Purification and characterization of elongation factor 2 (EF2) and cyclic AMP-independent protein kinases from soybean (Glycine max, L) cotyledons.

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PURIFICATION AND CHARACTERIZATION OF ELONGATION FACTOR 2 (EF$_2$) 
AND CYCLIC AMP-INDEPENDENT PROTEIN KINASES 
FROM SOYBEAN (GLYCINE MAX, L) COTYLEDONS

by

Siddarama Gowda

A DISSERTATION
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH 
THROUGH THE DEPARTMENT OF BIOLOGY 
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE 
DEGREE OF 
DOCTOR OF PHILOSOPHY 
AT THE UNIVERSITY OF WINDSOR 
WINDSOR, ONTARIO, CANADA 
1980
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to my parents
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Elongation factor 2 (EF2) from soybean cotyledons was isolated and purified to homogeneity. The purified EF2 was found to contain a single polypeptide chain of molecular weight 92,000. An increase in the content of EF2 was observed from dry seeds to 1 day old cotyledons after which it decreased during aging as measured by the diphtheria toxin dependent ADP-ribosylation. EF2 exists in both a free form, present in the post-ribosomal fraction and in a bound form, which is attached to a ribosome rich fraction. Two ADP-ribosylating species of EF2 were observed in the cotyledons. A high molecular weight form (EF2H M.W. 92,000) is the functional species which is active in protein synthesis, and a low molecular weight form (EF2L M.W. 60,000) which is without any functional properties of EF2. As the cotyledons age (10-20 days) another low molecular weight (29,000) EF2 which is capable of ADP-ribosylation appears. These low molecular weight EF2 species may represent degradation products of the functional EF2. Changes in the relative distribution of the light and the heavy forms of EF2 during aging may regulate the amounts of functional EF2. A partially purified protease isolated from soybean cotyledons completely degrades EF2.

Ribosomes and ribosomal subunits were prepared from 1 day old soybean cotyledons. Two dimensional polyacrylamide gel electrophoresis of 60S and 40S ribosomal subunits revealed 43 and 32 proteins respectively. Two cyclic-AMP-independent protein kinases (CK I and

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CK II) which phosphorylate casein were isolated and purified from the post-ribosomal supernatant of soybean cotyledons. CK I is a single subunit protein of M.W. 39,000 and CK II is made up of 3 subunits of molecular weight 52,000, 37,000 and 35,000 daltons. Both CK I and CK II use casein as substrate preferably over all the other substrates tested. CK II phosphorylates 40S subunits and 40S subunit proteins while CK I does not. Both the enzymes were found to undergo autophosphorylation in the presence of $^{32}$P-ATP, phosphorylating CK I and the 35,000 dalton protein band of CK II. A cyclic AMP-independent protein kinase which phosphorylates casein was found to be associated with ribosomal salt wash which phosphorylates casein. This enzyme exhibits similar chromatographic properties on DEAE and substrate specificities as CK II present in post-ribosomal supernatant. **In vitro** phosphorylation of 40S subunits by ribosomal casein kinase results in the phosphorylation of 4 subunit proteins.
ACKNOWLEDGEMENTS

I express my sincere gratitude to Dr. Datta T.N. Pillay for his helpful advice, encouragement and direction throughout the course of these studies. I would like to express my appreciation to the members of my dissertation committee, Dr. David A. Cotter, Dr. Michael J. Dufresne, Department of Biology, Dr. Norman F. Taylor, Department of Chemistry, University of Windsor, Windsor, Ontario and Dr. R.B. Van Huystee, Department of Plant Sciences, University of Western Ontario, London, Ontario, Canada. I like to thank Dr. A.H. Warner, Department of Biology, University of Windsor, Windsor, Ontario for his generous gift of Diphtheria toxin and Mrs. Z. Yablonka-Reuveni and Mrs. Viji Shridhar for their help in ADP-ribosylation assay. I extend my sincere thanks to Dr. B. Atkinson, Department of Zoology, University of Western Ontario, London, Ontario for his help in electrophoretic techniques. My special thanks to all my friends, who made my stay at Windsor pleasant and memorable.

I thank authorities of the University of Mysore, Mysore, India for granting study leave and the University of Windsor and the Government of Ontario for their financial support. Financial assistance from NSERC of Canada (Grant No. A-1984 to Dr. D.T.N. Pillay) is gratefully acknowledged.
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<tr>
<td>cAMP</td>
<td>Adenosine 3', 5'-cyclic monophosphate</td>
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<tr>
<td>A&lt;sub&gt;n&lt;/sub&gt;</td>
<td>absorbance at n nm</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BIS</td>
<td>N,N'-methylenebisacrylamide</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cm</td>
<td>centimeter</td>
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<tr>
<td>CPM</td>
<td>counts per minute</td>
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<tr>
<td>°C</td>
<td>degree centigrade</td>
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<td>DEAE</td>
<td>diethylaminoethyl-</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DOC</td>
<td>deoxycholate</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>g</td>
<td>grams</td>
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<tr>
<td>cGMP</td>
<td>guanosine 3', 5'-cyclic monophosphate</td>
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<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
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<td>if</td>
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<td>ml</td>
<td>milliliters</td>
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<td>ß-ME</td>
<td>ß-mercaptoethanol</td>
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mmol - millimole
M.W. - molecular weight
NAD - nicotinamide adenine dinucleotide
pmol - picomole
PMSF - phenazine methosulfate
poly (U) - polyuridylic acid (5')
POPOP - 1,4-bis 2-(5-phenyloxazonyl)- benzene
PPO - 2,5-diphenyloxazol
rpm - revolutions per minute
SDS - sodium dodecyl sulfate
TCA - trichloracetic acid
TNBS - trinitrobenzenesulfonic acid
Tris - tris (hydroxymethyl) aminomethane
PART I

Purification and Characterization of Elongation Factor 2 (EF₂) from Soybean Cotyledons (Glycine max, L)
I INTRODUCTION

Soybean cotyledons represent a highly differentiated tissue. During the germination of the seed, the cotyledons enlarge mainly by cell enlargement, are metabolically very active and nourish the growing embryonic axis. As the seedling grows older and becomes independent the cotyledons wither and fall off from the parent plant. In one of the previous studies from our laboratory (Pillay, 1977) a decline in the rate of protein synthesis was observed in aging cotyledons. It was suggested that formation of lesions in soluble factors during aging of cotyledons was responsible for the decline in protein synthesis. In order to study the role of soluble factors in the control of protein synthesis in aging cotyledons the following investigation was undertaken.

Requirement of two soluble factors for protein synthesis in eukaryotic systems was first recognized (Arlinghaus et al., 1963; Bishop and Schweet, 1961) and their similarity to prokaryotic \( E_{\text{T}} \) and \( E_{\text{G}} \) was demonstrated (Felicetti and Lipmann, 1968). In plants two aminoacyl transfer factors were isolated from rice embryo (App 1969). Factor I, present in the crude supernatant and on crude ribosomes, was released by DOC treatment, while Factor II was extracted by 0.5 M KCl from DOC treated ribosomes. Legocki and Marcus (1971) isolated two soluble factors from post-ribosomal supernatant of wheat germ homogenates, one catalyzes the GTP and Poly (U) dep-
endent binding of phenylalanyl-tRNA to ribosomes, while the second factor functions in the formation of peptidyl puromycin from non-
enzymatically bound phe-tRNA. The reactions for peptide chain elongation in plant cytoplasm appear to be similar to those observed in animal systems, since the early observation of the interchange-
ability of factors between plants and animals was reported (Parisi et al., 1967) and confirmed subsequently by the use of separated elongation factors (Yarwood et al., 1971). Since then much work has been done to demonstrate the presence, purification and functional properties of elongation factors - elongation factor-1 (EF$_1$) and elongation factor-2 (EF$_2$) in eukaryotes.

EF$_1$ has been thoroughly examined in rat liver (Gasior and Moldave, 1965; Collins et al., 1972; Iwasaki et al., 1973; Liu et al., 1974), wheat germ (Jerez et al., 1969; Golinska and Legocki, 1973; Tarrago et al., 1973; Bollini et al., 1974), Krebs ascite cells (Drews et al., 1974; Nolan et al., 1974), reticulocytes (McKeenan and Hardesty, 1969; Lin et al., 1969) and calf brain (Moon and Weissbach, 1972; Moon et al., 1972, 1973). In many eukaryotes EF$_1$ was observed to be present in high and low molecular weight forms (Weissbach et al., 1973; Bolla and Brot, 1975; Slobin and Moller, 1975, 1976; Nagata et al., 1976; Slobin and Moller, 1978; Grasmuk et al., 1978). Occurrence of EF$_1$ in multiple species was also observed by sucrose density gradient analysis (Moon and Weissbach, 1972; Moon et al., 1972, 1973). The heavy form of EF$_1$ referred to as EF$_{1H}$, and light form referred to as EF$_{1L}$, are both active in protein synthesis. EF$_{1H}$ and EF$_{1L}$ have been
purified to homogeneity from liver and calf brain by ammonium sulfate precipitation, column chromatography and gel adsorption (Moon et al., 1972, 1973). EF$_{1L}$ from calf brain was shown to have a molecular weight of 50,000 (Moon et al., 1973), while in pig liver EF$_{1L}$ was shown to have a molecular weight of 53,000 (Iwasaki et al., 1974). Support for the existence of two forms of EF$_1$ and the idea that EF$_{1H}$ is an aggregate of EF$_{1L}$ has come from the amino acid analysis of calf liver EF$_1$ (Liu et al., 1974). Lanzini and his associates (1974) studying the EF$_1$ from wheat embryos, isolated several forms of the factor but found that only the 'heavy' form with the molecular weight range of 240,000 - 500,000 can join directly in a binary complex formation with GTP. Further they showed that this heavy form was to be converted into the light molecular weight form before the ternary complex could be formed. The EF$_{1H}$ by itself can form the binary complex with GTP but was unable to form the ternary complex suggesting that transformation of heavy form to light form was necessary to obtain a stable ternary complex.

The observation that protein elastase could disaggregate EF$_{1H}$ in reticulocytes and Artemia salina focused attention on the role of proteolysis in disaggregation of EF$_1$ (Kemper et al., 1976; Nombela et al., 1976). Elastase and phospholipase were also shown to disaggregate EF$_{1H}$ into low molecular weight species (Twardowski et al., 1976; Legocki et al., 1974). However, it was shown that phospholipase contained considerable amounts of carboxypeptidase activity and that this latter enzyme was responsible for the conversion of EF$_{1H}$ to EF$_{1L}$.
(Twardowski et al., 1977). Variations in the relative amount of EF$^\text{IL}_{1}$ and EF$^\text{IL}_{\text{L}}$ have been observed in the case of the nematode Turbatrix acetii (Bolla and Brot, 1975) and Artemia salina (Slobin and Moller, 1978). In the latter case, in the dormant embryos, the predominant form was observed to be EF$^\text{IL}_{1}$ and was transformed into EF$^\text{IL}_{\text{L}}$ after the cysts hatch. In developing embryos of Artemia salina, Slobin and Moller (1976, 1978) have demonstrated the presence of EF$^\text{IL}_{\text{L}}$ that is similar to bacterial EF$_{\text{TS}}$ and EF$_{\text{TS}}$ and they further showed that brine shrimp EF$^\text{IL}_{1}$ is made of protein factors analogous to bacterial EF$_{\text{TS}}$ and EF$_{\text{TS}}$. Occurrence of multiple species of EF$_{1}$ leads one to visualize that interconversion between heavy and light forms of this enzyme could be a regulatory mechanism controlling the overall rate of protein synthesis (Miller and Weissbach, 1977).

Elongation factor 2 (EF$_{2}$) plays a major role in the process of translocation which comprises the following: movement of peptidyl-tRNA from the aminoacyl site to the peptidyl site and the movement of the ribosome precisely one codon closer to the 3' end of messenger RNA enabling translation of a new codon. Eukaryotic elongation factor 2 and its prokaryotic analogue (EF$_{\text{G}}$) have been described (Weissbach and Ochoa, 1976; Brot, 1977; Bermek, 1978). Earlier studies on EF$_{2}$ were performed mainly by the enzyme prepared from rabbit reticulocytes (Bishop and Schweet, 1971; Arlinghaus et al., 1963; Hardesty et al., 1963) and rat liver (Fessenden and Moldave, 1963; Gasior and Moldave, 1968a, 1968b; Raeburn et al., 1971). Purification of EF$_{2}$ has been achieved by several methods, including chromatography on hydroxylapatite or DEAE-cellulose and gel filtration.
fication of EF$_2$ was first achieved in rat liver (Galasinski and Moldave, 1969), after which it was purified to homogeneity from other eukaryote systems (Twardowski and Legocki, 1974; Yablonka-Reuveni and Warner, 1979). EF$_2$ has been observed to be made up of a single polypeptide chain with molecular weight ranging from 60,000 - 112,000 (Merrick et al., 1975; Galasinski and Moldave, 1969; Collins et al., 1971; Twardowski and Legocki, 1973; Mizumoto et al., 1974; Comstock and Van, 1977). EF$_2$ contains twenty two cysteine groups and the reduced state of the sulfhydryl groups has been found to be essential for the functional integrity of the protein (Mosteller et al., 1966; Sutter and Moldave, 1966). One of the unique features of eukaryotic EF$_2$ is its inactivation by diphtheria toxin (Collier, 1967; Goor and Pappenheimer, 1967a, 1967b). Diphtheria toxin inactivates EF$_2$ by catalysing the transfer of the adenosine diphosphate ribose (ADPR) moiety of NAD to the protein (Honjo et al., 1968; Goor and Maxwell, 1970), while the prokaryotic EF$_G$ is not sensitive to diphtheria toxin (Gill and Pappenheimer, 1971; Richter and Lipmann, 1970; Pappenheimer, 1977). Inactivation of EF$_2$ by diphtheria toxin has been used in the quantitative determination of EF$_2$ when isotopically labelled NAD is used (Raeburn et al., 1971; Collins et al., 1971; Smulson et al., 1970a, 1970b; Traugh and Collier, 1971; Gill and Dinus, 1973; Legocki, 1970; Yablonka-Reuveni and Warner, 1979), since one molecule of ADPR binds to one molecule of EF$_2$ in the presence of excess amounts of NAD and toxin (Raeburn et al., 1971; Honjo et al., 1974). The site of ADP-ribosylation in EF$_2$ from rat liver (Robinson et al.,
1974), yeast (Van Ness et al., 1978) and beef and wheat (Brown and Bodley, 1979) has been shown to be a new amino acid, amino acid X, of which one copy is present in each EF₂ molecule, and situated next to the basic arginine residue. This unidentified residue appears to be unique to EF₂ and presumably results from a post-transcriptional modification of a standard amino acid (Van Ness et al., 1978; Brown and Bodley, 1979).

Very little information is available on the regulatory nature of EF₂ in protein synthesis during plant development and senescence. Studies with animal systems, especially in sea urchin, have shown that EF₂ activity in post-ribosomal supernatant increases by 50% within two minutes after fertilization (Felicetti et al., 1972). During early development of Artemia salina it has been shown recently (Yablonka-Reuveni and Warner, 1979) that the amount of EF₂ in the particulate fraction decreases with a concomitant increase in the amount of soluble EF₂. Further it has been shown by the same authors that extracts from hatched embryos contain low molecular weight polypeptides which accept ADP-ribose whereas extracts from pre-hatched and dormant embryos are devoid of the low molecular weight polypeptides of EF₂. Studies by Pillay (1977) on protein synthesis in aging soybean cotyledons have indicated that soluble factors from younger cotyledons were more efficient in supporting protein synthesis than older cotyledons. Substitution of wheat embryo supernatant factors for soybean cotyledon supernatant resulted in several fold increase
in amino acid incorporation which is particularly significant in older cotyledons. Thus changes in the total content and/or activity of translation factors present in the cotyledon supernatants may account for the decrease in the protein synthetic ability of the cotyledons during senescence.

