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POLYSOME METABOLISM IN WINTER RYE (SECALE CEREALE CV PUMA)

by

André Laroche
Department of Plant Sciences

Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada
April 1987

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ABSTRACT

The effect of growth at low temperature on polysome metabolism in winter rye (Secale cereale cv Puma) has been investigated. A method for isolation of highly polymerized polysomes from mature leaf tissues has been developed. The degree of intactness of isolated polysomes was monitored by two independent but complementary methods: size class distribution on sucrose gradients and *in vitro* translation. The composition of the optimal polysome isolation buffer for mature rye leaves is similar for leaves grown at low (5°) or high (20°) temperature but different from that reported for young tobacco and pea leaves. The quantity of polysomes (per unit DNA) increases by a factor of 2.7 at low temperature. These polysomes are larger and their melting point is decreased by 3.7°. Analysis of ribosome composition by one- and two-dimensional electrophoresis revealed that only a few peripheral ribosomal proteins (and possibly a subunit of initiation factor 3) are modified. No significant change in the rRNA could be detected. Polysomes isolated from leaves grown at low temperature incorporate twice as much label as polysomes isolated from control plants, regardless of the temperature of translation. Polysomes from low temperature plants require higher magnesium levels for optimal translation and were more sensitive to detergent. Electrophoretic analysis of translation products revealed that some transcripts are newly expressed (16 kD to 170 kD), some are repressed (35

kD to 105 kD) and others increase or decrease in quantity. It is suggested that growth of rye at low temperature leads to alterations in both the ribosome conformation and peripheral proteins which in turn provides for a more efficient translation system. Enhanced translational activity does not appear to be due to the mRNA, although transcription does appear to be altered at low temperature. These changes are considered significant in the adaptation and growth of rye at low temperature.

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ABBREVIATIONS

A	adenine
ABA	abscisic acid
ATA	aurintricarboxilic acid
AUG	initiation codon
A_{235}	absorbance at 235 nanometer
A_{260}	absorbance at 260 nanometer
A_{280}	absorbance at 280 nanometer
BSA	bovine serum albumin
Ci	Curie
cpm	counts per minute
cv	cultivar
DEP	diethylpyrocarbonate.
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(ß-aminoethyl ether) N,N'tetraacetic acid
FW	fresh weight
G	guanidine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HP	polysomal size class of maximum absorbance
HPRI	human placental ribonuclease inhibitor
IEF	isoelectric focussing
kD	kilodalton

LP	large polysomes: hexamers and larger
M	monosomes
mA	milliampere
MB	membrane-bound
MF	membrane-free
Mr	relative molecular mass
mRNA	messenger RNA
NP-40	Nonidet P-40
P	polysomes: dimers and larger
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
poly-A'	polyadenylated
PMSF	phenylmethylsulfonylfluoride
RH	rye hardened
RNA	ribonucleic acid
RNase	ribonuclease
RNH	rye nonhardened
rpm	revolution per minute.
rRNA	ribosomal RNA
S	ribosomal subunit
SB	standard buffer
SP	small polysomes: dimers to pentamers
SDS	sodium dodecyl sulfate
T	total
TKMD	Tris, potassium, magnesium, DTT buffer
Tris	tris-hydroxymethylaminomethane
tRNA	transfert RNA

U	uridine
UAA	termination codon
UAG	termination codon
UGA	termination codon
1-D	one-dimensional
2-D	two-dimensional

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Growth and temperature.

The response of plants to the severity of their environment has occupied the man's attention long before the beginnings of the science of biology (Levitt, 1980). Temperature is one of the major environmental constraints governing the distribution of both wild and cultivated plants (Graham and Patterson, 1982). Effects of temperature on the development of plants have been observed for over a century (Levitt, 1980). Low temperature as well as high temperature has been shown to be detrimental to plant growth (Levitt, 1980) and to affect the growth kinetics of plants (MacDowall, 1974). Some plants, termed cold tolerant, are able to acclimate at low temperature by lowering the temperature at which the plant is damaged or killed. In others plants which are intolerant, the freezing tolerance is not lowered (Graham and Patterson, 1982).

The economic importance of cold acclimation studies should be obvious but is not always well recognized (Weiser, 1978). Since most crop plants are of tropical and subtropical origin and are usually cultivated up to their northern limit, an increase of 2°C in their degree of cold hardiness could extend their geographic distribution (Weiser, 1978). On the other hand, a decrease of only 1°C

globally would decrease rice crops by more than 40% (Weiser, 1982, 1978). Thus, there is a real practical importance to understanding the cellular mechanisms which allow plants to acclimate to low temperature.

1.2 Acclimation of plants to low temperature.

Generally, plants are cold acclimated by exposure to low temperature. Recently, the possibility of mimicking the low temperature effect, at least partly, has been reported by using the abscisic acid (ABA). The temperature at which plant were killed by freezing frost was decreased following an ABA treatment (Chen et al., 1983; Gusta et al., 1982; Orr et al., 1986).

Historically, two approaches have been used to study plant cold hardiness. One approch compares cold-acclimated with non-acclimated plants of the same species while the other compares different species within a genus with different inherent cold hardiness (Li, 1985). Unfortunately, a large amount of the cold hardiness literature is based on experiments in which mature plant material (eg., spinach or potato leaves) are subjected to short term low temperature treatments. In these treatments, no significant development occurs and the plant material is probably responding to a cold stress.

1.3 Low temperature and cellular metabolism.

Woody species have been used in early studies on the acquisition of cold hardening and an increase in RNA, protein, sugar and lipids content were noted in most cases (Gusta and Weiser, 1972; Levitt, 1980; Siminovitch *et al.*, 1968, 1967; van Huystee *et al.*, 1967). Later similar studies were extended to crop species with analogous results observed (Chen and Li, 1980; Chen *et al.*, 1983; Devay and Paldi, 1977; Gusta *et al.*, 1982; Kacperska-Palacz, 1978; MacDowall, 1974; Singh *et al.*, 1975; Trunova, 1982). Differences in the complement of proteins have also been reported between non-acclimated and acclimated species (Bixby and Brown, 1975; Brown, 1978; Brown and Bixby, 1975; Cloutier, 1983; Huner *et al.*, 1984; Kacperska-Palacz, 1978; Siminovitch *et al.*, 1968; Uemura and Yoshida, 1984). RNA or protein synthesis inhibitors have been shown to prevent cold acclimation (Chen *et al.*, 1983; Hatano, 1978; Hatano *et al.*, 1976; Kacperska-Palacz, 1978; Trunova, 1982). Since protein synthesis plays a major role in cell regulation, it is reasonable to expect that protein synthesis be involved in a metabolic process such as a development of cold hardiness (Brown, 1978). Despite the apparent importance of protein synthesis during cold acclimation, few studies have been directed toward the translational machinery. Ribosomes with a lowered melting point and a different complement of ribosomal proteins have been observed in cold-hardened black locust seedlings

(Bixby and Brown, 1975). However, contrasting results have been reported with regards to size class distribution of isolated polysomes and on amino acid incorporation. Larger polysomes have been observed in potato and barley leaves but not in mimosa epicotyls (Brown, 1972; Kenefick et al., 1974; Vigue et al., 1974). Similarly, protein synthesis has been reported to be activated in black locust tissues but to be decreased in wheat leaves (Brown, 1978; Rochat et Therrien, 1975). More recently, a decrease of polysomes activity and the expression of new genes products have been reported following a short exposure of plant seedlings to low temperature (Fehling and Weidner, 1986; Guy et al., 1986, 1985; Mahopatra et al., 1986; Meza-Basso et al., 1986). However, a single study on polysome melting point, size class distribution, composition and synthesizing activity in the same plant or same tissue following a cold acclimation has yet to appear.

1.4 Ribosomes and protein synthesis.

Since the effect of low temperature on ribosome function is to be addressed in this thesis, a brief review on ribosomes is presented. The only known function of ribosomes is to catalyze protein synthesis (Wool, 1979). In the cytoplasm of eucaryote cells, this organelle is composed by two subunits of 60 S and 40 S which are made up of proteins and rRNA. In plants, the larger subunit contains the 25 S, 5.8 S and 5 S rRNA and the small subunit

contains the 18 S rRNA (Davies and Larkins, 1980). The rRNA seems to be the scaffold on which the proteins are organized. In plants, the exact mechanism of protein synthesis is not known but it is thought to be very similar to that occurring in mammalian cells (Davies and Larkins, 1980; Weeks, 1981). In the absence of protein synthesis, the two subunits are separated. The decoding of the codons in mRNA occurs in three distinct phases. The three phases are initiation, elongation and termination. During the initiation step, the initiator species of methionine-tRNA (Met-tRNAi) binds to the 40 S subunit. Subsequently, the mRNA binds at the level of its initiation codon (AUG) to form a 40 S ribosome preinitiation complex. Finally, a 60 S ribosomal subunit couples to this complex to form a complete 80 S ribosomal initial complex. This 80 S complex with the Met-tRNAi at the P site is functional in peptide bond formation. At the elongation step, an aminoacyl-tRNA binds to triplet codon of mRNA at the A site. Then the formation of a peptide bound occurs between the two amino acids. Subsequently, this tRNA bearing the growing polypeptide chain is transferred from the A site to the P site with the concomitant movement of the ribosome on the mRNA by a distance of three nucleotides in the 5'---->3' direction. New cycles of elongation occur until the termination step. The codons UAA, UAG and UGA serve as signals for the termination of peptide chain elongation. At the termination step, the appropriate codon is thus

brought into position at the A site and a release factor is bound to the vacant A site. This allows the peptidyl transferase to cleave the bond between the tRNA and the terminal carboxyl group of the protein. This releases the finished protein. In a subsequent step, the mRNA is free to disengage from the ribosome and the two ribosomal subunits dissociate (Weeks, 1981).

When there is more than one ribosome traversing the same mRNA, such functional units are known as polyribosomes or polysomes (Davies and Larkins, 1980). The degree of polysome polymerization is usually analyzed by centrifugation of polysomes on sucrose gradient. Alterations in polysome size class distribution would reflect modification of the synthesizing activity of polysomes (Davies and Larkins, 1980; Gast <u>et al</u>., 1985).

There are two types of polysomes depending on their location in the cell. Some polysomes are found associated with endoplasmic reticulum membranes and form the rough endoplasmic reticulum. These polysomes are termed membrane-bound polysomes. Other polysomes appear free in the cytosol and are termed membrane-free polysomes. These membrane-free polysomes are in fact attached to the cyto-skeleton as revealed by studies using high voltage electron microscopy (De Robertis and De Robertis, 1980). Both membrane-bound and membrane-free polysomes form the total polysome fraction. Their relative proportions may vary depending on the tissue and the physiological stage of the

cell. A greater proportion of free polysomes rather than bound polysomes has been reported in plant cells and reticulocytes (Bielka, 1982; De Robertis and De Robertis, 1980; Pfisterer and Kloppstech, 1977). But a greater proportion of bound polysomes has been observed in plasma cells, exocrine pancreas and liver (De Robertis and De Robertis, 1980).

1.5 Winter rye (Secale cereale cv Puma) as a system for the study of low temperature effects.

Puma rye has been used for more than a decade to study responses to low temperature. Rye is characterized by its large increase of freezing tolerance during acclimation at low temperature. Rye has the highest known freezing tolerance among all the winter cereals. Futhermore, rye is sometimes used as a control in studies on acquisition of freezing tolerance involving different species or cultivar (Cloutier, 1983). Composition, function and development of chloroplasts have been thoroughly studied as a function of low temperature (see Huner, 1986 for a review). Differences in cell anatomy and morphology have also been observed (Huner et al., 1984, 1981). Rye has also been used for studies on the plasma membrane (Steponkus, 1985; Uemura and Yoshida, 1984) and freezing tolerance of isolated protoplasts and cells (Singh, 1981, 1979). Furthermore, and perhaps the most important point, the growth kinetics of rye grown at 5°C and 20°C have been

thoroughly documented (Krol et al., 1984). Consequently, it is possible to obtain material of predictable developmental stage regardless of the temperature at which the plants are grown.

Most of the work addressing acclimation at low temperature in the literature was done on mature leaves which are first grown at high temperature and then transferred to low temperature for period of time varying from a few days up to two or three weeks. This period of time is not long enough to allow growth of tissues but is long enough to induce necrosis in leaves. It has also been reported that leaves developed at 20°C but exposed at 5°C had a lower freezing tolerance and were senescing faster than leaves developed at 5°C (Krol et al., 1984). In many studies, the importance of testing plants of comparable developmental stage has been overlooked and the results obtained would reflect the capacities of tissues of differing developmental stages to fully adapt to low temperature. With rye these problems are circumvented. The results should then reflect changes due to acclimation and subsequent development at low temperature.

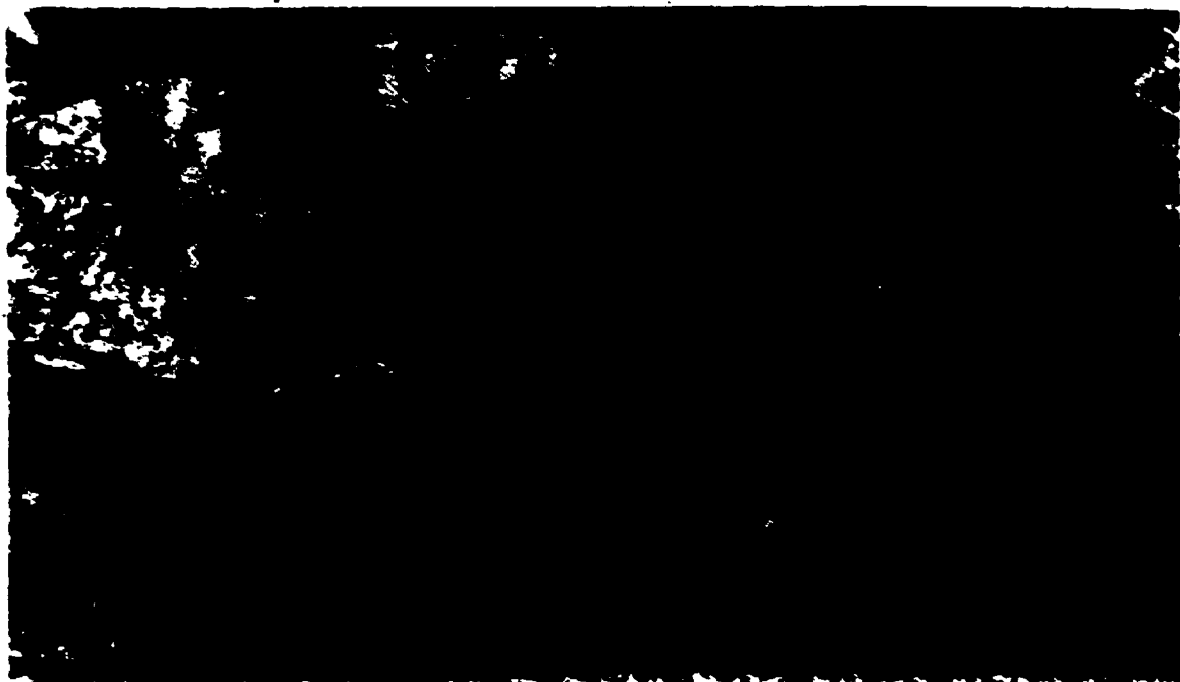
1.6 Proposed research and thesis objectives.

The objective of the research described in this thesis is to study the effect of growth at low temperature on the composition, function and translation products of polysomes isolated from rye. There were two main reasons for

initiating this research. First, my curiosity was stimulated by the observation showing a large increase of cytoplasmic polysomes in micrographs of tissues isolated from leaves grown at low temperature when compared to tissues isolated from leaves grown at higher temperature. In addition, polysomes in leaves grown at 5°C appeared larger than those in leaves grown at 20°C (Fig 1). Secondly, although it has been suggested that protein synthesis is affected during cold acclimation, there has been no systematic study addressing the effect of low temperature on polysomes metabolism.

Since intact polysomes are required for the subsequent study it was first necessary to optimize methods for isolation of intact cytoplasmic polysomes from mature rye seedlings grown at normal and low temperatures. In most, if not all, previous studies polysomes had been isolated from young plants and etiolated leaves. The rationale for using young materials was that RNase activity is known to increase with the age of leaves. Having defined the conditions necessary for isolating polysomes with a minimum of disruption, the second objective is to determine whether growth at low temperature affects polysome composition and function. Analysis of quantity, purity, size class distribution, melting point, ribosomal protein, rRNA and *in vitro* synthesizing activity of polysomes will be carried out. Finally, translation products will be resolved by 1-D and 2-D PAGE and revealed by fluorography in order to

Figure 1. Electron micrographs of rye leaves grown at 5°C (RH) and 20°C (RNH). Arrows indicate polysomes. The magnification is different for RNH and RH tissues.



determine whether gene expression is altered during growth at low temperature.

CHAPTER 2

ISOLATION AND *IN VITRO* TRANSLATION OF RNH AND RH POLYSOMES FROM THE LEAVES OF MATURE RNH AND RH SEEDLINGS

2.1 INTRODUCTION

Plant polysome metabolism is most often studied in young, immature leaf tissues which appear to yield polysomes with the least amount of degradation (Arglebe and Hall, 1969; Breen *et al*., 1971; Davies *et al*., 1972; Erkeev and Kudoyarova, 1981; Fehling and Weidner, 1986; Gray and Kekwick, 1974; Hogan *et al*., 1985; Jackson and Larkins, 1976; Larkins and Davies, 1975, 1973; Leaver and Dyer, 1974; Lin *et al*., 1966; Pearson, 1969; Ramagopal and Hsiao, 1973). The variety of tissues used for the isolation of polysomes is reflected in the diversity in the composition of isolation media employed (Anderson and Key, 1971; Arglebe and Hall, 1969; Brady and Scott, 1977; Breen et *al*., 1971; Davies *et al*., 1972; Dunlop and Walden, 1985; Erkeev and Kudoyarova, 1981; Fehling and Weidner, 1986; Gonzalez, 1980; Gray and Kekwick, 1974; Hogan *et al*., 1985; Jackson and Larkins, 1976; Larkins and Tsai, 1977; Leaver and Dyer, 1974; Lin *et al*., 1966; Pearson, 1969; Ramagopal and Hsiao, 1973). Often the media used for isolation of plant polysomes (Anderson and Key, 1971; Davies *et al*., 1972; Gray and Kekwick, 1974; Hogan *et al*., 1985; Lin *et al*., 1966; Pearson, 1969) are similar to those used for

mammalian tissues (Dunn, 1970; Heikkala and Brown, 1981; Venkatesan and Steele, 1971). However, polysomes isolated under these conditions show only a small proportion of large polysomes. A systematic study with young pea leaves has focused on improving the degree of polysome polymerization by reducing ribonuclease activity (Davies et al., 1972; Larkins and Davies, 1975) or by chelating divalent cations (Larkins and Davies, 1973). A study of etiolated barley seedlings has demonstrated the importance of KCl concentration and reducing agent (Breen et al., 1971). Thus it appears that methods for plant polysome isolation vary widely, depending on the tissue chosen and other factors. They are not in all cases interchangeable (Arglebe and Hall, 1969). It may be desirable for the purposes of some physiological studies to examine the metabolic role of polysomes in older plant tissues (Brady and Scott, 1977). Requirements may vary with the physiological age or status of the tissues to be extracted (Brady and Scott, 1977). It is known, for example, that ribonuclease activity increases with age (Ramagopal and Hsiao, 1973) and successful isolation of highly polymerized polysomes from older tissue may require even greater protection.

The degree of polysome polymerization will reflect the extent to which native mRNA is protected during isolation, although it is not clear to what extent *in vitro* translation is dependent on polysome polymerization. In a

study with maize (Ramagopal and Hsiao, 1973), in vitro amino acid incorporation did not appear to be affected by the degree of polysome integrity. On the other hand, animal studies have shown latent ribonuclease activity to be sufficient to inactivate in vitro translation (Dunn, 1970) and that in vitro amino acid incorporation increased when polysome populations with a higher proportion of large polysomes were used (Nowak et al., 1984). The traditional criterion for characterizing polysome integrity is the proportion of large polysomes (LP, hexamers and larger) relative to small polysomes (SP, dimers to pentamers) (Jackson and Larkins, 1976). While it may be assumed that the ratio LP/SP correlates with the accuracy with which polysomal in vivo protein synthesis is reproduced in vitro (Nowak et al., 1984), this correlation has not been clearly demonstrated for plant tissues (Davies and Larkins, 1980).

In the present part of this study on polysomes isolated from rye seedlings grown at low and high temperature but of comparable developmental stage, conditions necessary for isolation of highly polymerized polysomes from leaves of relatively old (20-22 days) rye seedlings were examined. In addition, polysomal profiles were correlated with kinetics of incorporation and SDS-PAGE analysis of the products of polysomal protein synthesis. Finally, size class distribution was different between RNH and RH polysomes.

2.2 MATERIALS AND METHODS

2.2.1 Plant Materials.

Winter rye seedlings (Secale cereale L. cv. Puma) were grown in vermiculite at low (5°C) or high (20°C) temperature at a light intensity of 200 µmol quanta $m^{-2}s^{-1}$ with a photoperiod of 16 h. The day/night temperature cycles were 5°C/5°C and 20°C/16°C respectively. Seedlings grown at low temperature were initially germinated for 7 days at 20°C then subsequently transferred to the growth regime of 5°C for 7 to 8 additional weeks. Plants were watered daily to replace water lost by evapotranspiration. Under these growth conditions, the rye seedlings (RH) were at their maximum of cold hardiness as demonstrated by Krol et al. (1984). Control, non hardened plants (RNH) were grown for a total of 20-24 days at the high temperature regime. In a systematic study by Krol et al. (1984), these RH and RNH plants have been shown to be of comparable developmental stage.

Triticale seedlings (OAC wintri, a hexaploid triticale variety) were grown at low temperature exactly as described for RH seedlings. Corn coleoptiles were isolated from corn seedlings (Zea mays cv Seneca 60) germinated on a moistened filter paper into a petri dish for 5 days at 31°C in darkness.

2.2.2 **Polysome isolation.**

Regardless of the composition of the medium, the following protocol was used for isolation of polysomes.. All glassware was sterilized or baked and all solutions sterilized. All work was carried out at 0-4°C. Disposable gloves were worn in order to minimize contamination of the extract with exogenous ribonuclease. Only the uppermost fully expended leaves were harvested from RNH and RH seedlings as previously described by Krol et al., (1984). Contamination by chloroplast ribosomes was minimized by homogenizing the leaves in a Waring blender (3x5 s bursts) with liquid nitrogen as reported by Rhodes and Kung (1981). After the liquid nitrogen evaporated and as soon as the temperature of the pulverized leaves reached -4°C to 0°C, the liquid nitrogen powder was resuspended in the appropriate buffer solution (3 ml/g fresh weight). In addition, isolation media included a high concentration of osmoticum (0.6 M for RNH or 0.9 M for RH) in order to further minimize chloroplast rupture and contamination by chloroplast polysomes as suggested by Arglebe and Hall (1969) and Ramagopal and Hsiao (1973). Preliminary work also indicated that it was easier to remove the chloroplasts in the presence of sorbitol rather than sucrose, probably because of the lower density of the sorbitol solution (1.036 g cm^{-3} vs 1.081 g cm^{-3}).

The homogenate was filtered through one layer of Miracloth and centrifuged at $5,000xg_{max}$ (10 min; HB-4

rotor) to remove the chloroplast fraction. This supernatant was the source of total polysomes. When membrane-free or membrane-bound polysomes were required, membrane-free (MF) polysomes were separated from membrane-bound (MB) polysomes by centrifugation of the 5,000xg_{max} supernatant at 81,500xg_{av}, 15 min (SW 41 rotor) as described (Davies et al., 1972). The MB polysomal pellet was resuspended in the extraction buffer. In order to free the polysomes from the membranes in total or MB, their membranes were solubilized with Triton X-100 and sodium deoxycholate; both at 1% (w/v) final concentration. To ensure an efficient recovery of MB polysomes from the membranes the samples were treated with 4 strokes of a glass-Teflon homogenizer. The detergent-insoluble fraction was removed by centrifugation (24,000xg_{max}, 10 min, SS-34 rotor). The 24,000xg_{max} supernatant was the source of MB or total polysomes. A polysomal pellet was obtained from MF, MB and total polysome fractions by centrifugation over a 1.5 M sucrose (RNase free) cushion (4 ml) containing 40 mM Tris-HCl, 10 mM $MgCl_2$, 20 mM KCl, 5 mM EGTA, 5 mM DTT, pH 8.5 (Solution B). Centrifugation was at 145,000xg_{max} for 4 hours. The supernatant was decanted and the tube walls washed with sterilized water and dried with a Kimwipe. The polysomes were resuspended in Solution B without sucrose and separated on a 12%-50% (w/v) linear sucrose gradient (40 mM Tris-HCl, 10 mM $MgCl_2$, 20 mM KCl; pH 8.5) for 90 min at 260,000xg_{max}. The sucrose gradients

were analyzed with a UV monitor (ISCO model UA-5, 254 nm) attached to an ISCO model 640 gradient fractionator. A scheme of polysome isolation is also presented (Fig. 2).

Under these isolation conditions, the degree of contamination of RNH and RH cytoplasmic polysomes by chloroplast polysomes was at the most 15%. Given the consideration that chloroplast polysomes have a lower proportion of large polysomes (Brady and Scott, 1977) and require a much higher Mg^{++} concentration for *in vitro* translation (Boardman et al., 1966) this degree of contamination was judged acceptable for the experiments to be carried out.

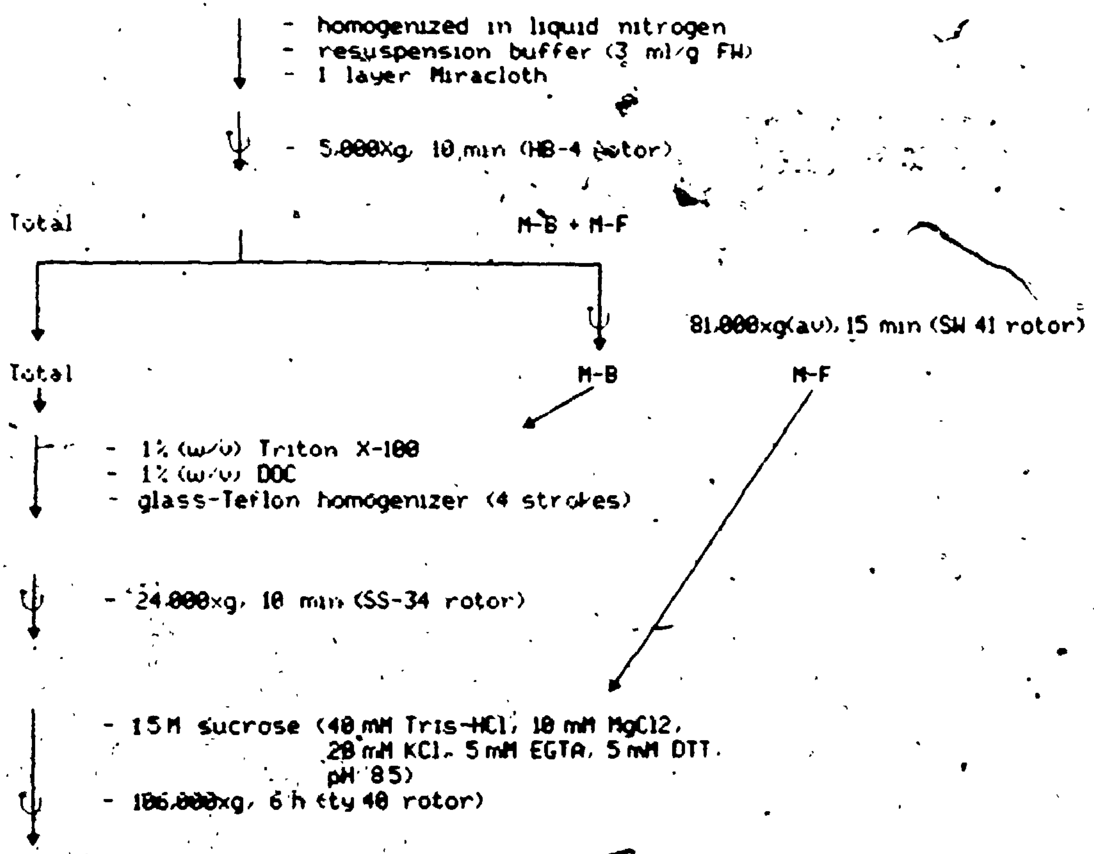
2.2.3 Polysome size class distribution.

Size distribution of polysomes was determined as the area under the curve in different regions of the polysomal profiles, using a LI-COR area meter (LAMBDA Instrument Corporation model LI-3000). These different regions are: the ribosome subunits (S), the monosomes (M), the small polysomes (SP) (material sedimenting faster than the monosomes but slower than hexamers), the large polysomes (LP) (material sedimenting faster than the pentamers), the polysomes (P) where P = SP + LP and the total (T) where T = S + M + P. Each profile was corrected for baseline, monitored with a blank gradient as reported by Davies *et al.*, (1972). From this data, the following ratios were determined: S/T, M/T, SP/T, LP/T, P/T and LP/SP. This last

Figure 2. Flowchart for polysome isolation.

Polysome isolation (Total, M-B, M-F)

Rye leaves



Polysomes

amount | translation

size class distribution

ratio has been reported the best criterion by which to describe polysome integrity, since the magnitude of the ratio indicates the extent of polysome preservation (Jackson and Larkins, 1976).

2.2.4 **Polysome purity.**

Polysome purity was monitored by measuring absorbance at 235, 260 and 280 nm (Petermann, 1964). All readings were corrected for Rayleigh light scattering (Freifelder, 1982). Values for 260/280 and 260/235 ratios higher than 1.6 and 1.4 respectively have been reported by Jolicoeur and Brakier-Gingras (1983), Leaver and Dyer (1974) and Venkatesan and Steele (1971) to indicate acceptably contaminant-free polysome preparations. The contaminants usually reported are cytoplasmic proteins adsorbed to polysomes.

2.2.5 **Polysome estimation.**

The quantity of polysomes was estimated using an $E^{0.1\%}_{260}$ = 11.1 (Martin et al., 1969) (1 A_{260} unit = 90 µg of polysomes).

2.2.6 **Cycloheximide, diethylpyrocarbonate and RNase treatments.**

Cycloheximide was added to the high Tris buffer prior to the resuspension of the liquid nitrogen powder. Concentration of 20 µg/ml and 50 µg/ml of buffer were

respectively used for isolation of RNH and RH polysomes. Diethylpyrocarbonate (DEP) was added to the high Tris buffer prior to the resuspension of the liquid nitrogen power at a concentration of 20 μg/ml of buffer. Polysomes were treated with both RNases A and T1 or with RNase A alone. With both RNases, 10 μg of each were added to 90 μg of polysomes resuspended in solution B without sucrose as described in section 2.2.2. The mixture was incubated for 12 min at 35°C, cooled down and loaded on the sucrose gradient for the separation of the different size classes of polysomes. In the second case, 2 μg of RNase A were added to 90 μg of polysomes resuspended in solution B without sucrose, incubated for 10 min at 25°C and 10 min at 4°C then loaded on the sucrose gradient. This second treatment is less severe than the first one.

2.2.7 Wheat germ extract preparation.

Wheat germ was purchased at a local mill and stored desiccated at 4°C. Extraction was carried out on ice in a cold room at 5°C (Reisfeld and Edelman, 1982). Wheat germ (6 g) was mixed with 6 g of glass powder and ground for one minute with a prechilled mortar and pestle. Fifteen ml of a solution containing 100 mM potassium acetate, 1 mM magnesium acetate, 6 mM β-mercaptoethanol (added fresh), 1 mM Hepes-KOH (pH 6.6), 0.1 mM PMSF were added and the extract was ground for an additional minute. The slurry was centrifuged (30,000xg_{max}, 10 min, SS-34) and the middle

third of the supernatant fraction was removed from the centrifuge tube. Twenty µl of 1 M Hepes-KOH (pH 7.6) was added per ml of supernatant to bring up the final concentration of Hepes to 20 mM. This extract was then applied to a Sephadex G-25 (medium) column (37 cm x 2.6 cm) equilibrated with 20 mM Hepes-KOH (pH 7.6), 120 mM potassium acetate, 5 mM magnesium acetate, 0.25 mM EDTA, 15 mM β-mercaptoethanol and 0.1 mM PMSF. The appropriate high molecular weight fraction was collected and centrifuged at 105,500xg_{av} for 4 hours (Spinco type 40 rotor) (Nirenberg and Matthaei, 1961;) to obtain the S-105 fraction. This high-speed supernatant (S-105) was divided into aliquots and stored in liquid nitrogen.

