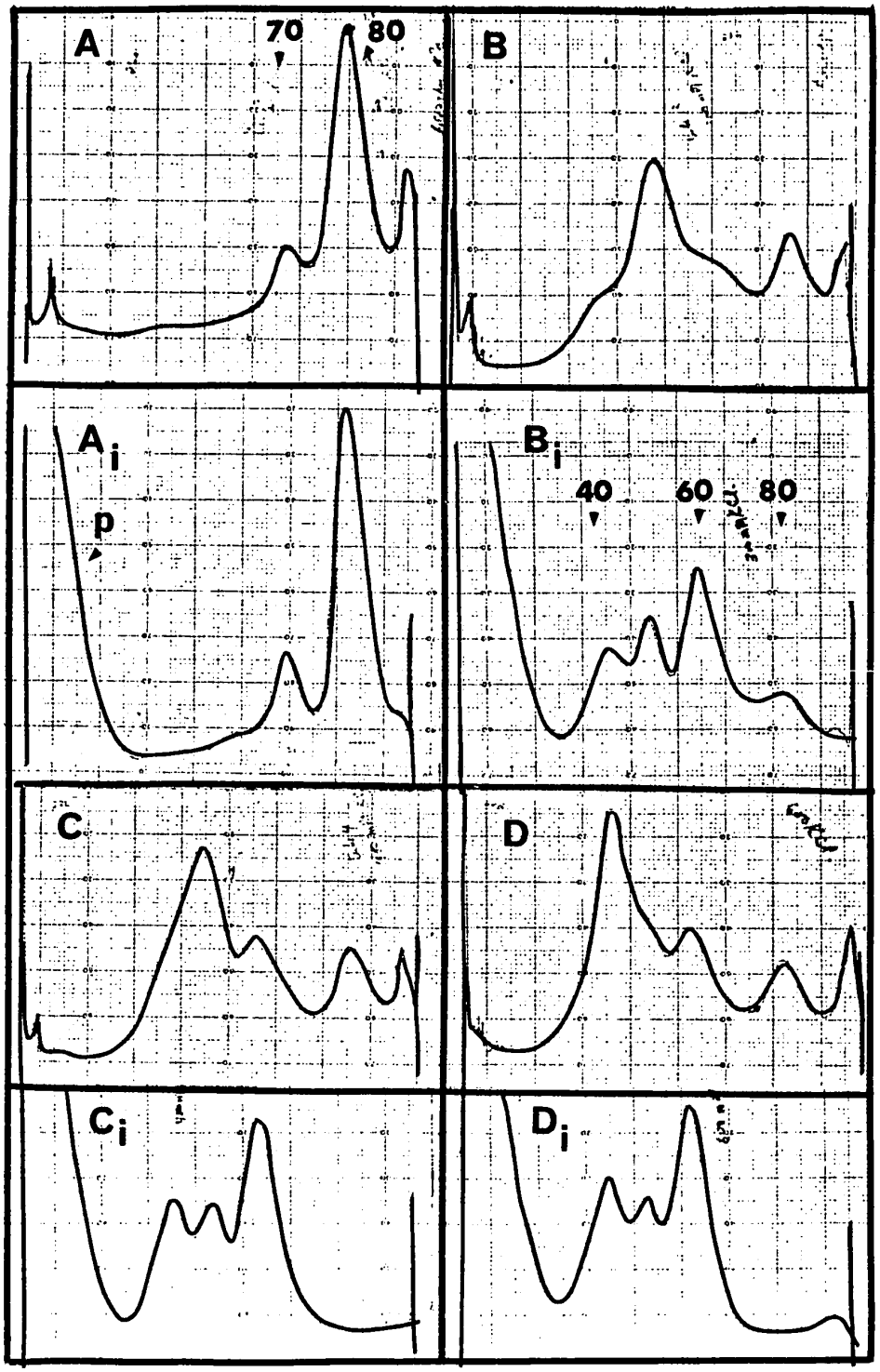
Figure 23. Puromycin stimulated dissociation of ribosomes prepared from 3.5 day-old wheat seedlings. 20 E_{260} units (A, C) of ribosomes (prepared with Buffer III, washed with 0.5 M NH_4Cl and suspended in Buffer II), in a volume of 0.2 ml, was added to 0.25 ml ice cold double concentration Buffer IV. 0.05 ml of 0.01 M puromycin pH 7.0 (C) or 0.05 ml H_2O (A) was added and this solution was held on ice for 15 minutes, and then aliquots containing 5 E_{260} units of ribosomal material was layered on top of a 5 to 20% linear sucrose gradient in Buffer IV. (B) 5 E_{260} units of the water-treated ribosomes was also layered on top of a 10 to 30% linear sucrose gradient in Buffer IV before they were all centrifuged 4¼ hours at 85,000 xg. The absorbance at 260 nm of the gradient was recorded.