In order to understand the role played by EF$_2$ in protein synthesis of aging cotyledons of soybean, a detailed study of EF$_2$ was undertaken. An attempt has also been made to study the nature and activity of EF$_2$ in aging cotyledons. Purification of EF$_2$ to homogeneity was also carried out. These studies should contribute to the understanding of the mechanism of protein synthesis regulation by EF$_2$ in aging soybean cotyledons.
II MATERIALS AND METHODS

1. Materials:

a. Seeds: Soybean seeds (Glycine max, var. Harcor) were purchased in bulk and stored in the cold at 4°C for later use.

b. Chemicals: Diphtheria toxin (lot No. D-298, 1800 if/ml) about 13.2 mg/ml purchased from Connaught (Toronto, Ontario) was kindly provided by Dr. A.H. Warner, Department of Biology, University of Windsor, Windsor, Ontario. Triton X-100, Poly (U), β-mercaptoethanol (β-ME), 2,4,6-trinitrobenzene sulfonic acid (TNBS), bovine serum albumin (BSA) were purchased from Sigma (St. Louis, Missouri). Ribonuclease free sucrose and ammonium sulfate were from Schwartz/Mann. (Orangeburg, New York). Liquid scintillation fluors, 1,4-bis 2- (5-Phenylloxazolyl)-benzene (POPOP), 2,5-diphenyloxazol (PPO) and toluene were from Sigma (St. Louis, Missouri). Materials for ion exchange columns, Sephadex G-25, Sephadex G-150 from Pharmacia Fine Chemicals (Piscathaway, N.J.). Phosphocellulose (Whatman P11), DEAE-32 and DEAE-52 (DE-52) from Mandel Scientific (Montreal, Quebec). Reagent for SDS-polyacrylamide gel electrophoresis was either from BioRad or BDH (Poole, England). Molecular weight marker kit containing six molecular weight marker proteins phosphorylase a (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and α1-lactalbumin (14,000) was from Pharmacia Fine Chemicals. Materials for fluorography, Kodak X-Omat, R. film XR-1, rapid
developer and fixer from Kodak (Toronto, Ontario). Dimethyl sulfoxide (DMSO) and PPO were from Sigma (St. Louis, Missouri). Glass fibre filters (Size 2.1 cm) were from Reeve Angle (Clifton, N.J.). $^3$H-Nicotinamide adenine (2,8) dinucleotide (NAD) (Specific activity 2.96 ci/mmol) was from New England Nuclear (Boston, Mass.), $^3$H-phenylalanine (Specific activity 1.0 ci/mmol), $^3$H-Guanosine triphosphate (10-20 ci/mmol) and NCS tissue solubilizer were from Amersham (Oakville, Ont.).

c. Buffers:

Buffer A. 50 mM Tris-HCl pH 7.7 (4°C), 5 mM MgCl$_2$, 20 mM KCl,
4 mM DTT and 0.05% ATP

Buffer B. 50 mM Tris-HCl pH 7.7 (4°C), 5 mM MgCl$_2$, 20 mM KCl,
4 mM DTT, 0.05% ATP and 0.5 M Sucrose

Buffer C. 1 mM Tris-HCl pH 7.7, 1 mM MgCl$_2$ and 4 mM DTT

Buffer D. 20 mM Tris-HCl pH 7.7 (4°C), 10 mM KCl, 5 mM MgCl$_2$,
4 mM β-ME and 5% glycerol

Buffer E. 20 mM Tris-HCl pH 7.7 (4°C), 5 mM MgCl$_2$, 500 mM KCl and
4 mM β-ME

Buffer F. 20 mM Tris-HCl pH 7.7 (4°C), 10 mM KCl.

Buffer G. 20 mM Tris-HCl pH 7.7 (4°C), 300 mM KCl, 3 mM β-ME

Buffer H. Phosphate buffer, 20-200 mM potassium phosphate buffer, pH 7.8,
3 mM β-ME and 1 mM PMSF

All other chemicals were either from Sigma or Fisher Scientific and were of ACS grade.

d. Scintillation Fluid:

0.4% PPO and 0.005% POPOP in Toluene
2. Methods:

a. Germination of seeds and harvesting of cotyledons. Soybean seeds were surface sterilized in 10% chlorox solution, soaked in water overnight and planted in moist vermiculite. Cotyledons from 1, 3, 5, 10 and 20 day dark grown seeds, were harvested and used immediately or stored in liquid nitrogen until used. Zero day old cotyledons were from dry seeds stored at 4°C.

b. Preparation of ribosomes and post-ribosomal supernatant ($S_{100}$). Exactly 100 grams of cotyledons were homogenized in 250 ml of grinding buffer (Buffer A) and the slurry was strained through 4 layers of cheese cloth and centrifuged at 27,000 xg for 10 min. The pellet was discarded and the supernatant was passed through two layers of Miracloth. The filtrate was centrifuged at 105,000 xg for 2 hours in a Beckman Analytical ultracentrifuge (Model L5-65). The supernatant was saved and the pellet was suspended in an extraction buffer, made to 0.1% with Triton X-100, layered over one-third the volume of buffer B and centrifuged at 105,000 xg for 3 hours. The two supernatants were pooled and used as $S_{100}$. The ribosomal pellet was suspended in buffer C, stirred gently in the cold for 30 min and centrifuged at 10,000 g for 10 min to remove the insoluble material. The ribosomal suspension was stored at 100 A$_{260}$ mg/ml at -20°C until used.

c. Preparation of Ribosomal Salt Wash

Low salt washed ribosomes prepared as outlined above were suspended in Buffer E and stirred at 4°C for 4-5 hours. The suspension was
clarified by centrifugation at 10,000 xg for 10 min, layered over buffer E containing 1 M Sucrose and centrifuged at 150,000 g for 3 hours. The pellet which represents the salt washed ribosomes was suspended in buffer C and stored at -20°C. The supernatant dialysed against Buffer F for 8-10 hours with several changes of the same buffer, represents the ribosomal salt wash.

d. Assays of EF₂

i) ADP ribosylation Assay of EF₂

Diphtheria toxin dependent ADP-ribosylation assay of EF₂ was performed according to the method of Collins et al., (1971) as modified by A.H. Warner. A reaction mixture of 0.1 ml contained 3.2 μg of protein equivalent diphtheria toxin, 186 pmol ³H-NAD (specific activity 2.96 Ci/μmol), 20 μl of Mg-DTT (1 mM-10 mM) and varying amounts of either purified EF₂, partially purified EF₂ or crude EF₂ preparations; the mixture was incubated at 28°C for 45 min. The reaction was stopped by the addition of two volumes of ice cold 5% trichloroacetic acid (TCA) containing 0.1 mM unlabelled NAD; 0.05 ml of BSA (10 mg/ml) was added as carrier protein. The mixture was kept on ice for one hour, the precipitate was collected on glass fiber filters and washed with three additional volumes of cold TCA. The filters were dried and the radioactivity determined after adding 5 ml of scintillation fluid.

ii) EF₂ and Poly (U) dependent polyphenylalanine synthesis assay.

The EF₂ dependent, Poly (U) directed polyphenylalanine synthesis assay was performed essentially according to the procedure des-
cribed by Twardowski and Legocki (1973). The assay is based on EF$_2$ dependent Poly (U) directed transfer of $^3$H-phenylalanine from $^3$H-phe-tRNA into material precipitable by hot TCA. Varying amounts of EF$_2$ from column fractions (DEAE-32) were added to the phenylalanine polymerization system in a final volume of 0.2 ml, containing the following components: 20 mM Tris-HCl buffer pH 8.05 (4°C), 72 mM KCl 6.5 mM Mg-Acetate, 250 µM GTP, 4.5 µM DTT 20 µg Poly (U), 36 pmol $^3$H-phe-tRNA (12,000 CPM), 2.5 µg of ribosomes and 4-12 µg of EF$_1$. The reaction mixture was incubated at 30°C for 15 min and the reaction was stopped by the addition of an equal volume of ice cold 10% TCA. The mixture was heated at 90°C for 15 min and filtered through glass fiber filters. The filters were washed twice with 5 ml of cold TCA, dried and the radioactivity was determined.

iii) Binding of $^3$H-GTP to EF$_2$ and ribosomes

EF$_2$ and ribosome dependent GTP binding assay was done according to Chuang and Weissbach (1972). A reaction mixture of 100 µl contained 10 mM Tris-HCl pH 7.4; 10 mM MgCl$_2$, 7.5 mM ammonium acetate, 1 mM DTT: $4.5 \times 10^{-6}$ M $^3$H-GTP, 2.5 µg EF$_2$ and 1.5 A$_{260}$ units of ribosomes. The mixture was incubated at 37°C for 10 min and binding of the nucleotide was assayed by a filter procedure using millipore filters.

e. Analysis of ADP-ribosylation products on Polyacrylamide gels.

After incubation of the ADP-ribosylation reaction mixture, the reaction was stopped by adding the sample application buffer
(50 mM Tris-HCl pH 7.7 (4°C), 5 mM MgCl₂, 0.1% SDS) and kept in an ice bath for 30 min. Then the sample was heated at 90°C for 5 min and applied to polyacrylamide gel slabs. The ADP-ribosylated protein was analysed on 10% polyacrylamide gel slabs (1.5 x 140 x 110 mm) containing 0.1% SDS as described by Laemmli (1970). Electrophoresis was carried out at 120 volts for 3 hours or until the bromophenol blue marker dye migrated to a position 0.5 cm from the bottom of the gel slab. The gels were stained in 0.25% coomassie brilliant blue G in 50% methanol and 9.2% acetic acid for an hour and destained in a solution containing 5% methanol and 7% acetic acid (Weber and Osborne, 1969). For the analysis of ³H radiolabelled products, the gel lane containing the labelled proteins was cut and sliced into 2 mm thin sections. The slices were transferred to glass vials and 0.6 ml of 90% NCS (v/v) tissue solubilizer was added and incubated at 50°C for 3 hours and the radioactivity in the slices was determined (Basch, 1968).

f. Fluorography of radioactive gels.

Fluorography of gels containing ³H radiolabelled protein bands was performed according to Bonner and Laskey (1974). Following electrophoresis, staining and destaining, the gel was soaked in 20 times its volume of DMSO for 30 min followed by a second and third soaking in a 20 fold volume of DMSO. This procedure removes all water from the gel. Next the gels were transferred to a PPO/DMSO solution (20% W/V) and soaked for two 1.0 hour periods. Following the third
soaking for 3 hours, the PPO in the gel was allowed to precipitate in situ by immersing the gel in water, and the water was changed several times. After one hour in water, when all the DMSO in the gel was removed, the gel was placed on a piece of Whatmann No. 3 filter paper and dried in a BioRad slab gel drier (Model 224). The dried gel was taped down to an appropriate size card board to keep it stiff, and exposed to Kodak-X-0 mat R film XR-1 for 3-5 days at -70°C. The X-ray film was developed by routine procedures of developing and fixing.

g. **Protease assay.**

Protease activity in the \( S_{100} \) was measured by the procedures described by Nagainis and Warner (1979). A 0.4 ml reaction mixture containing 1.5 mg BSA and 0.1 M potassium phosphate pH 6.0 was incubated at 28°C; enzyme samples of 100 ul were added to the above reaction mixture and incubation was continued at 28°C. At various intervals of time, samples were removed and the rate of proteolysis determined by TNBS reagent. One unit of protease activity was defined as the amount of enzyme that produces a change of 0.01 OD unit \( (A_{420}) \) in one minute.

h. **Preparation of protease from cotyledon.**

Partially purified protease was prepared from 1 day old cotyledons by the procedures of Garg and Virupaksha (1970) with modifications. Cotyledons were extracted with 2 volumes of acetone. The residue was washed with acetone and air dried. The acetone powder was extracted with 50 mM sodium phosphate buffer pH 6.0 and the super-
natant was brought to 80% saturation by the addition of solid ammonium sulfate. The precipitate was dissolved in extraction buffer and dialysed extensively against the same buffer overnight. The sample was applied to a 2 x 30 cm DEAE-32 cellulose column previously equilibrated with 100 mM phosphate buffer. The column was washed with one column volume of buffer after applying the sample and the enzyme was eluted by 400 ml linear gradient of 25 mM phosphate buffer pH 6.5 containing 0–0.6 M NaCl. About 80 fractions of 5 ml were collected and assayed for protease activity. The active fractions were pooled, dialysed against the starting buffer, concentrated and used as partially purified protease.

1. **Protein determination.**

Protein content was determined by the method of Lowry et al., (1951) using crystalline BSA as standard.
II RESULTS

1. Purification of EF\textsubscript{2} from Soybean Cotyledons

a) Steps of Purification

Purification of EF\textsubscript{2} was carried out in 1 day old soybean cotyledons. Preliminary attempts for purification consisted of using post-ribosomal supernatant (S\textsubscript{100}) fraction as the starting material. When it was observed that the ribosomal salt wash contained more than 30% of total EF\textsubscript{2} activity, all subsequent attempts in the purification of EF\textsubscript{2} involved the pooling of S\textsubscript{100} and ribosomal salt wash fractions in developing a purification procedure.

Step 1 - Ammonium sulfate precipitation of S\textsubscript{100} fraction and Sephadex G-150 column chromatography

S-100 and ribosomal salt wash fractions were prepared as outlined under Methods from 100 gm (fresh weight) of soybean cotyledons. To the pooled fractions solid ammonium sulfate was added slowly to 65% saturation and stirred for 30 min at 4°C. The resulting precipitate was collected, dissolved in 25 ml of buffer and dialysed extensively against buffer A for 8-10 hours. The dialysate was concentrated by vacuum dialysis. The concentrated sample (10 ml equivalent to 1,020 mg protein) was applied to a Sephadex G-150 (fine) column (4 x 90 cm) previously equilibrated with buffer D. After loading the same on to the column it was eluted with one L of buffer D. About 100 fractions of approximately 10 ml were collected and alternate fractions assayed for the presence of EF\textsubscript{2} by the ADP-ribosylation assay. Results pre-
Presented in Fig. 1 show that EF$_2$ elutes as a single peak. A second small peak of EF$_2$ activity was observed just after the main peak, which possibly represents some low molecular weight ADPR accepting protein. It was further observed that low molecular weight ADPR accepting proteins occur in aging soybean cotyledons; this phenomenon will be discussed later in detail. Fractions representing this small peak of EF$_2$ were not included in the purification studies. The fractions represented by a horizontal bar in Figure 1 were pooled and either used immediately in subsequent purification steps or stored at -20°C. No loss in EF$_2$ activity was observed even after four weeks of storage at this temperature.

**Step 2 - Ammonium sulfate precipitation and DEAE-cellulose chromatography**

To the active fractions from Sephadex G-150 column (equivalent to 440 mg protein) solid ammonium sulfate was added slowly to a final concentration of 57%. The precipitate was collected and dissolved in 25 ml of buffer D and dialysed extensively for 7-8 hours over several changes of buffer. The dialysed sample containing approximately 113 mg protein was concentrated to 8-10 ml by vacuum dialysis and applied to a DEAE-32 cellulose column (2 cm x 15 cm) previously equilibrated with buffer D.

After the sample entered the column, it was washed with two column volumes of buffer D and EF$_2$ was eluted with 100 ml of buffer G. About 50 fractions of 2 ml each were collected and 0.05 ml of the aliquots
Figure 1. Chromatography of combined post-ribosomal supernatant and ribosomal salt wash on Sephadex G-150. A ten ml sample equivalent to 1,020 mg protein from the ammonium sulfate precipitate, was applied to a 4 x 90 cm Sephadex G-150 column. The column was eluted with 1000 ml of Buffer D and 100 fractions of 10 ml were collected and tested for EF$_2$ by ADP-ribosylation assay (••••). Fractions were also analyzed for protein by measuring absorbance at 280 nm (0—0). Fractions represented by a horizontal bar containing EF$_2$ were pooled, and used in subsequent purification steps.
used for assay of EF$_2$ content by ADPR-ribosylation. Results of the elution profile of EF$_2$ from the DEAE-cellulose column are presented in Figure 2. Fractions containing EF$_2$ were pooled and used in the next step of purification.

**Step 3 - Ammonium sulfate precipitation and hydroxylapatite chromatography**

In this step solid ammonium sulfate was added slowly while stirring the pooled fractions from DEAE-cellulose (equivalent to 39 mg protein) until the final concentration was 50%. The precipitate was collected and dissolved in 10 ml of buffer F and dialysed against 100 volumes of buffer F for 5-6 hours. The concentrated sample (3-5 ml) representing approximately 12 mg protein was applied to a hydroxylapatite column (1 x 15 cm) and developed with a 200 ml linear gradient of 20-200 mM phosphate buffer pH 7.8 (Buffer H). About 60 fractions of 3 ml were collected and assayed for EF$_2$ content and the column fractions simultaneously monitored at 280 nm for the protein concentration. The hydroxylapatite column chromatography profile presented in Figure 3 shows that EF$_2$ elutes between 100-125 mM phosphate buffer. Active fractions were pooled, concentrated by vacuum dialysis, dialysed against several changes of buffer A for 6-8 hours, and used in the next step of purification.

**Step 4 - Sephadex G-150 gel filtration**

Active fractions from hydroxylapatite column (1-1.5 ml containing 3 mg protein) were applied to a Sephadex G-150 (fine) column (1 x 50 cm)
Figure 2. Chromatography of purified EF$_2$ from the Sephadex G-150 column on DEAE cellulose. The ammonium sulfate precipitate combining EF$_2$ from Sephadex G-150 column was dialyzed against Buffer D, concentrated to 8-10 ml by vacuum dialysis, and applied to a 2 x 15 cm DEAE-32 cellulose column. EF$_2$ was eluted with 100 ml of Buffer G. Fractions collected were monitored at A$_{280}$ for measuring protein concentration (0—0) and assayed for EF$_2$ content (●—●).
Figure 3. Chromatography of EF$_2$ on a hydroxylapatite column. Pooled fractions showing EF$_2$ activity from the DEAE column were precipitated with ammonium sulfate, the precipitate was dialysed against buffer F, concentrated to 3-5 ml and chromatographed on a 1 x 15 cm hydroxylapatite column. EF$_2$ was eluted with a 200 ml linear gradient (0.02-0.2 M) of buffer H(—). Fractions were assayed for EF$_2$ content (●—●) and monitored at 280 nm for protein content (○—○).
previously equilibrated in buffer D. After the sample entered the column, EF$_2$ was eluted with 60 ml of buffer D. Sixty 1 ml fractions were collected and 0.02 ml aliquots were assayed for EF$_2$ by ADP-ribosylation. Elution of EF$_2$ from a Sephadex G-150 column is shown in Figure 4. Fractions containing EF$_2$ were pooled, concentrated by vacuum dialysis and dialysed against buffer D. The total volume of 1-2 ml was stored at -20°C in 0.1 ml aliquots at 0.6 mg protein/ml.

b) Purity of EF$_2$

Table 1 summarizes the steps in the purification of EF$_2$ from the soybean cotyledons. A 265 fold purity of EF$_2$ was obtained with approximately 9% recovery. EF$_2$ purified by the above procedure was active in polyphenylalanine synthesis and also remained stable for 6-8 weeks at -20°C. Absence of any ADP-ribosylating band in addition to the EF$_2$ band, on SDS-polyacrylamide gel, indicated that there was no degradation during this storage period.