2.2.8 In vitro translation.

For *in vitro* translation, the polysomal pellets were resuspended in 50 mM Hepes-KOH (pH 7.8) containing 100 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM EDTA and 5 mM DTT. Translation in a cell-free protein-synthesizing system derived from wheat germ (Reisfeld and Edelman, 1982) was performed in sterile 0.5 ml microcentrifuge tubes. A typical reaction mixture in a final volume of 50 µl contained: 25 mM Hepes-KOH (pH 7.6), 3.5 mM magnesium acetate, 120 mM potassium acetate, 5 mM DTT, 1.3 mM ATP (disodium salt), 0.25 mM GTP (sodium salt), 15 mM creatine phosphate, 2 µg creatine phosphokinase (type 1), 0.66 mM spermidine, 25 µM each of 19 L-amino acids (without

methionine), 12.5 µCi of $[^{35}S]$methionine (>1000 Ci/mmol), 48.9 µM (non-radioactive) methionine, 2 µg of wheat germ tRNAs, 10 µl of the S-105 wheat germ extract and 45 µg of polysomes (20 µg of RNA). The reaction was carried out for 90 min at 25°C. Preliminary work showed the necessity of including the non-radioactive methionine in order to insure that the added polysomes, not methionine, was the limiting factor in the translation. The addition of human placental ribonuclease inhibitor (HPRI, 50 units) did not influence the total number of counts incorporated or the protein pattern after SDS-gel electrophoresis. This translation mixture used is also similar to the one reported by Morch et al. (1986) to obtain the synthesis of high molecular weight proteins in vitro using the wheat germ translation system. The in vitro translation was also carried out with the rabbit reticulocyte lysate system (Promega Biotec) accordingly to the manufacturer instructions. The translation was carried out in a 25 µl volume into a 0.5 ml microcentrifuge tube. To 17.5 µl of lysate were added 0.5 µl of 1 mM amino acid mixture (minus methionine), 2.5 µl of $[^{35}S]$methionine (>1000 Ci/mmole, 2.5 µCi/µl), 1.0 µl of polysomes (1 µg/µl), 3.0 µl of H_2O and 0.5 µl of 2.0 M KCl. The temperature of translation was 30°C for 60 or 85 min whether the translation were used for PAGE analysis or a time course experiment was carried on. In addition, in the case of time course experiments, 1.66 µl of 0.2 M

non-radioactive methionine was added to the translation mixture and the water volume was decreased concomitantly.

Incorporation of [^{35}S]methionine into polypeptides during the _in vitro_ reaction was measured by TCA precipitation of 2 or 5 µl aliquots on small paper disc (S & S #57-GH, 12.7 mm diameter) as reported by Mans and Novelli (1961).

2.2.9 Gel electrophoresis.

The translation products were separated by SDS-PAGE on a 7.5-17.5% linear gradient using the Laemmli discontinuous buffer system (Piccioni _et al_., 1982). Following electrophoresis, the gels were stained with Coomassie Blue R-250 and destained (Piccioni _et al_., 1982). The following protein standards were used: phosphorylase B, 94 kD; albumin serum bovine, 68 kD; ovalbumin, 45 kD; carbonic anhydrase, 29 kD; soybean trypsin inhibitor, 20 kD; cytochrome c, 12.5 kD.

2.2.10 **Fluorography.**

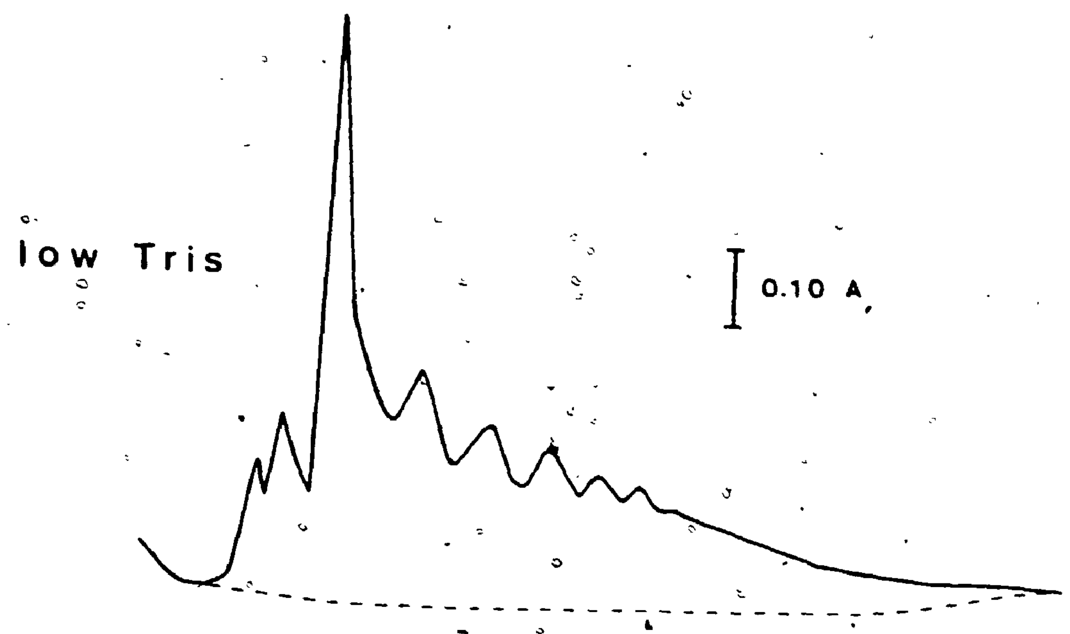
After destaining, gels were prepared for fluorography by treatment with PPO in DMSO prior to drying (Bonner, 1984; Laskey and Mills, 1975). Fluorography was performed on dry gels using preflashed Kodak XAR-5 X-ray film (Bonner, 1984; Laskey and Mills, 1975). The film was exposed at -70°C for an appropriate interval.

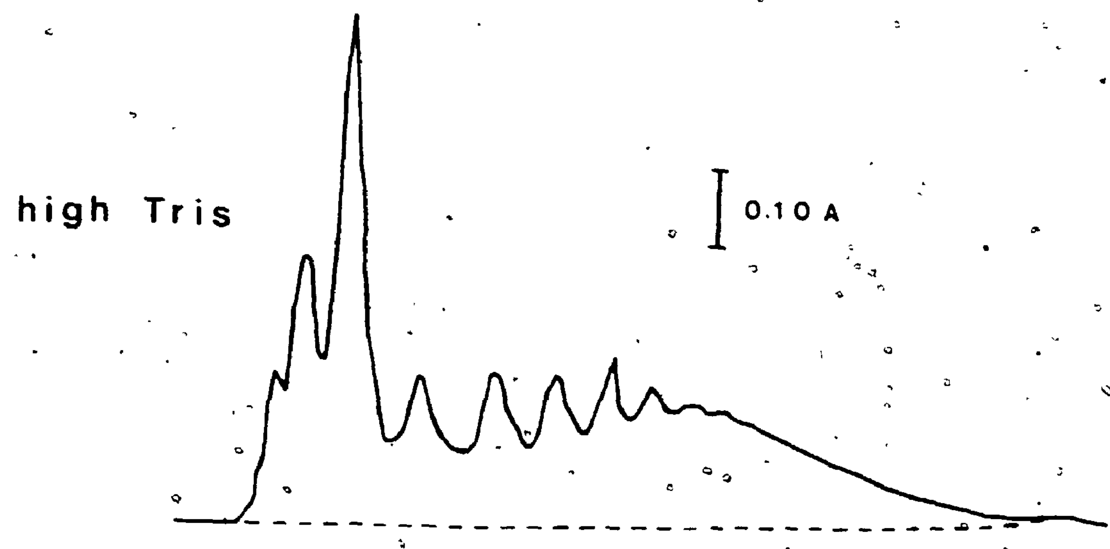
2.3 RESULTS

2.3.1 The effects of Tris concentration.

Two buffers differing by their Tris concentration (low and high) were selected in order to compare their efficacy for isolation of highly polymerized polysomes from mature RNH leaves. The low Tris buffer contained 0.25 M sucrose, 50 mM Tris-HCl, 250 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, pH 7.4, and is identical to the one designated TKMD and used previously for isolation of polysomes from animal and plant tissues (Hogan et al., 1985; Venkatesan and Steele, 1971). The high Tris buffer contained 200 mM Tris-HCl, 0.6 M sorbitol (0.9 M for RH), 60 mM KCl, 30 mM $MgCl_2$, 25 mM EGTA and 2 mM DTT, pH 8.5, and is similar to the one used for polysome isolation from tobacco leaves and corn kernels (Jackson and Larkins, 1976; Larkins and Tsai, 1977). The initial results (Fig. 3 and Table 1) show that the high Tris buffer is the more efficient buffer for isolation of polysomes from 20-22 day old rye leaves. The proportion of LP and the LP/SP ratio are both higher with the high Tris buffer. The polysome yield was markedly lower when RNH polysomes were isolated with the low Tris buffer. In the case of RH polysomes isolated with the high Tris buffer, although the yield was similar to the one obtained for RNH polysomes, the proportion of LP and the LP/SP ratio were higher (Fig. 4 and Table 2). In addition to a high Tris concentration, DEP, cycloheximide, EGTA, DTT, $MgCL_2$, KCl

Figure 3. Size class distribution of RNH polysomes isolated with low Tris, high Tris or standard buffer (SB).


low Tris
0.10 A


high Tris
0.10 A

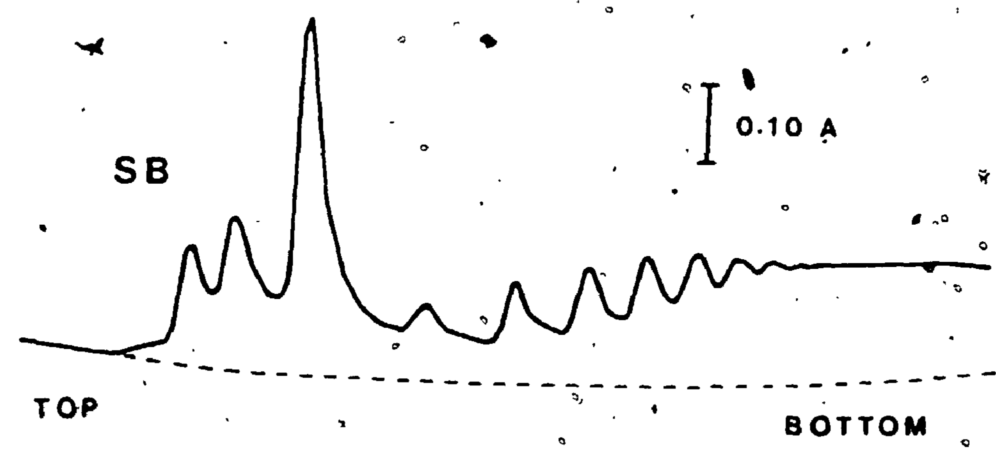
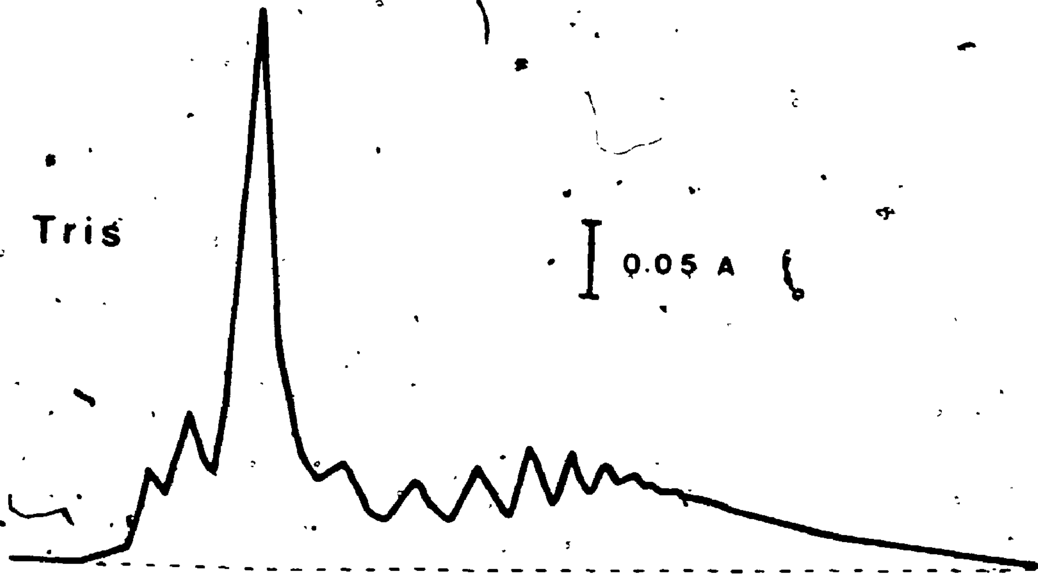

SB
0.10 A
TOP
BOTTOM

Table 1. Effects of three different resuspension buffers on the proportion of different size classes of RNH polysomes.

	low Tris	high Tris	SB
S/T	0.10	0.18	0.12
M/T	0.31	0.31	0.22
SP/T	0.37	0.23	0.23
LP/T	0.22	0.28	0.43
	1.00	1.00	1.00
P/T	0.59	0.51	0.66
LP/SP	0.58	1.22	2.01
HP	2	5	6
yield µg T/g FW	74	172	185
A_{260}/A_{280}	1.80	1.73	1.81
A_{260}/A_{235}	1.69	1.57	1.71

Figure 4. Size class distribution of RH polysomes isolated with high Tris or standard buffer (SB).


high Tris
0.05 A

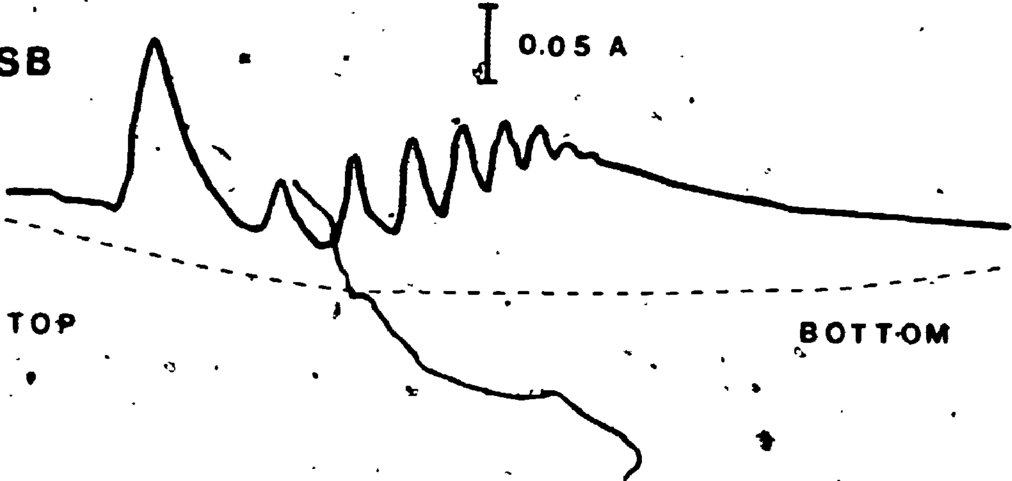

SB
0.05 A
TOP
BOTTOM

Table 2. Effects of two different resuspension buffers on the proportion of different size classes of RH polysomes.

	high Tris	SB
S/T	0.10	0.07
M/T	0.36	0.18
SP/T	0.21	0.23
LP/T	0.33	0.52
	1.00	1.00
P/T	0.54	0.75
LP/SP	1.58	2.27
HP	5	6
yield µg T/g FW	198	682
A_{260}/A_{280}	1.68	1.71
A_{260}/A_{235}	1.41	1.61

and high pH have also been reported for the preservation of polysome polymerization.

2.3.2 DEP, cycloheximide and RNases.

The influence of DEP and cycloheximide on the isolation of rye polysomes was examined since both have previously been recommended for plant polysome isolation (Anderson and Key, 1971; Breen et al., 1971; Weeks and Marcus, 1969). It was not possible to obtain significant quantities of large polysomes with the addition of DEP while the addition of cycloheximide at a concentration of 20 µg/ml or 50 µg/ml did not improve polysome profiles (Tables 3 and 4). In order to demonstrate that this method was effective in isolating polysomes rather than ribosomal aggregates, both RNH and RH polysomal fractions were treated with RNase(s). Following these treatments, subunits and monosomes rather than polysomes were observed on the sucrose gradient, as shown by the size class distribution (Table 4).

2.3.3 The effects of EGTA and DTT concentrations.

The effects of EGTA and DTT on polysome isolation from mature RNH and RH leaves are shown in Tables 5-8. Highest LP/SP and P/T ratios were obtained in the presence of 10 mM DTT with an EGTA concentration in the range of 12.5 mM to 25 mM for RNH polysomes (Table 5) although that range was between 0 and 25 mM for RH polysomes (Table 6). Increasing

Table 3. Effect of cycloheximide on the proportion of different size classes of RNH and RH polysomes isolated with high Tris buffer. Concentrations of 20 μg/ml and 50 μg/ml of cycloheximide were used for RNH and RH polysomes respectively.

	RNH	RH
S/T	0.26	0.15
M/T	0.31	0.37
SP/T	0.19	0.22
LP/T	0.23	0.26
	1.00	1.00
P/T	0.42	0.48
LP/SP	1.19	1.18
HP	2	5
A_{260}/A_{280}	1.73	1.66
A_{260}/A_{235}	1.49	1.67

Table 4. Effects of DEP, RNases A plus T1 and RNase A alone on the proportion of different size classes of RNH and RH polysomes isolated with high Tris buffer in the presence of 10 mM DTT or SB. With both RNAses, 10 µg of each were added to 90 µg of RNH polysomes. For the second treatment, 2 µg of RNase A were added to 90 µg of RNH or RH polysomes.

	RNH DEP	RH RNases	RH RNase A	RNH RNase A
S/T	0.36	0.93	0.20	0.34
M/T	0.33	0.07	0.43	0.51
SP/T	0.26	—	0.34	0.15
LP/T	0.05	—	0.03	--
	1.00	1.00	1.00	1.00
P/T	0.31	—	0.37	0.15
LP/SP	0.19	—	0.09	—

Table 5. Effect of EGTA concentration on the proportion of different size classes of RNH polysomes isolated with high Tris buffer in the presence of 10 mM DTT.

	EGTA (mM)				
	0	12.5	25	37.5	50
S/T	0.08	0.14	0.18	0.30	0.80
M/T	0.28	0.30	0.32	0.13	0.16
SP/T	0.32	0.22	0.20	0.23	0.04
LP/T	0.32	0.34	0.30	0.34	0.00
	1.00	1.00	1.00	1.00	1.00
P/T	0.64	0.56	0.50	0.57	0.04
LP/SP	1.00	1.52	1.50	1.45	—
HP	2	5	7	5	—
A_{260}/A_{280}	1.80	1.81	1.74	1.82	2.11
A_{260}/A_{235}	1.71	1.67	1.64	1.06	1.65

Table 6. Effect of EGTA concentration on the proportion of different size classes of RH polysomes isolated with high Tris buffer in the presence of 10 mM DTT.

	EGTA (mM)				
	0	12.5	25	37.5	50
S/T	0.04	0.07	0.12	0.12	0.27
M/T	0.25	0.33	0.25	0.25	0.14
SP/T	0.22	0.17	0.19	0.24	0.23
LP/T	0.49	0.43	0.44	0.39	0.36
	1.00	1.00	1.00	1.00	1.00
P/T	0.71	0.60	0.63	0.73	0.59
LP/SP	2.19	2.46	2.26	1.63	1.56
HP	5	6	5	2	4
A_{260}/A_{280}	1.73	1.72	1.73	2.03	1.72
A_{260}/A_{235}	1.61	1.59	1.53	1.60	1.49

the concentration of DTT to 15 mM in the presence of 25 mM EGTA significantly improved the LP/SP ratio for both RNH and RH polysomes (Tables 7 and 8).

2.3.4 The effects of pH, $MgCl_2$ and KCl.

Increasing the $MgCl_2$ concentration to 35 mM and pH to 9.0 resulted in a further increase in the LP/SP ratio (Tables 9 and 10). A still further increase in the LP/SP ratio was obtained by increasing the KCl concentration to 200 mM. However an additional increase in KCl concentration to 400 mM appeared to cause a significant dissociation of polysomes.

2.3.5 Standard Buffer.

On the basis of these results, a standard buffer (SB) was established for the isolation of highly polymerized polysomes from mature RNH and RH leaves. SB contains 200 mM Tris-HCl, 0.6 M sorbitol (0.9 M for RH), 200 mM KCl, 35 mM $MgCl_2$, 12.5 mM EGTA, 15 mM DTT, pH 9.0. The efficacy of this buffer for the isolation of rye polysomes is compared with the low and high Tris buffers in Table 1 and 2. Isolation of polysomes in SB gave the lowest proportions of M, the highest proportions of LP and P, the highest LP/SP ratio, the largest value for polysomal size class of maximum absorbency, and the best yield of recovered polysomes. The high values for the A_{260}/A_{280} and A_{260}/A_{235} ratios show that the 1.5 M sucrose cushion efficiently

Table 7. Effect of DTT concentration on the proportion of different size classes of RNH polysomes isolated with the high Tris buffer in the presence of 25 mM EGTA.

	DTT (mM)		
	2	10	15
S/T	0.18	0.18	0.14
M/T	0.31	0.32	0.21
SP/T	0.23	0.20	0.22
LP/T	0.28	0.30	0.43
	1.00	1.00	1.00
P/T	0.51	0.50	0.65
LP/SP	1.22	1.50	2.00
HP	5	7	6
A_{260}/A_{280}	1.73	1.74	1.76
A_{260}/A_{235}	1.57	1.64	1.66

Table 8. Effect of DTT concentration on the proportion of different size classes of RH polysomes isolated with the high Tris buffer in the presence of 25 mM EGTA.

	DTT (mM)		
	2	10	15
S/T	0.10	0.11	0.08
M/T	0.36	0.25	0.20
SP/T	0.21	0.19	0.20
LP/T	0.33	0.44	0.52
	1.00	1.00	1.00
P/T	0.54	0.63	0.72
LP/SP	1.58	2.26	2.60
HP	5	5	6
A_{260}/A_{280}	1.68	1.73	1.69
A_{260}/A_{235}	1.49	1.53	1.55

Table 9. Effects of KCl and $MgCl_2$ concentrations and pH on size class distribution of RNH polysomes. Buffer contained 200 mM Tris-HCl, 15 mM DTT and 12.5 mM EGTA. KCl, $MgCl_2$ and pH were as indicated below.

	60 mM KCl 30 mM $MgCl_2$ pH 8.5	60 mM KCl 35 mM $MgCl_2$ pH 9.0	200 mM KCl 35 mM $MgCl_2$ pH 9.0	400 mM KCl 35 mM $MgCl_2$ pH 9.0
S/T	0.20	0.21	0.21	0.32
M/T	0.34	0.36	0.31	0.28
P/T	0.18	0.16	0.17	0.19
LP/T	0.28	0.27	0.31	0.21
	1.00	1.00	1.00	1.00
P/T	0.46	0.43	0.48	0.40
LP/SP	1.56	1.70	1.87	1.14
HP	5	5	5	5
A_{260}/A_{280}	1.83	1.82	1.77	1.81
A_{260}/A_{235}	1.62	1.63	1.63	1.64

Table 10. Effects of KCl and $MgCl_2$ concentrations and pH on size class distribution of RH polysomes. Buffer contained 200 mM Tris-HCl, 15 mM DTT and 12.5 mM EGTA. KCl, $MgCl_2$ and pH were as indicated below.

	60 mM KCl 30 mM $MgCl_2$ pH 8.5	60 mM KCl 35 mM $MgCl_2$ pH 9.0	200 mM KCl 35 mM $MgCl_2$ pH 9.0	400 mM KCl 35 mM $MgCl_2$ pH 9.0
S/T	0.10	0.08	0.06	0.11
M/T	0.33	0.31	0.22	0.18
SP/T	0.18	0.20	0.20	0.27
LP/T	0.39	0.41	0.53	0.45
	1.00	1.00	1.00	1.00
P/T	0.58	0.62	0.73	0.71
LP/SP	2.15	2.03	2.63	1.66
HP	5	5	6	5
A_{260}/A_{280}	1.67	1.73	1.72	1.55
$A_{260}/A_{2[illegible]5}$	1.62	1.58	1.66	1.52

separated polysomes from the microsomal fraction, and that the ionic strength of SB was high enough to efficiently remove soluble proteins adsorbed on RNH and RH polysomes both during homogenization and resuspension procedures.

These results on intact RNH and RH polysomes reveal in addition some important features. Following growth at low temperature, the size class distribution of polysomes is skewed toward larger polysomes and there is an increase in the quantity of polysomes (Tables 1 and 2).

Polysomes from mature triticale leaves and corn coleoptiles were also isolated using SB. This buffer appeared to be suitable since in the case of triticale grown at low temperature, the proportion of LP is similar to the LP value reported for RH polysomes. In corn coleoptiles, the results showed that SB was more appropriate than the low Tris buffer to isolate polysomes as was verified for RNH polysomes (Table 11).

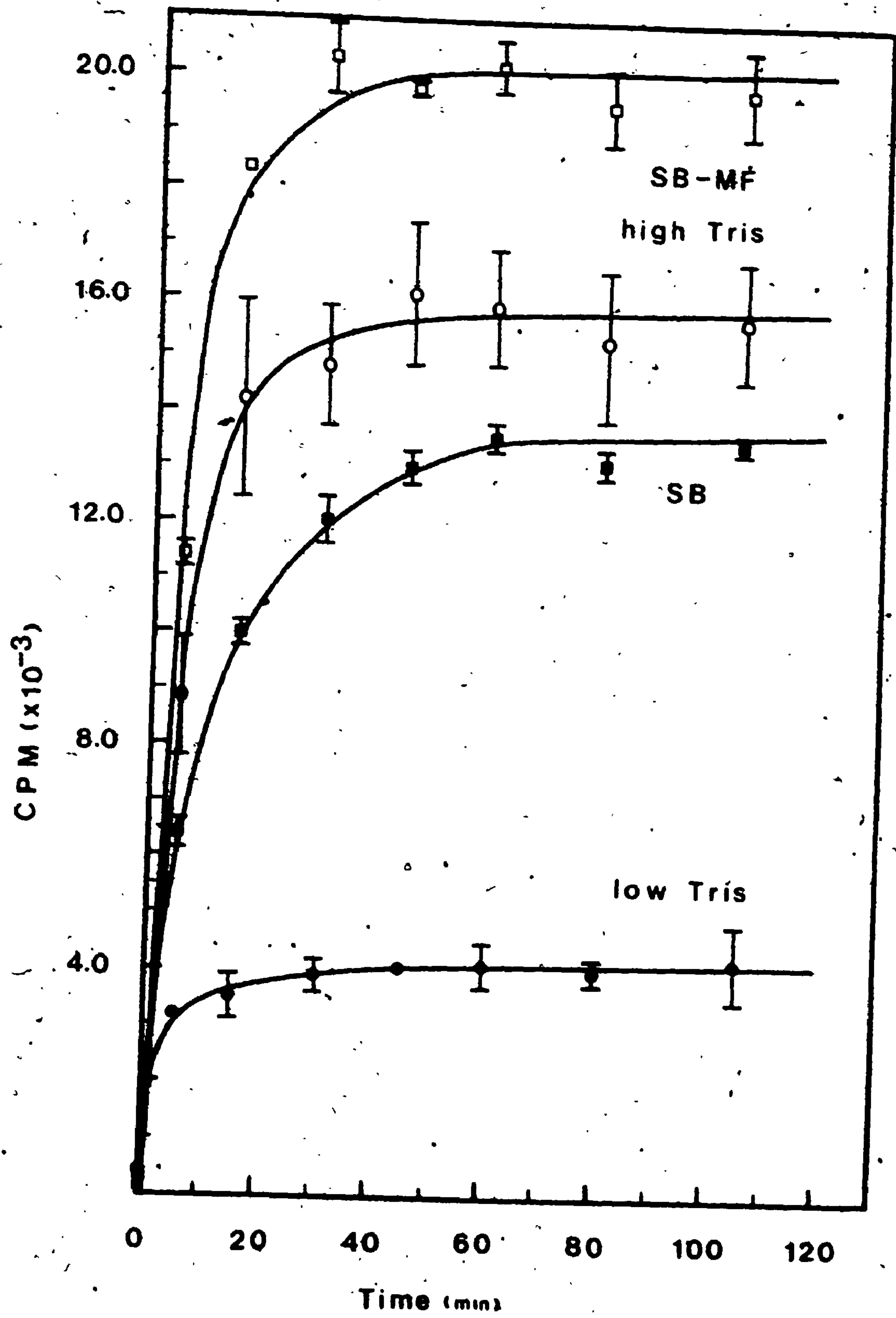
2.3.6 *In vitro* translation.

The kinetics of *in vitro* translation with polysomes isolated in the three buffers using the wheat germ system are compared in Fig. 5. Polysomes isolated with high Tris buffers were four times more active than those isolated with low Tris buffer (Fig. 5). There is no statistical difference between the total amino acid incorporation by the polysomes isolated with SB and high Tris buffer. Saturation was reached more quickly with polysomes isolated

Table 11. Effects of different resuspension buffers on the proportion of different size classes of corn coleoptile polysomes and size class distribution of polysomes isolated from leaves of triticale seedlings grown at low temperature.

	corn		triticale
	low Tris	SB	SB
S/T	0.02	0.03	0.06
M/T	0.23	0.29	0.14
SP/T	0.35	0.10	0.27
LP/T	0.40	0.58	0.53
	1.00	1.00	1.00
P/T	0.75	0.68	0.79
LP/SP	1.15	6.14	1.94
HP	3	10	5
A_{260}/A_{280}	1.79	1.73	1.77
A_{260}/A_{235}	1.52	1.51	1.67

Figure 5. Time course of [^{35}S]methionine incorporation into protein by polysomes isolated with low Tris, high Tris or SB at 25°C. Protein synthesis was carried out with the S-105 wheat germ fraction. The CPM values were determined by TCA precipitation of 5 µl aliquots on paper discs as reported (Mans and Novelli, 1961). The error bars represent the standard error from the average of three independent incorporations. All the values were corrected by subtracting the value of the appropriate blank. All the values for the blank obtained at different time were within 1050 ± 100 CPM.


SB-MF
high Tris
SB
low Tris
CPM (x10^{-3})
20.0
16.0
12.0
8.0
4.0
0
20
40
60
80
100
120
Time (min)

in low Tris buffer than with those isolated with high Tris buffers. In addition, the membrane-free polysomes isolated with SB were more active than the high Tris or SB total polysomes. When these polysomes were translated with the rabbit reticulocyte lysate, the differences in the incorporation of amino acids, previously observed using the wheat germ system, between the high Tris buffers polysomes and the low Tris buffer polysomes and the high Tris MF polysomes were further amplified (Fig. 6).

Translation products also differed among the three different polysome preparations (Fig. 7). Larger polypeptides were found in the translation products of polysomes isolated with both high Tris buffers, but the highest proportion of larger polypeptides were associated the polysomes isolated with SB. However, no difference was observed in the complement of translation products of both SB total and free polysomes.

Figure 6. Time course of [^{35}S]methionine incorporation into protein by polysomes isolated with low Tris, high Tris or SB at 30°C. Protein synthesis was carried out with the rabbit reticulocyte lysate. The CPM values were determined by TCA precipitation of 2 µl aliquots on paper discs as reported (Mans and Novelli, 1961). All the values were corrected by subtracting the value of the appropriate blank. All the values for the blank obtained at different time were varying between 1950 and 2850 CPM.