A
40
60
80
B
40
60
80
C

ribosomes dissociated into the expected ratio of 40S and 60S subunits, while without this pretreatment the 40-50S absorbing area was much too large. Increasing the KCl concentration above 300 mM did not further dissociate ribosomes that had not been pretreated, but led to a build-up of the 40S peak. Increasing the KCl concentration above 300 mM with puromycin pretreated ribosomes caused complete dissociation; however, at higher concentrations of KCl, the 40S peak appeared to increase while the 50S peak decreased. The high amounts of 40S observed in Figure 24 B, C, and D was contradictory to the results previously obtained from 3.5 day-old seedlings (Figure 22A). Thus this phenomenon cannot be explained as entirely due to the age of the plants. However, these large amounts of 40S were regularly observed when dissociating ribosomes obtained from 4.5 day-old plants. A suggestion, that some of the larger subunits had dimerized, was put forth. In the present research, there was not a notable peak sedimenting in front of the 80S parent species. However, it is possible that particles more dense than the 80S monomer would sediment through the 5 to 20% linear sucrose gradient during the centrifugation (Figure 23B).

An analytical ultracentrifugation (Figure 25A) verified that a sample of ribosomes washed with NH_4Cl had a major peak of 81S, while when treated with puromycin suspended in the dissociation buffer containing 300 mM KCl, it dissociated to give particles which had sedimentation values of 35, 45, and 60S (Figure 25B).

Figure 24. The dissociation effect of various concentrations of KCl on the ribosomes from 3.5 day-old wheat seedlings, treated or untreated with puromycin. Ribosomes were prepared with Buffer III, washed with 0.5 M NH_4Cl and suspended in Buffer II. The puromycin treatment (P) as well as the 5 to 20% linear sucrose gradient contained the various concentrations of KCl in Buffer IV. The samples were centrifuged 4¼ hours at 85,000 xg at 25°C and the gradient absorbance at 260 nm was recorded. A, 0 KCl; A_i, puromycin treated + 0 KCl; B, 300 mM KCl; B_i, P + 300 mM KCl; C, 400 mM KCl; C_i, P + 400 mM KCl; D, 600 mM KCl; D_i, P + 600 mM KCl.