Purified EF$_2$ tested for its homogeneity by electrophoresis under non-denaturing conditions resolves into a single protein band. The non-stained homologous gel was cut into 2 mm sections and the sections assayed for poly (U) dependent polyphenylalanine synthesis, ADP-ribosylation and ribosome dependent GTP binding. Data presented in Figure 5 indicate that EF$_2$ under non-denaturing conditions resolves into a single protein band. ADP-ribosylating region, poly (U) dependent phenylalanine polymerization region and ribosome dependent GTP binding region co-migrate with the stained protein band confirming the homogeneity
Figure 4. Sephadex G-150 gel filtration of EF$_2$. Fractions from hydroxylapatite column concentrated to 1-1.5 ml equivalent to 3.0 mg protein, were applied to a Sephadex G-150 column and eluted with Buffer D. 60 fractions of 1 ml were collected, monitored at A$_{280}$ (○—○) for protein elution profile and assayed for EF$_2$ by diphtheria toxin-NAD assay (■—■).
<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg) a</th>
<th>Specific Activity (Units/mg protein) b</th>
<th>Cumulative Purification (Fold)</th>
<th>Percent Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude Supernatant</td>
<td>3,190</td>
<td>2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2. (NH₄)₂SO₄</td>
<td>1,020</td>
<td>4</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>(65% Saturation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Sephadex G-150</td>
<td>440</td>
<td>7</td>
<td>3.5</td>
<td>48</td>
</tr>
<tr>
<td>4. (NH₄)₂SO₄</td>
<td>113</td>
<td>20</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>(57% Saturation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. DEAE-Cellulose</td>
<td>39</td>
<td>46</td>
<td>23</td>
<td>28</td>
</tr>
<tr>
<td>6. (NH₄)₂SO₄</td>
<td>12</td>
<td>116</td>
<td>58.0</td>
<td>22</td>
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<tr>
<td>(50% Saturation)</td>
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<td></td>
</tr>
<tr>
<td>7. Hydroxylapatite</td>
<td>3</td>
<td>297</td>
<td>149</td>
<td>14</td>
</tr>
<tr>
<td>8. Sephadex G-150</td>
<td>1.1</td>
<td>530</td>
<td>265</td>
<td>9</td>
</tr>
</tbody>
</table>

a. From 100 gm of 1 day old soybean cotyledons.

b. One unit corresponds to 1 pmol of phenylalanine polymerized.
Figure 5. Polyacrylamide gel electrophoresis of purified EF$_2$ under non-denaturing conditions. Twenty µg of EF$_2$ was applied to 6.2% polyacrylamide gel. Electrophoresis was performed for 3 hours at 4°C. The gel was stained in coomassie brilliant blue, destained in 7.5% acetic acid in 5% methanol and scanned at 560 nm in Ortec 4310 densitometer (----). The unstained co-electrophoresed lane containing EF$_2$ was sliced into 2 mm thin sections, EF$_2$ was eluted from the sections by EF$_2$ assay buffer, and EF$_2$ activity was determined by diphteria toxin-NAD assay (0—0), poly (U) dependent polyphenylalanine synthesis (0—0) and ribosome dependent GTP binding (X—X).
of purified EF₂.

c) Subunit Composition and Molecular Weight of EF₂

In an attempt to determine the subunit composition of EF₂, electrophoresis of the purified product was performed under denaturing conditions in the presence of SDS. The results presented in Figure 6 indicate that soybean cotyledon EF₂ is a single subunit with molecular weight of 92,000 daltons.

2. Activity of Elongation Factor 2 in aging Soybean Cotyledons

Pillay (1977) observed that in vitro protein synthesis decreases in aging soybean cotyledons. Further it was shown that partially purified supernatant factors from aging cotyledons in the presence of ribosomes from 1 day old cotyledons elicited a decreased response in protein synthetic activity. This suggested that decreased incorporation associated with aging of the cotyledons is independent of the age of the ribosomes since 1 day ribosomes were used in all cases. In order to evaluate the control of protein synthesis in aging cotyledons, it is necessary to determine the content of these factors present in the post-ribosomal supernatant.

Elongation factor-2 from supernatants of dry, and 1,3,5,10 and 20 day old cotyledons were partially purified by passing through a Sephadex column (G-25) and tested for ADP-ribosylation. Data presented in Table 2 show the EF₂ content in relation to age of the cotyledons. A higher EF₂ content was observed in 1 day old cotyledons.
Figure 6. Molecular weight and subunit composition determination by SDS-polyacrylamide gel electrophoresis of purified EF₂. Pure EF₂ was separated in 10% polyacrylamide slab gel containing 0.1% SDS. A semilog plot of migration of EF₂ relative to the migration of molecular weight markers. Molecular weight markers used were a) β-galactosidase (130,000) b) Phosphorylase b (94,000) c) Bovine serum albumin (67,000) d) Ovalbumin (43,000) and e) DNase (31,000).
Table 2.  **EF$_2$ in Aging Soybean Cotyledons**

<table>
<thead>
<tr>
<th>Age of Cotyledons</th>
<th>EF$_2$ content (pmoles of H-ADPR bound per/mg protein)</th>
<th>% EF$_2$ content compared to 1 day cotyledons</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>18</td>
<td>29</td>
</tr>
<tr>
<td>1 day</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>3 days</td>
<td>36</td>
<td>57</td>
</tr>
<tr>
<td>5 days</td>
<td>16</td>
<td>25</td>
</tr>
<tr>
<td>10 days</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>20 days</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

EF$_2$ content of 1 day cotyledon is taken as standard (100%). All other comparisons were made with 1 day EF$_2$ content.
compared to dry seed cotyledons. EF$_2$ content in 1 day old cotyledons was taken as standard (100%) and the relative EF$_2$ content from aging cotyledons was compared. Dry seeds possess about 30% of EF$_2$ found in 1 day cotyledons. As the cotyledons age, there is a measurable decrease in EF$_2$ content. In 3 and 5 day cotyledons a 43% and 75% decrease respectively was observed compared to 1 day old cotyledons. Total EF$_2$ suddenly decreases to 15% and 8% in 10 and 20 day old cotyledons respectively compared to 1 day old cotyledons.

3. Free and bound EF$_2$ in Cotyledons

EF$_2$ present in free and bound fractions of the aging soybean cotyledons was determined by the ADP-ribosylation assay. Free EF$_2$ was represented by the post-ribosomal supernatant and contained nearly 65% of total EF$_2$. The ribosome rich fraction, represented by the ribosomal pellet, was not active in ADP-ribosylation. The ribosomal pellet was suspended in buffer E, stirred at 4°C for 3-5 hours and the resulting suspension was layered over one-third of the volume of the buffer E containing 1 M sucrose and centrifuged at 105,000 xg for 1.5 hours. The resulting supernatant representing the ribosomal salt wash was found to be active in ADP-ribosylation. This suggested that the EF$_2$ in soybean cotyledons exist both in a free form in the post-ribosomal supernatant (S-100) and in a bound form attached to a ribosomal rich fraction, which was released by high salt. They were referred to as the free and the bound form of EF$_2$ respectively. Since it is known that only free EF$_2$ can undergo the ADP-ribosylation, treatment with high salt must have released the EF$_2$ from the ribosomal rich particulate fraction.
Free and bound fractions were isolated from dry, 1, 3, 5, 10 and 20 day old cotyledons and the presence of EF$_2$ was quantitatively determined by the toxin-NAD assay. Results presented in Table 3 indicate that the quantity of both free and bound EF$_2$ increase from dry seeds to 1 day old cotyledons and then gradually decrease during aging of the cotyledons. Free and bound EF$_2$ content in 1 day old cotyledons was taken as standard (100%) to compare the relative amounts of EF$_2$ in free and bound fractions in aging cotyledons. A 100% increase in both free and bound EF$_2$ was observed in 1 day old cotyledon compared to dry seeds. However between 1 day and 20 days an overall reduction in the amount of free and bound EF$_2$ was observed. During the period of 1 and 3 days the amount of free and bound EF$_2$ decreased by 32% and 58% respectively. Similarly, a 66% and 82% decrease between 1 and 5 days occurs in free and bound EF$_2$. As the cotyledons aged (between 1 and 10 days) EF$_2$ content decreased 32% and 88% respectively in the two fractions. Between 1 and 20 days the free and bound EF$_2$ content decreased by as much as 88% and 95% respectively. Total protein concentrations of the free and bound fractions were determined during the above periods. The results (Table 3) indicate that a gradual decrease in total protein occurs in 1 day to 20 day old cotyledons. By taking the 1 day old cotyledon protein content (100%) as standard, the dry seeds contained 53% and 66% protein in free and bound fractions. Table 3 further shows that the reduction in total protein content in these two fractions was gradual, when compared to the drastic decrease in EF$_2$ content in the aging cotyledons.
<table>
<thead>
<tr>
<th>Age of Cotyledons</th>
<th>Protein Content&lt;sup&gt;a&lt;/sup&gt;</th>
<th>EF&lt;sub&gt;2&lt;/sub&gt; Content&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Free/Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Bound&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Free</td>
</tr>
<tr>
<td>Dry</td>
<td>20.1 (53)</td>
<td>4.3 (66)</td>
<td>1.64 (58)</td>
</tr>
<tr>
<td>1 day</td>
<td>38.2 (100)</td>
<td>6.5 (100)</td>
<td>2.81 (100)</td>
</tr>
<tr>
<td>3 day</td>
<td>34.6 (90)</td>
<td>5.9 (91)</td>
<td>1.92 (68)</td>
</tr>
<tr>
<td>5 day</td>
<td>30.4 (80)</td>
<td>4.8 (74)</td>
<td>0.95 (34)</td>
</tr>
<tr>
<td>10 day</td>
<td>28.5 (25)</td>
<td>4.1 (63)</td>
<td>0.51 (18)</td>
</tr>
<tr>
<td>20 day</td>
<td>16.2 (42)</td>
<td>3.2 (50)</td>
<td>0.33 (12)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total protein present in 1 gm of cotyledon
<sup>b</sup> Protein in post-ribosomal supernatant
<sup>c</sup> Protein in ribosomal salt wash
<sup>d</sup> Pmoles ADPR bound to EF<sub>2</sub> present in 1 mg of protein present in free and bound fractions
4. **Existence of Multiple species of EF\textsubscript{2}**

Purified EF\textsubscript{2} from 3 day old cotyledons when ADP-ribosylated and separated on polyacrylamide gels in the presence of SDS migrates as a single band (M.W. 92,000). It is similar to the non-ADP-ribosylated EF\textsubscript{2}. Slicing the gel into 2 mm thin sections and determination of the radioactivity indicates that the radioactive band coincides with the stained band. This confirms the observation in other systems that ADP-ribosylation of EF\textsubscript{2} does not affect the migration on polyacrylamide gels containing SDS (Robinson et al., 1974; Van Ness et al., 1978). However, when either S-100, ribosomal salt wash or partially purified EF\textsubscript{2} from 3 day old cotyledons was ADP-ribosylated and the products separated on SDS-polyacrylamide gels, two major radioactive peaks corresponding to the two forms of EF\textsubscript{2} (a high molecular weight form 92,000 and a low molecular weight form 60,000) were observed. But only the high M.W. form co-migrates with the purified EF\textsubscript{2}. The high molecular weight form was called heavy EF\textsubscript{2} designated as EF\textsubscript{2H} and the low molecular weight species was referred to as the light EF\textsubscript{2} and designated as EF\textsubscript{2L}.

To test the participation of EF\textsubscript{2H} and EF\textsubscript{2L} in protein synthesis, it was necessary to fractionate the heavy and light forms of EF\textsubscript{2} without any cross contamination. To achieve this the post-ribosomal supernatant was passed through a column of Sephadex G-25 and the fractions active in ADP-ribosylation were pooled and chromatographed on a DEAE-32 column. Fractions collected were assayed for ADP-ribosylation and poly (U) dependent phenylalanine polymerization. Results

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presented in Figure 7 indicate that two regions are active in ADP-ribosylation. One of the regions active in ADP-ribosylation is also active in poly (U) directed phenylalanine incorporation. The second peak active in ADP-ribosylation does not exhibit any biological activity, in that it does not function in phenylalanine polymerization. When these two regions active in ADP-ribosylation were pooled separately, concentrated, and the molecular weight of the ADP-ribosylated product determined, it was found that the early eluting peak, from DEAE-cellulose, had a molecular weight of 92,000 similar to the purified EF£. The second region was found to have a molecular weight of 60,000. This clearly indicates that the early eluting peak identified with the heavy form of EF£ (EF£1H) functions both in ADP-ribosylation and phenylalanine polymerization, while the late eluting region is capable of only ADP-ribosylation. Since in eukaryotes only the free EF£ or EF£ fragments undergo ADP-ribosylation (Pappenheimer 1977), the region which functions only in ADP-ribosylation was identified with the light form of EF£ (EF£2L).

Since aging cotyledons possess decreasing amounts of total EF£, with an accompanying decline in protein synthetic activity, it was of interest to study the nature of EF£ in aging cotyledons and determine its level (of heavy and light forms). EF£ present in S-100 from dry, 1,3,5,10 and 20 day cotyledons was prepared, ADP-ribosylated and separated on polyacrylamide slab gels containing SDS. Results summarized in Figure 8 indicate the presence of variable amounts of EF£1H and EF£2L in cotyledons of different ages. In dry seeds there is a
Figure 7. Chromatography of partially purified EF₂ on a DEAE cellulose column.
Partially purified EF₂, equivalent to 50 mg protein (prepared as outlined in the text) was applied to a DE-32 column (1.5 x 15 cm). After sample application, the column was washed with two column volumes of Buffer D and EF₂ was eluted with 300 ml linear gradient of 10-300 mM KCl in buffer D. Fractions collected were assayed for EF₂ activity by ADP-ribosylation (0—0) and poly (U) directed polyphenylalanine synthesis (•—•). Protein content was determined by measuring A₂₈₀ (——).
Figure 8. Polyacrylamide gel electrophoresis of partially purified EF2 from aging soybean cotyledons. Partially purified EF2 from aging cotyledons was ADP-ribosylated and the products were applied to different lanes of 1.5 mm thick 10% polyacrylamide gel containing 0.1% SDS. Following electrophoresis, lanes containing EF2 were cut into 2 mm thin sections, and the radioactivity of the slices were determined.
Figure 9. Fluorogram of the ADP-ribosylated EF2 from aging cotyledons. ADP-ribosylated products from aging (a, dry, b, 1 day and c, 3 day old) cotyledons, separated on SDS-polyacrylamide gel as outlined in Fig. 8. Fluorography was done according to the procedures described in Methods. Following drying, the gel was exposed to Kodak X-0 mat R film for 5 days at -70°C.
higher preponderance of $\text{EF}_{2\text{L}}$, while in 1 day old cotyledons $\text{EF}_{2\text{H}}$ predominates with very little or no $\text{EF}_{2\text{L}}$. However, 3 and 5 day old cotyledons possess equal amounts of both heavy and light forms. In 10 and 20 day old cotyledons $\text{EF}_{2\text{H}}$ completely disappears and a very low molecular weight form of $\text{EF}_{2}$ (M.W. 29,000) appears along with $\text{EF}_{2\text{L}}$ (M.W. 60,000). This very low molecular weight ADP-ribosylating component also does not possess any biological activity. Its appearance parallels the disappearance of $\text{EF}_{2\text{H}}$ in both 10 and 20 day old cotyledons. The question now arises if this low molecular weight $\text{EF}_{2}$ is a degraded part of $\text{EF}_{2}$. Based on its ability for ADP-ribosylation, it may be suggested that it represents the degraded product of $\text{EF}_{2}$. A fluorogram of the gel showing the relative distribution of heavy and light forms in dry, 1 and 3 day old cotyledons is presented in Figure 9. The amount of radioactivity in ADPR bound proteins in 5, 10 and 20 day old cotyledons is too low to be detected on the fluorogram.

5. **Protease Activity in aging Cotyledon and possible involvement of protease (?) in the degradation of $\text{EF}_{2}$**

So far, it has been shown that in aging soybean cotyledons $\text{EF}_{2}$ exists in two forms (free and bound) and as two species (high and low molecular weight). It was also shown that only the heavy form of $\text{EF}_{2}$ functions biologically, while the low molecular weight form is inactive. Does the decreased availability of functional $\text{EF}_{2}$ in aging cotyledons contribute to the decline in protein synthesis is the key question? Therefore, it was of interest to determine, if some factor present in
the cotyledons during aging, could be responsible for regulating the relative amounts of this heavy and light form of EF$_2$ and the endogenous levels of functional EF$_2$.