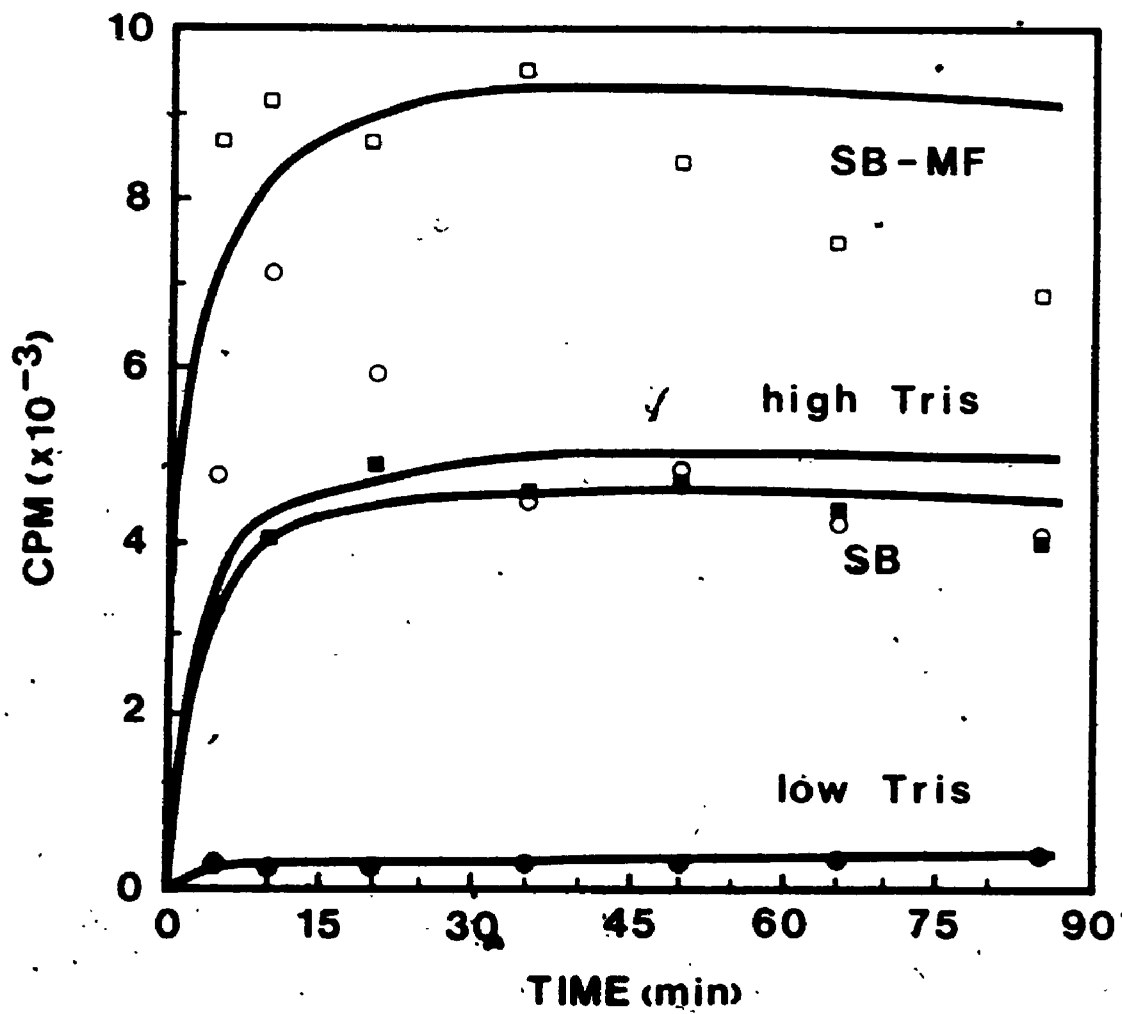
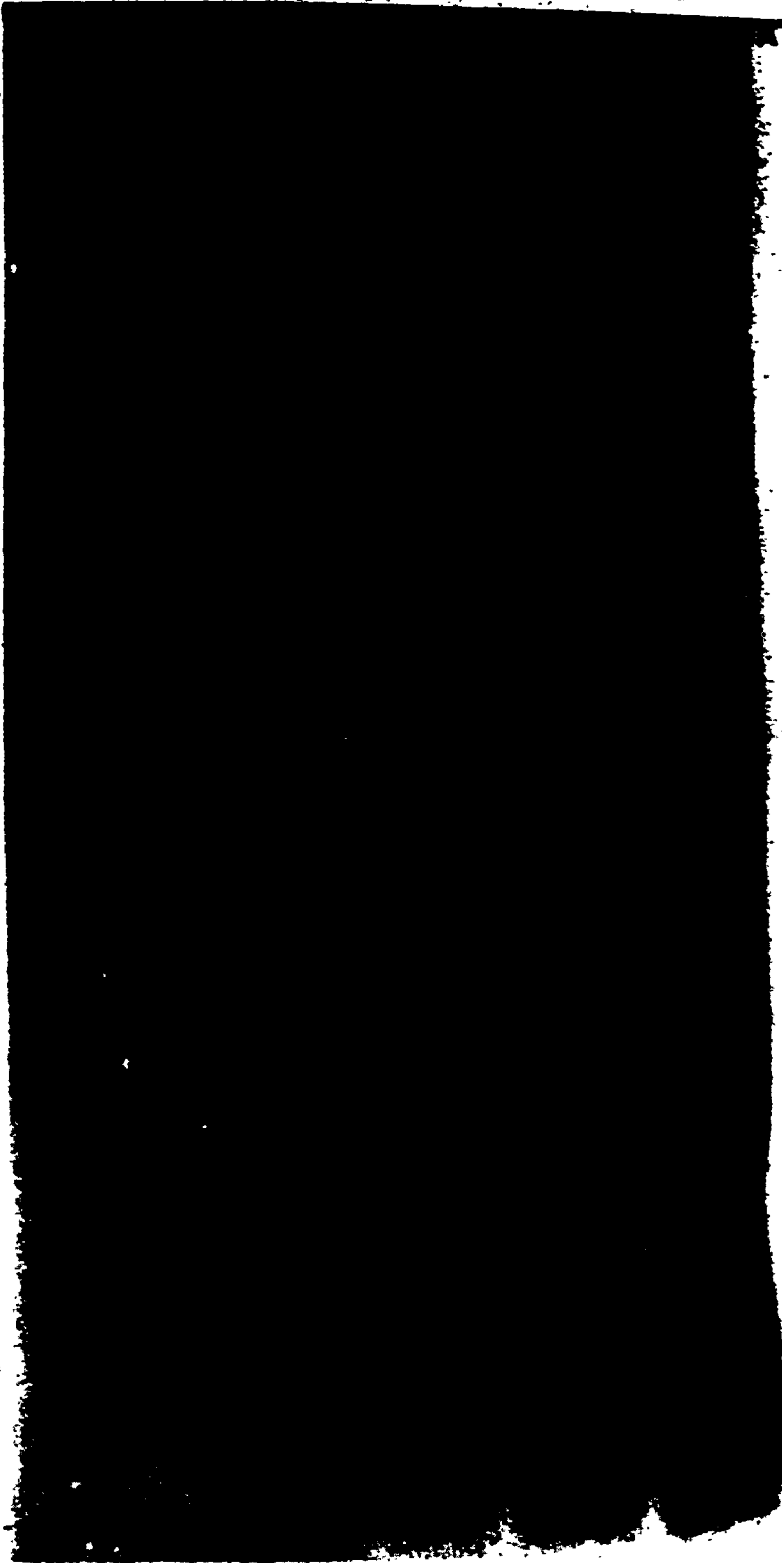

10
8
6
4
2
0
CPM (x10^{-3})
SB-MF
high Tris
SB
low Tris
0
15
30
45
60
75
90
TIME (min)

Figure 7. SDS-PAGE of translation products of rye polysomes isolated with low Tris, high Tris or SB. Products are detected by fluorography. Lane 1, blank; lane 2, low Tris total polysomes; lane 3, high Tris total polysomes; lane 4, SB total polysomes; lane 5, SB MF polysomes.


Mr
94
68
45
29
20
12.5
1
2
3
4
5

2.4 DISCUSSION

Most studies with plant polysomes have been carried out in young tissues. In all these studies two principal buffer systems have been used for the isolation of polysomes. One buffer, characterized by low Tris (50 mM or less) and low pH (7.4) and used extensively with mammalian systems (Heikkala and Brown, 1981; Venkatesan and Steele, 1971) has also been used for isolation of polysomes from corn pollen, coleoptiles and plumules (Baszczynski et al., 1984; Dunlop and Walden, 1985; Hogan et al., 1985). Polysomes from beans and radish have also been isolated with similar buffers (Anderson and Key, 1971; Gray and Kekwick, 1974; Lin et al., 1966; Pearson, 1969). Profiles of polysomes isolated in low Tris/low pH buffer have generally shown signs of degradation since an accumulation of monosomes and a low proportion of large polysomes are observed. The second buffer, characterized by high Tris (200 mM) and high pH (8.5) and initially developed for isolation of more highly polymerized polysomes from young etiolated pea leaves (Davies et al., 1972) yields polysomes with profiles containing a larger proportion of large polysomes. The presence of KCl, $MgCl_2$, EGTA, DTT, DEP and cycloheximide in addition to a buffer of high pH and ionic strength has been reported as effective during polysome isolation in a wide variety of young tissues (Davies and Larkins, 1980).

However, no report addresses the problem of isolation of intact polysomes from older, mature tissues. The results obtained confirm that high buffer concentration and high pH are necessary not only for isolation of polysomes from young tissues such as pea or tobacco (Davies _et_ _al_., 1972; Jackson and Larkins, 1976; Larkins and Davies, 1973) but also for isolation of highly polymerized polysomes from older, more mature rye leaves grown under different temperature regimes. The medium (SB) used for the isolation of polysomes from mature rye leaves is, however different from the media used for the isolation of polysomes from young pea and tobacco leaves with regard to the specific requirements for EGTA, DTT, $MgCl_2$, KCl and pH.

EGTA has been reported to prevent RNase activity by removing the endogenous calcium which activates RNases (Jackson and Larkins, 1976; Larkins and Davies, 1973). In the presence of EGTA a higher LP/SP ratio is obtained presumably due to an inhibition of the RNase activity. The optimal concentration of EGTA is 12.5 mM. It is also interesting to observe that an excess of EGTA (50 mM) causes virtually complete dissociation of RNH polysomes as previously reported for tobacco (Jackson and Larkins, 1976) but not in RH polysomes. Although these RNH and RH polysomes required similar optimal concentration of EGTA, they also showed subtle differences.

In general, the efficacy of DTT in preserving polysome integrity has been overlooked. DTT keeps the RNases reduced, further inhibiting their action (Breen et al., 1971). In some cases, 1 to 5 mM reducing agent (DTT or ß-mercaptoethanol) has been added on a routine basis (Arglebe and Hall, 1969; Baszczynski et al., 1984; Dunlop and Walden, 1985; Dunn, 1970; Gray and Kekwick, 1974; Heikkala and Brown, 1981; Hogan et al., 1985; Larkins and Tsai, 1977; Noll et al., 1963; Pearson, 1969; Ramagopal and Hsiao, 1973; Tucker and Laties, 1984; Venkatesan and Steele, 1971). On the other hand, since no beneficial effect of ß-mercaptoethanol could be demonstrated for pea polysomes (Davies et al., 1972), others have not included a reducing agent (Brady and Scott, 1977; Erkeev and Kudoyarova, 1981; Gonzalez; 1980; Jackson and Larkins, 1976; Larkins and Davies, 1975). However the importance of DTT during the isolation of polysomes from barley has been shown (Breen et al., 1971). The results obtained with DTT are in agreement with those found in barley (Breen et al., 1971), although, polysomes isolated from mature RNH and RH leaves require a concentration 50% higher.

Magnesium and potassium ions have also been reported to inhibit RNase activity (Davies and Larkins, 1980). It was found that, in mature RNH and RH leaves, the LP/SP ratio was higher with 35 mM $MgCl_2$ and a pH of 9.0. These values for $MgCl_2$ concentration and pH are similar for those required for tobacco (Jackson and Larkins, 1976) but higher

than those found optimal for pea (Davies _et al_., 1972). The optimal KCl concentration (200 mM) in rye is much lower than the 400 mM reported for polysome isolation from young barley and tobacco leaves (Breen _et al_., 1971; Jackson and Larkins, 1976). This difference in the KCL requirement could be due to differences inherent in the plant material (eg. age) or perhaps, to the difference in the ionic strength of the isolation buffer. The buffer used during the isolation of polysomes from barley leaves contained only 50 mM Tris (Breen _et al_., 1971). However in tobacco, the isolation buffer contained 200 mM Tris but, only 60 mM KCL and 400 mM KCl were compared (Jackson and Larkins, 1976). The observed dissociation of rye monosomes into subunits at high KCl concentration (Tables 9 and 10) is presumably an effect of the high ionic strength of the buffer (Davies and Larkins, 1980). In barley an excess of KCl in the isolation buffer also caused monosomes to dissociate (Breen _et al_., 1971).

Other additives such as DEP and cycloheximide have been used in isolation buffers in order to improve the profile of isolated polysomes (Anderson and Key, 1971; Breen _et al_., 1971; Weeks and Marcus, 1969). DEP, a highly effective inhibitor of RNases is known to inactivate a wide variety of enzymes (Davies and Larkins, 1980) and to react with RNA (Ramagopal and Hsiao, 1973) further rendering the polysomes unsuitable for _in vitro_ synthesis. Inclusion of DEP in the isolation medium did not protect polysomes

against degradation, but in fact led to markedly reduced polysome yields. Cycloheximide has been shown to prevent ribosome run-off in barley (Breen et al., 1971). However cycloheximide did not improve the polysome profiles in the present study, indicating that ribosome run-off was not a problem. Recently, a high concentration of Tris and basic pH were used for isolation of polysomes from wheat seedlings (Fehling and Weidner, 1986). However, EGTA was absent and the DTT concentration was low (5 mM). Since the proportion of polysomes was relatively high (75%), EGTA and a high DTT concentration were probably not required because these authors used younger leaves in which RNase activity is generally lower (Brady and Scott, 1977; Ramagopal and Hsiao, 1973).

The higher proportion of LP in RH when compared with RNH could result from the growth at low temperature. Formation of larger polysomes has been observed in potato leaves grown at low temperature (Vigue et al., 1974). An increase in the quantity of ribosomes and rRNA has also been reported during growth at low temperature (Bixby and Brown, 1975; Brown, 1978; Paldi and Devay, 1983; Sarhan and D'Aoust, 1975; Siminovicth et al., 1967; Siminovitch et al., 1968).

The proportion of polysomes (66%) in mature RNH leaves is lower than the values reported for young pea (80%-85%) and young wheat (75%) seedlings (Davies et al., 1972; Fehling and Weidner, 1986) although it is significantly

higher than the 42% reported for wheat seedlings of comparable age (Brady and Scott, 1977). The difference in the proportion of polysomes between rye and the younger tissues could reflect the difference in the age of tissues used.

However the higher proportion of polysomes from rye compared with wheat of similar age further emphasizes the need to provide adequate protection for the polysomes when isolated from older tissue.

It has been shown that the decrease in the proportion of polysomes was accompanied by a decline in protein synthesis activity as wheat grew older (Brady and Scott, 1977). The lower proportion of polysomes in mature rye leaves compared with those reported for younger tissues could also reflect a lower in vivo protein synthesis activity. This is substantiated by the observation that synthetic activity of isolated polysomes is directly related to the degree of polysome polymerization (Nowak et al., 1984). These results further show that more highly polymerized polysomes, when translated in vitro, better represent in vivo protein synthesis. Polysomes isolated with high Tris and high pH not only incorporated higher levels of amino acid, but also continued to incorporate methionine over a longer period of time and directed the synthesis of larger polypeptides. It is assumed that more highly polymerized polysomes indicate a greater intactness of the mRNA complement and that the synthesis of larger

polypeptides gives a more accurate representation of the synthesis of protein in vivo. It appears, then, that there is a direct relationship between the state of mRNA degradation during isolation and the capacity of that mRNA to direct protein synthesis. A similar correlation was not observed with polysomes from maize (Ramagopal and Hsiao, 1973). However, the results obtained with rye polysomes agree with those reported for polysomes from mammalian systems that a lower degree of integrity of polysomes will reduce the amino acid incorporation in vitro (Dunn, 1970; Nowak et al., 1984).

During isolation of polysomes in presence of detergent in animal systems, factors necessary for maximal polysome activity were removed, reducing the synthetic activity of these detergent treated polysomes when compared with non detergent treated polysomes. However the activity could be restored by adding the cell sap (Ramsey and Steele, 1976). SB membrane-free polysomes were found to be more active than SB total polysomes. These results suggest that the synthetic activity of membrane-free polysomes would be more representative of the original cellular activity than the activity of membrane-bound or total polysomes. Although detergents have been used during isolation of plant polysomes (Ramagopal and Hsiao, 1973; Häggman, 1986), it seems that this is the first time that a deleterious effect of detergent on synthetic activity of plant polysomes is pointed out.

In conclusion, two complementary approaches (<u>in vitro</u> translation and sucrose gradient centrifugation) have been used to evaluate different buffer systems for the isolation of highly polymerized polysomes from mature RNH and RH leaves. The components required for the isolation of polysomes from mature rye leaves which are similar for RNH and RH, differ from those required for the isolation of polysomes from young pea leaves. These differences probably reflect a higher RNase activity in older plant tissues. <u>In vitro</u> translation of RNH polysomes demonstrated the importance of using highly polymerized polysomes. These results reinforce the fact that the components of the isolation buffer must be critically evaluated when using polysome isolation buffers previously developed for plants physiologically different. On the other hand, these results have revealed that following the growth at low temperature of rye seedlings, there is more polysomes and these polysomes are skewed toward larger polysomes. Intact polysomes have been isolated from RNH and RH seedlings and appear to be different at least with respect to their size class distribution. These polysomes will be further characterized, as described in the next chapter, by evaluating their size class distribution with more details, quantities, melting points and their composition.

CHAPTER 3

POLYSOMES FROM WINTER RYE SEEDLINGS GROWN AT LOW TEMPERATURE. QUANTITY, SIZE CLASS DISTRIBUTION, MELTING POINT AND COMPOSITION.

3.1 INTRODUCTION

Growth at low temperature induces morphological and cellular modifications. These modifications include (i) an increase in the amount of cytoplasm (Huner et al., 1984), (ii) a decrease in leaf area and plant height (Krol et al., 1984), (iii) an increase in total protein and total RNA (Bixby and Brown, 1975; Brown, 1978; Brown and Bixby, 1975; Cloutier, 1983; Paldi and Devay, 1983; Sarhan and D'Aoust, 1975; Siminovitch et al., 1968, 1967) and (iv) a relative increase in ribosomal RNA (rRNA) (Brown, 1978). The observed parallel increase in protein and RNA was thought to reflect the proposed role of RNA in promoting protein synthesis (Siminovitch et al., 1967). Alterations to the complement of soluble and membrane polypeptides have also been reported in cold-hardened plants (Bixby and Brown, 1975; Brown, 1978; Brown and Bixby, 1975; Cloutier, 1983; Elfman et al., 1984; Griffith et al., 1982; Huner et al., 1984; Siminovitch et al., 1968). In addition, thermal melting profiles and ribosomal protein analysis have shown differences in ribosomes isolated from cold-hardened and nonhardened black locust seedlings (Bixby and Brown, 1975). Although the effect of various treatments on the degree of

polysome polymerization in plants is well documented (for a review see Davies and Larkins, 1980), the influence of low temperature on polysome size class distribution has not been extensively studied. Following growth at low temperature, polysome size class distributions in mimosa epicotyls and potato leaves show different trends (Brown, 1972; Vigue et al., 1974). While the size class distribution of polysomes was unaffected in mimosa, relatively larger polysomes were isolated from potato leaves after a short cold hardening treatment. All of these previous studies have been carried out on different plant tissues subjected to various periods of time at either cold-hardening or non-hardening growth temperatures. It is not clear whether the differences observed are due primarily to species differences or growth at low temperature or to differences in developmental stage of the plant used. In order to clarify these points, data on size class distribution, composition and stability of polysomes isolated from leaves of rye seedlings grown at low (5°C) and high (20°C) temperatures will be presented in this chapter.

3.2 MATERIALS AND METHODS

3.2.1 Polysome isolatión.

Polysomes from RNH and RH seedlings were isolated as described in section 2.2.2.

3.2.2 Polysome distribution.

The method for determining polysome size class distribution was described in section 2.2.3.

3.2.3 Purity and quantity of polysome.

Polysome purity and quantity of the membrane-bound, membrane-free and total fractions were evaluated as reported in sections 2.2.4 and 2.2.5. An $E_{260}^{0.1\%}$ = 25 was used for RNA determination.

3.2.4 Subunit isolation.

Large and small ribosomal subunits from RNH and RH polysomes were obtained by dissociation of polysomes with puromycin as reported (Blobel, 1971) except that 2 mM DTT was included in all solutions. Briefly, a polysome suspension (40 $_{260}$ units) was prepared to a volume of 0.5 ml with a buffered solution containing at final concentration 50 mM Tris-HCl (pH 7.5), 500 mM KCl, 5 mM $MgCl_2$, 2 mM DTT and 1 mM puromycin. The suspension was incubated for 15 min at 0°C and 10 min at 37°C, cooled down and 0.16 ml was loaded a 10 ml 5-20% linear sucrose (RNase free

grade) gradient in 50 mM Tris-HCl (pH 7.5), 500 mM KCl, 5 mM $MgCl_2$, 2 mM DTT (solution C). Gradients were centrifuged for 3.5 h at 272,000xg_{max} (SW 41 rotor). Gradients were analyzed with a UV monitor previously described (section 2.2.2). Fractions of 0.6 ml were collected. Central fractions of either small or large subunit peaks were pooled, diluted with 2 volumes of solution C without sucrose and centrifuged at 196,000xg_{max} for 3 h (Type 65 rotor). Large and small subunit pellets were resuspended with 0,6 ml of 50 mM Tris-HCl (pH 9.0), 10 mM EDTA and 0.1% SDS and the rRNA was extracted from aliquots of this suspension with phenol.

3.2.5 Polysome and rRNA stability

The melting point of the total polysome fraction was determined by a modification of a reported technique (Bixby and Brown, 1975). The melting point of large subunit rRNA was determined in 50 mM Tris-HCl (pH 7.5) buffer containing 0.08 mM $MgCl_2$ (Cammarano et al., 1983). A Unicam SP876 Series 2 Temperature controller in combination with a SP1800 UV Spectrophotometer was used. Temperature was increased at the rate of 1.0°C/min. Melting curves were plotted on a X-Y recorder (Omnigraphic 2000, Houston Instruments). Under these conditions, it was possible to resolve differences in melting point as small as 0.1°C. Changes of as little as 1% were recorded for the hyperchromicity.

3.2.6 Electrophoresis of ribosomal proteins.

3.2.6.1 One-dimensional electrophoresis.

The polypeptide complement of polysomes was characterized by SDS-6M urea-PAGE on a 10%-14% linear gradient using the Laemmli discontinuous buffer system as previously described by Piccioni *et al.* (1982). Gels were run at room temperature at 4.5 mA for 14 hours. To calibrate the SDS-urea gels, the following molecular weight protein standards were electrophoresed: bovine serum albumin, 68 kD; ovalbumin, 45 kD; carbonic anhydrase, 29 kD and cytochrome c, 12.5 kD. Following electrophoresis the gels were stained with Coomassie Blue R-250 (0.1% w/v) and destained with acetic acid-methanol-water (Piccioni *et al.*, 1982).

3.2.6.2 Two-dimensional electrophoresis.

In order to run a first-dimensional gel electrophoresis without SDS, ribosomal proteins were freed from the rRNA by the acetic acid method (Waller and Harris, 1961). Polysomes samples (1.1-1.3 mg) were brought to a volume of 0.5 ml with a solution of 40 mM Tris-HCl, 10 mM $MgCl_2$, 20 mM KCl, 5 mM EGTA, 5 mM DTT, pH 8.5. Then 0.5 ml of 0.4 M magnesium acetate was added. This suspension was stirred and 2 ml of cold glacial acetic acid were added slowly. The suspension was treated with 5 strokes of the pestle in a glass-Teflon homogenizer in order to disperse the precipitated RNA. This suspension was stirred for 1 h at

0-4°C and centrifuged at 20,000xg_{max} during 20 min (SS-34 rotor). The supernatant was saved and kept on ice while the pellet was re-extracted twice with 0.5 ml of 60 mM magnesium acetate and 1.0 ml of cold glacial acetic acid. The three supernatants were pooled, dialyzed overnight (16 h) against 1 M acetic acid and 5 mM β-mercaptoethanol and lyophilized. Acidic proteins were separated by isoelectric focussing (IEF) in the first dimension (O'Farrel, 1975). The following proportion of ampholines (LKB ampholine) was used: (pH 3.5-10, 1% (v/v); pH 5-7, 0.7% (v/v); pH 9-11, 0.3% (v/v)). The pH gradient for IEF gels was determined by measuring the pH on intact gel with a flat surface pH electrode. Basic proteins were electrophoresed to the cathode at pH 5.5 in the first dimension (system 1) as reported (Madjar et al., 1979). In either case, the second dimension was carried out on SDS-urea gels as described above.

3.2.7 Electrophoresis of ribosomal RNA.

Total RNA was extracted with phenol and the 18 S and 25 S rRNA were resolved on SDS-polyacrylamide gels (Dyer and Leaver, 1981). The molecular size of phenol extracted 18 S and 25 S rRNA was estimated by electrophoresis on agarose gel in the presence or absence of a denaturing agents (McMaster and Carmichael, 1977).

3.2.8 **Chloroform preparation.**

Iso-amyl alcohol (3-methyl-1-butanol) was added to chloroform to reduce foaming and facilitate separation of the two phases. The chloroform:iso-amyl alcohol proportion was 24:1 as specified by Maniatis et al. (1982).

3.2.9 **Phenol preparation.**

Phenol was prepared essentially as reported by Maniatis et al., (1982). White liquefied phenol was used and stored at -20°C in small aliquots under nitrogen gas. As needed, phenol was removed from the freezer, allowed to warm at room temperature and melted at 68°C. 8-hydroxy-quinoline which is an antioxidant, partial inhibitor of RNase and weak chelator of metal ions was added to the liquefied phenol at a final concentration of 0.1%. The phenol was then extracted several times with an equal volume of buffer (1.0 M Tris, pH 8.0) followed by a second buffer (0.1 M Tris (pH 8.0), 0.2% ß-mercaptoethanol) until the pH of the aqueous phase is greater than 7.6. The solution was stored under the equilibrium buffer at 4°C for periods up to 1 month.

3.2.10 **Phenol extraction.**

Aliquots of 0.2 ml of polysomes or subunits in 50 mM Tris-HCl (pH 9.0), 10 mM EDTA, 0.1% SDS were added to 0.2 ml of phenol in a 1.5 ml polypropylene microcentrifuge tube, mixed for 1 min and centrifuged in a Fisher

microcentrifuge model 235A (13,000xg_{max}) for 15 sec. The upper aqueous phase was removed, kept on ice and an equal volume of 50 mM Tris-HCl (pH 9.0), 0.1 % SDS was added to the interface and the organic phase, mixed and centrifuged. The two pooled aqueous phases were added to 0.4 ml of phenol:chloroform (1:1), mixed and centrifuged. The aqueous layer was then re-extracted with 0.4 ml chloroform, mixed and centrifuged. Then two volumes of cold 95% ethanol (-20°C) and 0.1 volume of 3.0 M sodium acetate (pH 6.0) were added to the aqueous phase. RNA was allowed to precipitate overnight (16 h) at -20°C and centrifuged at 12,000xg_{max} for 20 min. Pellet were drained and resuspended in sterile double distillated H_2O or with an appropriate buffer as specified.

3.2.11 DNA.

DNA was extracted using the perchloric acid (PCA) method (Volkin and Cohn, 1954). Briefly, RNH and RH leaves were ground in liquid nitrogen. After the liquid nitrogen had evaporated and as soon as the temperature of the pulverized leaves reached -4 to 0°C, the liquid nitrogen powder was resuspended with 5 ml of isolation buffer (section 2.3.5). One ml aliquots were mixed with 2.5 ml of cold 10% PCA and centrifuged (10 min at 12,000xg_{max}, SS-34 rotor). The supernatant was discarded while the precipitate was resuspended in 2.5 ml of cold 10% PCA and centrifuged. The supernatant was discarded and the tissue

residue was resuspended with 1.0 ml of H_2O, mixed with 4 ml of 95% ethanol and centrifuged. The tissue residue was resuspended in 5 ml of ethanol and centrifuged. The precipitate was then re-extracted three times with 3 ml of ethanol:ether (3:1) at room temperature. The precipitate was then treated with 0.1N KOH (10ml/g fresh tissue) at 37°C for 16 hours. Following the neutralization of the suspension with 6N HCl, DNA and protein were precipitated by 1 volume of 5% PCA. The supernatant was saved and the pellet was washed with 5% PCA and centrifuged. Both supernatants were pooled and saved to give the RNA fraction. Then the precipitate was resuspended with 5.0 ml of 5% PCA, heated for 15 min at 90°C, cooled and centrifuged (save supernatant). The precipitate was washed with 2.5% PCA and centrifuged. Both supernatants were pooled to form the DNA fraction. The remaining precipitate was retained as the protein fraction. DNA concentration was estimated by spectrophotometry using an $E_{260}^{0.1\%} = 50$ (Maniatis et al., 1982) or by the diphenylamine method (Volkin and Cohn, 1954).

3.3 RESULTS

3.3.1 Proportion and quantity of membrane-free, membrane-bound and total polysomes.

Both mature RNH and RH leaves contain a larger proportion of membrane-free than membrane-bound polysomes (Table 12). Growth at low temperature resulted in an increase in the quantity of polysomes in the three different polysome fractions. The quantities of bound, free and total polysomes in RH leaves increased by factors of 1.5, 2.4 and 2.7, respectively. Therefore, the free polysome fraction increased to a greater extent than the bound fraction. The larger increase in the membrane-free fraction relative to the membrane-bound fraction resulted in an increase in the MF/MB ratio from 5.5 in RNH to 8.5 in RH leaves. Although the sum of bound and free polysomes was similar to the amount of total polysomes isolated from RNH leaves, the sum of bound and free polysomes represent only 80% of the total polysome fraction isolated from RH leaves. It is not possible to ascertain whether this result represent an incomplete recovery of bound and/or free polysomes from RH leaves or an overestimation of the total polysome fraction. Whichever the case, the increase in the quantity of polysomes following the cold hardening treatment is clearly significant.

The dry matter of RH seedlings has been reported to increase from 12% to 29% over RNH seedlings of comparable

Table 12. Quantity of MB, MF and T polysomes isolated from RNH and RH leaves. The amount of polysomes is expressed per unit of DNA, fresh weight or dry weight. MB, MF and Total polysomes were obtained from separate isolations.

	MB	MF	Total
	µg polysomes/mg DNA		
RNH	100 ± 46	539 ± 121	675 ± 104
RH	151 ± 54	1284 ± 330	1843 ± 354
RH/RNH	1.5	2.4	2.7
	µg polysomes/g fresh weight		
RNH	27 ± 13	151 ± 34	189 ± 29
RH	56 ± 20	475 ± 122	682 ± 131
RH/RNH	2.1	3.1	3.6
	µg polysomes/g dry weight		
RNH	234 ± 109	1263 ± 284	1575 ± 242
RH	195 ± 70	1638 ± 420	2350 ± 450
RH/RNH	0.8	1.3	1.5

developmental stage (Krol et al., 1984). On the other hand, the DNA content appear to remain relatively constant during a cold-hardening treatment (Brown, 1978). Consequently the increase in the quantity of polysomes depends on the parameter selected to express the results. The increase was maximum when the results were expressed on a fresh weight basis but minimum when a dry weight basis was used (Table 12).

3.3.2 Purity of polysomes.

Polysome purity was estimated using 260/280 and 260/235 ratios which supply information on the homogeneity of polysome fractions (Petermann, 1964). Our results show no differences in the degree of purity between membrane-bound, membrane-free and total RNH' polysomes (Table 13). Polysomes isolated from RH plants also show an equivalent degree of purity. Finally, the high values of these ratios indicate that all these different polysome preparations are substantially free of contaminants.

3.3.3 Size class distribution of polysomes.

Polysome size class distribution was analyzed by centrifugation on sucrose gradients (Fig. 8). Each profile is characterized by a prominent peak toward the top of the gradient. This peak represents the ribosomes or monosomes while the other peaks on the right represent polysomes of increasing sizes. Growth at low temperature appears to

Table 13. Purity of RNH and RH polysomal preparations as expressed by 260/280 and 260/235 ratios.