A
70
80
B
Ai
p
Bi
40
60
80
C
D
Ci
Di

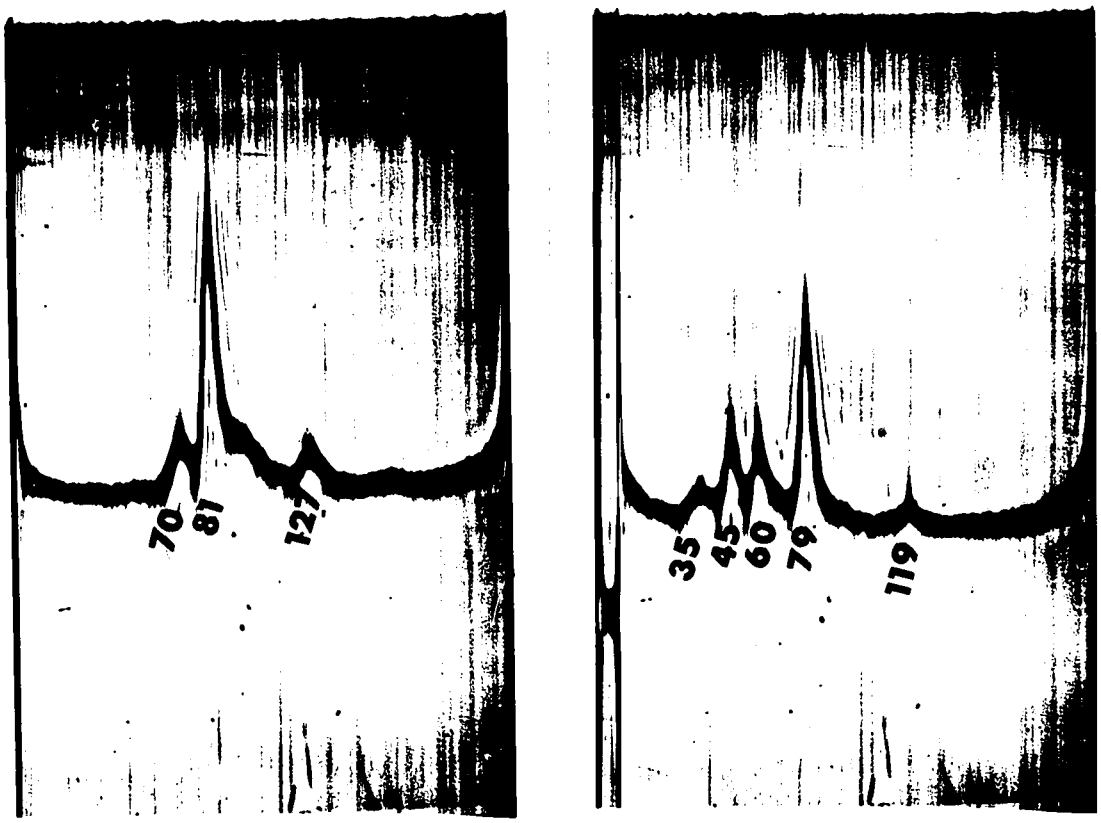


Figure 25. Sedimentation values of NH_4Cl washed ribosomes and their subunits from 3.5 day-old wheat seedlings. From analytical ultracentrifugation the sedimentation values of ribosomes and their subunits was calculated as is described in the "Methods". These pictures were taken 8 minutes after a speed of 39,460 rev/min was reached. A. 3.5 day-old wheat seedling ribosomes; B. these ribosomes dissociated by puromycin and Buffer IV containing 300 mM KCl.

E. Recombination and phenylalanine incorporation of subunits

Ribosomes from 3.5 day-old leaves were pretreated with puromycin before being centrifuged through a dissociation buffer, containing 300 mM KCl, in a zonal rotor. Figure 26 indicates that the dissociation was almost 100%, and that the area of the 40-50S peak was close to the expected. The fractions separated from this zonal separation were pure 60S or 40-50S, as was seen by a sucrose gradient analysis through the dissociation gradient (Figure 27 A, and B). If the subunits were sedimented through the gradient containing the incorporation buffer (high $MgCl_2$), the sedimentation values of the 60S particles increased to 68S, while the 40-50S particles increased to 55-62S. As has been mentioned before in this thesis, the increased sedimentation value can be explained as a compacting or an aggregation of the subunits caused by the high magnesium concentration. Shifts of sedimentation value caused by increasing magnesium concentration have recently been reported by Reboud et al. (1972). Rat liver ribosomes in a buffer containing 0.3 M KCl, dissociated into 40S and 60S subunits which when centrifuged through a gradient containing a higher concentration of magnesium, would partially reform respectively into 55S and 90S particles. Delihas et al. (1972) dissociated euglena cytoplasmic ribosomes in buffers containing various concentrations of KCl and they noted that at 0.3 M KCl an additional component (46S) appeared which they attributed to a transition of the larger subunit.

In the present study, when the two subunits were mixed in a ratio of 2:1 (60:40S) and centrifuged through the incorporation

Figure 26. Zonal dissociation and separation of ribosomes from 3.5 day old seedlings. 3000 E_{260} units of ribosomes suspended in buffer II, were pretreated with puromycin and Buffer IV (containing 300 mM KCl) before being layered on the same Buffer IV zonal gradient. After centrifuging for 4 hours at 25°C, the gradient was analyzed at 290 nm and fraction I and II were collected and assumed to be respectively 40-50S and 60S.