Cotyledons of higher plants, leguminous plants in particular, synthesize and store large amounts of protein during development. These proteins will be rapidly hydrolyzed during the time of germination of the seed and nourish the growing embryonic axis. The hydrolysis of the reserve proteins present in the cotyledons has been observed to occur mainly by hydrolyzing enzymes during germination of leguminous seeds has been well documented. Therefore, it was of interest to study whether any relationship exists between the production of proteases during aging and the amount of EF$_2$ present in the cotyledons.

In order to quantitatively estimate protease activity in cotyledons, assays were carried out at 28°C at pH 6.0. Initially protease activity was estimated at 5°C, 28°C and 40°C. At 5°C protease activity was not detected in the cotyledon, while assays carried out at both 28°C and 40°C yield similar results. Therefore all subsequent experiments to quantitatively determine proteases were done at 28°C.

Protease activity and the relative ratio of light to heavy form of EF$_2$ as a function of cotyledon age is presented in Table 4. Dry seeds exhibit only slight amount of protease activity. With the onset of germination protease activity increases steadily up to a maximum in 10 day old cotyledons (40 units/mg protein of S-100) which
Table 4. Protease Activity of Aging Cotyledons and its Relation to the Ratio of Light and Heavy Forms of EF$_2$

<table>
<thead>
<tr>
<th>Age of Cotyledons</th>
<th>Protease activity$^a$ (units/mg protein)</th>
<th>EF$_2$ $^b$ Light/Heavy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>0.02</td>
<td>2.4</td>
</tr>
<tr>
<td>1 day</td>
<td>0.43</td>
<td>0.06</td>
</tr>
<tr>
<td>3 days</td>
<td>16.03</td>
<td>1.1</td>
</tr>
<tr>
<td>5 days</td>
<td>36.00</td>
<td>0.83</td>
</tr>
<tr>
<td>10 days</td>
<td>40.24</td>
<td>8.6</td>
</tr>
<tr>
<td>20 days</td>
<td>26.33</td>
<td>9.2</td>
</tr>
</tbody>
</table>

$^a$ One unit of protease activity is defined as the amount of enzyme that produces a change of 0.01 OD unit ($A_{420}$) in one minute.

$^b$ CPM from heavy and light EF$_2$ regions of Fig. 8 were quantitated and the results were expressed as relative amount light/heavy forms of EF$_2$. 

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is approximately a 21 fold increase in protease activity over protease present in dry seeds. A 6 fold increase in protease activity was observed between 1 and 3 day old cotyledons. In 5, 10 and 20 day old cotyledons a 125%, 150% and 62% increase respectively was observed compared to the 3 day old cotyledons. The data presented in the table further shows the relative ratio of light to heavy form of EF2 in aging cotyledons and its relationship to protease activity. Except for the dry seeds, in aging cotyledons an increase in the relative ratio of light to heavy form of EF2 is observed. Dry seeds contain a light/heavy EF2 ratio of 2.4 which indicates the preponderance of EF2L. On germination EF2H becomes the predominant species in 1 day cotyledons having a light/heavy EF2 ratio of 0.06. In 3, and 5 day old cotyledons there is an equitable distribution of EF2L and EF2H having a light/heavy form of EF2 ratio at approximately 1.0. As the cotyledons age, EF2L is the predominant species (See also Figures 8 and 9).

To study the effect of protease on EF2, an acid protease from 3 day old cotyledon was isolated as outlined in Chapter II, and incubated at 30°C with EF2 from 3 day old cotyledons prior to ADP-ribosylation. The partially purified protease used was devoid of any EF2. The incubation products were ADP-ribosylated, and the resulting products were separated on SDS-polyacrylamide slabs. The ADP-ribosylated EF2 without prior incubation with protease was run as control. The radioactivity of the gel slices were determined and the results are presented in Figure 10. It is clear, that EF2 which was not incubated with protease separates into EF2H and EF2L while that incubated with protease and ADP-ribosylated completely degrades.
Figure 10. Effect of protease from soybean cotyledons on partially purified EF$_2$. Partially purified protease from 3 day old soybean cotyledons was incubated with partially purified EF$_2$ from 3 day cotyledons. Following incubation of the mixture at 30°C for 15 min, the product was ADP-ribosylated and separated on SDS-polyacrylamide slab gel. The lanes containing EF$_2$ were cut into 2 mm thin sections and radioactivity was determined (□——□). Control is represented by EF$_2$ ADP-ribosylated without prior incubation with protease (0——0).
IV DISCUSSION

In a study concerning protein synthesis in aging soybean cotyledons (Pillay, 1977) it was demonstrated that supernatant factors prepared from younger cotyledons were more effective in phenylalanine incorporation in vitro than factors isolated from older cotyledons. It was further noticed that substitution of wheat embryo supernatant factors for soybean cotyledon supernatant factors resulted in a several fold increase in amino acid incorporation in ribosomes from all ages of soybean cotyledons. This clearly demonstrated the possible existence of lesions in the supernatant factors which may be responsible for decrease in phenylalanine incorporation. Although the above studies did not include the supernatant factors and ribosomes from dry seeds to evaluate the presence of active protein synthesizing system, it was assumed that dry seeds also contained an active protein synthesizing system. An active protein syntehsizing system in dry non-viable pea embryonic axes has been observed (Bray and Chow, 1976) to support in vitro poly (U) directed polyphenylalanine synthesis. Studies on the activity of elongation factors in aging soybean cotyledons (Gowda and Pillay, 1978) indicated that they play a regulatory role in controlling the protein synthesis rate. In order to ascertain whether EF$_2$ regulates protein synthesis in aging cotyledons a detailed study of elongation factor 2 was carried out.

Soybean cotyledons represent a highly differentiated tissue with very little or no meristematic activity. Cotyledons store a lot of reserve food materials and during the process of seed germination feed
the embryonic axis. At the time of germination, cotyledons possess an active protein synthetic machinery and assist in the synthesis of food to some extent and mobilize stored food material in an available form to the growing embryo. Growth and expansion of the cotyledons in size during germination are mainly due to cell enlargement and not cell division. During advanced stages of seed germination, metabolism in cotyledons consists primarily of catabolic events. As the seedling grows older and becomes independent, cotyledons wither and fall off from the parent plant. Thus cotyledons in early stages of seed germination possess both anabolic and catabolic activity, while catabolic processes become most predominant as they age. Therefore, cotyledons serve as a unique system to study biochemical changes during the process of senescence or aging.

The presence of Elongation factor 2 was determined by diphtheria toxin dependent ADP-ribosylation assay. One of the unique features of eukaryotic EF2 is its inactivation by diphtheria toxin. It was noticed that the inhibition of protein synthesis by diphtheria toxin required the absolute presence of NAD (Collier, 1967; Goor and Pappenheimer, 1967). The diphtheria toxin catalyzes the transfer of ADPR moiety of NAD to EF2 causing inactivation (Honjo et al., 1968). Since one molecule of ADPR binds to one molecule of EF2 in the presence of excess amounts of NAD and toxin, the diphtheria toxin catalyzed binding of ADPR moiety to EF2 has been used in the quantitative determination of eukaryotic EF2 in the presence of radiolabelled NAD. ADP-ribosylation assay is specific only towards eukaryotic EF2, while
prokaryotic organellar EF G is not sensitive (Gill and Pappenheimer, 1971; Pappenheimer, 1977). Thus, the above technique proved to be a specific and a convenient assay system to quantitatively determine EF2 content in the soybean cotyledon and simultaneously eliminate any organellar EF G interference.

Using diphtheria toxin-NAD assay to monitor EF2 present in various column fractions, a simple and convenient method of purification of EF2 from 1 day old soybean cotyledons was established. ADP-ribosylation assay was also used to quantitatively determine the amount of EF2 present in aging cotyledons and to evaluate if it occurs in a free or bound form. These studies indicated -

a) A shift in the relative amount of heavy and light forms of EF2 during cotyledon aging.
b) Changes in the content and activity of EF2 in aging cotyledons, and
c) Presence of proteases (produced in cotyledons during germination) as possible regulating factors of endogenous EF2 levels.

**Purification of EF2 from Cotyledons**

EF2 from 1 day old soybean cotyledons was purified to homogeneity. The above purification steps employed here are similar to those used for the purification of EF2 from wheat germ (Twardowski and Legocki, 1973) and *Artemia salina* (Yablonka-Reuveni, 1979). One of the early steps in the purification of EF2 involved the use of DEAE-cellulose anion exchange column. This step was very useful in the purification of EF2 since at low ionic concentrations EF2 adsorbs strongly, and this
enables complete separation from EF₁, which does not bind to DEAE-cellulose under low ionic conditions (Twardowski and Legocki, 1973). Phosphocellulose chromatography has been widely used in the purification of eukaryotic EF₂. In the present investigation, EF₂ containing fractions from DEAE-cellulose when chromatographed on phosphocellulose showed that most of the contaminant proteins elute together with EF₂. No significant improvement in either the extent of purification or yield was achieved using this step in the purification of EF₂. A similar observation was reported in the purification of EF₂ from Artemia salina (Yablonka-Reuveni, 1979). Therefore the use of phosphocellulose chromatography in purification of EF₂ from soybean cotyledons was not employed. The molecular weight and subunit composition of soybean EF₂ is in general agreement with EF₂ from other eukaryotes (Table 5) and prokaryotic EF G (Arai et al., 1978).

Purification of EF₂ by affinity chromatography was employed recently (Lam and Heintz, 1978), where EF₂ from reticulocytes was separated by its binding to an agarose column containing covalently bound NAD and diphtheria toxin. This method of purification may be the most convenient single step purification achieving most satisfactory isolation of EF₂ from crude homogenates.

Free and bound Elongation Factor 2 in Aging Cotyledons

In soybean cotyledons EF₂ is present, both in free and bound form. The bound form is associated with the ribosome rich fraction. Low salt washed ribosomes do not exhibit any ADP-ribosylation. But high
Table 5. Molecular Weight of EF2 from Eukaryotes (adopted from Bermek, 1978)

<table>
<thead>
<tr>
<th>Source</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit reticulocytes</td>
<td>95,000 (Merrick et al., 1975)</td>
</tr>
<tr>
<td></td>
<td>97,000 (Merrick et al., 1975)</td>
</tr>
<tr>
<td>Rat liver</td>
<td>89,000 (Collins et al., 1971)</td>
</tr>
<tr>
<td></td>
<td>95-105,000 (Collins et al., 1971)</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>70,000 (Twardowski and Legocki, 1973)</td>
</tr>
<tr>
<td>Artemia salina</td>
<td>95,000 (Yablonka-Reuveni, 1979)</td>
</tr>
<tr>
<td></td>
<td>90,000 (Nombela and Ochoa, 1973)</td>
</tr>
<tr>
<td>Human tonsil</td>
<td>93,000 (Bermek, 1978)</td>
</tr>
<tr>
<td>Pig liver</td>
<td>95,000 (Mizumoto et al., 1974)</td>
</tr>
<tr>
<td></td>
<td>110,000 (Mizumoto et al., 1974)</td>
</tr>
<tr>
<td>Hen oviduct</td>
<td>93,000 (Comstock and Van, 1977)</td>
</tr>
<tr>
<td></td>
<td>96,000 (Comstock and Van, 1977)</td>
</tr>
<tr>
<td>Soybean cotyledons</td>
<td>92,000*</td>
</tr>
</tbody>
</table>

a - Determined by SDS-polyacrylamide gel electrophoresis
b - Determined by gel filtration
* - present investigation
salt (0.5 M KCl) wash ribosomal fraction is very active in ADP-ribosylation. Dissociation reagents like ammonium chloride and EDTA have been used in releasing bound EF$_2$ (Smulson et al., 1970; Traugh and Collier, 1971; Gill and Dinus, 1973). Nearly 30% of total EF$_2$ in soybean cotyledons is in the bound form. Similar values for bound EF$_2$ were reported in *Artemia salina* (Yablonka-Reuveni and Warner, 1979), rabbit reticulocytes (Traugh and Collier, 1971b), and HeLa cells (Smulson and Rideau, 1970). Association of EF$_2$ to a particulate fraction other than ribosomes has been reported for rabbit reticulocytes (Hradec and Dusek, 1978). It has been suggested, in the reticulocytes, that the 20S particles present in the post-mitochondrial fraction stimulates protein synthesis, suggesting that EF$_2$ may be bound to these particles. More recently, experiments involving *Artemia salina* demonstrate the association of EF$_2$ with particulate fractions other than ribosomes (Yablonka-Reuveni and Warner, 1979). Protein synthesizing initiation factors, have been reported to be associated with some high molecular weight particles other than ribosomes (Hradec and Dusek, 1978; Hellerman and Shafritz, 1975). It is difficult for us to conclude from our data that EF$_2$ was associated with one of the above particulate fraction other than ribosomes.

With the onset of germination both free and bound forms of EF$_2$ decreased in soybean cotyledons, whereas in 1 day old cotyledons a 100% increase was observed compared to the dry seeds. This increase in free and bound EF$_2$ activity from dry seeds to 1 day old cotyledon probably reflects a high protein synthetic activity reported in 1 day old cotyledons (Pillay, 1977). Similar reports in increase of free EF$_2$
with concomitant increase in protein synthesis has been observed
As the seeds germinate, both free and bound EF$_2$ quantitatively decrease,
with decrease being more pronounced after 5 days. Protein in free
and bound fractions showed nearly a 70-80% increase from dry seeds to
1 day imbibed cotyledons and later showed a 60% decrease from 1 day to
20 days. However, the decrease in protein content was only gradual
when compared to the rate of decrease of EF$_2$. This may be due to the
synthesis of many hydrolytic enzyme proteins that hydrolyze the reserve
food in the aging cotyledons to nourish the growing embryonic axis.
The plausible reasons for the decrease in the concentration of EF$_2$ in
the aging cotyledons may be due to a decrease in synthesis of EF$_2$
and/or the degradation of both bound and free EF$_2$ by hydrolyzing enzymes
which are produced in large quantities in aging cotyledons.

**EF$_2$ content of aging cotyledons**

Increase in soybean EF$_2$ content from dry seeds to 1 day germinated
seeds may represent an increased synthesis. As the cotyledons age, a
decrease in protein synthesis could be attributed to a decrease in EF$_2$
along with other components of protein synthesis. It must be cautioned
here that the content of EF$_2$ measured by ADP-ribosylation assay may
not represent biologically active EF$_2$ in the seeds, because both free
EF$_2$ and EF$_2$ fragments undergo ADP-ribosylation. As stated earlier, EF$_2$
present in S-100 from soybean cotyledons exists in two forms (heavy and
light). Only the heavy form (EF$_{2H}$) functions biologically and the light
form (EF$_{2L}$), though functions in ADP-ribosylation, does not exhibit
any biological activity. (See next section). Decline in protein syn-
thesis in aging cotyledons could be attributed to 1) decrease in functional EF2, 2) transformation of functional EF2 to a non-functional form and/or 3) complete degradation of EF2 into fragments. The appearance of an additional low molecular weight EF2 fragment and the complete disappearance of EF2H in 10 and 20 day old cotyledons supports the above view.

Existence of multiple ADPR accepting (EF2) polypeptides, and the possible role of proteases in the degradation of EF2

The two species of EF2 (EF2H and EF2L) observed in soybean have a differential biological function (Figure 7) and occur in different amounts in aging cotyledons (Figure 8). A similar observation was made in Artemia salina where only EF2H functions in poly (U) directed protein synthesis and ribosome dependent GTP binding (Yablonka-Reuveni and Warner, 1979). The possibility that the presence of light and heavy forms of EF2 in soybean cotyledons could have a regulatory function in protein synthesis, needs further rigorous examination. Dry seeds contain higher proportions of EF2L. With the onset of germination, within one day there is a greater proportion of EF2H. It is speculated here that the increased availability of EF2H in one day old cotyledons reflects an increased synthesis of EF2H. The occurrence of higher amounts of EF2L in dry seeds may probably reflect the pattern of EF2 synthesis during embryogenesis and seed ripening stages. The events occurring at the time of final stages of seed ripening may also be responsible for the higher quantity of EF2L in dry seeds. It would be interesting to study the exact time at which the synthesis of EF2H begins.
and determine the nature of transformation of EF$_{2H}$ to EF$_{2L}$ with the aging of the cotyledons. The occurrence of an additional very low molecular weight EF$_2$ fragment (M.W. 29,000) may suggest that it is a degradation product of EF$_{2L}$. Further work is necessary to establish if this is the case. The appearance of low molecular weight EF$_2$ in aging cotyledons may therefore account for the loss in protein synthetic capacity in aging cotyledons.

EF$_2$ fragments (M.W. 60,000 and 29,000) observed in 10 and 20 day old cotyledons may account for the degradation of EF$_{2H}$ (92,000 M.W.) since the summation of the molecular weights of fragments is about equivalent to the molecular weight to EF$_{2H}$. Collins et al., (1971) observed a higher value (M.W. 108,000 compared to M.W. 95,000) on summation of the EF$_2$ fragments (M.W. 67,000 and M.W. 41,000) obtained as the degradation products of rat liver EF$_2$.