	260/280 ratios	
	RNH	RH
MB	1.75 ± 0.04	1.61 ± 0.08
MF	1.80 ± 0.02	1.76 ± 0.02
T	1.81 ± 0.04	1.74 ± 0.03
	260/235 ratios	
	RNH	RH
MB	1.66 ± 0.02	1.53 ± 0.06
MF	1.72 ± 0.02	1.66 ± 0.01
T	1.71 ± 0.03	1.61 ± 0.02

2

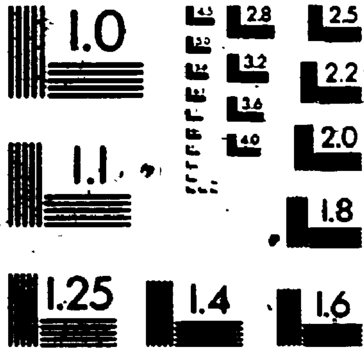
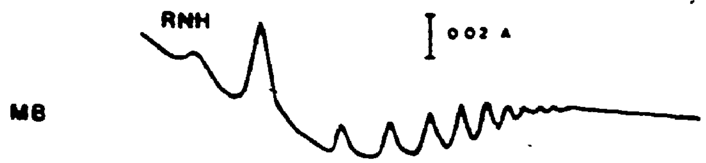
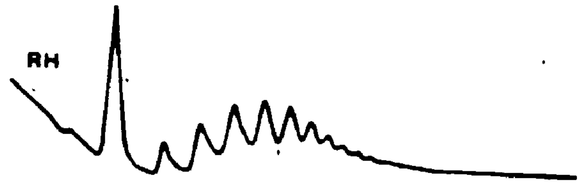
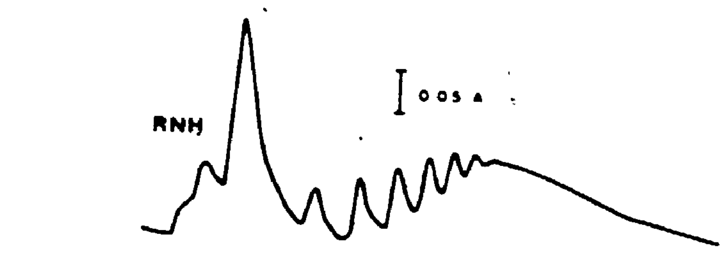


Figure 8. Density gradient profiles of RNH and RH membrane-bound (MB), membrane-free (MF) and total polysomes. The base line of each gradient is represented by the dashed line. A 2.5 times more sensitive scale was required to obtain profiles of membrane-bound polysomes during fractionation of sucrose gradients indicating a relatively smaller quantity of membrane-bound polysomes compared to the quantity of membrane-free or total polysomes.

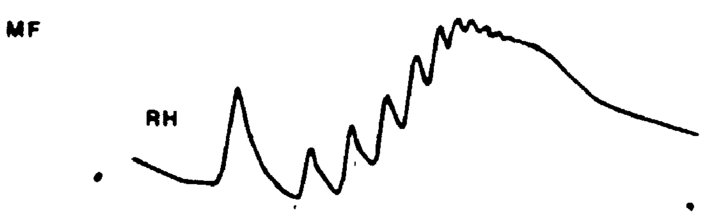
MB

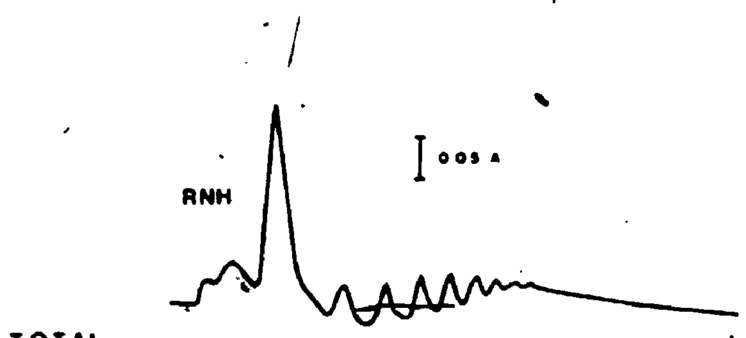






MF





TOTAL

TOP BOTTOM

affect monosomes and polysomes inversely. The proportion of monosomes clearly decreases and the proportion of large polysomes increases in bound, free and total polysome fractions from RH leaves.

The proportion of each size class for bound, free and total polysome fractions were relatively similar for RNH leaves (Table 14). Monosomes account for approximately 20% of the total RNH polysomes whereas small polysomes and large polysomes collectively account for 70% of the total. In addition, the ratio of large to small polysomes (LP/SP) indicates that polysome integrity was adequately protected. These results also show that during growth at low temperature there is an increase in the formation of large polysomes at the expense of monosomes in membrane-bound, membrane-free and total polysome fractions. This increase in the proportion of large polysomes is more obvious in the membrane-free fraction and is reflected by the substantial increase in the LP/SP ratio from 2.08 to 3.39 (table 14). Although the free polysomes represent more than 70% of total polysomes in RH leaves, the size class distribution of total polysomes is more similar to the distribution observed in membrane-bound polysomes than in membrane-free polysomes.

A difference between membrane-free polysomes and both membrane-bound and total polysomes fractions is that the free fraction is not detergent treated while the two others are. In order to verify whether this difference in the

Table 14. Size class distribution of membrane-bound (MB), membrane-free (MF) and Total polysomes isolated from RNH and RH leaves.

	MB		MF		Total	
	RNH	RH	RNH	RH	RNH	RH
S/T	0.13' ± 0.07#	0.07 ± 0.05 ns	0.10 ± 0.02	0.04 ± 0.02 **	0.12 ± 0.04	0.07 ± 0.03 ns
M/T	0.18 ± 0.04	0.13 ± 0.02 ns	0.21 ± 0.02	0.10 ± 0.02 **	0.22 ± 0.05	0.18 ± 0.01 ns
SP/T	0.22 ± 0.06	0.24 ± 0.03 ns	0.23 ± 0.02	0.20 ± 0.01 *	0.2[illegible] ± 0.02	0.23 ± 0.01 ns
LP/T	0.47 ± 0.06	0.56 ± 0.06 *	0.46 ± 0.01	0.66 ± 0.03 **	0.43 ± 0.02	0.52 ± 0.03 **
LP/SP	2.22 ± 0.30	2.27 ± 0.18 ns	2.08 ± 0.17	3.39 ± 0.28 **	2.01 ± 0.28	2.27 ± 0.18 ns

' = average of 5 (RNH) or 3 (RH) values.
= ± standard deviation.

Student's t test. *, $P<0.05$
**, $P<0.01$
ns, non signifiant

S = subunits
M = monosomes
SL = small polysomes (dimers to pentamers)
LP = large polysomes (hexamers and larger)
T = total

size class distribution resulted from an artefactual effect of detergent, Triton X-100 and DOC were added to 1% final concentration to aliquots of membrane-free polysomes. Membrane-free polysome aliquots were incubated for 10 min at 4°C and centrifuged on sucrose gradient (2.2.3). The results clearly show that RH polysomes are detergent sensitive. Consequently, the observed difference in the size distribution between membrane-free and total polysomes isolated from RH leaves reflects the great sensitivity of polysomes to detergents following growth at low temperature (Table 15). These results also demonstrate that only the membrane-free polysomes should be used to describe the influence of low temperature on the size class distribution.

3.3.4 Melting point of rRNA and polysomes.

Melting points were determined for both RNH and RH polysomes and the rRNA isolated from RNH and RH large ribosomal subunits. A decrease in the stability of RH polysomes and RH rRNA isolated from the large ribosomal subunit is reflected by melting points 3.7°C and 3.2°C lower, respectively, than the melting points obtained for RNH polysomes and rRNA (Table 16). However, no difference was observed either between RNH and RH polysomes and RNH or RH rRNA with respect to the hyperchromicity.

Table 15. Influence of Triton X-100 (1%) and sodium deoxycholate (1%) on size class distribution of membrane-free (MF) polysomes isolated from RNH and RH leaves. MF polysomes were incubated for 10 min at 4°C with detergents prior the centrifugation on sucrose gradients.

	control		+ detergents	
	RNH	RH	RNH	RH
S/T	0.10	0.06	0.14	0.10
M/T	0.21	0.10	0.17	0.17
SP/T	0.23	0.19	0.23	0.21
LP/T	0.46	0.65	0.46	0.52
	1.00	1.00	1.00	1.00

Table 16. Melting point and hyperchromicity of RNH and RH polysomes and rRNA isolated from the large ribosomal subunits isolated from RNH and RH polysomes.

	melting point (°C)	hyperchromicity
RNH polysomes	57.8 ± 1.5	1.38 ± 0.03
RH polysomes	54.1 ± 0.5	1.41 ± 0.06
RNH rRNA	65.3 ± 0.9	1.23 ± 0.04
RH rRNA	62.1 ± 0.6	1.25 ± 0.05

3.3.5 **Polysomal proteins.**

RNH and RH ribosomal polypeptides were analyzed to determine whether modifications to the protein complement occurs concomitantly with the differences observed with respect to size class distribution and melting points of RNH and RH polysomes. Analysis by 1-D gel electrophoresis revealed changes in polypeptides larger than 70 kD. A polypeptide of 117 kD observed in the RNH polysomes was absent from RH polysomes. On the other hand, a polypeptide of 140 kD observed in the RH polysomes was absent from RNH polysomes. These two high molecular weight proteins were dissociable from the ribosomes with 0.6 M KCl. No differences were observed in molecular weights among the ribosomal proteins less than 70 kD (Fig. 9). The basic ribosomal proteins were analyzed by two dimensional electrophoresis using an acidic pH gel for the first dimension and a SDS gel for the second dimension. Under these conditions, the complement of basic ribosomal proteins from RNH and RH polysomes were found to be virtually identical (Fig. 10). The acidic ribosomal proteins were analyzed by two dimensional electrophoresis using an IEF gel for the first dimension and a SDS gel for the second dimension. Among the twelve RH acidic ribosomal proteins detectable on the gel, three polypeptides differ from the RNH acidic proteins. Two RH polypeptides of isoelectric points (pIs) of 7.6 and 7.3 and of Mr of 37 kD and 10 kD were observed. However these two polypeptides

Figure 9. Electrophoretic separation of ribosomal proteins on a 10%-14% linear gradient SDS-6M urea polyacrylamide gel. A:non KCl-washed RNH and RH polysomes, B: KCl-washed RNH and RH polysomes. Protein standards, 68 kD: BSA; 45 kD: ovalbumin; 29 kD: carbonic anhydrase; 12.5 kD: cytochrome c.

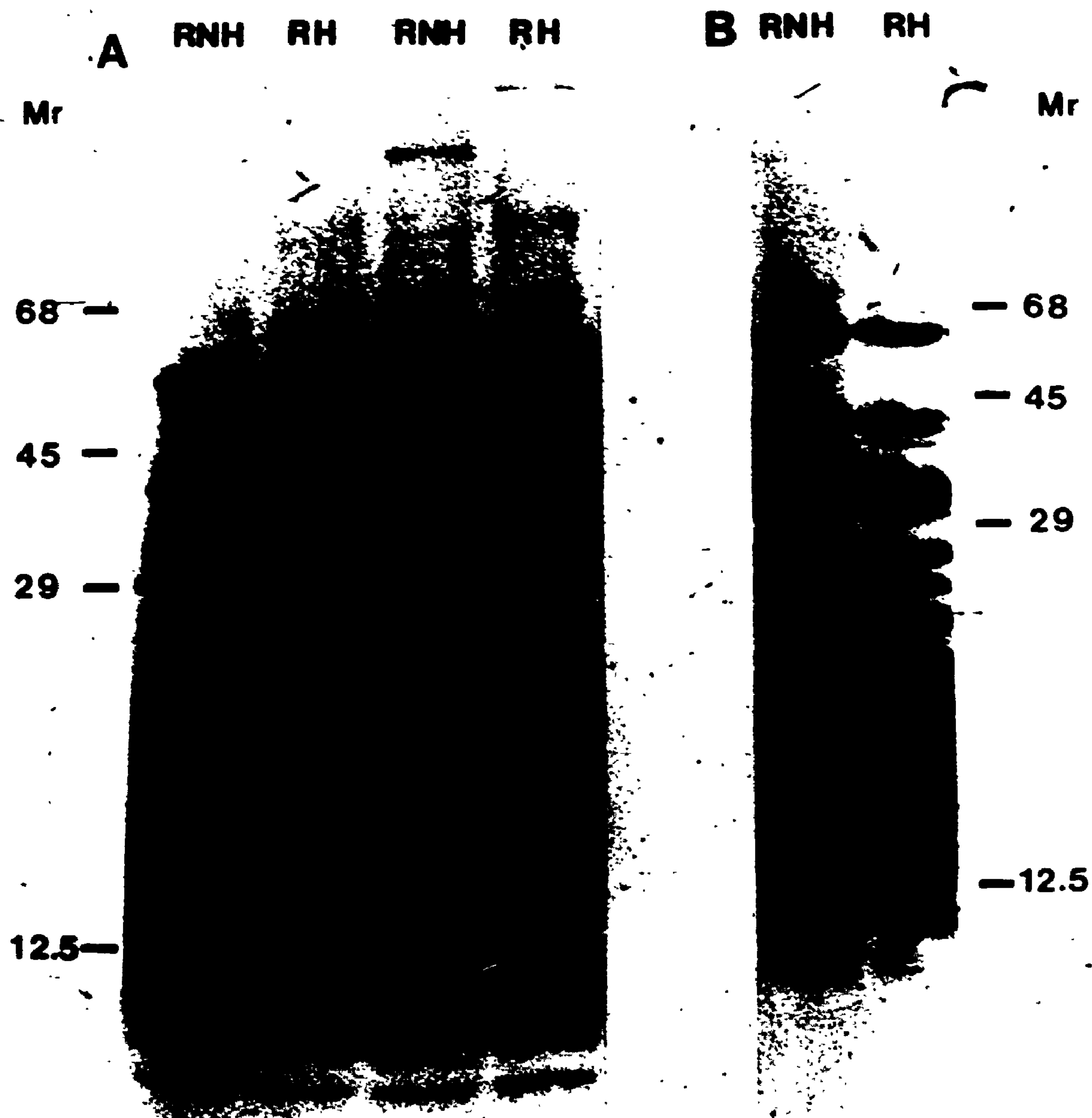
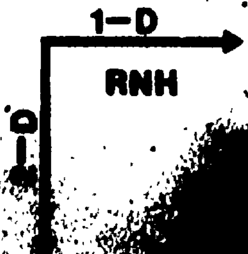
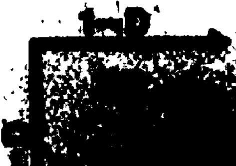

A
RNH
RH
RNH
RH
B
RNH
RH
Mr
68
45
29
12.5
Mr
68
45
29
12.5

Figure 10. 2-D electrophoresis of basic ribosomal proteins extracted from KCl-washed RNH and RH polysomes. The first dimension is a separation on the basis of mobility of the proteins at acidic pH in the presence of 8M urea (4% polyacrylamide gel). The second dimension is on the basis of the Mr of the proteins in presence of SDS with 6M urea (10%-14% linear gradient polyacrylamide gel). The same protein standards as reported in Figure 9 were used.


1—D
2—D
RNH
Mr
—68


1—D
Mr
—68
—45
—29
—12.5

were absent in RNH gels although similar polypeptides of pIs of 7.9 and 7.5 and of Mr of 38.5 kD and 11 kD were present instead. Finally, the RNH polypeptide of pI of 5.6 and Mr of 15.5 kD was also different from the RH polypeptide of pI of 5.7 and Mr of 15 kD (Fig. 11).

3.3.6 Polysomal rRNA.

The mobility of 18 S and 25 S rRNA were compared by electrophoresis on 2.6% gel in presence of SDS. No differences were observed in the mobility of the 18 S and 25 S subunits from RNH and RH rRNA (data not shown). In addition, the Mr determination of the 18 S and 25 S rRNA from RNH and RH were the same regardless of the absence or presence of the denaturing agents DMSO and glyoxal (Fig. 12). The molecular sizes of these 2 rRNAs were 0.69×10^6 and 1.30×10^6 daltons respectively.

Figure 11. 2-D electrophoresis of acidic ribosomal proteins extracted from non KCl-washed RNH and RH polysomes. The first dimension is a separation on the basis of mobility of the isoelectric point of the acidic proteins in the presence of 8M urea (4% polyacrylamide gel). The second dimension is on the basis of the Mr of the proteins in presence of SDS with 6M urea (10%-14% linear gradient polyacrylamide gel). The same protein standards as reported in Figure 9 were used.

Mr
68
45
29

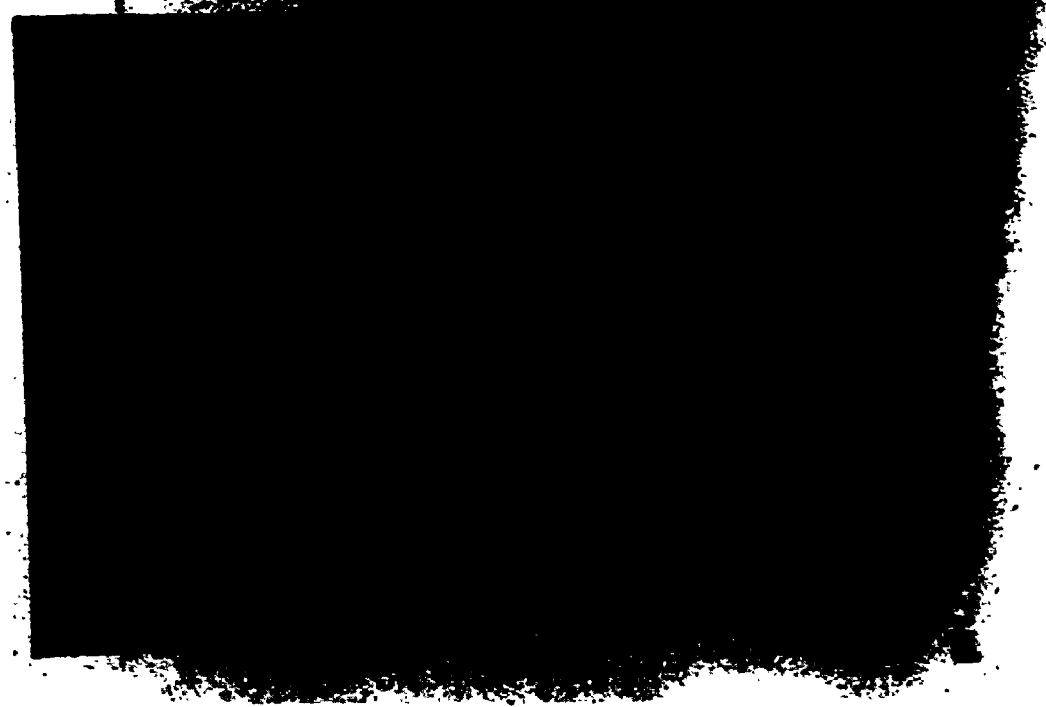
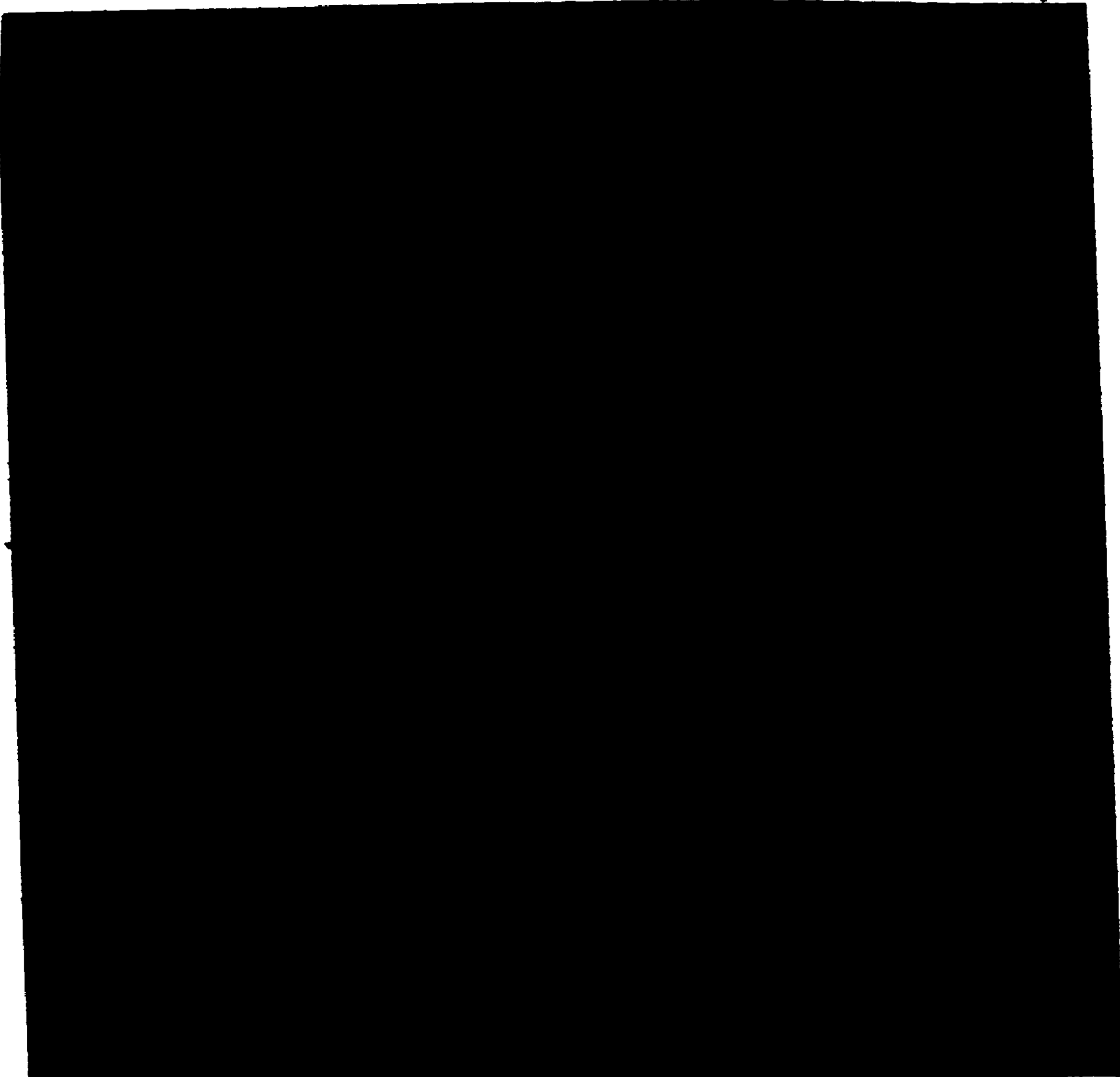

Mr
68

Figure 12. Electrophoresis of ribosomal RNA extracted from RNH and RH polysomes. The electrophoresis was done under native and denaturing condition on a 1.5% agarose gel. Lanes 1-4, native conditions; lanes 5-12, denaturating conditions; lanes 1, 5, 16 S and 23 S rRNA; lanes 2, 6, tRNA; lanes 3, 8, 10, RH rRNA; lanes 4,9,11, RNH rRNA.



3.4 DISCUSSION

Only a few studies have addressed the influence of low temperature on RNA or ribosome metabolism. An increase in the quantity of rRNA or ribosomes has been reported in the stem and living bark of black locust (Bixby and Brown, 1975; Brown, 1978; Siminovitch et al., 1968, 1967) and leaves of wheat seedlings and potato (Devay and Paldi, 1977; Paldi and Devay, 1983; Sarhan and D'Aoust, 1975; Vigue et al., 1974) following a low temperature treatment for various lengths of time. An increase in the quantity of ribosomes has been reported for overwintering pine buds (Häggman et al., 1985). All these reports suffer from several deficiencies. One of the major problem is that neither was preceded by thorough developmental study. Consequently, the low temperature plants were at a different developmental stage than the controls. Given the general observation that the rate of plant development is temperature dependent and the specific observation that ribosome metabolism is subject to age related changes (Davies and Larkins, 1980), it seems important that in any study of temperature effects on ribosome metabolism, the test and control plants be of comparable developmental stage. For this report, plants which on the basis of previous, systematic developmental studies (Krol et al., 1984) appear to satisfy that requirement have been used.

This study shows that the quantity of ribosomes in rye seedlings grown at low temperature (RH) is 2.7 and 3.6

times higher than for ribosomes in seedlings grown at high temperature (RNH) when expressed relative to DNA and fresh weight respectively. However, on a dry weight basis, there appears to be no significant difference. It has been shown that, following development of rye seedlings at low temperature, the leaves are characterized by larger cells with decreased vacuolar volume and increased proportion of dry matter (Krol et al., 1984). Our results indicate that the ribosomes constitute the same proportion of cytoplasm in both cold grown and control plants. The apparent increase in the quantity of ribosomes on a DNA or a fresh weight basis thus merely reflects this relatively larger proportion of cytoplasmic volume in the cold grown plants. This lest point may have been overlooked when a DNA basis was suggested to express results obtained during studies on acclimation at low temperature (Chen and Li, 1980; Singh et al., 1975). On this basis, a simple increase in the quantity of ribosomes would not appear to be a significant factor with respect to acclimation to growth at low temperature.

In spite of the above discussion, several observations point toward subtle changes in the architecture of the ribosome or mRNA/ribosome complex which could relate more directly to acclimation to growth at low temperatures. RH polysome profiles are definitely skewed toward larger polysomes. Variations in polysome size class distribution have been reported for other tissues in response to auxin

or gibberellin applications or during different stages of development (Davies and Larkins, 1980). Following growth at low temperature, larger polysomes have been observed in barley and potato leaves (Kenefick et al., 1974; Vigue et al., 1974) but not in mimosa (Brown, 1972). Larger polysomes were also observed upon rehydratation of the moss Tortula ruralis at 2°C rather than at 20°C (Malek and Bewley, 1978). Although larger polysomes were observed by EM in intact tissues of overwintering pine buds, smaller polysomes were obtained on sucrose gradient (Häggman et al., 1985). On the basis of the observations regarding the sensitivity of polysomes to detergent (Table 15), the loss of large polysomes previously reported may possibly be attributed to the use of detergent in the isolation of the free as well as the bound polysomes from pine buds. Although low temperature did not appear to affect polysome size class distribution in mimosa (Brown, 1972), larger polysomes have been detected in leaves of rye, barley (Kenefick et al., 1974) and potato (Vigue et al., 1974) following growth at low temperature. These larger polysomes suggest that polysomal activity is modified in rye grown at low temperature. Larger polysomes could result from either a more rapid initiation or a slower termination of protein synthesis (Davies and Larkins, 1980). It was demonstrated recently that larger polysomes in mammalian tissues were correlated with a higher polysomal activity (Nowak et al., 1984). Furthermore, it

is useful to note that few KCl-dissociable and/or acidic proteins were modified in RH polysomes while none of the basic (core) ribosomal proteins were affected during growth at low temperature. Some of these KCl-dissociable and/or acidic proteins (also identified as peripheral ribosomal proteins) have been reported to have an enzymatic function or to be factors required for protein synthesis and consequently to affect ribosomal function (Browning et al., 1985; Yurina et al., 1983). Whether the presence of larger polysomes results from the modification of these proteins can not be assessed at this time but a KCl-dissociable polypeptide of 116 kD has been identified as one of the subunits of initiation factor 3 (eIF3) in wheat germ (Browning et al., 1985). It is interesting to speculate that the 117 kD polypeptide present on RNH polysomes which is apparently replaced by a 140 kD on RH polysomes could be a subunit of eIF3. If this true, the RH eIF3 could have a different conformation which might be more active than RNH eIF3. A more active eIF3 would (1) contribute to a higher initiation rate of protein synthesis, (2) explain the increase in the proportion of large polysomes and (3) suggest a higher synthetic activity for these polysomes.

Since the isolation of membrane-bound polysomes requires the presence of a detergent (Davies and Larkins, 1980; Venkatesan et al., 1971) and since free polysomes from RH leaves were sensitive to detergents, the results obtained for size class distribution of both RH bound and

total polysomes could be artefactual. Until a new method is available to free the polysomes from the membranes, it will not be possible to verify whether an increase in size class distribution of membrane-bound and total polysome fractions similar to the one observed in the free polysome fraction occurs at low temperature. Given the present isolation conditions, the free polysome fraction more correctly reflects the influence of growth at low temperature on polysome size class distribution than rather membrane-bound or total polysome fractions.

Other modifications to ribosomes such as a decrease in the melting point, an increase in the degree of hyperchromicity and modifications to the complement of basic ribosomal proteins have been reported in black locust following growth at low temperature (Bixby and Brown, 1975). In wheat seedlings grown at low temperature, the rRNA base composition does not appear to be affected (Sarhan and D'Aoust, 1975). On the other hand, an increase in the melting point of ribosomes concomitant with the increase in the maximal growing temperature, but without any change in their degree of hyperchromicity has been reported for thermophilic bacteria (Cammarano et al., 1983). In bihelical rRNA, unlike in DNA, A and U bases and C and G bases are not all paired and the extent of pairing is variable. A higher degree of hyperchromicity is generally believed to reflect a higher proportion of paired A-U bases while a lower melting point is a measure of

structural stability indicating a lower proportion of paired G-C bases (Bixby and Brown, 1975; Cammarano et al., 1983). A lower melting point, which results from a lower proportion of paired G-C bases could result from (1) a difference in the rRNA base sequence, (2) a difference in the proportion of paired G-C bases or (3) a change in the rRNA-ribosomal protein association. The latter could result from changes in the complement of basic proteins which bind to the rRNA or from other factors such as divalent cations and/or polyamines which may modify the secondary structure of the rRNA (Cammarano et al., 1983). Finally, it has been shown that the secondary structure of RNA can be predicted by selecting the base pairing scheme which is favored in terms of free energy level (Tinoco et al., 1973). The secondary structure of RNA predicted by both sequencing and free energy level are very similar (Tinoco et al., 1973; Noller and Woese, 1981; Stiegler et al., 1981) although some exceptions were observed specifically with regards to rRNA (Noller and Woese, 1981; Stiegler et al., 1981). The temperature at which the secondary structure is considered affects the free energy. At lower temperature, the free energy values for A-U and G-C base pairs decrease (Tinoco et al., 1971). Given the same proportion of paired G-C bases, a bihelical RNA structure would then be more stable at 0°C than at 40°C.

Following growth of rye seedlings at low temperature, the melting points of both the ribosomes and the isolated

rRNA decreased. There was no change in the degree of hyperchromicity. There was also no change in the electrophoretic mobility of RNH and RH rRNA analyzed under different conditions or in the complement of basic ribosomal proteins. Taken together, these results suggest that the rRNA primary sequence is not affected during growth at low temperature. This suggestion is supported by the absence of any modification to the rRNA base composition in wheat seedlings subjected to a similar cold-hardening treatment (Sarhan and D'Aoust, 1975). It seems probable then that the observed decrease in the melting points of ribosomes and rRNA following growth at low temperature would result from a lower proportion of paired G-C bases in ribosomes and in rRNA from RH leaves. A lower proportion of paired G-C bases could itself result from changes in the binding of cations and/or polyamines to the rRNA during rRNA maturation (Cammarano et al., 1983).

There is another possible explanation for the apparent decrease in the proportion of paired G-C bases and consequent decrease in melting point. It is based on temperature effects on the free energy level of G-C and A-U base pairs (Tinoco et al., 1971). Data of free energy level for a fragment of R17 virus expressed for a temperature of 25°C has been reported (Tinoco et al., 1973). I have recalculated the free energy level of this fragment of R17 virus for temperature of 5°C and 20°C. The free energy level of -21.8 kcalories assessed for that R17

RNA fragment (Tinoco *et al.*, 1973) was lowered by 11% at 20°C (-24.3 kcalories) and by more than 44% at 5°C (-31.5 kcalories). A similar event could be invoked to explain a lower proportion of paired G-C bases in ribosomes during growth at low temperature. At 5°C, a lower proportion of paired G-C bases during either the maturation of rRNA and/or the assembly of ribosomes would be necessary in order to obtain similar free energy level and consequently a similar degree of stability than would be necessary at 20°C. Both the results obtained with rye in these experiments and their intérpretation differ from those previously reported for black locust seedlings where the melting point decrease was thought to result from the substantial differences observed in the complement of ribosomal proteins which would affect the rRNA/protein interaction (Bixby and Brown, 1975).