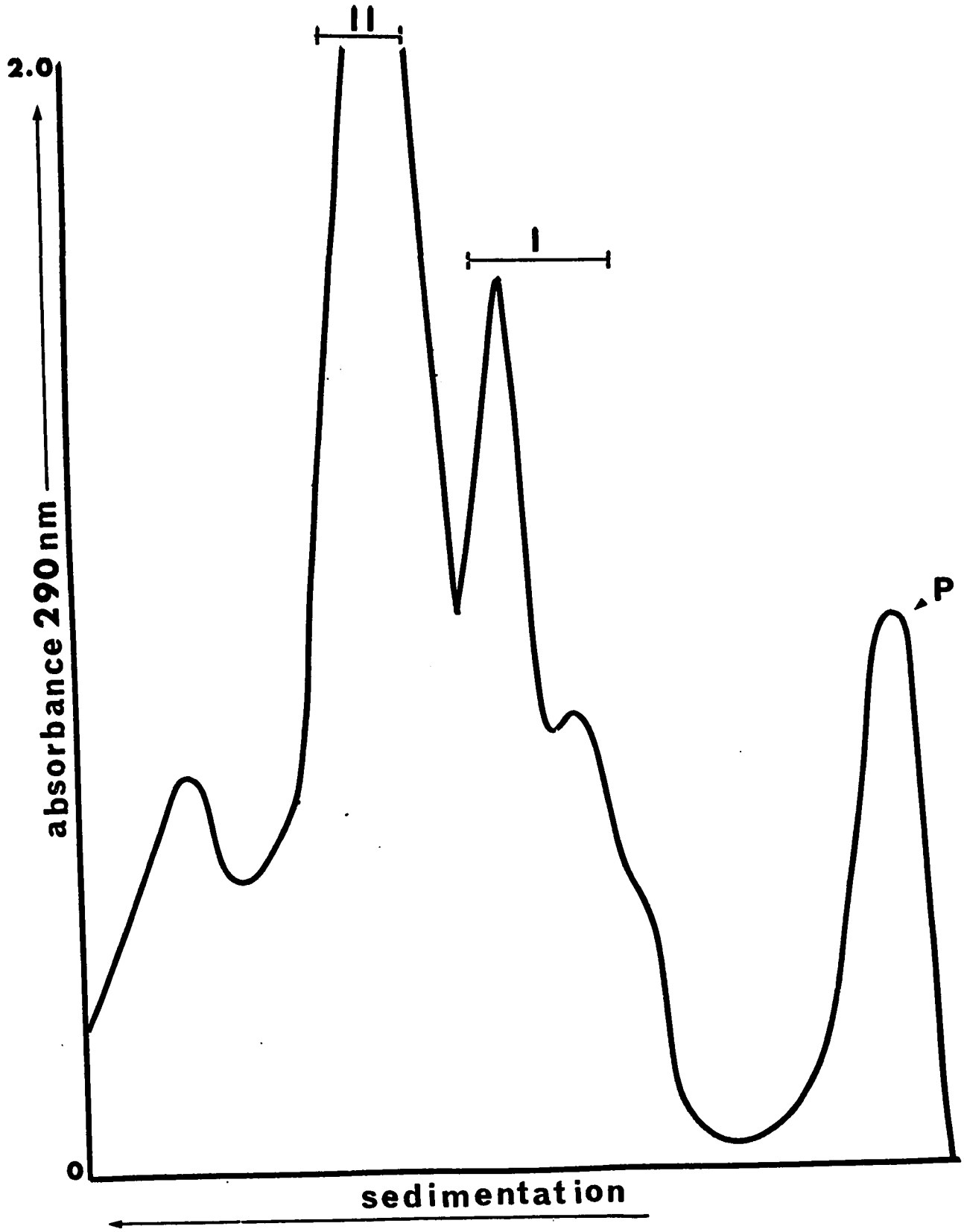