Since higher plants synthesize large amounts of storage protein during seed development and hydrolyze them during germination (Ihle and Dure, 1972) by proteases and hydrolases produced in the cotyledons, it was of interest to determine the protease activity in aging cotyledons. Results presented here show that an acid protease is present in soybean cotyledons. The protease activity increased as the cotyledons age (Table 4). A similar pattern of protease activity was observed in bean seeds (Prisco et al., 1975). Appearance of carboxypeptidase activity in cotton seed (Ihle and Dure, 1972) and some hydrolysing enzymes in mung bean (Chrispeels et al., 1976) follow the
same pattern. An increase in the proportion of the light to the heavy form of EF$_2$ in aging cotyledons (Table 4) closely corresponds with the increase in protease activity.

An acid protease partially purified from 3 day old cotyledons completely degrades a partially purified EF$_2$ (Figure 10). This clearly suggests that proteases control the level of functional EF$_2$ in the cotyledon. It may be possible that the crude preparations of protease contain a specific hydrolyzing enzyme, responsible for the degradation of EF$_2$. Twardowski et al., (1977) have suggested that EF$_{1H}$ from *Artemia* embryos disaggregate into functional EF$_{1L}$ by a factor present in the extracts of nauplii; the sensitivity of this factor to PMSF suggested that it was a protease. The observation that phospholipase C (Legocki et al., 1974) also caused disaggregation of EF$_{1H}$ into EF$_{1L}$ suggested that phospholipids are involved in the formation or the stability of EF$_1$ aggregates, was argued against, since purified EF$_{1H}$ from reticulocytes contained no phospholipids (Kemper et al., 1976). The ability of phospholipase C to disaggregate EF$_{1H}$ was observed to be due to the presence of a carboxypeptidase as a contaminant in the preparation of phospholipase C (Twardowski et al., 1977). This enzyme removes a small fragment from the C-terminal end of EF$_1$ active polypeptide, which does not have any effect on the enzymic activity. The involvement of elastase (Twardowski et al., 1976) in the disaggregation of EF$_{1H}$ might represent the protease mediated control in the mechanism of disaggregation of EF$_1$. These findings may imply involvement of proteases or specific hydro-
lases in the regulation of EF$_2$. In *Artemia salina* the presence of a specific hydrolysing enzyme in the regulation of the levels of EF$_2$ has been suggested (Yablonka-Reuveni and Warner, 1979). Data presented in this investigation support the presence of some factor in aging cotyledons that might regulate endogenous EF$_2$. Further studies in the isolation of this factor may contribute significantly to the understanding of the regulation of protein synthesis in aging soybean cotyledons.

The foregoing studies indicate that the pattern of EF$_2$ activity in aging soybean cotyledons is somewhat similar to that observed by Yablonka-Reuveni and Warner (1979) in developing embryos of *Artemia salina*. Although in one case it is a plant system and the other an animal system, the similarity in EF$_2$ activity in these two eukaryotic systems is extremely interesting, though coincidental. However, it is not possible to speculate at this time, how one could observe such similarities in two totally different systems. In any case, this close parallel in EF$_2$ activity in plants and an animal system does tempt one to conclude that possibly there is unity in diversity. Further work may provide additional interesting information.
PART II

Cyclic AMP-Independent Protein Kinases from Soybean Cotyledons (*Glycine max, L*)
Protein Kinases

Protein kinases are groups of enzymes which catalyze the transfer of phosphoryl moieties to the protein substrate by covalent modifications (Rubin and Rosen, 1975; Walsh and Krebs, 1973; Krebs, 1972; Rabinowitz 1962). The phosphoryl moiety is transferred to seryl or threonyl residues of the substrate protein; the transfer to a seryl residue is more common. The resulting phosphoryl esters are generally not stable to alkaline hydrolysis, therefore they are referred to as base labile phosphates (Rabinowitz, 1962). The phosphotransferase reaction usually requires the presence of a cation Mg++, although some enzymes prefer Ca++ or Mn++ to Mg++ (Rubin and Rosen, 1975). Amino acids other than serine and threonine may act as phosphate acceptors. The lysyl group of histone H1 and histidinyl residue of histone H4 are also phosphorylated by protein kinases which are specific for such reactions both in vitro (Chen et al., 1974) and in vivo (Smith et al., 1974). The resulting phosphate formed through a P-N bond is more labile to acid hydrolysis, unlike the ester bond established in serine or threonine phosphates and therefore they are referred to as acid labile phosphates (Rubin and Rosen, 1975).

It was first observed by Walsh et al., (1968), that the activity of these protein kinases was stimulated by cyclic adenosine 5'-monophosphate (cAMP). Since then, evidence from a number of laboratories has accumulated in favour of the existence of protein kinases influenced
by cAMP levels (for a review see Rubin and Rosen, 1975). Protein kinases whose activities are stimulated by cyclic nucleotides (cAMP or cGMP) are termed as cyclic nucleotide-dependent protein kinases, while those protein kinases which are not influenced by either cyclic nucleotides are termed cyclic nucleotide-independent protein kinases.

The discovery of cAMP dependent protein kinases is the result of studies concerning the enzymes of glycogen metabolism. Glycogen phosphorylase was first identified to be the enzyme concerned with the breakdown of glycogen as being regulated by changes in its state of phosphorylation (Cohen, 1978). Phosphorylase kinase and glycogen synthase were subsequently observed to be regulated by phosphorylation (Cohen, 1978). The existence of cAMP-dependent protein kinases was demonstrated after the initial discovery of phosphorylase kinase (Krebs et al., 1964; Walsh et al., 1968). Cyclic AMP was found to stimulate the activity of this enzyme. It was later observed that this enzyme was capable of phosphorylating other substrates in addition to phosphorylase kinase, therefore, it was appropriately called cAMP-dependent protein kinase (Walsh et al., 1968).

Cyclic AMP-dependent protein kinases are composed of two dissimilar subunits, the regulatory subunit (R) and the catalytic subunit (C) (Borstrom, et al., 1970; Gill and Garen, 1970; Tao, et al., 1970; Kumon, et al., 1970). Each regulatory subunit is made up of two identical subunits of M.W. 49,000-55,000 and each catalytic subunit is composed of two identical peptides of M.W. 41,000 (Rubin and Rosen, 1975), thus
the holoenzyme has the formula \( R_2C_2 \). cAMP activates the holoenzyme (\( R_2C_2 \)) by forming a complex with regulatory subunit thereby releasing the catalytic subunit to bind 2 moles of cAMP per mole or 4 moles of cAMP per holoenzyme (Corbin et al., 1978; Dosekland, 1978). Cyclic AMP-dependent protein kinases have been further classified into type I and type II enzymes based on their chromatographic behaviour on DEAE cellulose columns (Corbin et al., 1975). Type I kinases elute between 0.08–0.1 M NaCl, they are easily dissociated by 0.5 M NaCl, and reassociate slowly following removal of cAMP after cAMP induced dissociation. Type II enzymes elute from DEAE cellulose columns with 0.15–0.2 M NaCl, dissociate slowly upon addition of histone or salt and reassociate rapidly following cAMP induced dissociation. All the tissues examined contain both types of protein kinases and the proportion of each depends on the tissue and species (Corbin et al., 1975; Lee et al., 1976). Type II protein kinase from bovine cardiac muscle has been observed to undergo autophosphorylation of the regulatory subunits (Rosen et al., 1977, Erlichman, 1974). This modification has no effect on cAMP binding capacity but facilitates the dissociation of the holoenzyme (Rangel-Aldo and Rosen, 1977).

Cyclic GMP-dependent protein kinase was first isolated from lobster tail (Kuo and Greengard, 1970), and later found to be present in mammalian pancreas (Van Leemput-Courtez et al., 1973) and cerebellum (Hoffman and Sold, 1972). It was found to be similar to cAMP-dependent enzyme in its mode of activation but unlike cAMP-dependent protein...
kinase, the binding of cGMP-dependent protein kinase does not result in subunit dissociation (Lincoln et al., 1977; Gill et al., 1977).

The holoenzyme having a molecular weight ranging between 140,000-165,000 contains two similar subunits of molecular weight 70,000-82,000 and possesses one catalytic and a cGMP binding site per subunit. Unlike cAMP-dependent protein kinases, cGMP-independent protein kinases exist only in one form. cGMP protein kinases modify histones in preference to casein, but the phosphorylation site of the phosphorylated substrate differ from that phosphorylated by cAMP-dependent protein kinases and they also have been known to undergo autophosphorylation (Erlichman et al., 1974; Lincoln et al., 1977; Gill et al., 1977).

It is clear from the above discussion that cAMP and cGMP-dependent protein kinases share many common properties. It has been suggested that the similarities between the two types of enzymes reflect the evolutionary relationship between them (Lincoln and Corbin, 1978; Gill et al., 1976). It was suggested that both the enzymes are derived from the same ancestral protein kinase or that the cGMP-dependent protein kinase is the ancestral form from which cAMP-dependent protein kinase is derived.

In contrast to the cyclic nucleotide dependent protein kinases, another distinct group of protein kinases have been observed, whose activity is independent of cyclic nucleotides. Protein kinases that are not stimulated by cyclic nucleotides fall into this category of enzymes and are known as cyclic nucleotide independent protein kinases.
They phosphorylate limited types of substrates unlike cAMP-dependent enzymes. Therefore, they are referred to by the nature of the substrates they phosphorylate.

Casein/phosphvitin kinases are protein kinases that phosphorylate casein and phosphvitin which are the two nutritional proteins. Casein and phosphvitin kinases have been observed in rabbit reticulocytes and erythrocytes (Hathaway et al., 1979; Kunar and Tao, 1975; Issinger, 1977), human lymphocytes (Kemp et al., 1975), rat liver (Takeda et al., 1971; Matsumura and Takeda, 1972; Baggio and Moret, 1971; Rudden and Anderson, 1972; DesJardens et al., 1972; Dastugue et al., 1974; Maragoudakis and Han in, 1977; Clari et al., 1976; Thornburg and Lindell, 1977; Thornburg et al., 1978), Novikoff ascites cells (Dahmus, 1976; Dahmus and Natzle, 1977), calf brain (Walinder, 1973) chicken oviduct (Keller et al., 1976) pea shoots (Keates 1973), peas and Lemna (Keates and Trewavas, 1974), and soybean hypocotyl, (Lin and Key, 1978).

Casein kinases (CK I and CK II) have been purified from reticulocytes (Hathaway et al., 1979; Hathaway and Traugh, 1979), rat liver nuclei (Thornberg and Lindell, 1977; Thornberg et al., 1978), and Novikoff ascites nuclei (Dahmus, 1976; Dahmus and Natzle, 1977). CK I which uses ATP as phosphoryl donor is made up of a single polypeptide chain except in rabbit liver nuclei. Molecular weights of 37,000, 50,000 and 47,000 were observed in reticulocytes, rat liver nuclei and Novikoff ascites nuclei respectively.
CK II, the major form of casein kinase, has a molecular weight of 130,000 in reticulocytes (Hathaway et al., 1979; Hathaway and Traugh, 1979), 123,000 from rat liver nuclei (Thornberg and Lindell, 1977) and 122,000 from Novikoff ascites nuclei (Dahmus, 1976; Dahmus and Natzle, 1977). These enzymes use both ATP and GTP as phosphoryl donors. They were found to be tetrameric and composed of four dissimilar subunits in a 1:1:2 configuration.

Isolated casein kinases undergo autophosphorylation (Hathaway et al., 1979, Thornberg and Lindell, 1977; Dahmus, 1976, Dahmus and Natzle, 1977). The significance of this modification in relation to the activity of these enzymes is not known. In CK I, which is made up of a single subunit, it is not clear whether autophosphorylation results in activation or inactivation of the enzyme. In CK II, which is made up of more than one subunit, phosphorylation may result in subunit aggregation or disaggregation. Results of such changes in the activity of the enzyme are not known.

Cyclic nucleotide independent protein kinases which are specific towards basic proteins such as histone or protamine have been found to be present in the nucleus. The activity of these protein kinases is not attributed to the presence of the free catalytic subunit as seen in cAMP-dependent protein kinases, since the free regulatory subunit is not able to inhibit the activity of the enzyme (Rubin and Rosen, 1975; Walsh et al., 1973; Ashby and Walsh, 1972). All the major histone fractions (H1, H2A, H2B, H3 and H4) are phosphorylated as shown by the in vivo labelling studies in trout spermatocytes (Dixon et al.,...
1975; Sung and Dixon, 1976; Louie and Dixon, 1973; Louie et al.,
1973). However, some of the sites in these histones are phosphory-
lated by cAMP-dependent protein kinases and other sites are phos-
phorylated by cAMP-independent protein kinases.

Basic proteins such as protamines are also phosphorylated \textit{in vitro}
by cAMP-dependent protein kinases (Walsh and Krebs, 1973; Rubin and
Rosen, 1975); however, protamine phosphorylation \textit{in vivo} is mainly
cytoplasmic and cAMP-independent (Marushige et al., 1969). Protamine
phosphorylation occurs at multiple sites on each polypeptide (Ingles
and Dixon, 1967) which suggests the existence of protamine specific
kinases.

Two cAMP-independent histone kinases have been reported (Takai
et al., 1977a, 1977b; Inoue et al., 1977; Yamamoto et al., 1978).
These protein kinases are activated by mild proteolysis with trypsin or
homologous calcium stimulated protease (Takei et al., 1977a; Inoue et
al., 1977; Yamamoto et al., 1978). The activity of these enzymes is
found to be present in a variety of tissues (Inoue et al., 1977; Lin
and Key, 1976), and appears to possess different site specificity when
assayed against histone subfractions (Takai et al., 1977b; Inoue et al.,
1977). The calcium activated protease also activates glycogen phos-
phorylase and glycogen synthase (Kishimoto et al., 1977, 1978); however, the extent of activation is less when compared to cAMP-inde-
pendent protein kinases. Recently, two protease activated cAMP-
independent protein kinases referred to as PKA I and PKA II have been
isolated from rabbit reticulocytes (Tahara, 1979; Tahara and Traugh,
1979). These use ATP as the phosphoryl donor and phosphorylate histone. PKA I phosphorylates $H_4$ at 10 mM $Mg^{++}$ and shows decreasing amounts of phosphorylating capacity towards $H_{2B}$ and $H_3$ and does not phosphorylate $H_1$, $H_{2A}$, casein, protamine and phosvitin. It also phosphorylates $eIF_3$ (130,000 molecular weight subunit), $eIF_{4B}$, $eIF_5$ and ribosomal protein $S_{10}$. PKA II was found to phosphorylate the subunit of $eIF_2$ (molecular weight 53,000) and ribosomal protein $S_6$.

In addition to the protein kinases mentioned above there have been some reports on protein kinases which phosphorylate only very specific substrates. The best example is that of phosphorylase kinase, which phosphorylates specifically glycogen phosphorylase (Rubin and Rosen, 1975; Nimmo and Cohen, 1978). Glycogen synthase is another enzyme which is phosphorylated by a specific protein kinase (Cohen, 1978). Myosin protein light and heavy chains have their own specific protein kinases (Schlender and Reimann, 1975; Soderling et al., 1977). Two subunits of eukaryotic initiation factor 2 (eIF 2) have been shown to be phosphorylated by heme regulated repressor (HCR) or heme controlled inhibitor (HCl) (Tahara et al., 1978; Kramer et al., 1976; Farrel et al., 1977; Ranu and London, 1976; Gross and Mandelewski, 1977, 1978; Traschel et al., 1978) and dsRNA activated inhibitor (DAI) (Farrel et al., 1977; Levin and London, 1978). HCR and DAI have not been shown to have any other known substrates.

Phosphorylation of ribosomal subunits

Ribosomes isolated from rat liver (Loeb and Blat, 1970) and rabbit reticulocytes (Kabat, 1970) were found to contain proteins
which undergo phosphorylation when $^{32}$P was injected into these animals. *In vitro* phosphorylation of ribosomal proteins has been widely observed. The radioactive phosphate group is found attached in the phosphorylated protein through O-phosphoryl linkage to seryl and/or threonyl residues. When proteins were extracted from the total ribosomal population and subjected to electrophoresis in gels containing SDS, variable number of radioactive bands were observed. However, when the ribosomes were dissociated into subunits using high salt buffer, phosphorylated *in vitro* and analyzed for the phosphorylated proteins, only a few radioactive protein bands were found (Traugh et al., 1973). Proteins removed by high salt washing of ribosomes represented the proteins associated with the ribosomes and it is quite possible that some of these proteins represent factors involved in protein synthesis and are phosphorylated (Traugh et al., 1973). The rate of phosphorylation of ribosomal proteins has been shown to be modified by the presence of protein kinases which are present bound to the ribosomes or found free in the cytoplasm (Cawthon et al., 1974).