Since a decrease in the proportion of paired G-C bases affects the secondary structure of rRNA (Tinoco *et al.*, 1971), it would clearly alter the ribosome conformation and consequently the ribosomal activity (Davies and Larkins, 1980). The adaptation of ribosomes for fidelity and efficacy of translation at different temperature (Bixby and Brown, 1975; Cammarano *et al.*, 1983) would certainly be of biological significance.

In conclusion, rye polysomes are modified during growth at low temperature. Although the increase in the number of polysomes does not represent per se an adaptation for

growth at low temperature, other more subtle adaptations such as larger polysomes, lower melting point of polysomes, different complement of peripheral ribosomal proteins take place. These modifications would be necessary to allow rye seedlings to grow and develop at low temperature.

CHAPTER 4

GROWTH AT LOW TEMPERATURE MODIFIES *IN VITRO* TRANSLATIONAL ACTIVITY OF POLYSOMES AND mRNA.

4.1 INTRODUCTION

Interest is increasingly directed toward the protein synthesis machinery and translation products following growth at low temperature. Recently results on the *in vitro* translation of polysomes from 6 days old wheat seedlings and *in vitro* synthesis of new polypeptides from spinach both exposed at 4°C for 48 h have been reported (Fehling and Weidner, 1986; Guy *et al*., 1985). But, in spite of this new information, these *in vitro* systems have not been characterized yet. Acidic ribosomal proteins and modifications to ribosomal proteins (eg., phosphorylation, ADP-ribosylation, acetylation) have been reported as mechanisms for regulating protein synthesis (Bielka, 1982; Browning *et al*., 1985; Clemens, 1983; Floyd and Traugh, 1979; Hathaway *et al*., 1979; Mumby and Traugh, 1979; Nishimura and Deuel, 1983; Nygard and Nilsson, 1985; Ochoa and de Haro, 1979; Scharf and Nover, 1982; Traugh and Sharp, 1979; Tuhackova *et al*., 1985; Yurina *et al*., 1983).

In the previous chapter, it was suggested that the decrease in the melting point of RH polysomes and the occurrence of larger polysomes in RH leaves with four different peripheral and/or acidic proteins reflected a

different ribosome conformation and a higher initiation rate of protein synthesis leading to more active polysomes. As previously pointed out, these differences between RNH and RH polysomes could also affect the polysome translational activity. Initiation is the rate limiting step in protein synthesis and has been considered the major point of regulation of translational activity (Kramer et al., 1980; Ochoa and de Haro, 1979; Weissbach, 1980). In addition, protein synthesis on cytoplasmic ribosomes has been reported as indispensable for the development of frost hardiness in Chlorella ellipsoidea and higher plants (Chen et al., 1983; Hatano, 1978; Hatano et al., 1976; Kacperska-Palacz, 1978; Trunova, 1982).

To investigate the synthesizing capacity of polysomes in higher plants, in vitro cell-free systems are more appropriate than in vivo systems. An in vitro system helps to avoid the uncertainty related to variable precursor permeability and pool size and to distinguish the changes occurring at various steps of protein synthesis (Klyachko et al., 1982). Generally, during polysome translation with a wheat germ in vitro system, the reinitiation is very low or nonexistent (Clemens, 1984; Jolicoeur and Brakier-Gingras, 1983; Klyachko et al., 1982). Consequently, the synthesizing activity of polysomes is a measure of the elongation rate (Jolicoeur and Brakier-Gingras, 1983; Klyachko et al., 1982). Initiation rate can also be inferred from this elongation system when results are

expressed in terms of polysome specific activity (Klyachko *et al*., 1982). On the other hand, the translation of mRNA with a rabbit reticulocyte lysate is a measure of the effectiveness of mRNA to direct protein synthesis (Clemens, 1984).

In order to determine whether the function of rye polysomes and mRNA were altered following growth at low temperature, the requirements for their optimal *in vitro* translation were determined. Since these *in vitro* assays were designed to focus on ribosomes or mRNA, it is essential that polysome or mRNA be the limiting factor in the *in vitro* assay. These results show that the requirements are different for optimal translation of polysomes and mRNA isolated from non-acclimated and cold-acclimated rye seedlings of comparable developmental stage.

4.2 MATERIALS AND METHODS

4.2.1 Polysomes isolation.

Membrane-free polysomes from RNH and RH seedlings were isolated as described in section 2.2.2, resuspended in 50 mM Hepes (pH 7.8), 100 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM EDTA and 5 mM DTT (section 2.2.8) and aliquoted in 50 µl sample, frozen in liquid nitrogen and kept at -70°C.

4.2.2 mRNA isolation.

Cytoplasmic mRNA was isolated essentially as described by Cashmore (1982) excepted that the RNH and RH leaves were homogenized as for polysomes isolation (section 2.2.2) and the liquid nitrogen powder was resuspended in SB buffer (section 2.3.5). Once the mitochondrial fraction was removed, the buffer was made 1% SDS and 50 mM EDTA and the RNA extracted with phenol-chloroform and chloroform as described (Cashmore, 1982) with the exception that the first organic phase was back extracted with 50 mM Tris (pH 8.5), 0.2% SDS. Both aqueous phases were pooled. The RNA was then precipitated with ethanol overnight, centrifuged and resuspended in 0.5 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) and 0.1% SDS at a final concentration of 1 mg/ml. Poly-A$^+$ fractions were obtained by affinity chromatography on poly-U Sepharose.

Polysomal mRNA was obtained by phenol extraction of RNH and RH polysomes as described in section 3.2.10.

The amount of cytoplasmic and polysomal mRNA was estimated by hybridization with [^{3}H]poly-U. The poly-A content of the mRNA was assumed to be 6% (Buffard *et al* ., 1982).

4.2.3 Poly-U affinity chromatography.

Affinity chromatography was done as reported by Cashmore (1982). One g of poly-U sepharose was swollen in an excess of 0.5 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) and 0.1% SDS (S buffer), washed with 90% formamide, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, re-equilibrated with S buffer and poured into a small column (10 ml disposable syringe previously plugged with glass wool). The formamide was deionized with Biorad AG501-X8 mixed bed resin by stirring 20 ml of formamide with 2 g of resin for 1 h. The RNA sample in the S buffer was heated for 10 min at 65°C to dissociate aggregates, cooled on ice and filtered through the column of poly-U sepharose at 4°C. To increase the efficiency of the technique, the poly-A$^-$ fraction was recirculated a second time through the column. The column was washed with 10 ml of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), the poly-A$^+$ fraction was eluted with 4 X 2 ml of 90% formamide containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) and ethanol precipitated twice. The column was stored at 4°C in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.2% SDS and 0.02%

sodium azide until further use and re-equilibrated with buffer S just before a new poly-A⁺ fractionation.

4.2.4 [3H]Poly-U preparation.

[^{3}H]Poly-U was synthesized essentially as described by Buffard (1980). The reaction was carried out in a final volume of 1 ml containing 150 µCi of [^{3}H]UDP lyophilized, 1.3 mM of non-labelled UDP, 6 units of polynucleotide phosphorylase (PNPase) and the volume was brought to 1 ml with 100 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$ and 0.1 mM EDTA. Polymerization of UDP was complete after 30 min at 37°C. Following the addition of 50 µg of yeast tRNA, the [^{3}H]poly-U was precipitated with two volumes of cold ethanol (-20°C) and 0.1 volume of 3 M sodium acetate (pH 6.0). After 2 hours at -20°C, and a centrifugation, the [^{3}H]poly-U pellet was resuspended with 0.5 ml of 50 mM Tris-HCl, pH 7.4, 10 mM NaCl and purified on Sephadex G-75 (5 ml, 8.6 cm x 0.5 cm) equilibrated in the same resuspension buffer. The [^{3}H]poly-U fractions (0.5 ml) eluted in the void volume was monitored by counting the radioactivity of 10 µl aliquots. Fractions containing [^{3}H]poly-U were pooled and frozen at -20°C until needed.

4.2.5 [^{3}H]Poly-U-mRNA hybridization.

Hybridization was done with an excess of [^{3}H]poly-U. [^{3}H]poly-U (4µl) and diluted mRNA or poly-A (up to 20 µl) were added to 76 µl of 2xSSC (1xSSC= 0.15 M NaCl, 0.015 M

sodium citrate). The mixture was incubated at 45°C during 20 min, cooled on ice for 5 min and treated with 10 μg of RNase A during 30 min at 4°C. [^{3}H]poly-U-poly-A hybrids were then TCA precipitated (6.7% final concentration) in presence of carrier (50 μg DNA) during 15 min at 4°C and collected on GF/C filters (Buffard <u>et</u> <u>al</u>., 1982). Filters were then rinsed with 20 ml of cold 10% TCA and 10 ml 95% ethanol, dried under a heat lamp and counted with 10 ml of a toluene base cocktail (Econofluor-2, NEN) in a Beckman LS-230 scintillation counter. Under these conditions, a linear standard curve was obtained with 0 to 400 ng of poly-A.

4.2.6 Wheat germ preparation.

Wheat germ extract was prepared as reported by Reisfeld and Edelman (1982) and was described in section 2.2.7.

4.2.7 <u>In vitro</u> translation.

RNH and RH membrane-free polysomes were translated <u>in vitro</u> with the S-105 wheat germ fraction as source of soluble enzymes while RNH and RH mRNA were translated with a rabbit reticulocyte lysate (Promega Biotec) as described in section 2.2.8 with some modifications for both as described in the coming results section. The translation was carried out at 10°C and 25°C for polysomes and 30°C for mRNA. In order to make sure that polysomes or mRNA were the limiting factors during <u>in vitro</u> assays, some non-

labelled methionine was added in the translation mixture as shown in the results section.

During translation of polysomes with wheat germ, human placenta ribonuclease inhibitor (1 unit/µl of translation mixture; Clemens, 1984; Jolicoeur and Brakier-Gingras, 1983; Morch *et al*., 1986; Scheele and Blackburn, 1979) and aurintricarboxylic acid (ATA) at a concentration of 50 µM (Jolicoeur and Brakier-Gingras, 1983; Fresno and Vasquez, 1979) were added to determine the activity of RNase in the translation mixture and the capacity of that translation system for reinitiation of protein synthesis.

4.3 RESULTS

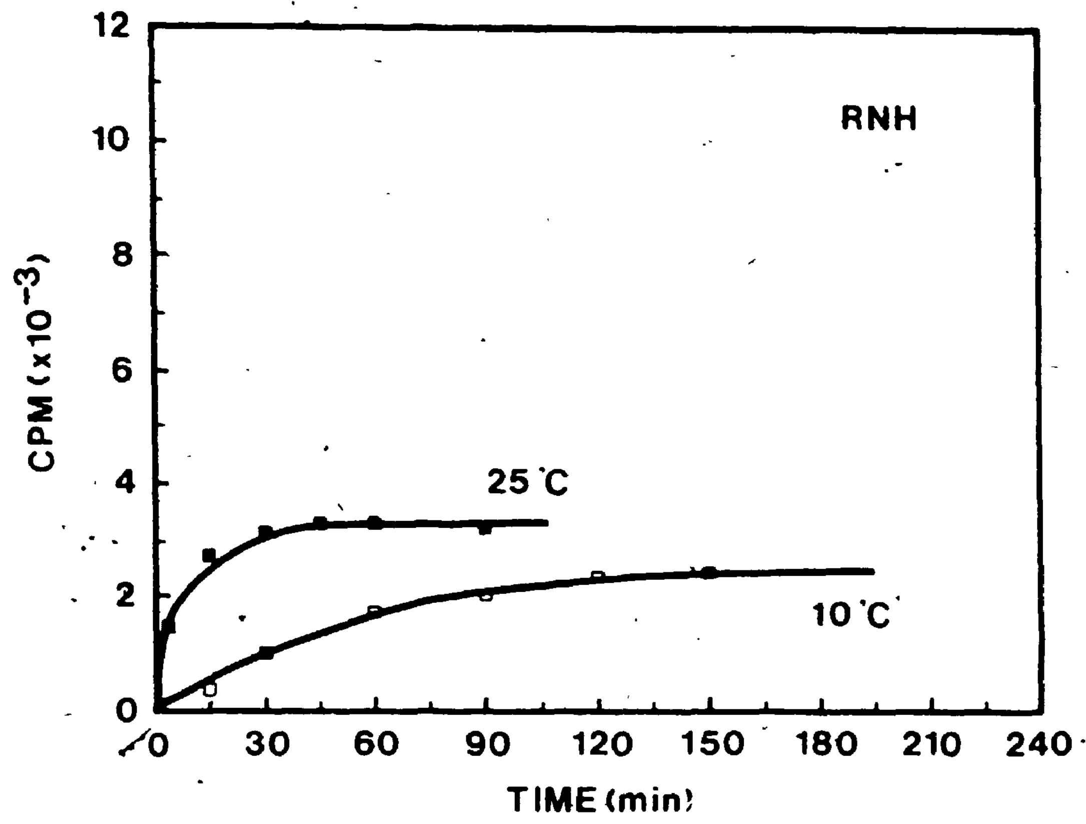
4.3.1 Translational activity of polysomes.

For these experiments, membrane-free polysomes were used. Membrane-bound and total polysome fractions were not used because detergent interferes with size class distribution (section 3.3.3) and translation (section 2.3.6). It is generally accepted that the temperature at which *in vitro* translation is carried out will affect the translation. A temperature of 25°C has been reported optimal for translation with the wheat germ system (Clemens, 1984; Reisfeld and Edelman, 1982). In this present study, it was desirable to examine translation at temperatures near the growing temperature. RNH and RH polysomes were therefore translated at 10°C and 25°C which are 5°C above the growing temperature of RNH and RH seedlings, respectively.

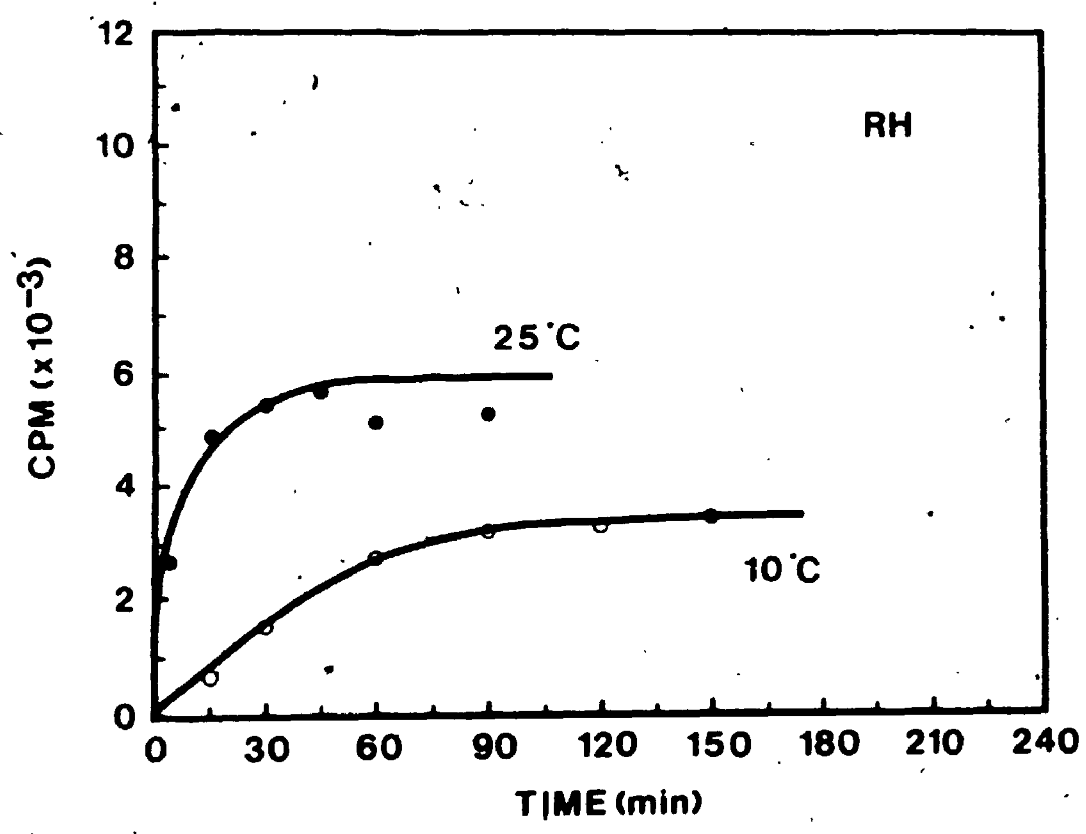
4.3.1.1 Effect of temperature.

Incorporation of methionine by RNH and RH polysomes at 10°C was reduced and slower when compared to the results obtained at 25°C (Fig. 13 and 14). For both RNH and RH polysomes, a plateau was reached after 45 min at 25°C instead of 125 min at 10°C (Fig.13).

Figure 13. Time-course of amino acid incorporation by RNH and RH polysomes (45 μg) at 10°C and 25°C with 1.3 picomoles of labelled [^{35}S]methionine. The volume of the *in vitro* translation mixture was 50 μl from which aliquots of 5 μl were removed at given times. The Mg^{++} and K^{+} final concentrations were 3 mM and 120 mM respectively. Results are from one experiment. This experiment was repeated once with similar results.

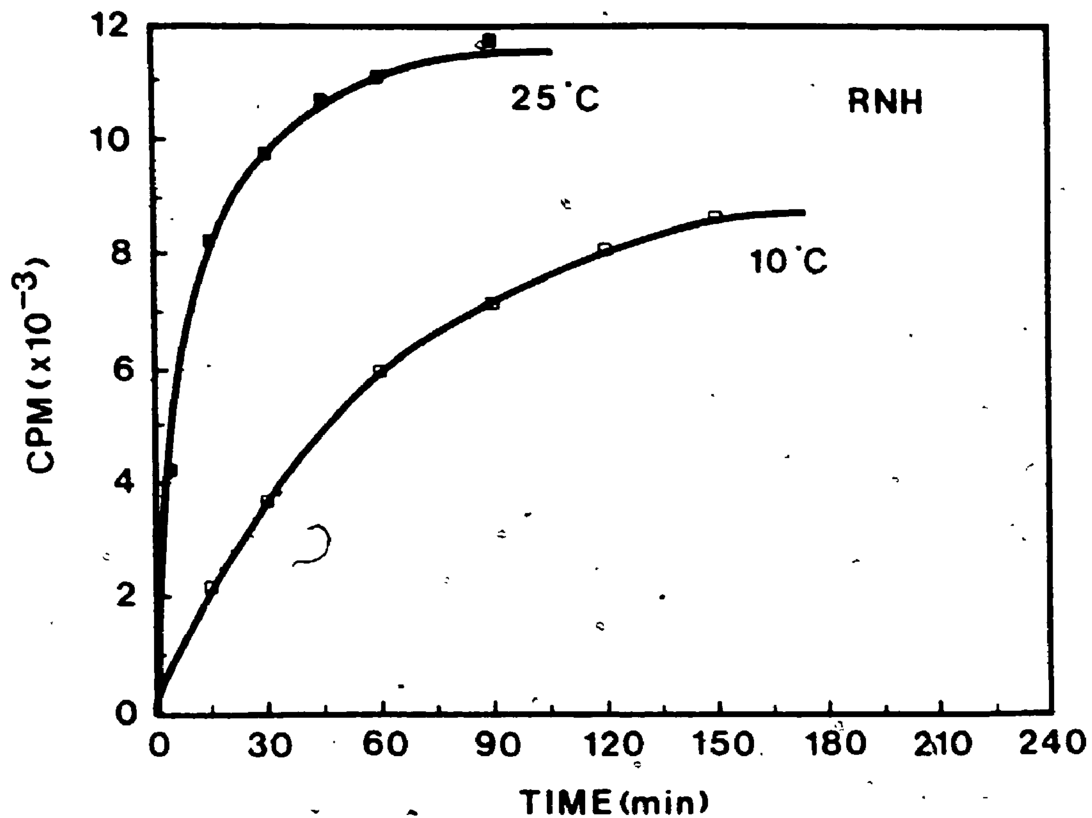


RNH
25°C
10°C
CPM (x10⁻³)
TIME (min)
0
2
4
6
8
10
12
0
30
60
90
120
150
180
210
240

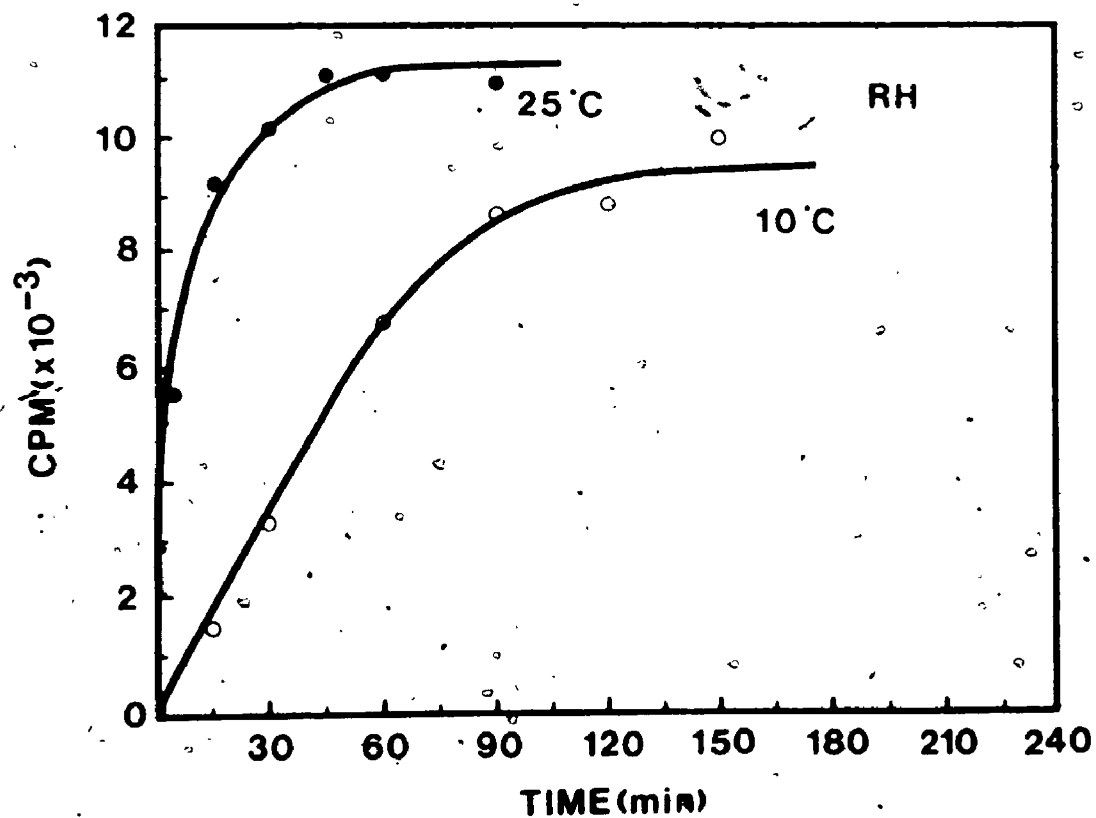


RH
25°C
10°C
CPM (x10⁻³)
TIME (min)
0
2
4
6
8
10
12
0
30
60
90
120
150
180
210
240

Figure 14. Time-course of amino acid incorporation by RNH and RH polysomes (45 µg) at 10°C and 25°C with 2.6 picomoles of labelled [^{35}S]methionine. The volume of the *in vitro* translation mixture was 50 µl from which aliquots of 5 µl were removed at given times. The concentration of labelled [^{35}S]methionine was 2.6 picomoles. The Mg^{++} and K^{+} final concentrations were 3 mM and 120 mM respectively. Results are from one experiment. This experiment was repeated once with similar results.



25°C
RNH
10°C
CPM (x10^{-3})
TIME (min)



25°C
RH
10°C
CPM (x10^{-3})
TIME (min)

4.3.1.2 **Effect of methionine concentration.**

The incorporation of methionine by RNH and RH polysomes was also regulated by the amount of methionine available in the translation mixture (Fig. 13 vs Fig. 14). RNH and RH polysomes were affected differently by a limiting amount of methionine. Total incorporation by RNH polysomes was increased by a factor of 3.5 times when the amount of labelled methionine was doubled from 1.3 to 2.6 picomoles regardless of temperature. Comparable values for RH polysomes were 2.8 and 2.0 times at 10°C and 25°C, respectively. At a concentration of 1.3 picomoles of methionine, RH polysomes were 1.4 (10°C) and 1.7 (25°C) times more active than the RNH polysomes to synthesize polypeptides. At 2.6 picomoles of methionine, RNH and RH polysomes incorporated similar quantities of methionine at 25°C (Fig. 13 and 14). The incorporation at 10°C by RH polysomes was less reduced than for the RNH polysomes.

The capacity of RNH and RH polysomes to incorporate methionine varied with the increase in the concentration of the labelled amino acid. In order to identify whether RNH and RH polysomes had the same synthetic capacities <u>in vitro</u>, the determination of the minimal quantity of methionine to saturate the incorporation and the optimization of the translational mixture for RNH and RH polysomes appeared necessary. Concentrations of 1.3 and 2.6 picomoles of methionine were clearly suboptimal. Saturation in the incorporation of methionine was reached

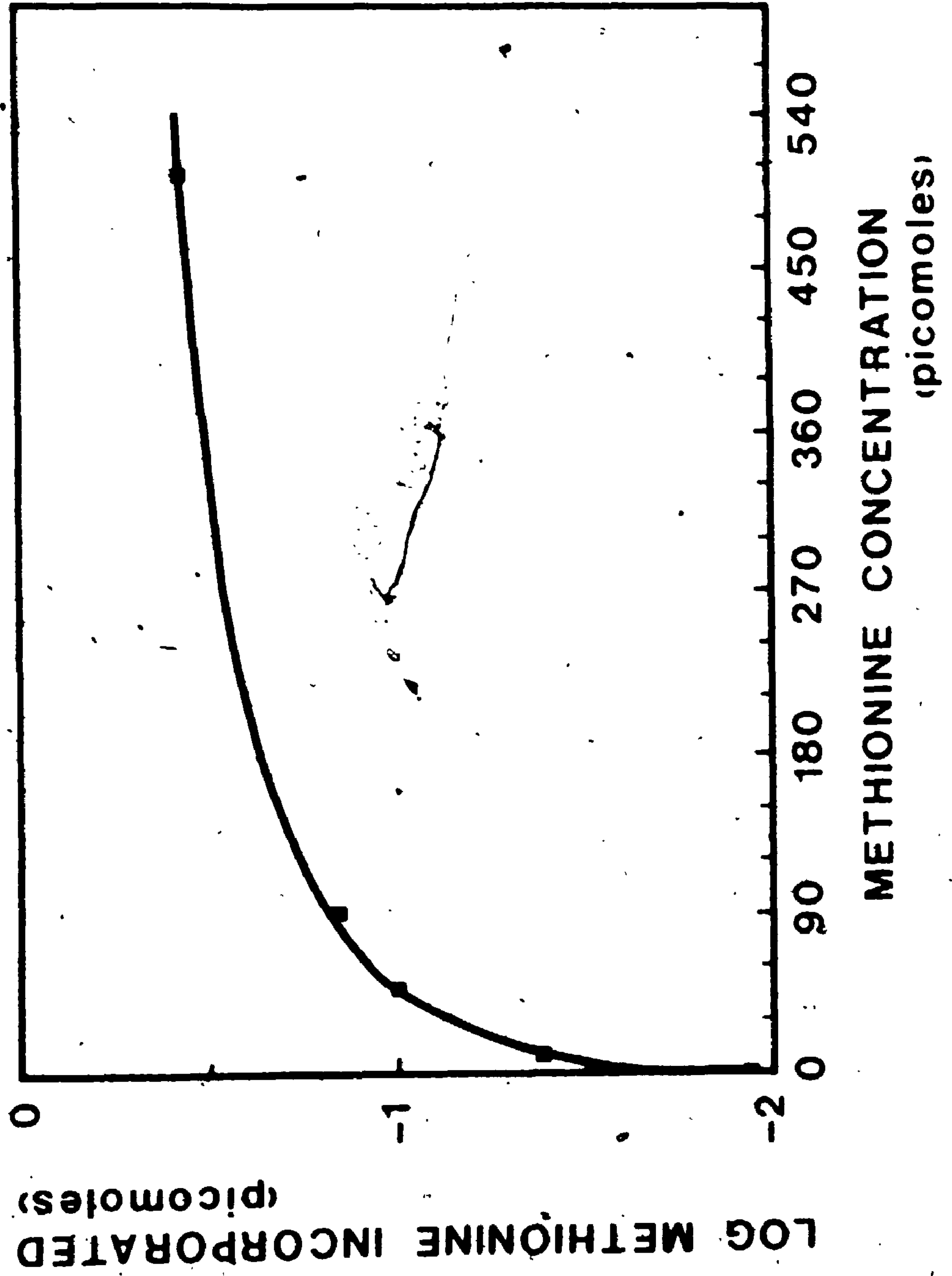
when 300 picomoles of methionine were present in the translational mixture for RNH polysomes (Fig. 15). Taking into account the concentration of the other amino acids (1500 picomoles) in the translation mixture, 1000 picomoles of methionine were added in subsequent translations. In order to achieve that concentration of methionine, non-labelled methionine was added to the translation mixture in the following proportion 26:974 (labelled:non-labelled). This lowered the specific activity of methionine and the TCA-precipitable counts but increased the quantity of incorporated picomoles of methionine in subsequent experiments.

4.3.1.3 **Effects of magnesium and potassium.**

Magnesium titration revealed that the optimal Mg^{++} concentration appears to be temperature and polysome dependent (Fig. 16 and Table 17). At 10°C, 2.2 mM Mg^{++} was optimal for the translation of both RNH and RH polysomes. However at 25°C, optimal Mg^{++} concentrations were 3.5 mM and 5.5 mM for translation of RNH and RH polysomes respectively. In addition, RH polysomes appear to be less susceptible than RNH polysomes to an excess of magnesium during translation.

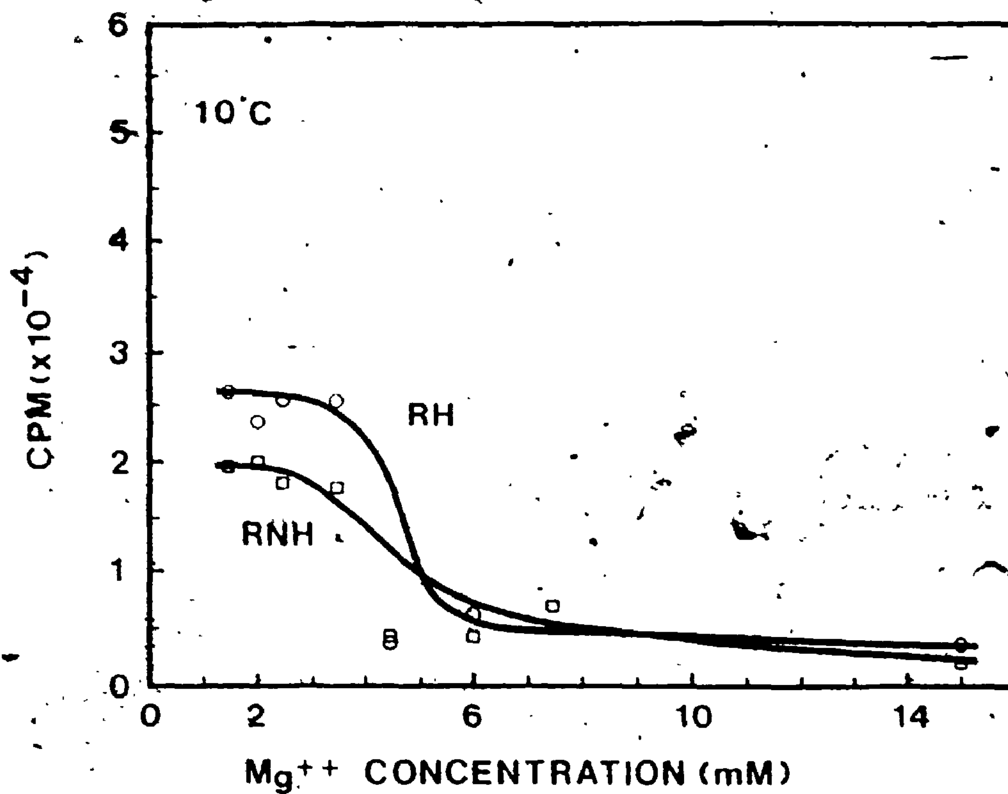
The optimal concentration of K^{+} was different for RH polysomes at 10°C and 25°C (Fig. 17). At 10°C, the optimal K^{+} concentration for *in vitro* translation was 130 mM for both RNH and RH polysomes. However, at 25°C, the optimum

Figure 15. Saturation curve for methionine incorporation by RNH polysomes (45 μg) at 25°C. The methionine concentration was increasing from 1.3 to 510 picomoles. The volume of the in vitro translation mixture was 50 μl from which 2 aliquots of 5 μl each were removed after 90 min of incubation. The Mg^{++} and K^{+} final concentrations were 3 mM and 120 mM respectively. Results are from one experiment. This experiment was repeated once with similar results.