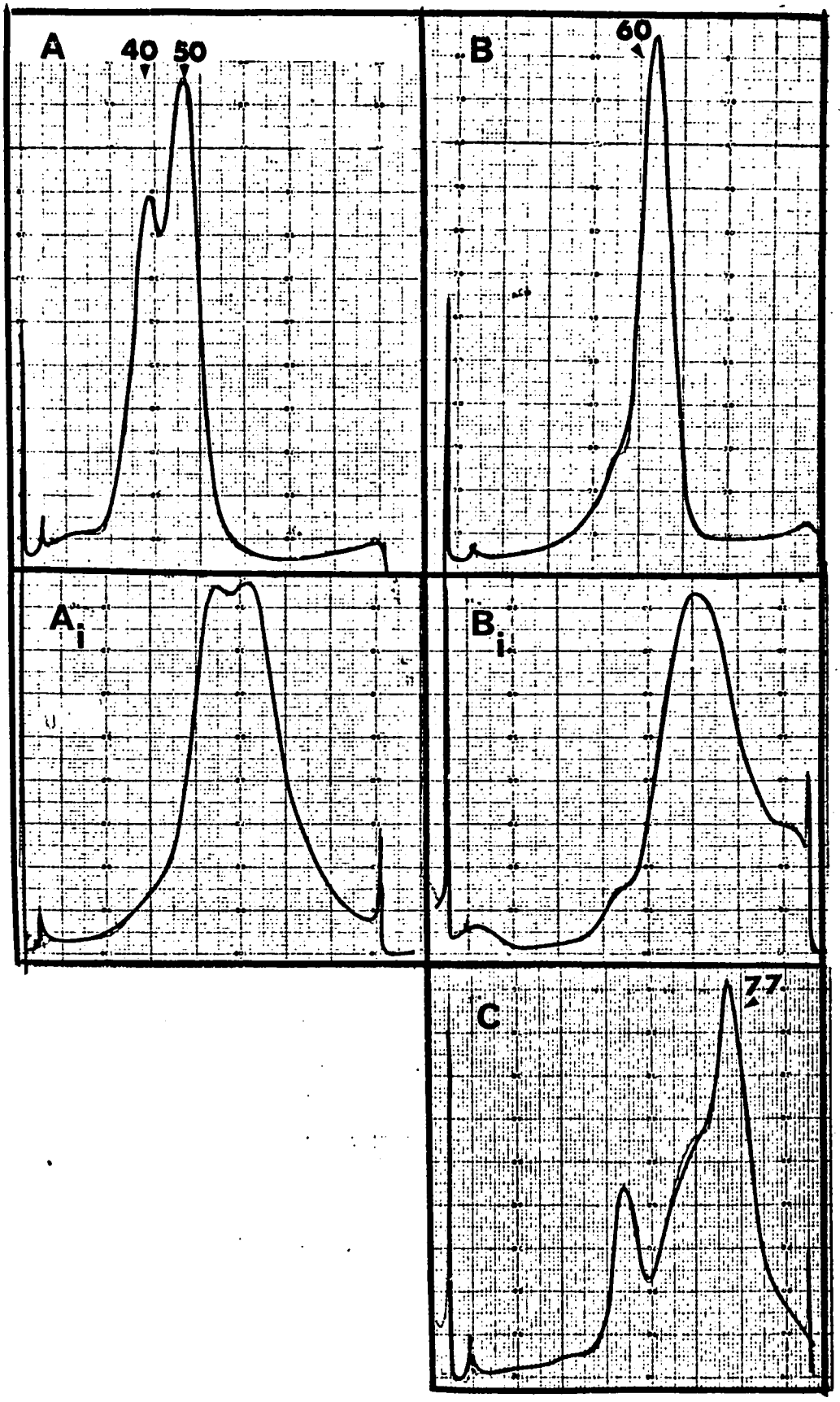