Two cAMP regulated protein kinases present in the post-ribosomal free supernatant of rabbit reticulocytes have been found to have different specificities for the ribosomal proteins of both subunits (Traugh et al., 1973). Ribosomal subunit proteins have been shown to be preferentially phosphorylated together with other soluble proteins, by cAMP-dependent protein kinase (Herbert et al., 1977; Rankine, 1977; Sikorski et al., 1979). Three to six proteins of large ribosomal subunit were found to undergo phosphorylation (Herbert et al., 1977; Sikorski et al., 1979).
The presence of phosphorylated proteins and protein kinases in plants have not been studied in as much detail as is the case in the animal systems. Kuo and Greengard (1969) have reported the absence of histone kinase from plants. Phosphorylation of ribosomal proteins has been observed in sterile cultures of *Lemna minor* (Trewavas, 1973) and isolated ribosomes from peas and *Lemna* (Keates and Trewavas, 1974).

In *vivo* phosphorylation of ribosomes result in the phosphorylation of less number of proteins than *in vitro* (Rubin and Rosen, 1975; Belanger, et al., 1979). Kabat (1971) indicated that the extent of protein kinase dependent phosphorylation remains the same, both *in vitro* and *in vivo*. Phosphorylation of additional proteins *in vitro* may be the result of exposition of new sites on the ribosomes during their isolation and purification of the subunits. Therefore it is necessary to correlate *in vitro* observations with *in vivo* data to determine the physiological validity of the phosphorylation phenomena.
VI MATERIALS AND METHODS

1. Materials

a. Seeds. See Chapter II.

b. Chemicals

Source of most of the chemicals is presented in Chapter II. In addition, the following were utilized: DEAE-52 cellulose and phosphocellulose (Pll Whatman), obtained through Mandel Scientific Company (Montreal, Quebec). Phosvitin, casein (dephosphorylated), histone type II A, CTP, UTP, GTP, cAMP, cGMP were from Sigma Chemical Company (St. Louis, Missouri). Kodak no-screen medical X-ray film was from Kodak (Toronto, Ontario). 

\[ ^{32} \text{P-ATP} \]

purchased from Amersham (Oakville, Ontario), was diluted upon arrival to a specific activity of 2000 mci/mmol and stored in 50% ethanol at -20°C. Counts per minute (CPM) per mole was calibrated for each experiment.

c. Buffers

Buffer A: 50 mM Tris-HCl pH 7.7 (4°C), 5 mM MgCl₂, 500 mM KCl, 4 mM DTT and 0.05% ATP.

Buffer B: 50 mM Tris-HCl pH 7.7 (4°C), 5 mM MgCl₂, 500 mM KCl, 4 mM DTT, 0.05% ATP and 1 M sucrose.

Buffer C: 1 mM Tris-HCl pH 7.7 (4°C), 1 mM MgCl₂ and 4 mM DTT

Buffer D: 20 mM Tris-HCl pH 7.7 (4°C), 3 mM MgCl₂, 20 mM KCl and 4 mM DTT.

Buffer E: 20 mM Tris-HCl pH 7.7 (4°C), 3 mM MgCl₂, 500 mM KCl 4 mM DTT.
2. **Methods**

a. Germination of seeds and harvesting cotyledons. See Chapter II.

b. Preparation of ribosomes and post-ribosomal supernatant. See Chapter II.

c. **Preparation of salt washed ribosomes and ribosomal subunits**

Low salt washed ribosomes prepared as outlined previously (Chapter II), were suspended in buffer A at a concentration of 100 $A_{260} / \text{ml}$ and stirred at 4°C for 4-5 hours. The suspension was clarified by centrifugation at 10,000 xg, and the supernatant layered carefully over one-third volume of buffer B and centrifuged at 150,000 g for 3 hours in a Beckman analytical ultracentrifuge. The pellet was suspended in buffer C and stored at a concentration of 100 $A_{260} / \text{ml}$. This represents salt washed ribosomes.

Ribosomal subunits from low salt washed ribosomes were prepared by zonal centrifugation using a Beckman Ti 15 zonal rotor according to the method of Sypherd and Wireman (1974) which permits easy loading and unloading of the zonal rotor. All solutions and the rotor were precooled to 4°C. The rotor was initially filled with buffer D and brought to 3,000-4,000 rpm with the upper bearing and rulon sealing assembly in place. The sample (80S ribosomes, 2,000 $A_{260}$ at 100 $A_{260} / \text{ml}$ concentration) in buffer D containing 5% sucrose was pumped into the rotor through rotor edge with a Pharmacia peristaltic pump (Model p-3). The sample layers beneath the buffer and displaces an equal volume of buffer through the
rotor centre. A total of 800 ml of 7.5-30% sucrose in buffer E was used for generating the gradient. A hyperbolic sucrose gradient was generated using the Beckman Model 141 gradient pump and the gradient was pumped through the edge of the rotor. This displaces an equal volume of buffer from the rotor centre core. The loading was completed by pumping 400 ml of 45% sucrose through the outer vein as a cushion. After all loading operations were completed, the rotor was run at 25,000 rpm for 10 hours. To recover the ribosomal subunits, the rotor was brought to 3,000-4,000 rpm, the upper bearing and rulon seal assembly was repositioned and 60% sucrose was pumped through the rotor edge while the contents of the rotor will be displaced through the rotor centre. The first 600 ml of solution was discarded and 20 ml fractions were collected until the 60% sucrose emerges from rotor. Absorption of fractions was monitored at 260 nm. Fractions containing 40S and 60S subunits were pooled and their purity was tested by analytical sucrose density gradient centrifugation in a Beckman ultracentrifuge using the SW 41 rotor.

Because either high salt or sucrose interferes with the precipitation of ribosomal subunits by ethanol, the pooled fractions containing subunits were dialysed against low salt buffer (Buffer A), (Kaulenas, 1971). One volume of 95% ethanol was added to the dialysed subunits and the suspension was left at -20°C for 3-4 hours to ensure complete precipitation of subunits. They were collected by centrifugation at 10,000 xg for 10 min, suspended in
buffer A and stored at -20°C at a concentration of 75-100 A\textsubscript{260}/ml until used.

d. **Isolation of ribosomal proteins**

Proteins from ribosomes, ribosomal subunits or phosphorylated ribosomes were extracted according to the procedure of Hardy et al., (1969). To the samples containing 100-500 A\textsubscript{260} of ribosomes or ribosomal subunits in buffer A, one-tenth volume of 1 M MgCl\textsubscript{2} and two volumes of glacial acetic acid were added in rapid succession. The mixture was stirred in an ice bath for one hour and the precipitated RNA was removed by centrifugation at 10,000 xg for 10 min. The pellet was washed with an acetic acid mixture and recentrifuged. The combined supernatants were pooled, dialysed extensively against buffer A to remove acetic acid and protein was concentrated by vacuum dialysis.

e. **Polyacrylamide gel Electrophoresis of Ribosomal proteins**

A two dimensional polyacrylamide gel electrophoresis system in which acid-urea gel (Reisfield et al., 1962) in the first dimension and discontinuous gel system of Laemmli (1970) in the second dimension was used in the separation of ribosomal proteins and phosphorylated ribosomal proteins. Both dimensions were run in a slab gel electrophoresis apparatus similar to the one described by Studier (1973). Slab gels were constructed from two glass plates 160 x 130 mm in which one plate had a wide notch 8-9 mm deep. Three teflon spacers of desired thickness (1.5 mm for first dimension
and 3 mm for second dimension) were inserted between the glass plates along the side and bottom edges and sealed with lubriseal (No. 8690-B12 Arthur Thomas Company, Philadelphia, Pa.). Binder clips were used to hold the plates together, while the separating gel was poured in. After polymerization of the separating gel, the stacking gel was poured. A teflon comb with ten teeth (8 x 30 mm) was used to form lanes for the first dimension. The final dimension of the separating gel was 140 x 110 mm. The 8% separating gel was prepared according to Leboy et al., (1964). Both the separating gel and the stacking gel contained 6 M urea. The sample containing 6 M urea was incubated for 30 min at 30°C to assure that proteins were reduced and 3 mM β-ME was added along with a tracking dye pyronin Y. The sample containing 80-100 µg of proteins in a total volume of 50 µl was kept in a boiling water bath for 2 minutes and carefully introduced into the lanes of the stacking gel using Pederson pipettes. The bridge buffer consisted of B-alanine-acetic acid mixture (31.2 gm B-alanine, 8 ml glacial acetic acid per L and diluted 10 times). The electrophoresis was performed at 120 volts for 8 hours or until the tracking dye migrated within 0.5 cm of the bottom of gel. When electrophoresis was completed, the gel was removed and equilibrated against equilibrating solution (50 mM Tris-HCl pH 7.6, containing 0.5% SDS and 6 M urea) for 30 min, and the individual lanes were cut lengthwise and placed on the second dimension gel.

The second dimension gel (3.3 mm thick) was made up of a 10%
separating gel and a 5% stacking gel into which the lane from the first dimension was introduced and embedded by pouring additional stacking gel solution and allowing it to polymerize so that the gel is 0.5-1 cm below the notched portion of the plate. The second dimension separating gel system (Laemmli, 1970) was composed of 10% acrylamide buffered with 0.37 M Tris-HCl pH 8.8 containing 0.03% SDS. The stacking gel was made up of 3% acrylamide and 0.083% BIS buffered with 0.1 M Tris-HCl pH 6.8 containing 0.03% SDS. The gel was placed in the electrophoresis apparatus containing 0.25 M Tris-glycine buffer pH 8.3 containing 0.1% SDS. Electrophoresis was performed at room temperature at 120 volts for 4 hours. After electrophoresis, the gel was removed and stained for 3 hours in a staining solution (0.25% coomassie brilliant blue G in 50% methanol and 0.2% acetic acid) and destained with several changes of destaining solution (Weber and Osborne, 1969) and stored in 7% glacial acetic acid (V/V).

f. Polyphenylalanine synthesis by ribosomal subunits

Poly (U) directed polyphenylalanine synthesis by ribosomes and ribosomal subunits was performed according to Twardowski and Legocki (1973) with minor modifications. The reaction mixture (200 ul) contained 20 mM Tris-HCl pH 8.05 (4°C), 70 mM KCl, 6.5 mM Mg-acetate, 250 μM GTP, 4.5 mM DTT, 20 μg Poly (U), 36 pmol ³H-Phe-tRNA (10,000 cpm), 130 μg of 80S or 100 μg of 40S or 120 μg 60S ribosomal subunits and 60-70 μg protein equivalent soybean cotyledon post-ribosomal supernatant (Pillay, 1977). The reaction
mixture was incubated for 15 min at $30^\circ$C and the reaction was stopped by adding TCA to a final concentration of 10%. The resulting mixture was heated at $90^\circ$C for 15 min, filtered through glass fibre filters and the radioactivity determined.

g. **Analysis of rRNA of ribosomal subunits by polyacrylamide gel electrophoresis**

rRNA of ribosomal subunits was extracted by the method described by Parish and Kirby (1966) and analyzed by polyacrylamide gel electrophoresis according to the method outlined by Loening (1969). Gels containing 2.6% acrylamide were cast in 9 x 0.5 cm perspex cylinders and were preelectrophoresed for 30 min at 5 mA/gel before applying the sample. Electrophoresis was conducted for 1.5 hours at 3 mA/tube. The gels were scanned at 260 nm in a Gilford Spectrophotometer Gel Scanner.

h. **Casein Kinase assay**

Assay for casein kinase was performed as outlined by Hathaway et al., (1979) with modifications. The reaction mixture (100 µl) contained 20 mM Tris-HCl pH 7.7, 10 mM MgCl$_2$, 100 mM KCl, 2 mM ß-ME, 7.5 mg/ml casein and enzyme equivalent to 50-60 µg protein. The reaction was started by adding $^{32}$P-ATP. After incubation for 30 min at $30^\circ$C the reaction was stopped by the addition of 1 ml of 10% TCA and 50 µl of carrier protein (BSA, 1.25 mg in 2.2 ml). The mixture was heated at 90-95°C for 15 min. The hot TCA insoluble precipitate was collected on Whatman glass fibre filters, trans-
ferred to glass vials, dried and 5 ml of scintillation fluid was added and the radioactivity determined.

i. Phosphorylation assay of ribosomes

Phosphorylation assay of ribosomes was performed according to Li and Amos (1971). The reaction mixture (200 µl) contained 20 mM Tris-HCl pH 7.7, 5 mM MgCl$_2$, 100 mM KCl, 2 mM DME, 2.5 x 10$^{-4}$ M $\gamma$-P-ATP and 10 A$_{260}$ units of ribosomes. The reaction was carried out at 30°C for 30 min and stopped by the addition of 10 volumes of 10% TCA containing 50 µg BSA. The mixture was heated at 90-95°C for 15 min and the hot TCA insoluble precipitate was collected on glass fibre filters, dried and radioactivity measured. Phosphorylation of ribosomes in vitro for the analysis of phosphorylated ribosomes was done by the procedures outlined by Li and Amos (1971) with the following modifications. The ribosomes present in the reaction mixture containing protein kinase and $\gamma$-P-ATP was precipitated after incubation by the addition of 0.2 ml of ice cold 1 M magnesium acetate and chilled for 30 min. Ribosomes were collected by centrifugation at 10,000 xg for 10 min. They were again resuspended in 10 mM Tris-HCl pH 7.7 (4°C), 100 mM KCl and 5 mM MgCl$_2$ and centrifuged again. Protein extraction (Hardy et al., 1969) and polyacrylamide gel electrophoresis (Laemmli, 1970) were performed as described earlier.

j. Autoradiography of ribosomal proteins

Following SDS-polyacrylamide gel electrophoresis the phosphorylated
ribosomal proteins were prepared for autoradiography by the following procedures. After electrophoresis staining and destaining the gel was transferred onto a piece of Whatman No. 3 filter paper and dried in a BioRad slab gel dried Model 224. The dried gel was taped down to a piece of appropriate sized cardboard to keep the gel stiff, covered with saran wrap and exposed to Kodak no screen medical X-ray film for a period of 1-8 days.
1. Isolation of ribosomal subunits from ribosomes

The ribosomal subunits were separated by using hyperbolic sucrose gradient centrifugation in a Beckman zonal rotor as outlined in Chapter II. A typical optical density profile of the subunits present in the sucrose fraction is shown in Figure 11. To avoid mutual contamination of 60S and 40S subunits only the specific fraction representing each peak as indicated by the shaded areas in Figure 11 were collected. Purity of ribosomal subunits was further tested by subjecting 60S and 40S subunits to analytical sucrose density gradient centrifugation in a Beckman ultracentrifuge using a SW41 rotor. The optical density profile obtained is shown in Figure 12a. It is clear that 40S and 60S subunits are relatively pure without much cross contamination.

To further substantiate the purity of the subunits ribosomal RNA was extracted from the subunits and separated on 2.6% polyacrylamide gel electrophoresis. The results obtained are shown in Figure 12b. Examination of the isolated rRNA by SDS PAGE indicates that both subunit fractions have very little contamination.

The 7.5-30% sucrose gradient used in the isolation of subunits by zonal centrifugation was chosen to obtain the best possible resolution. Experiments involving the use of 5-20% and 12-40% sucrose gradients showed that they could also separate greater amounts of dissociated subunits, but the 60S and 40S peaks were very broad in 5-20% gradients and very sharp in 12-40% gradients.
Figure 11. Separation of soybean cotyledon ribosomal subunits by zonal centrifugation. Details of isolation of ribosomes and centrifugation in a Beckman Ti 15 zonal rotor as described in Methods. Fractions 18-23 representing 40S subunits and 37-45 representing 60S subunits were pooled separately. The ribosomal subunits were recovered by precipitation in ethanol.
Figure 12. a). Sedimentation profiles of 40S and 60S ribosomal subunits by analytical ultracentrifugation. Five A$_{260}$ units of each of the subunits were applied to a 12 ml, 7.5-30% linear sucrose gradient in Buffer D and centrifuged at 105,000xg for 1.5 hours in a Beckman SW 41 rotor. The absorbance profiles were recorded in an ISCO density gradient fractionator equipped with a UV monitor. Direction of sedimentation is from right to left. b). SDS-polyacrylamide gel electrophoresis of rRNA isolated from 60S and 40S ribosomal subunits isolated by zonal centrifugation. One A$_{260}$ of 60S rRNA and 0.6 A$_{260}$ of 40S rRNA are used in electrophoresis. Direction of migration is indicated by the arrow.
By using the above procedure, the yield of the ribosomal subunits was slightly less than 25% of the total amount of sample applied. In a typical run approximately 2000 $A_{260}$ units were applied. The yield of the subunits after dialysis, precipitation and resuspension was only 350 $A_{260}$ units for the 60S and 40 $A_{260}$ units for the 40S subunits.

Ribosomes and ribosomal subunits prepared as outlined above were tested for their biological activity by poly (U) directed polyphenylalanine synthesis, and the results presented in Table 6. The reconstituted 80S ribosomes were obtained by mixing the 60S and 40S subunits while 60S subunits served as controls. It was observed that ribosomes and ribosomal subunits prepared in the manner described above were intact and no loss in activity was detected in polyphenylalanine synthesis.