LOG METHIONINE INCORPORATED
(picomoles)
0
-1
-2
0
90
180
270
360
450
540
METHIONINE CONCENTRATION
(picomoles)

Figure 16. Effect of Mg^{++} concentration on _in vitro_ _translation_ of RNH and RH polysomes (45 µg) at 10°C and 25°C. The Mg^{++} concentration was increased from 1.5 to 15 mM. The volume of the _in vitro_ translation mixture was 50 µl from which 2 aliquots of 5 µl each were removed after 90 min (25°C) or 180 min (10°C) of incubation. The final concentration of K^{+} was 120 mM while 26 picomoles of [^{35}S]methionine and 974 picomoles of non-labelled methionine were present in the translation mixture. Results are from one experiment. This experiment was repeated three times with similar results.


10°C
RH
RNH
CPM (x10^{-4})
0
1
2
3
4
5
6
0
2
6
10
14
Mg++ CONCENTRATION (mM)

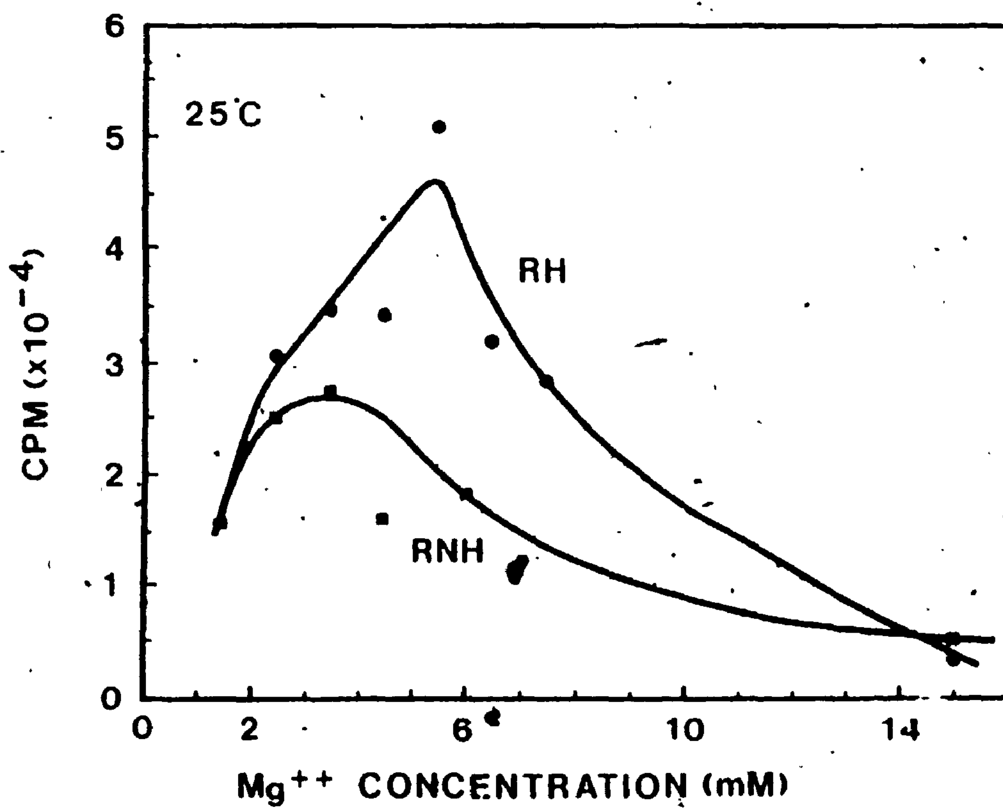
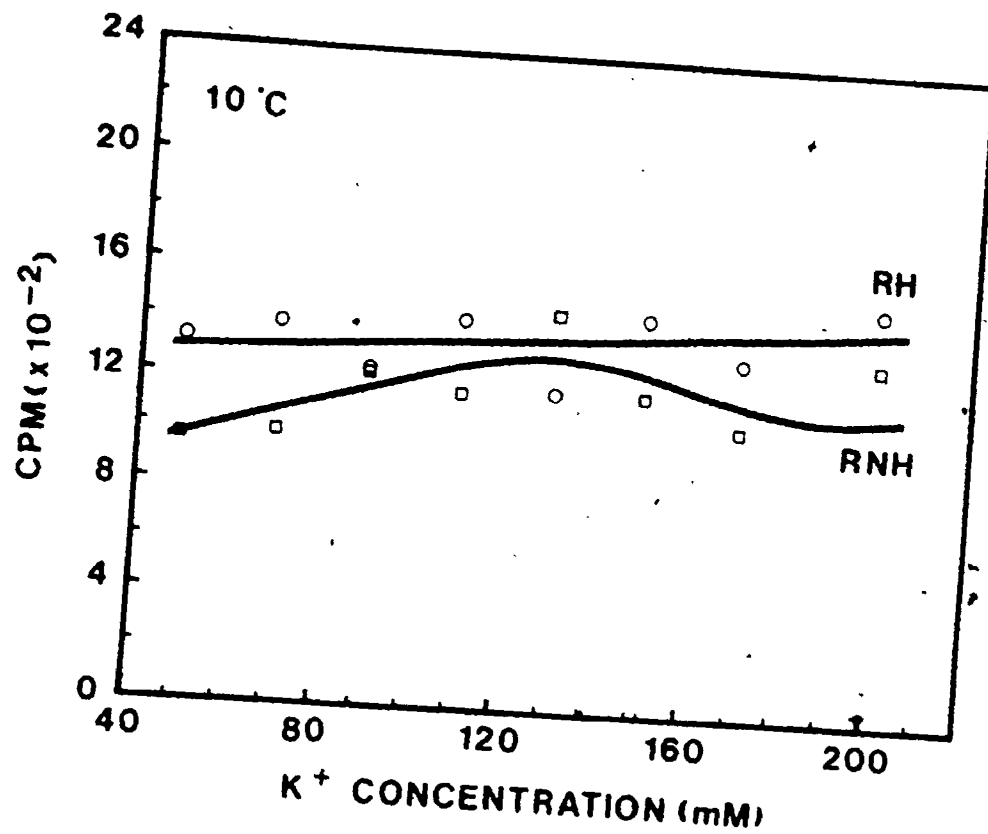
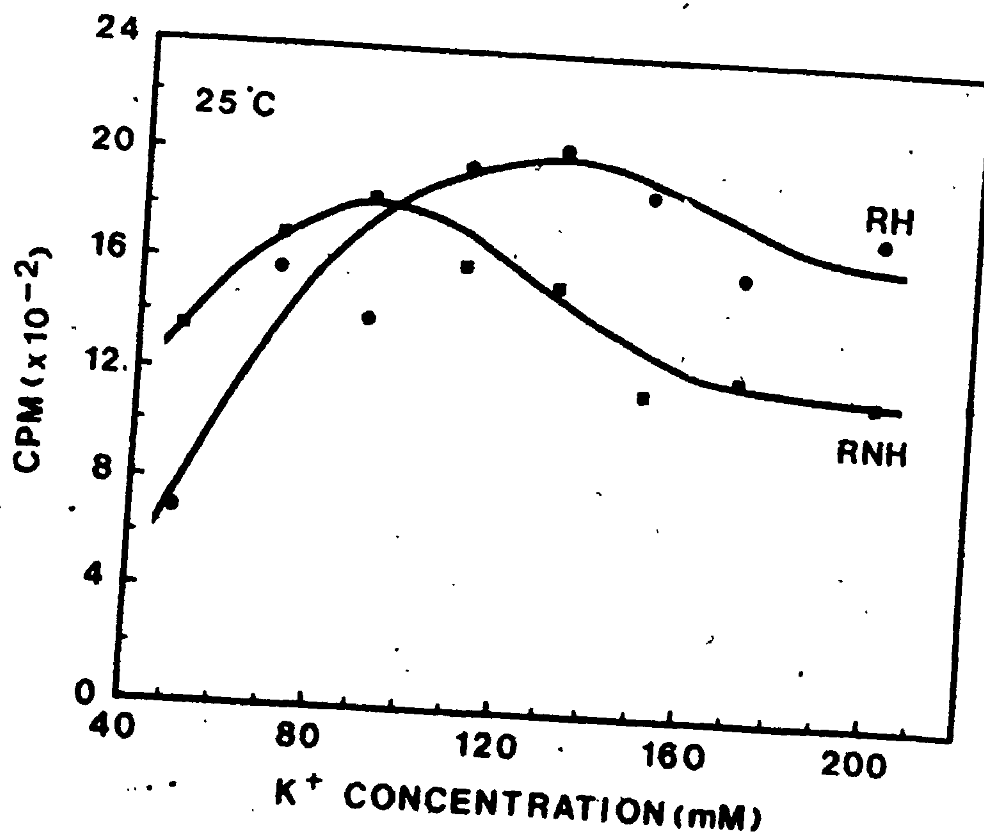

25°C
RH
RNH
CPM (x10^{-4})
0
1
2
3
4
5
6
0
2
6
10
14
Mg++ CONCENTRATION (mM)

Table 17. Optimal Mg^{++} concentration (mM) for *in vitro* translation of RNH and RH polysomes (20 µg) at 10°C and 25°C. The Mg^{++} concentration was increased from 1.5 to 15 mM. The volume of the *in vitro* translation mixture was 50 µl from which 2 aliquots of 5 µl each were removed after 90 min (25°C) or 180 min (10°C) of incubation. The final concentration of K^{+} was 120 mM while 26 picomoles of [^{35}S]methionine and 974 picomoles of non-labelled methionine were present in the translation mixture. Each point is the mean of four different experiments.

	RNH	RH
10°C	2.2	2.2
25°C	3.5	5.5

Figure 17. Effect of K+ concentration on *in vitro* translation of RNH and RH polysomes (45 µg) at 10°C and 25°C. The K^+ concentration was increased from 50 to 200 mM. The volume of the *in vitro* translation mixture was 50 µl from which 2 aliquots of 5 µl each were removed after 90 min (25°C) or 180 min (10°C) of incubation. Optimal concentrations of Mg^{++} were used for RNH and RH polysomes as described in Table 17. 26 picomoles of [^{35}S]methionine and 974 picomoles of non-labelled methionine were present in the translation mixture. Results are from one experiment. This experiment was repeated once with similar results.


10 °C
RH
RNH
CPM (x10⁻²)
24
20
16
12
8
4
0
40
80
120
160
200
K⁺ CONCENTRATION (mM)


25 °C
RH
RNH
CPM (x10⁻²)
24
20
16
12.
8
4
0
40
80
120
160
200
K⁺ CONCENTRATION (mM)

value was 130 mM for RH polysomes while 90 mM was optimal for RNH polysomes. In all cases, an excess of potassium is not as inhibitory as magnesium on the translational activity.

4.3.1.4 Saturation by polysomes.

Methionine incorporation by RNH and RH polysomes at 10°C and 25°C increased linearly with the quantity of polysomes indicating that polysomes were the limiting factor during translation (Fig. 18).

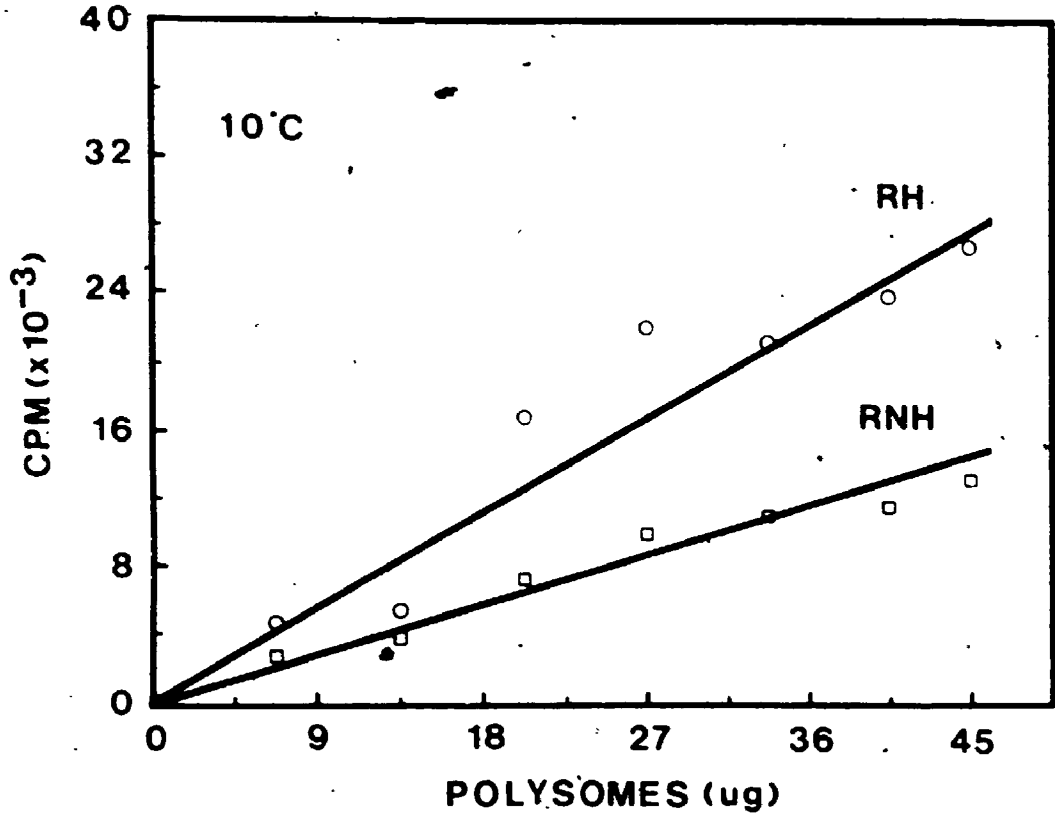
4.3.1.5 Effect of human placenta ribonuclease inhibitor.

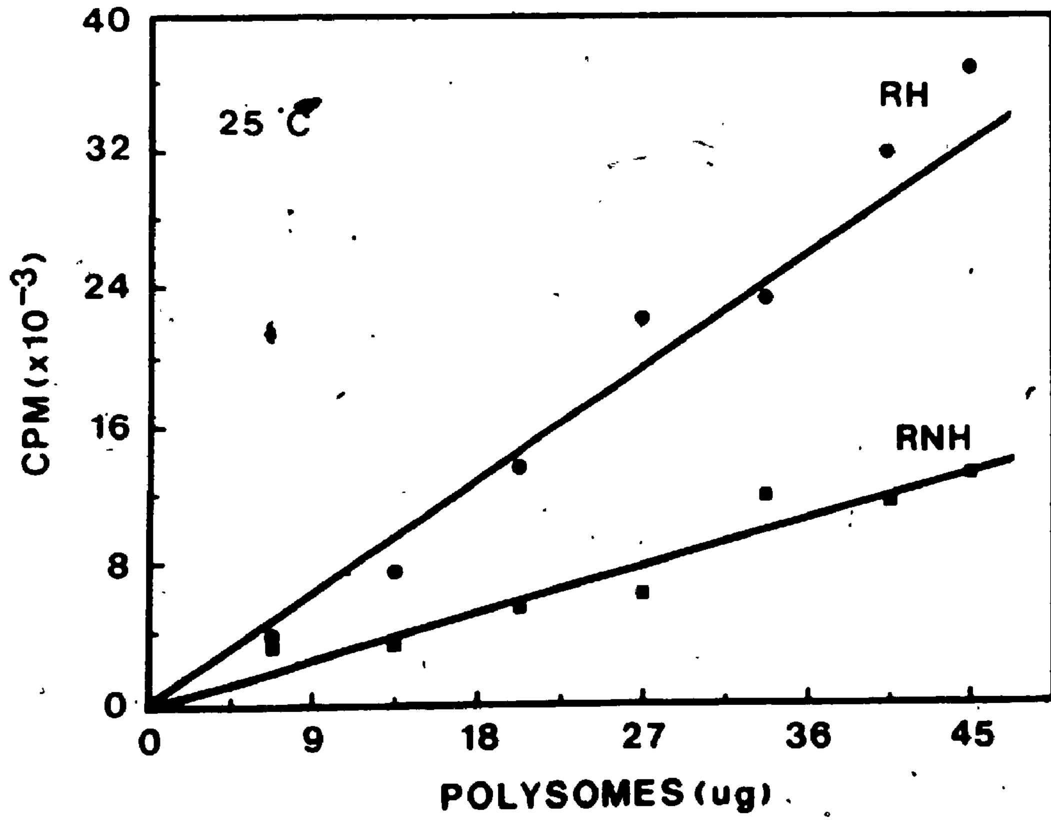
Addition of human placenta ribonuclease inhibitor (HPRI) at the recommended concentration of 1 unit/µl of translation mixture (Clemens, 1984; Jolicoeur and Brakier-Gingras, 1983; Morch et al.., 1986; Scheele and Blackburn, 1979) did not increase the total incorporation by either RNH or RH polysomes. Apparently, ribonucleases were not present in the S-100 wheat germ fraction used as source of soluble fraction for the in vitro translation assay.

4.3.1.6 Effect of aurintricarboxylic acid.

During translation of RNH and RH polysomes in presence of 50 µM aurintricarboxylic acid (ATA), the total incorporation by RNH and RH polysomes was decreased by only 12%. This result demonstrates that the S-100 wheat germ

Figure 18. Effect of polysome concentration on methionine incorporation at 10°C and 25°C. RNH and RH polysome quantities were increased from 0 to 45 µg. The volume of the *in vitro* translation mixture was 50 µl from which 2 aliquots of 5 µl each were removed after 90 min (25°C) or 180 min (10°C) of incubation. Optimal concentrations of Mg^{++} and K^{+} were used for RNH and RH polysomes as described (section 4.3.1.2). 26 picomoles of [^{35}S]methionine and 974 picomoles of non-labelled methionine were present in the translation mixture. Results are the mean of two experiments.


10°C
RH
RNH
CPM (x 10^{-3})
0
8
16
24
32
40
0
9
18
27
36
45
POLYSOMES (ug)

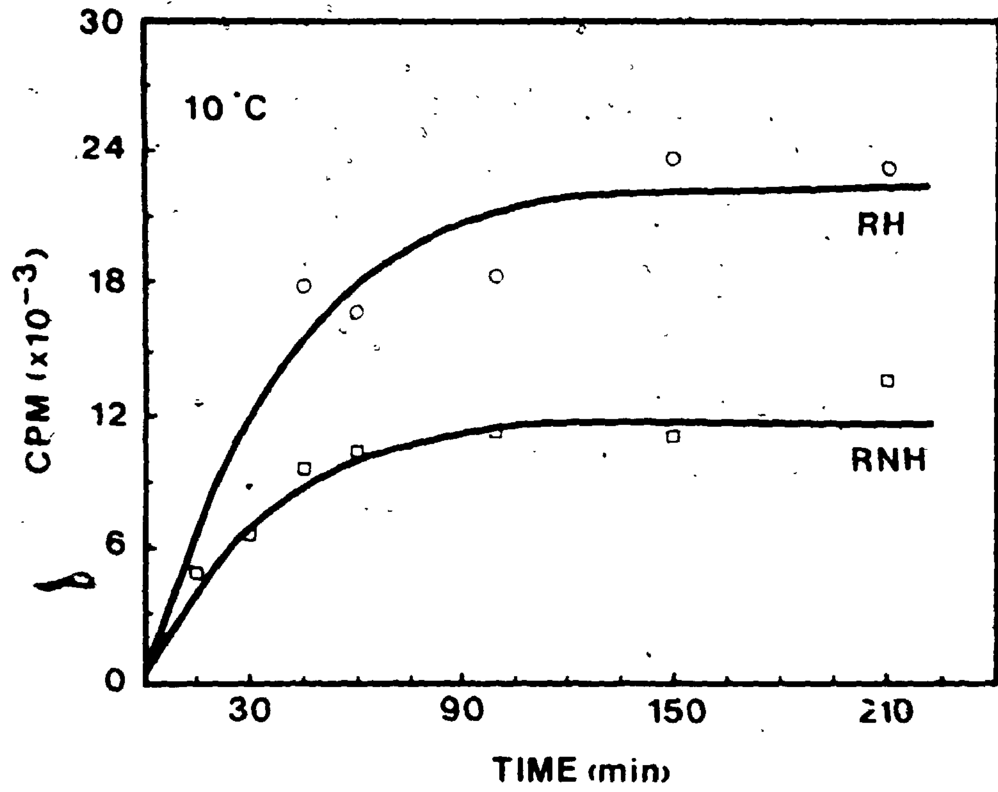

25 °C
RH
RNH
CPM (x 10^{-3})
0
8
16
24
32
40
0
9
18
27
36
45
POLYSOMES (ug)

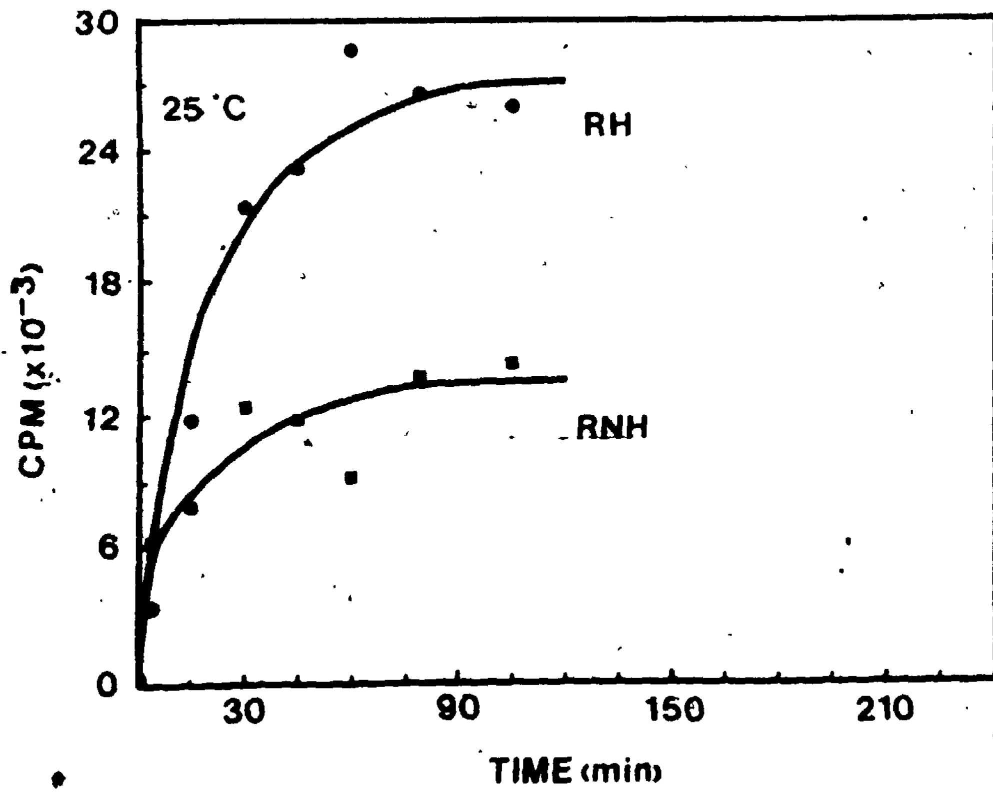
fraction was unable to effectively reinitiate protein synthesis.

4.3.1.7 Incorporation under optimal conditions.

Under optimal conditions, RH polysomes were always twice as active as RNH polysomes and regardless of temperatures. In addition, the total incorporation by RH polysomes at 10°C was 1.7 times higher than the RNH one at 25°C. The time required to incorporate half of the total incorporated methionine was longer for RH than for RNH polysomes at 10°C and 25°C. For both RNH and RH polysomes, total incorporation was reduced by less than 15% at 10°C while saturation occurred after 105 min at 25°C instead of 150 min at 10°C (Fig. 19). In order to estimate whether the initiation step was affected for polysome fractions with different proportion of large-polysomes and translated with S-100 wheat germ system, the results for the amino acid incorporation by polysomes should be expressed in terms of specific activity of polysomes (Klyachko _et al_., 1982). The specific activity of polysomes is the ratio of the total incorporation of polysomes by the proportion of small and large polysomes in the preparation of polysomes used (Klyachko _et al_., 1982). Expressed in terms of specific activity of polysomes, the synthesizing activity of RH polysomes was increased by more than 50% when compare to RNH polysome activity and regardless of the temperature of translation.

Figure 19. Time-course of methionine incorporation by RNH and RH polysomes (45 µg) under optimal conditions at 10°C and 25°C. The volume of the <u>in</u> <u>vitro</u> translation mixture was 50 µl from which aliquots of 5 µl were removed at given times of incubation. Optimal concentrations of Mg^{++} and K^{+} were used for RNH and RH polysomes as described (section 4.3.1.2). 26 picomoles of [^{35}S]methionine and 974 picomoles of non-labelled methionine were present in the translation mixture. Results are from one experiment. This experiment was repeated once with similar results.


10 °C
RH
RNH
CPM (x10^{-3})
TIME (min)
0
6
12
18
24
30
30
90
150
210


25 °C
RH
RNH
CPM (x10^{-3})
TIME (min)
0
6
12
18
24
30
30
90
150
210

4.3.2 Translational activity of mRNA.

Since growth at low temperature affected polysome activity, it was interesting to compare the effectiveness of mRNA from RNH and RH leaves with respect to directing *in vitro* translation. Messenger RNA was isolated and translated with a rabbit reticulocyte lysate.

4.3.2.1 Effect of methionine concentration.

Due to differences in the preparation of the wheat germ and rabbit reticulocyte lysate systems, the endogenous pool of methionine is removed in wheat germ system but not in rabbit reticulocyte lysate (Clemens, 1984). The concentration of methionine has been established as 5 µM in rabbit reticulocyte lysate (Clemens, 1984). Although this value is close to the saturation point observed during polysome translation, the influence of the methionine concentration was nevertheless verified. This was necessary mainly because the rabbit reticulocyte lysate, in contrast to the wheat germ system is able to reinitiate protein synthesis (Clemens, 1984). The saturation point was reached around 300 picomoles of methionine (Fig. 20).

4.3.2.2 Effect of magnesium and potassium.

The optimal Mg^{++} concentration was different for RNH and RH mRNA (Fig. 21). Magnesium titration of RNH mRNA showed a narrow profile with a maximum of translational activity at 0.7 mM Mg^{++} while the profile for RH mRNA was

Figure 20. Saturation curve of methionine incorporation by RNH polysomal mRNA (0.7 µg). The methionine concentration was increasing from 3 to 500 picomoles. Protein synthesis was carried out with the rabbit reticulocyte lysate at 30°C. The volume of the *in vitro* translation mixture was 25 µl from which 2 aliquots of 2 µl each were removed after 60 min of incubation. The Mg^{++} and K^{+} final concentrations were 0.7 mM and 145 mM respectively. Results are from one experiment. This experiment was repeated once with similar results.

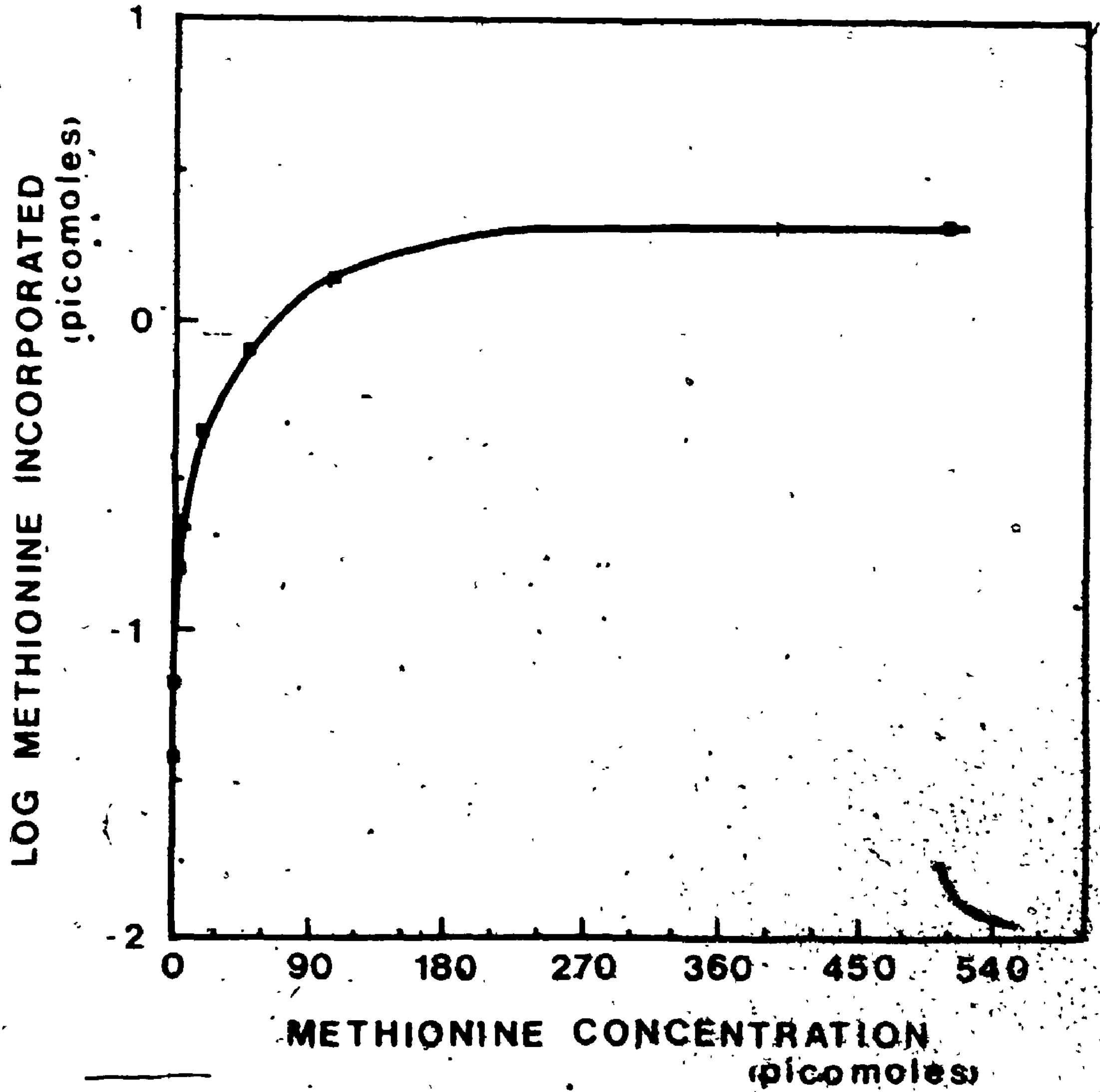
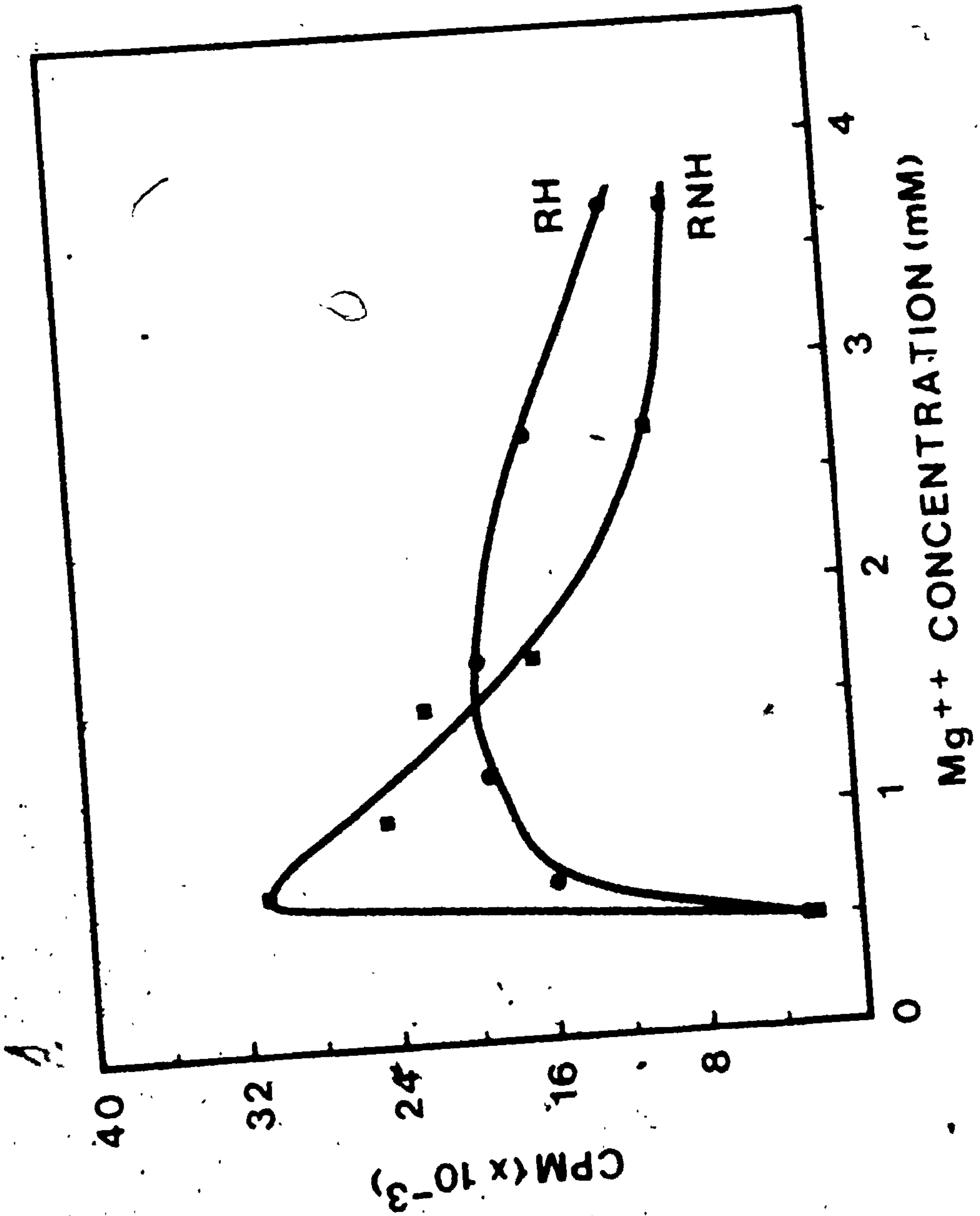

LOG METHIONINE INCORPORATED
(picomoles)
1
0
-1
-2
0
90
180
270
360
450
540
METHIONINE CONCENTRATION
(picomoles)

Figure 21. Effect of Mg^{++} concentration on in vitro translation of RNH and RH cytoplasmic mRNA (3.5 µg). The Mg^{++} concentration was increased from 0.5 mM to 3.7 mM. Protein synthesis was carried out with the rabbit reticulocyte lysate at 30°C. The volume of the in vitro translation mixture was 25 µl from which 2 aliquots of 2 µl each were removed after 60 min of incubation. The final concentration of K^{+} was 100 mM while 8.8 picomoles of $[^{35}S]$methionine were present in the translation mixture. Results are the mean of two experiments.