II
I
2.0
absorbance 290 nm
P
0
sedimentation

Figure 27. Reassociation of and cation effect on ribosome subunits from 3,5 day-old wheat seedlings. 4 E_{260} units of zonal separated 40-50S and 60S subunits suspended in Buffer IV were layered respectively (A_i, B_i) and together in a ratio of 1:2 (C), on top of a 5 to 20% linear sucrose gradient buffer containing 10 mM tris-HCl pH 7.5, 16 mM $MgCl_2$ and 25 mM KCl. Similarly the 40-50S and 60S subunits were layered (respectively A, B) on top of a 5 to 20% linear sucrose gradient containing Buffer IV. After centrifuging 4¼ hours at 85,000 xg at 25°C the absorbance at 260 nm was recorded.


A
40
50
B
60
A_i
B_i
C
77

buffer, they did recombine as is shown in Figure 27 C; the 50S and 58S peaks representing respectively compacted 40S and 60S, and the 77S representing the reformed parent species. However, as shown in Table 9 the reformed monomers were not active in poly U-directed phenylalanine incorporation.

Table 9. Incorporation by subunits of high salt dissociated (300 mM KCl), 80S ribosomes obtained from 3.5 day-old wheat seedlings and pretreated with puromycin. The 40-50S and 60S subunits were isolated from zonal fractions, but the undissociated 80S control was taken directly from the ribosome suspension and was untreated with puromycin or high salt. The respective samples contained in E_{260} units: 80S, 45; 40-50S, 15; 60S, 30; 40-50S + 60S, 15 + 30.

	Ribosomal Material				
	Undissoc't 80S	40S	60S	40+60S	H_2O
dpm	654	129	157	143	105
%	100	20	24	22	16

A possible reason for the inability of the reformed monomers to synthesize polyphenylalanine might be that among other things elongation factors needed for incorporation could have been removed during the dissociation. If these were not replaced in the incorporation mixtures, incorporation could not proceed.

SUMMARY

^{14}C-phenylalanine incorporation was induced by homogeneous preparations of wheat-seedling cytoplasmic and chloroplast ribosomes which had been isolated by zonal centrifugation. The sedimentation values of these two major ribosome species, calculated from analytical ultracentrifugation were 79S and 68S respectively. The final suspensions were centrifuged at 30,000 xg for 20 minutes to yield a preparation containing less that 10^4 bacteria per ml. Wheat embryo synthetase enzymes in the presence of yeast stripped tRNA and ^{14}C-phenylalanine, produced ^{14}C-phenylalanyl tRNA which was used in the incorporation system. The incorporation by cytoplasmic 79S ribosomes required high concentrations of ribosomes, high concentrations of magnesium, low concentrations of tris-HCl buffer pH 7.6, and was complete after 45 minutes incubation at 37^{o}C. This transfer of ^{14}C-phenylalanine from ^{14}C-phenylalanyl tRNA into protein was not dependent upon added energy factors. Inhibitor studies showed: incorporation by both species to be inhibited by puromycin, incorporation by chloroplast species to be inhibited by chloramphenicol, and incorporation by neither species of wheat-seedling ribosomes to be inhibited by cycloheximide. The sedimentation values, calculated from analytical ultracentrifugation, of the cytoplasmic species and its subunits in a high K^+- low Mg^{+2} buffer were respectively 76, 56, 46, and 37S. The parent species produced by the recombination of 37S and 56S subunits would not induce polyphenylalanine synthesis. The poor dissociation by the high-salt treatment was overcome by pretreating the cytoplasmic ribosomes with puromycin. However, the recombined monomers still would not induce

polyphenylalanine synthesis.

Preparations of ribosomes produced by homogenizing wheat leaves in a buffer containing 100 mM KCl, contained both types of ribosomes. Washing these ribosomes twice with 0.5 M NH_4Cl removed nucleases, but did not alter the sedimentation characteristics of the ribosomes. Furthermore, washing the ribosomes with 0.5 M NH_4Cl did not change the incorporation factor requirement and, in fact, when the incorporation system contained only tris buffer, $MgCl_2$, NH_4Cl washed ribosomes and ^{14}C-phenylalanyl tRNA; 80% activity was still obtained. Incorporation could not be further stimulated by adding isolated translocational factors to the incubation mixture. Unlike the ribosomes from 4.5 day-old wheat seedlings, those from 3.5 day-old wheat plants dissociated in high-salt to give the required ratio of smaller to larger subunit. A pretreatment with puromycin produced total dissociation into subunits with a calculated S value of 60, 45, and 35S. When the isolated 35-45S fraction was mixed with the isolated 60S fraction, the parent species was reformed. However, this reformed monomer would not induce polyphenylalanine synthesis.

This incorporation system, for wheat seedling ribosomes, was not dependent upon added messenger RNA, energy factors, nor supernatant factors. Cycloheximide, an inhibitor which specifically inhibits the formation of an initiation complex, had no effect. Ultracentrifugation analysis of preparations from seedlings of various ages were the same and established high proportions of monosomes relative to polysomes. The difficulties encountered in the dissociation of these monosomes could be overcome by a pretreatment with puromycin.

These facts have contributed to the conclusion that the homogenization and separation procedures had cleaved the polysomes into monosomes which contained not only mRNA fragments, but nascent protein as well. This cleavage is probably mechanical, although the observed presence of RNase on the ribosomes likely fragments the polysomes to some degree. The observed large ratio of the smaller subunit compared to the larger subunit and the unexpected occurence of the 50S particles, demonstrates the need for further research into the eukaryotic ribosome.

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