2. Preparation of ribosomal proteins and their separation on polyacrylamide gels

Ribosomal proteins were isolated from ribosomes and ribosomal subunits with 66% acetic acid (Hardy et al., 1969). The basic ribosomal proteins were completely soluble in acid, while the insoluble rRNA was precipitated. This method extracts over 95% of ribosomal proteins but leaves about 0.5% contamination of rRNA (Hardy et al., 1969). The proteins extracted by this method had an absorbance ratio of 1.55 ($A_{280}/A_{260}$) and was considered over 99.5% pure (Layne, 1957). The final volume of the acetic acid extracted proteins after dialysis was
Table 6. Synthesis of Poly(U)-directed Polyphenylalanine by Ribosomal Subunits prepared by Zonal Centrifugation

<table>
<thead>
<tr>
<th>Ribosomes / Ribosomal subunits</th>
<th>$^3$H-Phenylalanine polymerized (pmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80S</td>
<td>18.6</td>
</tr>
<tr>
<td>40S</td>
<td>1.3</td>
</tr>
<tr>
<td>60S</td>
<td>1.5</td>
</tr>
<tr>
<td>60S + 40S</td>
<td>17.2</td>
</tr>
</tbody>
</table>

Reaction mixture in 200 ul contained 20 mM Tris-HCl pH 8.05 (4°C), 70 mM KCl, 6.5 mM Mg-acetase, 250 μM GTP, 4.5 mM DTT, 20 μg poly (U), 36 pmol of $^3$H-phe-tRNA (10,000 cpm), 130 μg of 80S, or 100 μg 40S or 120 μg 60S ribosomal subunits. The experiment involving 60S and 40S subunits contained equal quantities (80 μg each) 60S and 40S subunits. Post-ribosomal supernatant equivalent to 60-70 μg protein was added and the complete reaction mixture was incubated at 30°C for 15 min.
about 12-15 ml. Since the protein concentration in this sample was low, it was concentrated before electrophoresis.

In earlier experiments, the subunit proteins were lyophilized. This procedure was not satisfactory because proteins that were soluble in sample buffer before lyophilization were insoluble in the same solution after lyophilization. Since the decrease in solubility of lyophilized ribosomal proteins has also been observed in bacterial systems (Kaltschmidt and Wittmann, 1970b), the concentration of protein by lyophilization was abandoned. Concentration of the protein sample by vacuum dialysis was found most satisfactory. The protein sample was concentrated from 12-15 ml to 1.0-1.5 ml in 3-4 hours. These smaller samples after equilibration with sample gel buffer and reduction with β-ME or DTT, were concentrated further to 200-250 μl without loss of protein.

In an attempt to improve the resolution of low molecular weight proteins, the methods of Reisfeld et al., (1962) and Laemmli (1970) were combined. Both these methods employ the stacking gel system. The first dimension slab gel system used 4% acrylamide with a pH of 6.8 (Reisfeld et al., 1962) in the stacking gel and 8% acrylamide in separating gel with a pH of 4.3. The second dimension slab gel system (Laemmli, 1970) contained 10% acrylamide with a pH 8.3 in the separating gel and 3% acrylamide with a pH 6.8 in the stacking gel. Both separating and stacking gels contained 0.1% SDS. As in Martini and Gould's (1971) system, separation was mainly by charge in the first dimension and size in the second dimension. When the ribosomal proteins were analyzed
by this method resolution of high molecular weight protein was good and that of the low molecular weight proteins improved. The stacking gel system, both in the first dimension and second dimension concentrates proteins into their starting zones and the diffusion of smaller proteins was appreciably reduced.

The 40S subunit fraction obtained by zonal centrifugation (Figure 11) was pure and free of any 60S subunit contamination. Two dimensional polyacrylamide gel electrophoresis of 40S subunit protein revealed the presence of 32 protein spots as shown in Figure 13 a. 60S subunits isolated by zonal centrifugation were free of any contaminating 40S subunits. The extracted proteins were analyzed by two dimensional polyacrylamide gel electrophoresis under standard conditions. The protein pattern of 60S subunits is shown in Figure 13 b. The 60S subunit proteins resolved into 43 different protein spots.

3. Purification and characterization of CK I and CK II cyclic AMP independent protein kinases

In an attempt to purify cyclic AMP independent protein kinases, it became apparent that casein kinases are present in the post-ribosomal supernatant of cotyledon cytosol. It was previously observed (Hathaway and Traugh, 1979) that the initial ammonium sulfate precipitation of crude post-ribosomal supernatant eliminated the activity of CK I. Thus preliminary attempts to isolate these proteins was performed by eliminating the ammonium sulfate precipitation step. However, subsequent experiments using the ammonium sulfate precipitation step indicated
Figure 13. Two dimensional polyacrylamide gel electrophoresis of 40S (a) and 60S (b) ribosomal subunit proteins and their schematic representation. Details of preparation of subunit proteins and electrophoretic conditions are given in Materials and Methods. Direction of migration is indicated by arrow.
it did not in any way interfere with CK I activity. Thus all subsequent experiments involving purification of CK I and CK II were carried out after the initial ammonium sulfate precipitation step (see below).

Step 1: Separation of protein kinase by DEAE-cellulose Chromatography

Post-ribosomal supernatant (prepared as outlined in Chapter II) was made 6 mM with B-mercaptoethanol. The pH of the sample was adjusted to 5.0 with glacial acetic acid and the precipitate removed by centrifugation. The pH of the supernatant was adjusted to 7.0 by adding 0.5 N KOH and brought to 45% saturation by the slow addition of solid ammonium sulfate and stirred for 30 min at 4°C. The ammonium sulfate precipitate was collected by centrifugation and dissolved in TM buffer (10 mM Tris-HCl pH 7.7, 6 mM β-ME) and dialysed against TM buffer for 16 hours. Protein kinases present in the dialysed sample were adsorbed to hydroxyapatite by batch chromatography. Hydroxyapatite 5% (dry wt.) was suspended in TM buffer and the dialysed sample was added slowly to the slurry in a ratio of gel to protein (1:1) and stirred for 30 min. Unbound proteins were discarded by low speed centrifugation and the hydroxyapatite was washed twice with TM buffer. The protein kinase activity was eluted with 0.25 M potassium phosphate buffer pH 8.0. The elution was repeated twice. The combined eluates were dialysed against TM buffer containing 10% glycerol (TMG) for 12 hours, and concentrated to approximately 10-15 ml by vacuum dialysis. The concentrate was applied to a 2.5 x 20 cm DEAE-52 cellulose column previously washed and equilibrated in TMG buffer. The column was
Figure 14. Separation of casein kinase by DEAE-52 cellulose chromatography. Soybean cotyledon post-ribosomal supernatant, after pH 5.0 fractionation, ammonium sulfate precipitation and batch chromatography on hydroxylapatite was applied to a DEAE-52 cellulose column and chromatographed as outlined in the text. 325 fractions of 2 ml were collected and 50 µl of the aliquots were assayed for casein kinase (●—●) activities. The fractions were monitored at A_{280} for protein content (●—●) (0—0).
washed with one column volume of TMG and the protein kinase was eluted with a 700 ml linear gradient of 0.05-0.4 M NaCl in TMG. Absorbance of fractions at 280 nm was monitored and alternate fractions assayed for casein kinase as outlined in Chapter II.

The results of DEAE-cellulose chromatography of cAMP independent protein kinases are shown in Figure 14. Two peaks of cAMP independent protein kinase activity have been detected which phosphorylate casein and use ATP as phosphate donor. The enzymes were eluted from DEAE-cellulose in the range of 0.07 to 0.125 and 0.16 to 0.23 M NaCl and were designated casein kinase I (CK I) and casein kinase II (CK II) according to their order of elution. The CK I and CK II activities were pooled separately, dialysed against TMG buffer and concentrated by vacuum dialysis.

**Step 2. Phosphocellulose Chromatography**

The CK I and CK II fractions from the DEAE-cellulose column were rechromatographed on phosphocellulose columns (1.5 x 15 cm) previously equilibrated with 25 mM potassium phosphate buffer pH 7.0 containing 6 mM P-ME. Following sample application the columns were washed with two column volumes of buffer and enzyme activities were eluted with a linear gradient of buffer containing 0.2 - 1.5 M NaCl. Elution profiles of CK I and CK II from phosphocellulose columns are presented in Figure 15. CK I elutes as a single peak between 0.6-0.8 NaCl (Figure 15 a) and CK II elutes as a peak between 0.85-0.95 M NaCl (Figure 15 b).
Figure 15. Phosphocellulose chromatography of casein kinase I(a) and CK II (b). A sample from DEAE-cellulose column containing CK I and CK II was applied to a phosphocellulose column and the column was washed with 2 column volumes of equilibrating buffer. The protein was eluted with 300 ml linear gradient of 0.2-1.5 M NaCl in 25 mM phosphate buffer pH 7.0 (—) and 150 fractions of 2 ml each were collected. Of every third fraction 0.05 ml was removed and assayed for casein kinase activity (●—●). All fractions were also monitored at 280 nm to determine protein content (0—0).
Hathaway et al., (1979) have observed an anomalous behaviour of CK II on phosphocellulose columns where 75-90% of CK II activity does not bind to the column. They employed a second phosphocellulose column equilibrated at high salt concentrations to which most of the CK II binds in the purification of CK II from reticulocytes. We have successfully used a phosphocellulose column equilibrated with 25 mM potassium phosphate buffer without any difficulty. It may be possible that the initial pH 5.0 fractionation of post-ribosomal supernatant removes a high molecular weight contaminant that interferes with CK II purification by phosphocellulose under low salt concentrations (Hathaway and Traugh, 1979).

Step 3. Hydroxylapatite column chromatography

Pooled fractions of CK I and CK II from phosphocellulose columns were dialysed against 20 mM potassium phosphate buffer pH 7.9 containing 6.0 mM β-ME, concentrated by vacuum dialysis and applied to hydroxylapatite columns (1.5 x 20 cm) previously equilibrated with 20 mM phosphate buffer pH 7.9 containing 6.0 mM β-ME. The columns were washed with one column volume of buffer and CK I and CK II were eluted in 300 ml of a linear gradient of 50-500 mM phosphate buffer. Fractions were diluted 1:20 times before assay in order to decrease phosphate concentration, which is known to inhibit CK I and CK II activity when present higher than 20 mM (Hathaway and Traugh, 1979). Elution profiles of CK I and CK II are shown in Figure 16. CK I elutes between 200-300 mM potassium phosphate (Figure 16 a) and CK II elutes between 150-270 mM potassium phosphate (Figure 16 b).
Figure 16. Hydroxylapatite chromatography of CK I (a) and CK II (b). 10 ml of CK I equivalent to 65 mg protein and CK II equivalent to 80 mg protein respectively from the phosphocellulose columns were applied to separate hydroxylapatite columns and washed with one column linear gradient of 0.05-0.5 M phosphate buffer (---). 125 fractions of 2 ml were collected and 50 µl of aliquots were used in CK I and CK II assay (●—●). The fractions were monitored at A280 for protein content (0—0).
Cas. Kinase 1
Hydroxylapatite

Cas. Kinase 2
Hydroxylapatite

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The active fractions were pooled, dialysed against TMG and concentrated by vacuum dialysis.

**Step 4. Sephadex G-150 column chromatography**

Concentrated casein kinases from the previous step were applied to a Sephadex G-150 (fine) column (1.0 x 50 cm) equilibrated with TMG buffer and CK I and CK II were eluted in the same buffer. The fractions were assayed for casein kinase activities. The active fractions were pooled and concentrated. The proteins were purified considerably by employing this step and it has no effect on the subunit composition of CK I and CK II. The elution profiles of CK I and CK II from this step are shown in Figure 17.

**Step 5. Glycerol gradient centrifugation**

CK I and CK II from step 4 (approximately 150 ug and 120 ug respectively) were applied to a 12 ml glycerol gradient (10-40%) in TMG buffer. The gradients were centrifuged at 105,000 xg for 7 hours at 4°C in Beckman SW 41 rotor. Fractions of 0.3 ml were collected by pumping the gradient through an ISCO Model 614 gradient fractionator and CK I and CK II activity was assayed (Figure 18). It can be seen that both CK I and CK II elute as sharp, clear peaks. Only the relevant fractions showing the activity were pooled, concentrated by vacuum dialysis, dialysed against TMG buffer and used as the source of pure enzyme.

A summary of the steps of purification of CK I and CK II is
Figure 17. Sephadex G-150 chromatography of CK I (a) and CK II (b). Active fractions from hydroxylapatite column were pooled, dialysed against TMG buffer, concentrated to 5-6 ml (3.8 mg and 12.2 mg protein equivalent of CK I and CK II respectively), applied to Sephadex G-150 columns (1 x 50 cm) and eluted with 150 ml of TMG buffer. 70 fractions of 2 ml were collected, 10 µl of aliquots from alternate fractions were assayed for casein kinase activity (○—○). Fractions were monitored at $A_{280}$ for protein content (0—0).
Figure 18. Purification of CK I (a) and CK II (b) by sedimentation in glycerol gradients. Centrifugation conditions were as described in the text. Fractions of 0.3 ml were collected by pumping the gradient through ISCO gradient fractionator, 5 µl of aliquots were used in 50 µl of reaction mixture for determination of CK I and CK II activity (•—•). Fractions were monitored at $A_{280}$ for protein content (0——0).
presented in Tables 7 and 8. Since it was not possible to calculate CK I and CK II activity independently in the S 100, pH 5.0 fraction and the ammonium sulfate precipitate, the purification of CK I and CK II was calculated on the basis of activity observed after DEAE-cellulose chromatography. The CK I and CK II proteins were purified 350 and 285 fold respectively.

4. **Substrate specificity of CK I and CK II**

Results presented in Table 9 demonstrate that CK I and CK II phosphorylate casein efficiently. Ribosomes and ribosomal subunit proteins were also found to be phosphorylated. However, nearly 80-85% of the phosphorylation of ribosomes and ribosomal proteins obtained can be accounted for the phosphorylation of 40S ribosomal subunits and 40S subunit proteins. The order of efficiency of other proteins tested as substrates for CK I and CK II is: phosvitin, histone Type II A and bovine serum albumin. Phosphorylation of salt washed 80S ribosomes by CK I and CK II, was less efficient compared to low salt washed ribosomes.

5. **Effect of Nucleoside Triphosphates on CK I and CK II**

The effect of other nucleotide triphosphates - GTP, CTP, UTP was tested on casein phosphorylation by CK I and CK II and results are presented in Table 10. The nucleoside triphosphates were tested by a competition assay (using unlabelled nucleotide and labelled ATP) at 5 and 10 μM of GTP, CTP and UTP in the presence of 10 μM $^{32}$P-ATP. If other nucleotides are used for the phosphorylation of casein, it
Table 7. Summary of steps of purification of CK I from soybean cotyledons

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (units mg/protein)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DEAE</td>
<td>1.800</td>
<td>950</td>
<td>1</td>
</tr>
<tr>
<td>2. Phosphocellulose</td>
<td>65</td>
<td>12,400</td>
<td>13</td>
</tr>
<tr>
<td>3. Hydroxylapatite</td>
<td>3.8</td>
<td>28,500</td>
<td>300</td>
</tr>
<tr>
<td>4. Sephadex G-150</td>
<td>1.1</td>
<td>299,000</td>
<td>315</td>
</tr>
<tr>
<td>5. Glycerol gradient</td>
<td>0.05</td>
<td>332,000</td>
<td>350</td>
</tr>
</tbody>
</table>

a. One unit of enzyme activity is defined as the amount of enzyme required for the incorporation of one pmol of phosphate into casein per minute.

Casein kinase I was purified as described in the text from 100 gm (fresh weight) of 1 day old cotyledons.
Table 8. Summary of steps of purification of CK II from soybean cotyledon

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein mg</th>
<th>Specific Activity units mg/protein (^a)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DEAE</td>
<td>2400</td>
<td>1220</td>
<td>1</td>
</tr>
<tr>
<td>2. Phosphocellulose</td>
<td>89</td>
<td>23,450</td>
<td>19</td>
</tr>
<tr>
<td>3. Hydroxylapatite</td>
<td>12.2</td>
<td>212,000</td>
<td>174</td>
</tr>
<tr>
<td>4. Sephadex G-150</td>
<td>3.1</td>
<td>318,000</td>
<td>261</td>
</tr>
<tr>
<td>5. Glycerol gradient</td>
<td>0.04</td>
<td>347,000</td>
<td>285</td>
</tr>
</tbody>
</table>

\(^a\) One unit of enzyme activity is defined as the amount of enzyme required for the incorporation of one pmol of phosphate into casein per minute.

Casein Kinase II was purified as described in the text from 100 gm (fresh weight) of 1 day old soybean cotyledons.
### Table 9. Substrate Specificity of CK I and CK II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$^{32}\text{P}$-incorporated CK I</th>
<th>$^{32}\text{P}$-incorporated CK II</th>
</tr>
</thead>
<tbody>
<tr>
<td>80S ribosomes</td>
<td>$136^a$</td>
<td>$102^a$</td>
</tr>
<tr>
<td>80S ribosomes (Salt Washed)</td>
<td>$25^a$</td>
<td>$80^a$</td>
</tr>
<tr>
<td>60S ribosomal subunits</td>
<td>$11^a$</td>
<td>$73^a$</td>
</tr>
<tr>
<td>40S ribosomal subunits</td>
<td>$8^a$</td>
<td>$34^a$</td>
</tr>
<tr>
<td>80S ribosomal proteins</td>
<td>$46^b$</td>
<td>$1848^b$</td>
</tr>
<tr>
<td>40S subunit proteins</td>
<td>$48^b$</td>
<td>$1783^b$</td>
</tr>
<tr>
<td>60S subunit proteins</td>
<td>$38^b$</td>
<td>$146^b$</td>
</tr>
<tr>
<td>Casein</td>
<td>$1932^b$</td>
<td>$1635^b$</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>$567^b$</td>
<td>$370^b$</td>
</tr>
<tr>
<td>Histone Type II A</td>
<td>$9^b$</td>
<td>$12^b$</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>$1.3^b$</td>
<td>$9^b$</td>
</tr>
</tbody>
</table>

- $^a$ pmoles/A$_{260}$/min.
- $^b$ pmoles/mg protein substrate/min.