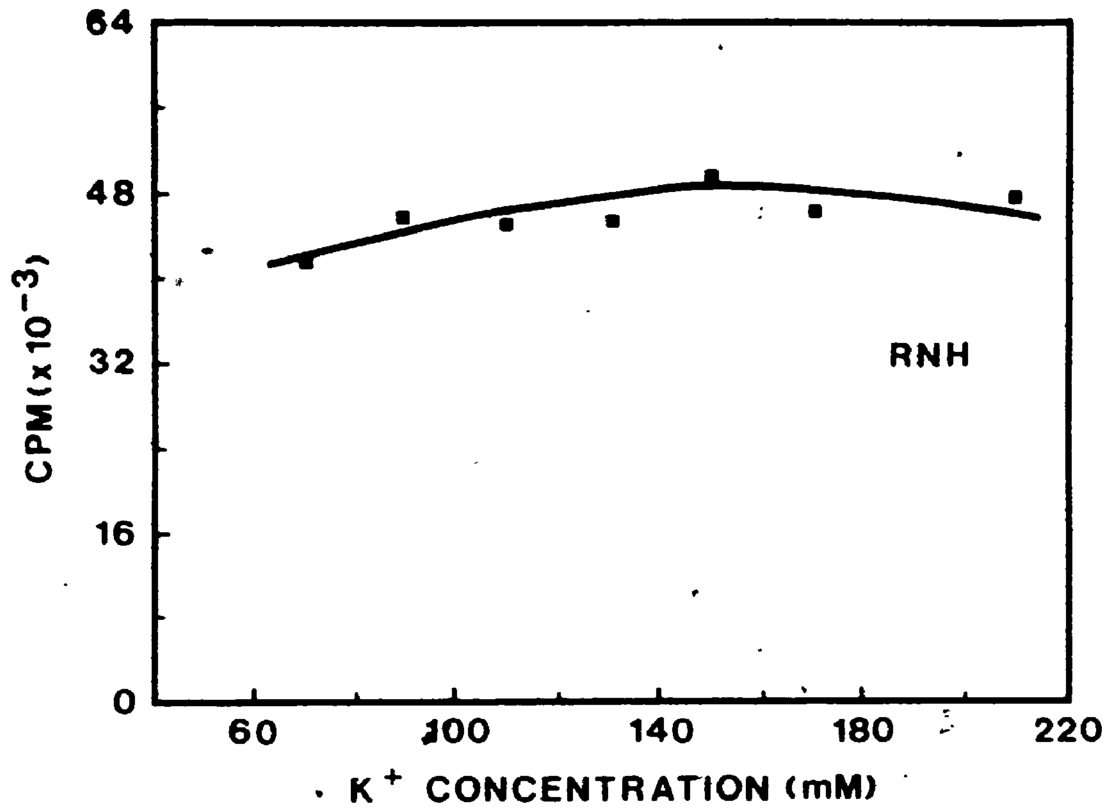

40
32
24
16
8
CPM (x 10⁻³)
0
1
2
3
4
Mg++ CONCENTRATION (mM)
RH
RNH

much broader and maximum at 1.7 mM Mg^{++}. Incorporation of methionine by RNH and RH mRNA decreased sharply at magnesium concentrations less than 0.7 mM and was almost completely inhibited at a concentration of 0.5 mM Mg^{++}. On the other hand, an increase of magnesium concentration from 0.7 to 1.7 mM resulted in a 50% reduction of amino acid incorporation by RNH mRNA. In contrast, incorporation of methionine by RH mRNA was reduced by only 17% when the magnesium concentration increased from 1.7 mM to 2.7 mM. This reduced sensitivity to excess magnesium and the higher optimal Mg^{++} concentration were similar to that observed for RH polysomes (Fig. 16). Differences were also observed in the K^{+} titration between RNH and RH mRNA (Fig. 22). Although the differences were not as great as for magnesium, optimal mean values of 145 mM and 135 mM K^{+} for RNH and RH mRNA were obtained. Specific requirements for K^{+} appear to be different for polysomes and mRNA since RH polysomes and RNH mRNA need a higher optimal K^{+} concentration.

4.3.2.3 **Saturation by mRNA.**

The optimal quantity of mRNA to direct amino acid incorporation was twice as low for RNH mRNA (1 µg) than for RH mRNA (2 µg) (Fig. 23). An excess of RH mRNA did not inhibit the incorporation of amino acids as severely as RNH mRNA.

Figure 22. Effect of K+ concentration on <u>in vitro</u> translation of RNH and RH cytoplasmic mRNA (3.5 µg). The K+ concentration was increased from 70 to 210 mM. Protein synthesis was carried out with the rabbit reticulocyte lysate at 30°C. The volume of the <u>in vitro</u> translation mixture was 25 µl from which 2 aliquots of 2 µl each were removed after 60 min of incubation. Optimal concentrations of Mg^{++} of 0.7 and 1.7 mM were used for RNH and RH mRNA while 8.8 picomoles of [^{35}S]methionine were present in the translation mixture. Results are from one experiment. This experiment was repeated once with similar results.


64
48
32
16
0
CPM (x 10⁻³)
RNH
60
100
140
180
220
K + CONCENTRATION (mM)

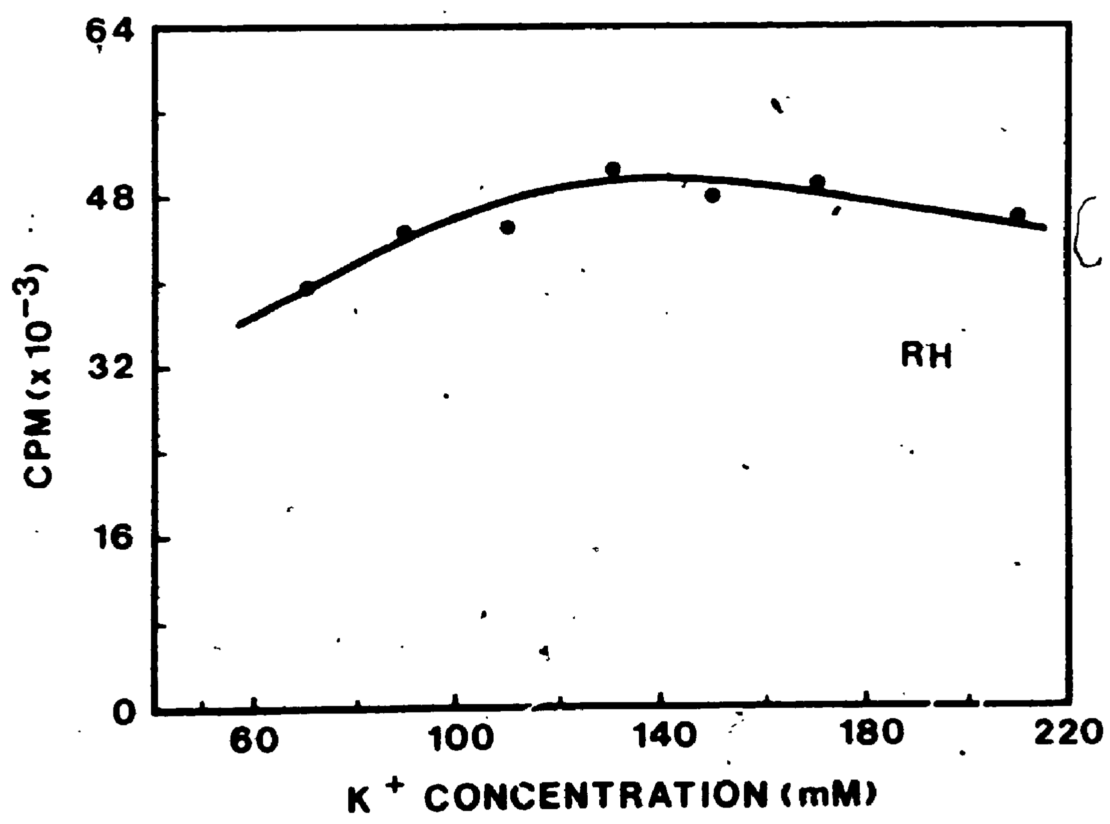
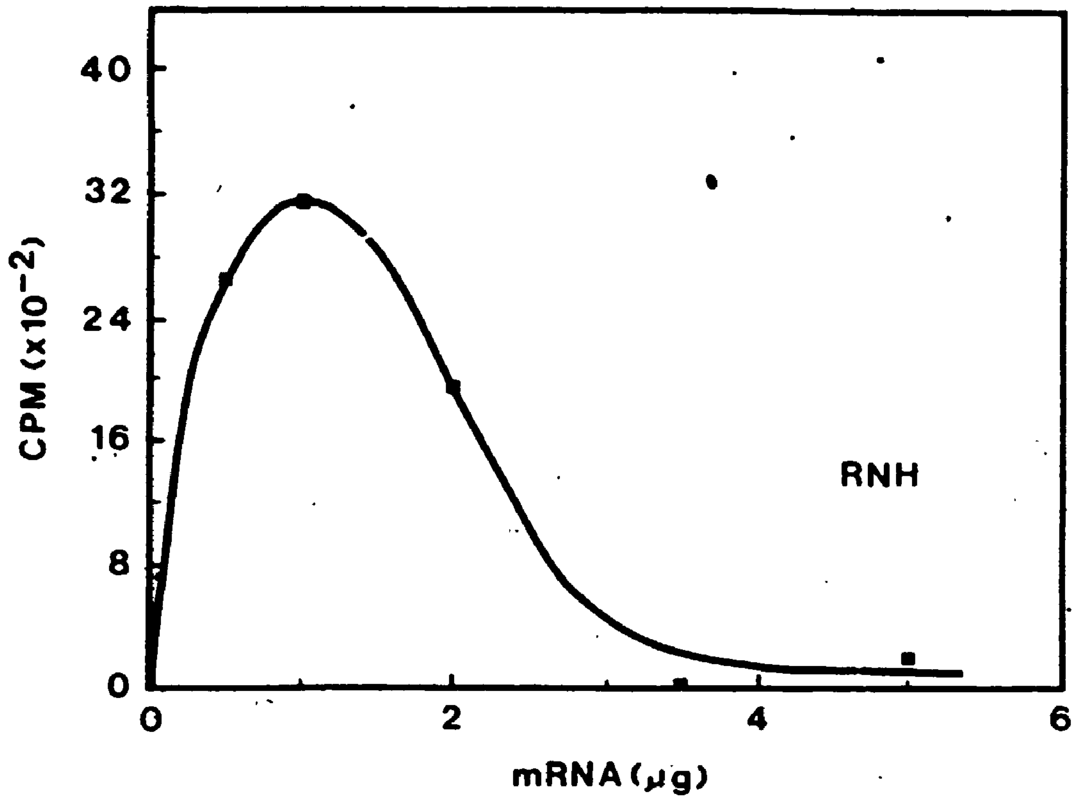
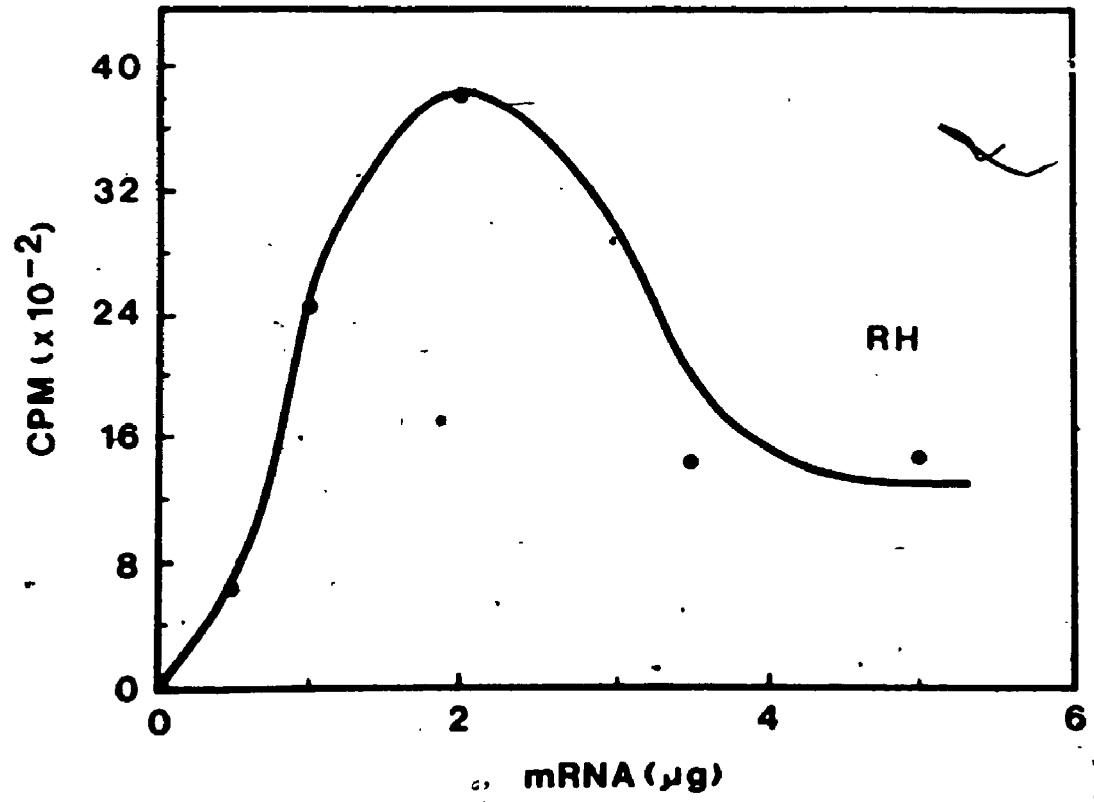

64
48
32
16
0
CPM (x 10⁻³)
RH
60
100
140
180
220
K + CONCENTRATION (mM)

Figure 23. Effect of mRNA concentration on methionine incorporation. RNH and RH polysomal mRNA quantities were increased from 0 to 5 µg. Protein synthesis was carried out with the rabbit reticulocyte lysate at 30°C. The volume of the *in vitro* translation mixture was 25 µl from which 2 aliquots of 2 µl each were removed after 60 min of incubation. Optimal concentrations of Mg^{++} and K^{+} were used for RNH and RH mRNA as described (section 4.3.2.2) while 6 picomoles of [^{35}S]methionine and 334 picomoles of non-labelled methionine were present in the translation mixture. Results are the mean of two experiments.



CPM (x10^-2)
40
32
24
16
8
0
0
2
4
6
mRNA (μg)
RNH

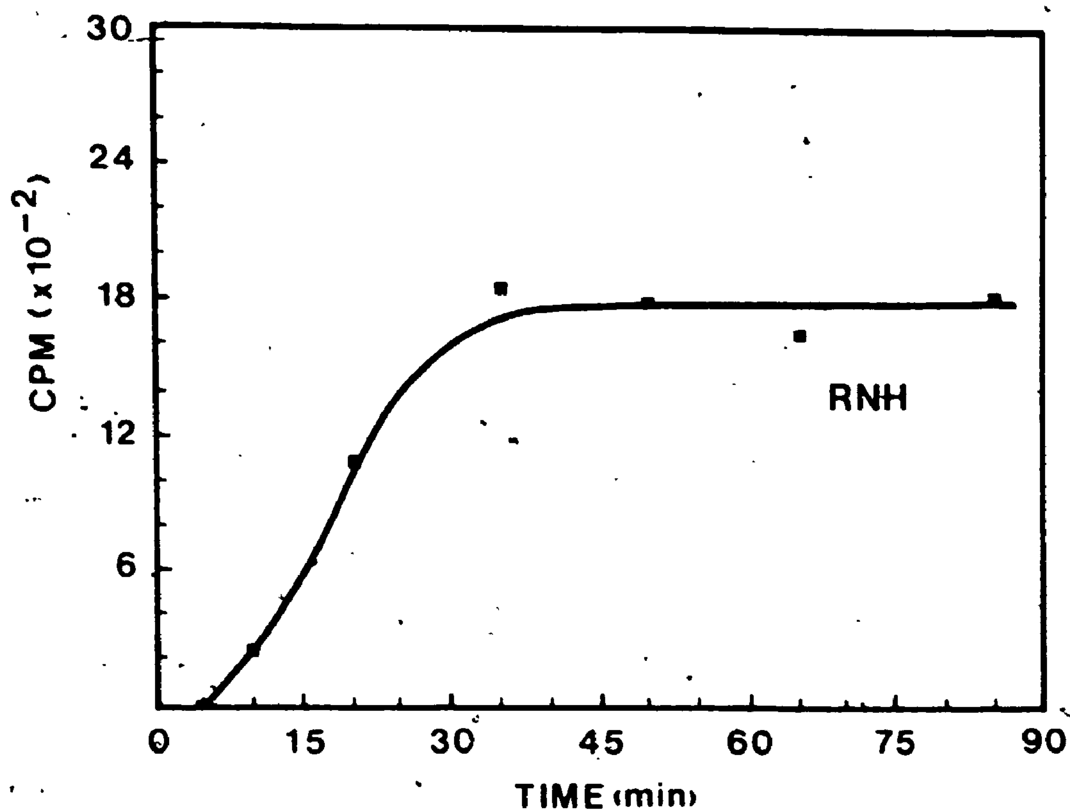


CPM (x10^-2)
40
32
24
16
8
0
0
2
4
6
mRNA (μg)
RH

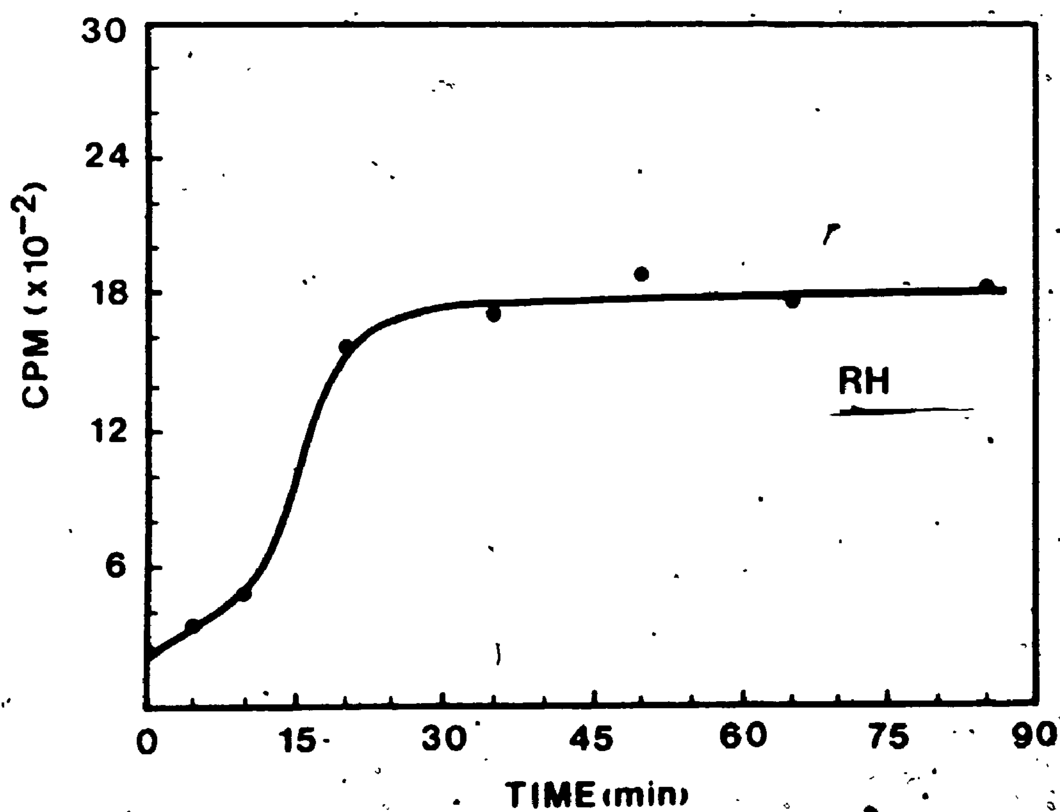
4.3.2.4 Incorporation under optimal conditions.

At a concentration of 1 µg of mRNA/25 µl of translation mixture, the capacity to incorporate the methionine was similar for both RNH and RH polysomal mRNA contrasting with the previous results obtained with polysomes (Fig. 24).

Figure 24. Time-course of methionine incorporation by RNH and RH polysomal mRNA (1 μg) under optimal conditions. Protein synthesis was carried out with the rabbit reticulocyte lysate at 30°C. The volume of the *in vitro* translation mixture was 25 μl from which aliquots of 2 μl were removed at given times of incubation. Optimal concentrations of Mg^{++} and K^{+} were used for RNH and RH polysomes as described (section 4.3.2.2) while 3 picomoles of [^{35}S]methionine and 167 picomoles of non-labelled methionine were present in the translation mixture. Results are the mean of two experiments.



CPM (x10⁻²)
30
24
18
12
6
0
15
30
45
60
75
90
TIME (min)
RNH



CPM (x10⁻²)
30
24
18
12
6
0
15
30
45
60
75
90
TIME (min)
RH

4.4 DISCUSSION

In vitro translation of protein using a wheat germ system is usually carried out at 25°C (Clemens, 1984; Reisfeld and Edelman, 1982) but translation at temperatures as low as 10°C and even 0°C have been reported (Fehling and Weidner, 1986; Gast *et al*., 1985). It was desirable if not necessary in a study on polysomes isolated from leaves of rye seedlings grown at low and high temperatures, to attempt translation of these isolated polysomes at/or near the same temperatures. A temperature of 25°C was selected because this temperature is optimal for translation with a wheat germ system (Clemens, 1984; Reisfeld and Edelman, 1982). As the low temperature of translation, 10°C was selected because the translation of isolated wheat polysomes has been reported to be almost completely inhibited at 5°C (Fehling and Weidner, 1985).

Total incorporation appears to be affected by the temperature of translation. A decrease of 15% to 35% as well as a lower rate of incorporation were observed at 10°C for both RNH and RH polysomes when compared to results obtained at 25°C (Fig. 13 and 14). But before drawing any conclusions about the effect of temperature on synthesis *in vitro*, it is first necessary to verify whether the incorporation of amino acid by both RNH and RH polysomes was carried out under optimal conditions.

Several aspects of the results presented support the conclusion that the establishment of optimal conditions for

each temperature of translation was necessary before trying to assess polysome activity. Optimal concentration of Mg^{++} and K^{+} were found to be temperature dependent and were different for RNH and RH polysomes. Magnesium concentration had a greater effect on the translation than that of potassium. It was also found that total incorporation was limited by the quantity of polysome present in the translation mixture. A low concentration of methionine regulated amino acid incorporation and RNH and RH polysomes were affected differently by methionine concentration. Finally, the rate of amino acid incorporation was temperature dependent.

The results obtained on the influence of Mg^{++} and K^{+} on polysomes isolated from RNH and RH leaves show that the Mg^{++} and K^{+} requirements differ for RNH and RH polysomes and are temperature dependent. Values of 2.2 mM Mg^{++} and 130 mM K^{+} for RNH and RH polysomes translated at 10°C and 3.5 mM Mg^{++} and 90 mM K^{+} for RNH polysomes and 5.5 mM Mg^{++} and 130 mM K^{+} for RH polysomes translated at 25°C are in the range of optimal values reported in other systems (Boardman _et al._, 1966; Clemens, 1984; Häggman, 1986; Jolicoeur and Brakier-Gingras, 1983; Morch _et al._, 1986; Reisfeld and Edelman, 1982). These results also confirm that the optimal concentration range of K^{+} is much broader than for Mg^{++} (Clemens, 1984) and consequently an excess of K^{+} is not as inhibitory as a Mg^{++} excess on the translational activity.

The importance for optimal concentration of Mg^{++} and K^{+} during *in vitro* translation is well recognized. Determination of optimal concentration of Mg++ and K+ for the translation of mRNA isolated from different species is always recommended in technical papers describing method for *in vitro* translation (Clemens, 1984; Reisfeld and Edelman, 1982) and by suppliers of translation kits (BRL, Dupont, Promega Biotec). Optimal Mg^{++} concentration has been shown and reported to give the optimal ribosome conformation for optimal biosynthesis (Boardman *et al.*, 1966; Clemens, 1984; Jolicoeur and Brakier-Gingras, 1983; Reisfeld and Edelman, 1982; Sim and Klambt, 1973). For optimal translation of mRNA with wheat germ, concentrations varying between 1.5-3.5 mM Mg^{++} and 70-125 mM K^{+} have been reported as optimal (Clemens, 1984; Morch *et al.*, 1986; Reisfeld and Edelman, 1982). Concentrations of 4 mM Mg^{++} and 120 mM K^{+} and 3.5 mM Mg^{++} and 140 mM K^{+} for respectively pine polysomes and polysomes isolated from hamster skeletal and heart muscles have been reported optimal for translation with the S-100 wheat germ fractions (Häggman, 1986; Jolicoeur and Brakier-Gingras, 1983). Similarly optimal concentration of 5 mM Mg^{++} and 140 mM K^{+} and 10 mM Mg^{++} and 50 mM K^{+} for cytoplasmic tobacco and corn polysomes have been reported during translation with the S-100 fraction in homologous systems (Boardman *et al.*, 1966; Sim and Klambt, 1973).

Increase in the optimal Mg^{++} concentration from 3.5 mM to 6.5 mM for in vitro translation between polysomes isolated from skeletal muscle and heart of normal and dystrophic hamster has been reported while the optimal concentration of K^{+} was not affected (Jolicoeur and Brakier-Gingras, 1983). Polysome fractions from dystrophic hamster tissues were also less sensitive to an excess of Mg^{++} than the control and consequently more active at a concentration of 10 mM Mg^{++} (Jolicoeur and Brakier-Gingras, 1983).

On the other hand, it is not known whether a high temperature treatment will induce a different ion requirement for in vitro translation of polysomes in a similar way as low temperature. In the only known study on the influence of the increasing growing temperature of seedlings on polysome activity, this point was not apparently verified (Fehling and Weidner, 1986, 1985).

Within the range of RNH and RH polysomes (0 to 45 µg) translated, the amino acid incorporation was proportional to the amount of polysomes present. Apparently large quantities of polysomes are necessary to saturate such incorporating systems (C. Rees, personal communication) although concentration higher than 18 µg of polysomes have been reported to saturate a poly-U incorporating system (Fehling and Weidner, 1985).

The capacity of RNH and RH polysomes to incorporate amino acid into polypeptides was dependent on the

concentration of methionine present. At 1.3 picomoles of methionine, RH polysomes had higher synthetic activity than RNH polysomes. However at 2.6 picomoles, the capacity of RNH and RH polysomes to synthesize proteins was similar. Even if the exact meaning of these results is difficult to evaluate, they do show that RNH and RH polysomes differ with respect to the kinetics of incorporation. While the minimal concentration of labelled amino acid is critical for *in vitro* translation and has been pointed out (Clemens, 1984; Nowak *et al.*, 1984), there is no particular study addressing the regulation of translational capacity of polysomes by a particular amino acid in limited quantity. Methionine was clearly limiting the incorporation and at least 300 picomoles were necessary to saturate the incorporating system. In a study on ribosome translational capacity, one must ensure that enough amino acid is present in the reaction mixture to prevent limitation of total incorporation (Clemens, 1984; Nowak *et al.*, 1984), particularly when labelled amino acid of high specific activity is used (Clemens, 1984). This problem is also more apparent when wheat germ is used rather than rabbit reticulocyte lysate since during wheat germ preparation, amino acids and low molecular weight components are removed during the gel filtration step while this step is absent from the preparation of rabbit reticulocyte lysate (Clemens, 1984).

The establishment of optimal ion requirements support the conclusion that growth at low temperature alters ribosome conformation.

The ribosome is presently viewed as a dynamic structure that is capable of assuming a variety of conformations which are stabilized by interactions among its RNA and protein components. These interactions are, in turn, greatly affected by the ionic environment. Cations, in particular, have been shown to play a central role in maintaining the stability and activity of the ribosome. They are critical for all aspects of protein biosynthesis and affect essentially every step of this process from the association of the subunits to the individual events in amino acid incorporation (Moore and Spremulli, 1985). For example, Mg^{++} and the polyamines have been shown to promote the association of ribosomal subunits while the monovalent cations clearly promote dissociation (Moore and Spremulli, 1985). Recent studies using wheat germ ribosomes have also shown that a critical number of Mg^{++} ions must be present on the ribosomes if they are to maintain their activity in protein biosynthesis (Sperrazza and Spremulli, 1983). In addition, variation in the millimolar range for Mg^{++} and in a higher range for K^{+} were able to induce modifications in the conformation of wheat germ ribosomes and its subunits (Moore and Spremulli, 1985).