120 μg of CK I and 150 μg of CK II were used in assay.
Table 10. **Effect of Nucleoside triphosphates (GTP, CTP, UTP) on the Phosphorylation of Casein by $^{32}$P-ATP in the presence of CK I and CK II**

<table>
<thead>
<tr>
<th>Additions</th>
<th>CK I (pmol)</th>
<th>CK II (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None *</td>
<td>502 (100)</td>
<td>482 (100)</td>
</tr>
<tr>
<td>GTP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 $\mu$M</td>
<td>410 (81)**</td>
<td>445 (92)</td>
</tr>
<tr>
<td>10 $\mu$M</td>
<td>318 (63)</td>
<td>321 (66)</td>
</tr>
<tr>
<td>CTP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 $\mu$M</td>
<td>498 (99)</td>
<td>503 (104)</td>
</tr>
<tr>
<td>10 $\mu$M</td>
<td>508 (100)</td>
<td>490 (100)</td>
</tr>
<tr>
<td>UTP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 $\mu$M</td>
<td>518 (103)</td>
<td>479 (99)</td>
</tr>
<tr>
<td>10 $\mu$M</td>
<td>501 (100)</td>
<td>456 (95)</td>
</tr>
</tbody>
</table>

* 250 $\mu$g of Casein, 10 $\mu$mol of $^{32}$P-ATP, 2.5 $\mu$g CK I or 3.0 $\mu$g of CK II

** Figures in parentheses represent the per cent incorporation compared to control experiment with $^{32}$P-ATP alone.
would result in dilution of the isotope and cause an apparent
decrease in rate of $^{32}$P-incorporation into casein. It is clear
from the results presented in Table 6 that only GTP competes with
ATP as phosphate donor in the presence of both CK I and CK II. The
other nucleotides such as CTP and UTP do not show any reduction in
radioactivity incorporation. It can be assumed therefore that CK I
and CK II employ ATP and to a relatively lesser extent GTP as phos­
phate donor.

6. Molecular Weight and autophosphorylation of CK I and CK II

Both CK I and CK II were purified 350 and 285 fold respectively.
The molecular weight and the subunit composition of CK I and CK II
have been determined by polyacrylamide gel electrophoresis containing 0.1%
SDS (Laemmli, 1970). CK I migrated as a single peak with a molecular
weight of 39,000 (Figure 19,1 a) which suggests that it is a single
subunit enzyme. CK II resolved into 3 major bands of molecular
weight 52,000, 37,000 and 35,000 as shown in Figure 19, 2a. CK I and
CK II when phosphorylated in vitro in the presence of $^{32}$P-ATP undergo
self-phosphorylation. Separation of phosphorylated CK I on SDS poly­
acrylamide gel electrophoresis and subsequent autoradiography results
in a phosphorylated band of 39,000 daltons which coincides with the
coomassie blue stained band (Figure 19, 1 b). This suggests that
CK I undergoes self-phosphorylation and incorporates the phosphate into
its protein chain. When CK II was self-phosphorylated, separated on
SDS polyacrylamide gel electrophoresis and autoradiography per­
formed, the radioactivity was found associated mainly with the 35,000
Figure 19. SDS-polyacrylamide gel electrophoresis of purified CK I (1a) and CK II (2a) and autoradiography of autophosphorylated CK I (1b) and CK II (2b). Purified CK I (12 μg) and CK II (16 μg) were electrophoresed in SDS-polyacrylamide slab gel and autoradiography was performed as outlined in the text.
dalton protein band (Figure 19, 2b).

7. **Cyclic AMP-independent ribosomal protein kinase in ribosomal fraction from soybean cotyledons and its similarity to CK II**

Low salt washed ribosomes from soybean cotyledons when incubated in the presence of \(^{32}P\)-ATP and in the absence of any added protein kinase incorporate radioactive phosphate indicating the presence of endogenous protein kinase. However, when low salt washed ribosomes were incubated in buffer containing 0.8 M KCl for 3-4 hours at 4°C, most of the endogenous protein kinase activity in the resulting ribosome fraction was lost (Table I). This suggests that high salt washing removes protein kinase bound to the ribosomes. To identify the nature of ribosome associated protein kinase, ribosomal salt wash was assayed for phosphorylation of casein protein substrate. Results presented in Table I indicate that ribosome associated protein kinase phosphorylates casein efficiently compared to histone, suggesting cyclic AMP independent nature of ribosome associated protein kinase.

To ascertain further the relation of this ribosome associated casein kinase to CK I and CK II, the elution pattern of ribosomal casein kinase from DEAE-cellulose column was studied. Ribosomal salt wash fraction was dialysed with several changes of TMG buffer, and applied to a DEAE-cellulose column, following pH 5.0 fractionation and ammonium sulfate precipitation. Conditions of DEAE-cellulose chromatography were similar to those described for casein kinases in
Table 11. Phosphorylation of ribosomes, casein and histone by ribosomal salt wash

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$^{32}$P-incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>80S ribosomes</td>
<td>12.1$^a$</td>
</tr>
<tr>
<td>80S ribosomes (salt washed)</td>
<td>1.2$^a$</td>
</tr>
<tr>
<td>80S ribosomes (salt washed) and ribosomal salt wash</td>
<td>11.3$^a$</td>
</tr>
<tr>
<td>Casein and ribosomal salt wash</td>
<td>92.8$^b$</td>
</tr>
<tr>
<td>Histone and ribosomal salt wash</td>
<td>4.6$^b$</td>
</tr>
</tbody>
</table>

$^a$ - pmol/A$_{260}$

$^b$ - pmol/mg protein substrate
Chapter II. Fractions were assayed for cAMP independent protein kinase activity using casein and ribosomal kinase activity using salt-washed ribosomes as substrates. Results presented in Figure 20 demonstrate that both ribosomal kinase activity and casein kinase activity overlap indicating a similarity of ribosomal kinase with casein kinase II. No similar casein kinase I activity was observed on DEAE-cellulose chromatography of ribosomal salt wash. Fractions representing ribosomal kinase activity were pooled, dialysed against TMG buffer, concentrated, and further purified by passing through Sephadex gel filtration and glycerol gradient centrifugation. The behaviour of ribosomal kinase in these steps was identical to casein kinase II. The fractions representing ribosomal kinase from glycerol gradient centrifugation were used as the source of enzyme in further studies.

Effects of ribosomal casein kinase on the rate of phosphorylation of ribosomal proteins, casein, phosvitin, histone type IIA and bovine serum albumin were tested and the results presented in Table 12 indicate that phosphorylation of ribosomal protein was mostly accounted for by the phosphorylation of 40S ribosomal proteins. Casein was the only protein that undergoes phosphorylation compared to all other protein substrates tested.

8. Phosphorylation of ribosomal proteins by ribosomal casein kinase

40S ribosomal subunits were phosphorylated in vitro in the presence of ribosomal casein kinase. Ribosomal subunits were isolated, and the ribosomal subunit proteins were prepared as outlined in
<table>
<thead>
<tr>
<th>Substrate</th>
<th>$^{32}$P-incorporated pmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>80S ribosomal proteins</td>
<td>1500</td>
</tr>
<tr>
<td>40S ribosomal proteins</td>
<td>1383</td>
</tr>
<tr>
<td>60S ribosomal proteins</td>
<td>87</td>
</tr>
<tr>
<td>Casein</td>
<td>2240</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>35</td>
</tr>
<tr>
<td>Histone Type II A</td>
<td>28</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 20. Separation of ribosomal casein kinase on DEAE-52-cellulose. Soybean cotyledon ribosomal salt wash, after pH 5.0 fractionation, ammonium sulfate precipitation and batch chromatography on hydroxylapatite was applied to a DEAE-52 cellulose column and chromatographed as outlined in the text. Fifty µl of the aliquots were assayed for casein kinase (X—X) and ribosomal kinase (•—•) activities. Fractions were also monitored at 280 nm for protein content (0—0).
Chapter II. The proteins were separated on one dimensional polyacrylamide gel electrophoresis, stained, destained and the autoradiography was performed by exposing the gel to X-ray film (Chapter II). Autoradiograph of the 40S ribosomal subunit proteins is shown in Figure 21. It can be seen that 4 proteins in the 40S subunit were phosphorylated.
Figure 21. Phosphorylation of 40S ribosomal subunit proteins by ribosomal casein kinase. Ten A_{260} of 40S ribosomal subunit were phosphorylated \textit{in vitro} in the presence of \textsuperscript{32}P-ATP and ribosomal casein kinase. Proteins were extracted and separated on SDS-polyacrylamide slab gel. a) Coomassie blue stained gel and b) autoradiograph of the stained gel. Direction of migration is indicated by arrow.
Soybean Cotyledon Ribosomal Proteins

Eukaryotic ribosomes successfully dissociate into subunits under high salt concentrations of potassium ion (0.3 - 1.0 M). In the present investigation 0.6 M KCl was used to dissociate the ribosomes into 40S and 60S ribosomal subunits and the dissociated subunits were separated on hyperbolic sucrose gradients using zonal centrifugation (Eikenberry, et al., 1978). A total of 75 ribosomal proteins in the soybean cotyledons were observed. This number is considerably closer to the number of ribosomal proteins observed for other eukaryotes (Sheraton and Wool, 1974; Kanda et al., 1974; Martin and Gould, 1971; Liu and Wool, 1974; Otaka and Kobata, 1978; Sikorski et al., 1979).

Casein Kinase I and Casein Kinase II from Soybean Cotyledons

Based on the criteria listed for the classification of Protein Kinases from rabbit reticulocytes (Traugh and Traut, 1974), soybean cotyledon protein kinases could be categorized into type III (cAMP independent protein kinases), since they utilize both ATP and GTP to phosphorylate casein. They are referred in this study as Casein I (CK I) and Casein Kinase II (CK II) based on their elution pattern from DEAE cellulose column.

Procedures of purification employed were similar to steps employed for the purification of rabbit reticulocyte casein kinases (Hathaway and Traugh, 1979). However, under low ionic strength casein kinases tend
to aggregate (Kumar and Tao, 1975; Murray et al., 1978), therefore, gel filtration at high ionic strength was used in the purification of these enzymes. Casein kinases from soybean cotyledons clearly separable by DEAE cellulose chromatography, eluted into two discrete peaks, making possible the further purification without much cross contamination.

Soybean cotyledon CK I was observed to be a single subunit protein (M.W. 39,000), similar to CK I isolated from rabbit reticulocytes (M.W. 37,000) (Hathaway and Traugh, 1979), while a higher molecular weight has been reported for CK I of rat liver nuclei (M.W. 50,000) (Thornberg and Lindell, 1977) and Novikoff ascites cells (M.W. 57,000) (Dahmus, 1976). CK II is a multimeric protein containing 3 subunits of molecular weight 52,000, 37,000 and 35,000, and is in conformity with the observations made in CK II of rabbit reticulocytes (Hathaway and Traugh, 1979), rat liver nuclei (Thornberg and Lindell, 1977) and Novikoff ascites cells (Dahmus, 1976). Apparent high molecular weights observed for GTP:CK I and GTP: CK II of rabbit erythrocytes (Kumar and Tao, 1975) may be due to aggregation of these enzymes under low salt conditions.

Soybean cotyledon CK I and CK II undergo autophosphorylation. It is not known whether autophosphorylation leads to activation, as in the case of bovine cardiac muscle cAMP dependent protein kinase, where autophosphorylation leads to alteration in the rate of subunit reassociation (Farell et al., 1977; Rosen et al., 1977), inactivation of the enzymes or the existence of specific phosphatases involved in the
dephosphorylation of the phosphorylated species. However, it may be possible that existence of such a system leads to a fine regulatory mechanism similar to cAMP dependent protein kinase regulated enzymes. Autophosphorylation of casein kinases observed in rabbit reticulocytes (Hathaway and Traugh, 1979), rat liver (Thornberg and Lindell, 1977) and Novikoff ascites cells (Dahmus, 1976) and soybean cotyledons suggest that phosphorylation of casein kinases may be a general mechanism of regulation of these enzymes in eukaryotes. In plants neither the presence of cAMP, cAMP dependent protein kinases nor the operation of adenylcyclase system have been unequivocally demonstrated. Plant protein kinases have not been shown to have sensitivity to cAMP (Trewavas, 1976) in contrast to animal systems where the effect of cAMP is generally mediated by ubiquitous cAMP dependent protein kinases.

In animals calmodulin is another regulatory protein of the second messenger system mediating various cellular processes in a manner analogous to cAMP dependent protein kinases in handling of cAMP signal (Wang and Waisman, 1979). Recent demonstration of the presence of calmodulin (Taylor, W.A., personal communication) and operation of calmodulin in pea (Anderson and Cormier, 1978), cotton seed (Wallace and Cheung, 1979) and spinach leaves (Van Edlik, et al., 1980), indicate that calmodulin or a calmodulin type regulator protein, may be involved in modifying the activities of cAMP independent protein kinases in plants.

Neither the importance of cAMP independent protein kinase nor the
role of cAMP-dependent protein kinase in plants is completely understood. Phosphorylation of certain initiation factors from rabbit reticulocytes (Hathaway, et al., 1979) suggested the involvement of cAMP-independent protein kinase in the regulation of ribosomal activity, by modifying certain ribosomal proteins in reticulocyte system (Traugh and Traut, 1974) and in Pea and Lemna preparations (Keates, 1973; Keates and Trewavas, 1974). Protein kinase regulated protein synthesis in rabbit reticulocytes during heme deficiency and the identification of a hemin controlled repressor (HCR) as a cyclic AMP independent protein kinase (Tahara et al., 1978; Kramer et al., 1976 and Gross and Mendelewski, 1977) point out the importance of this group of enzymes.

Ribosomal Casein Kinase

Protein kinase associated with the ribosome has been reported in pea stem and Lemna (Keates and Trewavas, 1974) and rabbit reticulocytes (Issinger, 1977; Traugh and Traut, 1974). Low salt washed ribosomes from soybean cotyledons were found to contain a protein kinase. Cyclic AMP independent nature of the ribosome associated kinase was confirmed by its ability to phosphorylate casein and inability to phosphorylate histone protein, which is normally phosphorylated by cAMP dependent protein kinases (Traugh and Traut, 1974; Issinger, 1977).

The elution profile of ribosomal kinase from a DE-52 cellulose column is similar to the casein kinase II present in the post-ribosomal supernatant. The phosphorylation of 40S ribosomal subunits and 40S
subunit proteins by ribosomal casein kinase also provides additional evidence for the homology of ribosomal casein kinase with casein kinase II. This indicates that there is a spatial diversity of the distribution of casein kinases in soybean cotyledons. CK I is present as a soluble enzyme in the post-ribosomal supernatant, and CK II is present both as a soluble enzyme and in bound form found attached to ribosomes. CK II, in addition to phosphorylation of ribosomal proteins, may also be involved in the phosphorylation of non-ribosomal proteins. The phosphorylation of ribosome associated proteins by a cyclic AMP independent protein kinase has been recently isolated from Krebs II mouse ascites cells (Issinger and Reichert, 1979).

A number of reports exist which show that the 40S subunit is phosphorylated by cAMP independent protein kinase (Hammet and Key, 1977; Keates and Trewavas, 1974; Sikorski, et al., 1979; Trewavas, 1973). In the soybean cotyledon ribosomal casein kinase phosphorylates four proteins of the 40S ribosomal subunit. In the case of *Lemna*, *in vitro* phosphorylation of ribosomes resulted in the phosphorylation of at least one protein (Trewavas, 1973). In the case of wheat germ, Sikorski, et al., (1979) have demonstrated the phosphorylation of 5 proteins of the 40S subunit and four proteins of the 60S subunit when phosphorylated by a heterologous protein kinase. The position of one of the phosphorylated ribosomal proteins of soybean cotyledons resembles the *S*₆ ribosomal protein of wheat germ, but this assumption is only superficial since different protein kinases were used for phosphorylation.
and different electrophoretic conditions were employed for the separation of ribosomal proteins.

The phosphorylation of ribosomal proteins in plants and animals (Trewavas, 1973; Keates and Trewavas, 1974; Issinger, 1977), the phosphorylation of ribosome associated protein (Issinger and Reichert, 1979) and the phosphorylation of eukaryotic initiation factors (Issinger, et al., 1976) by cAMP-independent ribosomal casein kinase clearly suggests the important role of this enzyme in the regulation of cellular metabolism. It must be pointed out that ribosomal casein kinase plays a significant role since it brings about the phosphorylation of one of the important components of protein synthesizing machinery, namely ribosomes.
IX BIBLIOGRAPHY


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