This different requirement with respect to Mg^{++} and K^{+} ions for maximal translation suggest differences in the

conformation of RNH and RH polysomes and that conformation appears to be temperature dependent for both RNH and RH polysomes. Consequently, temperature in addition to the ionic environment also affects ribosome conformation and protein synthesis. However, these results do not allow one to determine whether RNH and RH polysomes had different conformations or need a different conformation to translate mRNA from RNH or RH leaves. These results also show for the first time that temperature influences ribosome conformation. This complements previous reports on the modification of ribosome conformation by different concentration of Mg^{++} and K^{+} and by post-translational modification of ribosomal proteins (Hallberg et al., 1981; Moore and Spremulli, 1985; Sim and Rho, 1985). These conformational changes in addition to conferring the optimal spatial organization for mRNA translation, might also reduce the accessibility to ribonuclease- or protease-sensitive sites, thus increasing the life time of the ribosomes. Longer life times have been used to explain the observed increased ribosomal content in different tissues (Goldspink and Goldspink, 1977; Li, 1980; Melvin et al., 1976). Post-transcriptional modifications of ribosomal proteins, such as changes in the degree of phosphorylation (Bielka, 1982; Browning et al., 1985; Clemens, 1983; Floyd and Traugh, 1979; Hathaway et al., 1979; Leader, 1980; Mumby and Traugh, 1979; Nishimura and Deuel, 1983; Ochoa and de Haro, 1979; Scharf and Nover, 1982; Tuhackova et al., 1985;

Yurina et al., 1983; Wool, 1979) could also induce conformational changes in the ribosomes and consequently regulate protein synthesis (Hallberg et al., 1981; Sim and Rho, 1985). Although no study was done on the phosphorylation of rye ribosomes, the different Mg^{++} and K^{+} requirements for translation of RNH and RH polysomes at 25°C could be due at least partly to differences in the degree of ribosomal protein phosphorylation. However, differences in the degree of phosphorylation of ribosomal proteins between RNH and RH polysomes is surely not the only factor which might explain conformation differences between RNH and RH polysomes since (1) the optimal ion requirements are similar for translation of RNH and RH polysomes at 10°C but different at 25°C and (2) the melting point of both RH rRNA and RH ribosomes is lowered as reported in the previous chapter. At this present time, it is not possible to explain why the optimal Mg^{++} requirement was the same for RNH and RH polysomes at 10°C but different at 25°C. Although it is also impossible to assess clearly whether the difference in ribosome conformation would result from (1) the assembly of ribosomes or (2) from the effect of the temperature, the former possibility appears more probable.

The results presented support the conclusion that under optimal conditions RH polysomes were twice as active as RNH polysomes in incorporating amino acids regardless of the temperature of translation. In the previous chapter, it was suggested that RH polysomes which had a higher

proportion of large polysomes than RNH polysomes would reflect polysomes with a higher synthesizing capacity due to a higher initiation rate rather than a lower synthesizing capacity resulting from a lower termination rate. In addition, different conformations for RNH and RH polysomes might also confer a different translational capacity between RNH and RH polysomes.

The in vitro system used, measured the elongation step of protein synthesis. Unless the termination step is blocked, the initiation step is the regulatory step of protein synthesis in eucaryote systems (Kozak, 1983; Kramer et al., 1980). Using a similar elongation system (Klyachko et al., 1982), the synthesizing capacity of polysomes isolated from plants under different physiological conditions and differing in the proportion of small and large polysomes was expressed in terms of specific activity of polysomes. This specific activity of polysomes is the ratio of the total incorporation by polysomes to the proportion of small and large polysomes in the preparation of polysomes used. A parallel percentage increase in specific activity and in the proportion of small and large polysomes would indicate an increase in elongation activity. A higher percentage increase in the specific activity over the percentage increase in the proportion of small and large polysomes would characterize a polysome preparation with both higher elongation and initiation activity (Klyachko et al., 1982).

Under optimal conditions, the RH polysomes were always twice as active as RNH polysomes with respect to incorporation of amino acid at 10°C and 20°C. The incorporation at 10°C was reduced by less than 15% when compared to the values obtain at 25°C for both RNH and RH polysomes although the rate of incorporation was slower. Total incorporation was 1.7 times higher for RH polysomes at 10°C than RNH polysomes at 25°C. To the extent that the *in vitro* system measures primarily measures the elongation step in protein synthesis (Jolicoeur and Brakier-Gringras, 1983) these results would suggest that the elongation step was stimulated in polysomes isolated from RH seedlings. The specific activity of RH polysomes compared to RNH polysomes increased by more than 50% which is more than the increase (17%) in the proportion of polysomes in RH leaves (86%) compared to RNH leaves (69%) (Table 14). This would suggest that in addition to the proposed increase in the synthesizing activity at the elongation step, the initiation step would also be more active in RH polysomes.

These results differ from those reported for polysomes isolated from wheat seedlings grown at 20°C in which phenylalanine incorporation during 60 min by wheat polysomes was minimal at 10°C and with a Q_{10} (15°C-25°C) higher than 3.0 (Fehling and Weidner, 1986). The difference in the results between the synthetic activity of rye and wheat polysomes may suggest that rye polysomes or the wheat germ system used are not as sensitive to a low

translation temperature as the wheat polysomes or wheat germ used (Fehling and Weidner, 1986). But there is another possible explanation. Apparently no verification on the saturation time required for complete translation of wheat polysomes was done (Fehling and Weidner, 1986). In addition, in that report on adaptation of wheat ribosomes to different temperature in which the incorporation of [^{3}H]phenylalanine with a wheat germ extract was assayed from 10°C to 40°C by increments of 5°C, the optimal ion requirement was not determined at each temperature (Fehling and Weidner, 1986). The weak incorporation at 10°C reported for wheat ribosomes (Fehling and Weidner, 1986) could result from an excess of Mg^{++} and a slower incorporation at 10°C. The results obtained with RNH and RH polysomes showed that a lower optimal concentration of Mg^{++} was required for translation at 10°C rather than at 25°C. At 10°C and in presence of 5 mM Mg^{++}, the translation of polysomes isolated from RNH and RH leaves was inhibited to a large extent. Apparently, the fact that temperature clearly regulates the translational activity of polysomes by slowing down the incorporation has not been taken in consideration in previous studies, with the exception of the use of a low temperature to slow down the elongation in E. coli ribosomes (Gast et al., 1985).

Finally, these results also demonstrate that polysomes isolated from tissues which are maximally hardened do not exhibit the same characteristics as those from tissues

exposed to low temperature for only 48 hours (Fehling and Weidner, 1986). While RH polysomes were always twice as active as RNH polysomes, polysomes isolated from wheat seedlings adapted at 5°C for 2 days were always less active than the control polysomes isolated from seedlings grown at 20°C.

Some similarities between polysomes isolated from tissues of dystrophic animal (Jolicoeur and Brakier-Gingras, 1983) and RH polysomes regarding the ions requirement for maximal translation have been pointed out above.. However, dystrophic animal polysomes translated under optimal conditions were not more active than the control polysomes. By contrast, RH polysomes were twice as active as RNH polysomes.

The absence of reinitiation of protein synthesis during translation of polysomes with the S-100 wheat germ fraction in presence of 50 μM ATA was predicted since that concentration of ATA has been widely used to show that the S-100 wheat germ fraction is generally non-reinitiating (Fresno and Vasquez, 1979; Jolicoeur and Brakier-Gingras, 1983; Vasquez and Jimenez, 1980).

The S-100 wheat germ fraction used as source of soluble factors for translation was also free of RNase activity. Incorporation of amino acid in presence of HPRI was not enhanced. Consequently the modifications brought by Reisfeld and Edelman (1982) to the original method (Marcu

and Dudock, 1974; Roberts and Paterson, 1973) to prepare the wheat germ extract were useful.

Similarly as for polysomes, several aspects of the results presented with respect to mRNA support the following conclusion. The establishment of optimal conditions for translation is necessary before trying to assess the effectiveness of mRNA to direct protein synthesis. First, optimal concentrations of Mg^{++} and K^{+} were different for RNH and RH mRNA. Magnesium concentration had a greater influence on the translation than that of potassium. Second, the total incorporation was limited by the optimal quantity of mRNA present in the translation mixture. An excess of mRNA inhibited the translation. Third, a low concentration of methionine was regulating the amino acid incorporation.

An optimal concentration of Mg^{++} (0.7 mM) and a narrow peak of maximal activity have been reported for optimal translation of most mRNA (Clemens, 1984; Promega Biotec). This value 0:7 mM Mg^{++} is much lower than the values observed for optimal translation of RNH and RH polysomes even if the endogenous Mg^{++} concentration present in rabbit reticulocyte lysate translation kit as been established at 1 mM (Clemens, 1984). The lower required Mg^{++} concentration when mRNA rather than polysomes directs amino acid translation reflects that the two translation systems are different. During translation with mRNA, in addition to the elongation and termination step, the initiation step

is also involved. Lower concentration of Mg^{++} have been shown to be required in an initiating system rather than for only an elongating system (Merrick, 1979).

Although the optimal Mg^{++} requirement and the peak of maximal activity for RNH mRNA were similar to those reported for most mRNA, a higher optimal Mg^{++} concentration (1.7 mM) was required for optimal translation of RH mRNA. In addition, the peak of maximal activity was much broader. These data clearly indicate that these two mRNA are different. The data also suggest that a higher concentration of Mg^{++} is necessary for RH mRNA than for RNH mRNA in order to obtain a more effective recognition of the codon AUG at the initiation step. These results also show that the effect of growth at low temperature is different from the heat shock response on mRNA. In the latter case, no difference was observed in the requirement in Mg^{++} for optimal translation of mRNA isolated from control and heat shock treated corn seedlings (Baszczynski, 1984). In addition, no difference in the incorporation of amino acid was observed between 0.6 mM and 1.0 mM Mg^{++} for both control and heat treated mRNA while incorporation by RNH mRNA was decrease by more than 23% when the optimal Mg^{++} concentration (0.7 mM) was increase to 1 mM.

Optimal concentration of mRNA for <u>in vitro</u> translation has been reported to vary from 5 to 100 µg/ml of translation mixture (Clemens, 1984) On the other hand, the effectiveness of mRNA to direct protein synthesis

reportedly depends on the 5' cap structure of translated mRNA (Kozak, 1983; Moldave, 1985). Optimal quantities of RNH and RH mRNA for in vitro translation were 40 µg/ml and 80 µg/ml of translation, respectively. This is another difference between RNH and RH mRNA. The higher quantity of RH mRNA to saturate the incorporating system could result from a difference at the initiation level due to alteration at the 5' terminal end of RH mRNA.

The endogenous pool of methionine in rabbit reticulocyte lysate has been established at 5 µM (Clemens, 1984) and it was then suggested that methionine was not a limiting factor of translation when a rabbit reticulocyte lysate is used (Clemens, 1984). Even though experiments with BMV RNA indicated a relatively high level of methionine in the translation mixture, methionine was still limiting for RNH mRNA just as it was for RNA polysomes. The necessity to include enough methionine may reflect the higher capacity of rabbit reticulocyte lysate to polymerize amino acid due to the possibility of reinitiation of protein synthesis in that system.

The establishment of optimal ions requirements support the conclusion that growth at low temperature alters mRNA structure. A higher magnesium concentration suggests that the 40 S_N preinitiation complex must have a different conformation to effectively bind to RH mRNA.

The results presented support the conclusion that under optimal conditions of translation, RNH and RH mRNA were

equally effective in directing protein synthesis. This observed result would indicate that the differences observed in the activity of RH and RNH polysomes would be due more to the ribosomes than to the mRNA.

In conclusion, these results clearly show the importance of determining the optimal conditions for translations of both polysomes and mRNA before any assessment of their translational capacity is possible. An adequate level of methionine is required and the optimum requirements for translation are temperature dependent and different for both RNH and RH polysomes and mRNA. Finally, translational capacity of RH polysomes is twice as high as RNH polysomes regardless of the temperature of translation. RNH and RH mRNA had a similar capacity to direct amino acid incorporation suggesting that the difference observed in the activity of RH and RNH polysomes would result from observed modifications of ribosomes rather than mRNA.

CHAPTER 5

PRODUCTS OF *IN VITRO* TRANSLATION OF POLYSOMES AND POLYSOMAL mRNA.

5.1 INTRODUCTION

The structure and function of cells are largely determined by the proteins they contain. The pattern of growth and differentiation of an organism can thus be visualized as a combination of environmental and genetic factors which dictate the kinds and amount of proteins sequentially produced during the life of the organism (Weeks, 1981). Given the previous observations of anatomical and morphological modifications of RH cells (Huner *et al*., 1984; 1981), modifications to the complement of proteins during growth at low temperature would be expected. Both qualitative and quantitative changes to the complement of soluble and membrane-bound polypeptides have been reported in cold-hardened plants (Bixby and Brown, 1975; Brown, 1978; Brown and Bixby, 1975; Cloutier, 1983; Huner *et al*., 1984; Rochat et Therrien, 1975; Siminovitch *et al*., 1968, 1967). Studies with inhibitors of RNA synthesis have shown that RNA transcription was necessary for cold acclimation (Hatano *et al*., 1976; Scheele and Blackburn, 1979). Furthermore, studies with an inhibitor of protein synthesis by cytoplasmic ribosomes have clearly shown both that the increase in total protein results from

de novo protein synthesis and that *de novo* synthesis is also required for cold acclimation (Chen *et al*., 1983; Hatano, 1978; Hatano *et al*., 1976; Kacperska-Palacz, 1978; Trunova, 1982). Recently, differences in *in vivo* and/or *in vitro* translation products following a short cold treatment of spinach and brassica have been reported (Guy *et al*., 1985; Meza-Basso *et al*., 1986).

In order to determine whether the complement of polypeptides synthesized in RH seedlings was altered, an *in vitro* translation study was carried out with both polysomes and mRNA. Polysomes were translated with a S-100 wheat germ system while mRNA were translated with a rabbit reticulocyte lysate.

5.2 MATERIALS AND METHODS

5.2.1 Polysome isolation.

Membrane-free polysomes were isolated from RNH and RH seedlings as described in section 2.2.2, resuspended in the translation buffer and stored as described in section 4.2.1. Polysome quantity was estimated as described in section 2.2.5.

5.2.2 Messenger RNA isolation.

Messenger RNA from RNH and RH seedlings was isolated as described in detail in section 4.2.2. mRNA quantity was estimated as described in section 4.2.5.

5.2.3 Polysome and mRNA *in vitro* translation.

In vitro translation of polysomes and mRNA using respectively the S-100 wheat germ system and a rabbit reticulocyte lysate were described in section 2.2.2 and 4.2.5.

5.2.4 Analysis of translation products.

Translation products were separated by one-dimensional SDS-PAGE as described in section 2.2.9. Usually, 50,000 acid-precitable counts were loaded on each lane of the gel. Translation products also analyzed by two-dimensional PAGE were separated by IEF in the first dimension (3.2.6.2) and by SDS gel for the second dimension (2.2.9). For the first

dimension, the proportion of ampholines (LKB ampholine) used was 1.6% pH range 5-8 and 0.4% pH range 3.5-10. In preliminary experiments, an unacceptably large quantity of non-solubilized proteins remained near the top of the IEF gel. For this reason, aliquots of translation mixture were first solubilized by adding 0.2 volume of 10% SDS (2% final concentration) and boiled for 1 min in water and cooled on ice. NP-40 was added to the mixture to give a NP-40:SDS ratio of 8:1 (16%) followed by 4 volumes of a solution containing 9.5 M urea, 2% ampholines (pH range 5-8) and 5% β-mercaptoethanol. This mixture was incubated for 30 min at room temperature and loaded on a IEF gel. Usually, 100,000 acid-precitable counts were loaded on each IEF gel. Electrophoresis was carried out as reported (section 3.2.6.2) except that after 16 h at 400 volts, the voltage was increased to 800 volts for 1 h.

5.3 RESULTS

5.3.1 Analysis of translation products by 1-D PAGE.

Translations products analyzed by 1-D SDS PAGE revealed that some products were altered although most of them were unaffected during growth at low temperature. Among the altered polypeptides, seven new polypeptides characterized by the following molecular mass: 171, 166, 163, 152, 145, 110, and 75 kD were translated at both 10°C and 25°C (Fig. 25). In addition, the quantities of five polypeptides of molecular mass of 134, 100, 85, 15.5 and 14 kD increased, the quantity of a polypeptide of 72 kD decreased considerably while a polypeptide of 105 kD was no longer detected. Finally, a 24 kD polypeptide was translated only at 10°C in both RNH and RH polysomes (Fig. 25) while a few polypeptides translated from both RNH and RH polysomes were expressed in lower quantity at 10°C.

Similar results were obtained from translation of mRNA with the rabbit reticulocyte lysate. Most of the polypeptides were unaffected during growth at low temperature. However, five new polypeptides of 167, 161, 158, 151 and 16 kD were translated. In addition, the quantities of five other polypeptides of 110, 107, 102, 42 and 40 kD increased while four polypeptides of 80, 40, 44 and 28 kD decreased in quantity and one of 33 kD disappeared (Fig. 26).

Figure 25. Fluorograms of 1-D SDS-PAGE séparations of translation products of RNH and RH polysomes. Polysomes were translated with an optimized S-100 wheat-germ fraction at 10°C or 25°C. Lanes 1,2,6,7, RH; lanes 3,4,8,9, RNH; lanes 5,10, blanks; lanes 1,3,5,6,8,10, translation at 25°C; lanes 2,4,7,9, translation at 10°C. Lane 1-5 were exposed twice as long as lane 6-10. ➔, new polypeptides (171, 166, 163, 152, 143, 110, 75 kD); ⇾, polypeptides with increased intensity (134, 100, 85, 15.5, 14 kD); ◊, polypeptide with decreased intensity (72 kD); ◆, polypeptide not any longer detectable (105 kD); →, polypeptide translated at 10°C only (24 kD). Protein standards, 94 kD: phosphorylase b; 68 kD: BSA; 45 kD: ovalbumin; 29 kD: carbonic anhydrase; 12.5 kD: cytochrome c.

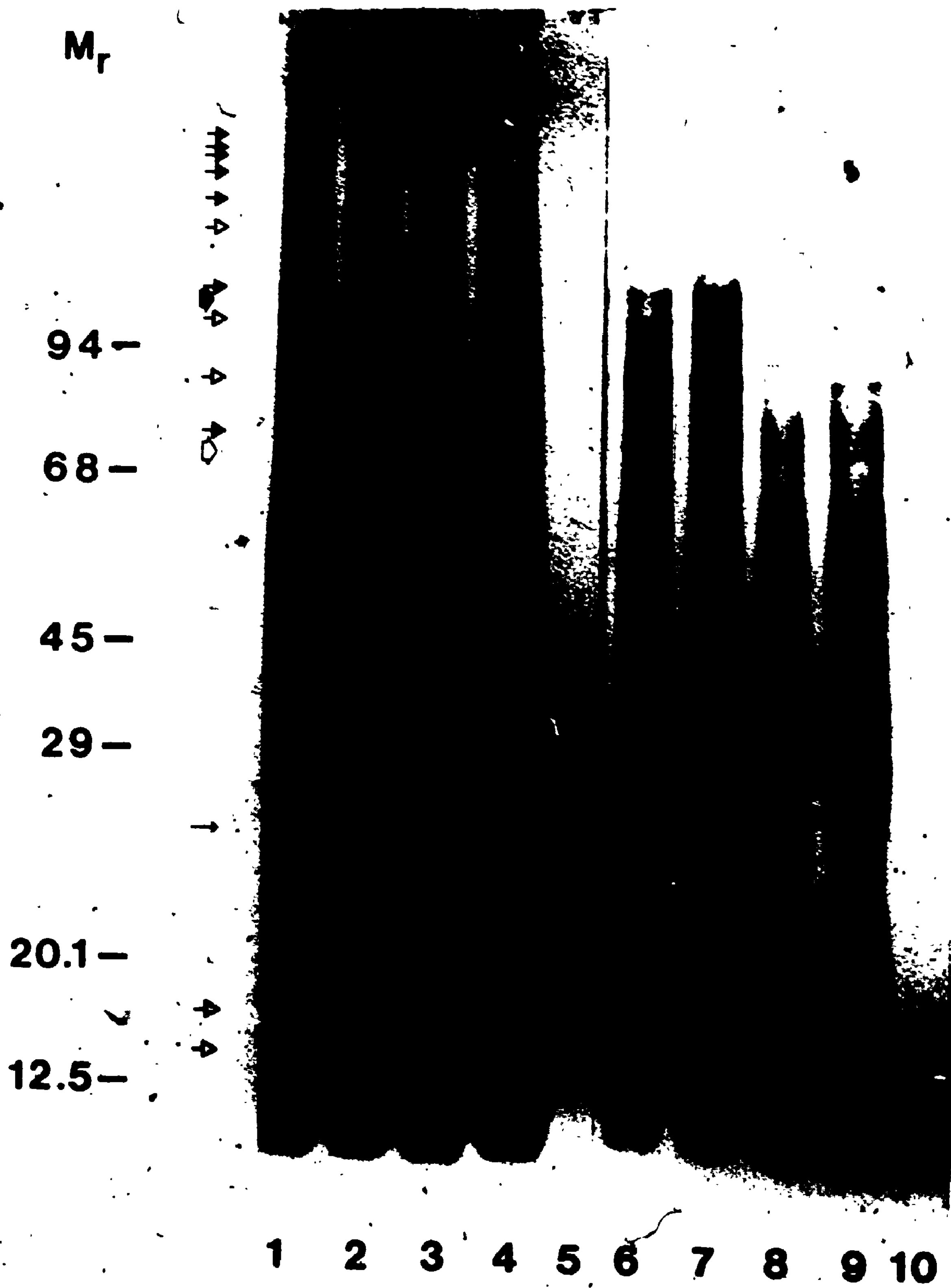
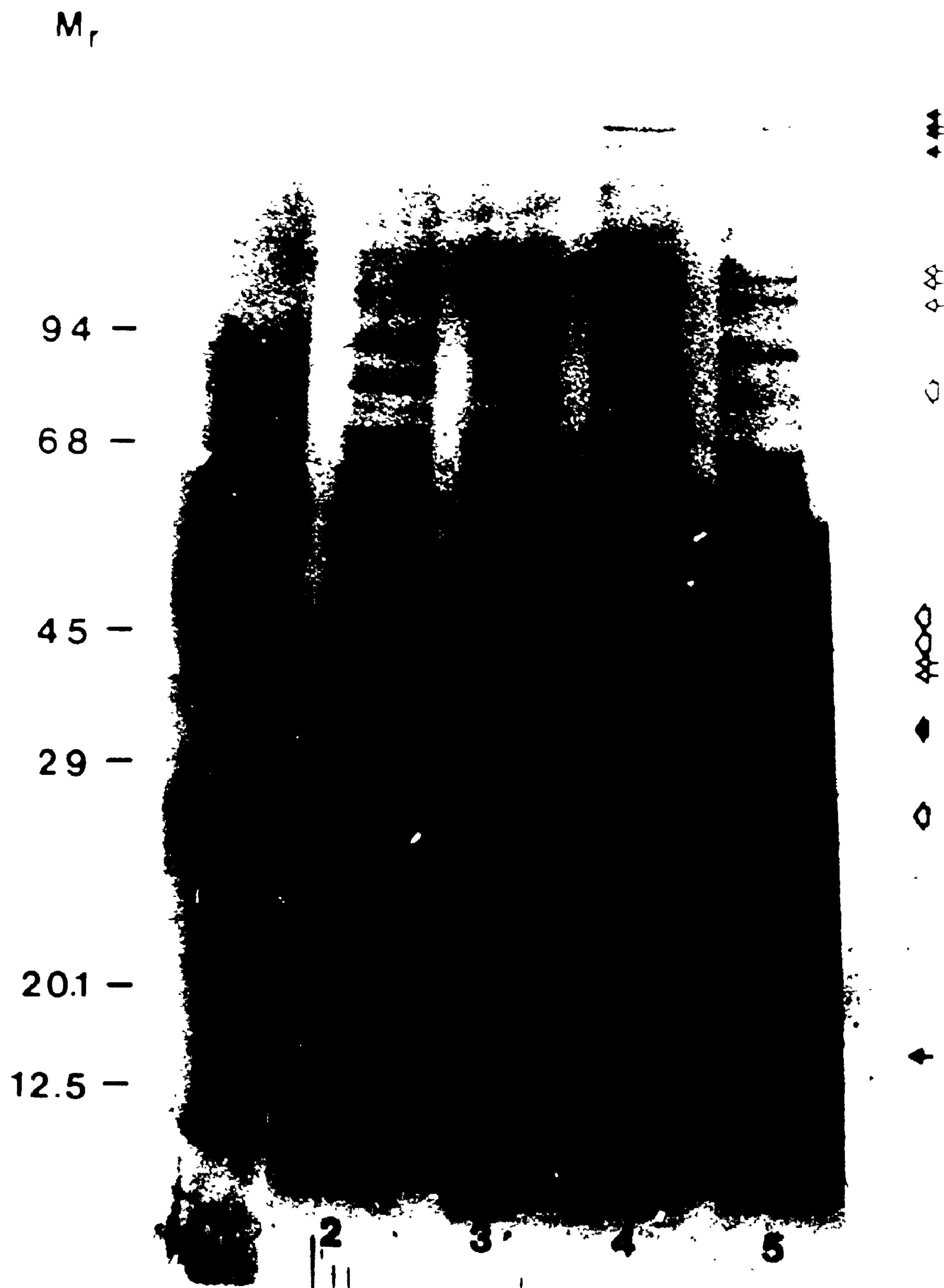

Mr
94
68
45
29
20.1
12.5
1 2 3 4 5 6 7 8 9 10

Figure 26. Fluorogram of 1-D SDS-PAGE separations of translation products of RNH and RH polysomal mRNA. mRNA were translated with an optimized rabbit reticulocyte lysate at 30°C. Lane 1, blank; lanes 2,3, RNH mRNA; lanes 4,5, RH mRNA; →, new polypeptides (167, 161, 158, 151, 16 kD); ⇾, polypeptides with increased intensity (111, 107, 102, 42, 40 kD); ◊, polypeptides with decreased intensity (80, 46, 44, 28 kD); ♦, polypeptide not any longer detectable (43 kD). The same protein standards as reported in Figure 25 were used.


Mr
94
68
45
29
20.1
12.5
2
3
4
5

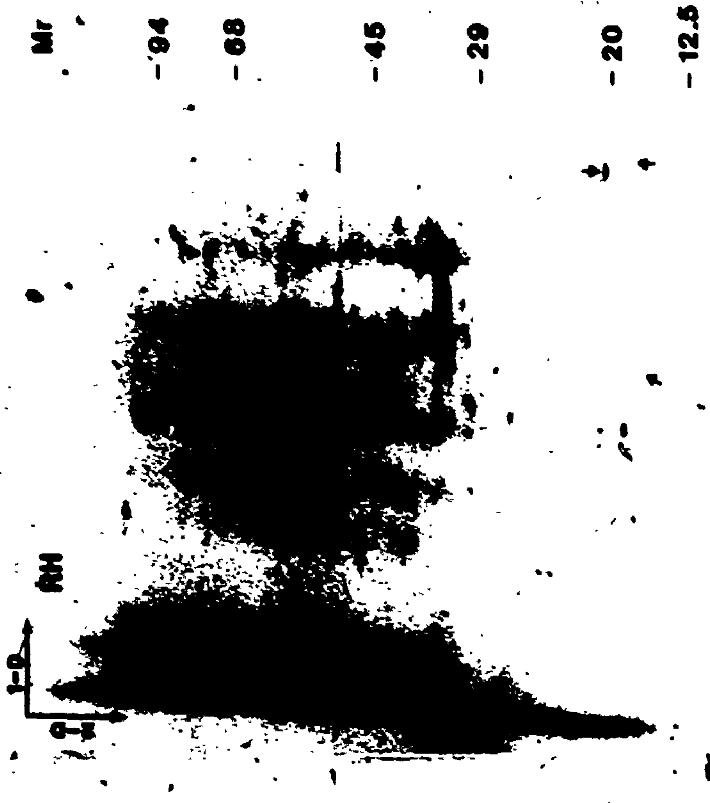
The resolution of the 1-D gels did not allow a precise determination with precision of the number of polypeptides affected. This was especially true for the translation products from polysomes. Thus, in order to improve resolution, translation products were compared using a two-dimensional gel electrophoresis system.

5.3.2 Analysis of translation products by 2-D PAGE.

Results obtained by 1-D PAGE were confirmed and extended by 2-D PAGE analysis. Due to the higher resolution obtained by 2-D PAGE than by 1-D PAGE, additional differences in translation products were observed with both polysomes and mRNA.

Additional differences, both qualitative and quantitative, in the translation products obtained at 10°C and 25°C from RNH and RH polysomes were revealed by 2-D PAGE analysis (Fig. 27 and 28). Some proteins among those newly expressed or translated in larger quantities appeared to be affected by the temperature of translation since they showed up as new proteins at 10°C but as protein in larger quantities at 25°C. The new polypeptides of high molecular size observed in 1-D PAGE were absent from these fluorograms although they were present when 2-D fluorograms were overexposed as for 1-D fluorograms. The fact that numerous polypeptides around 12.5 kD were observed on 1-D gels but were apparently absent on 2-D gels would suggest that most of these proteins would be basic. Similarly,

Figure 27. Fluorograms of 2-D IEF-SDS-PAGE separations of products of RNH and RH polysomes translated at 10°C. The translation was carried out with an optimized S-100 wheat-germ fraction. →, new polypeptides; ⇾, polypeptides with increased intensity; ⇩, polypeptides with decreased intensity; ⬇, polypeptide not any longer detectable. The same protein standards as reported in Figure 25 were used.


Mr
-94
-68
-45
-29
-20
-12.5
RH
1-D
pH
7.1
6.4
5.9
5.1

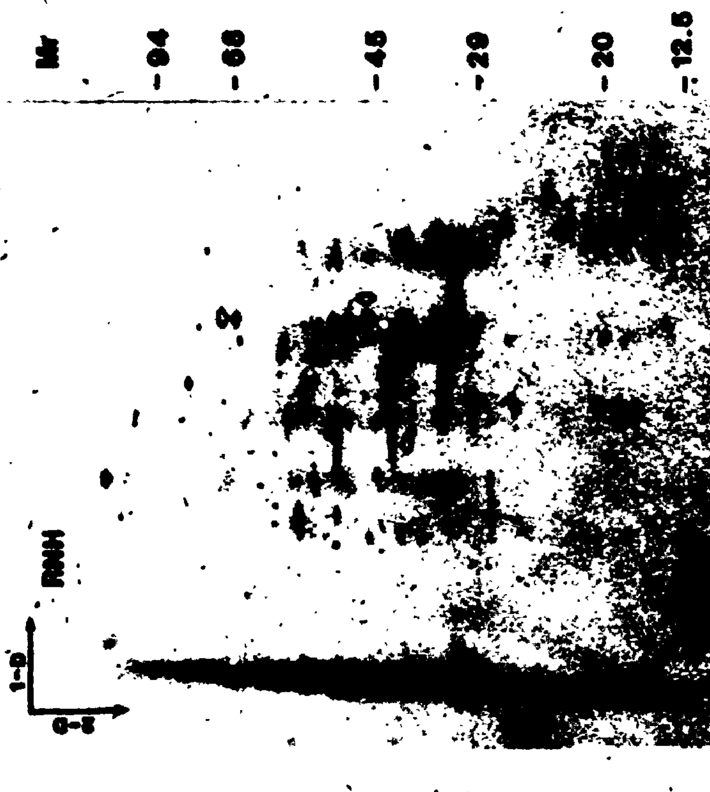

Mr
-94
-68
-45
-29
-20
-12.5
RNH
1-D
pH
7.1
6.4
5.9
5.1

Figure 28. Fluorograms of 2-D IEF-SDS-PAGE separations of products of RNH and RH polysomes translated at 25°C. The translation was carried out with an optimized S-100 wheat-germ fraction. →, new polypeptides; ⇾, polypeptides with increased intensity; ◊, polypeptides with decreased intensity; ♦, polypeptide not any longer detectable. The same protein standards as reported in Figure 25 were used.

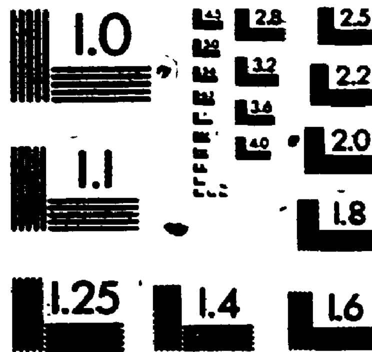
Mr

94
68
45
29
20
12.5

pH 7.1 6.4 5.9 5.1

1-D
2-D

3 of/de 3

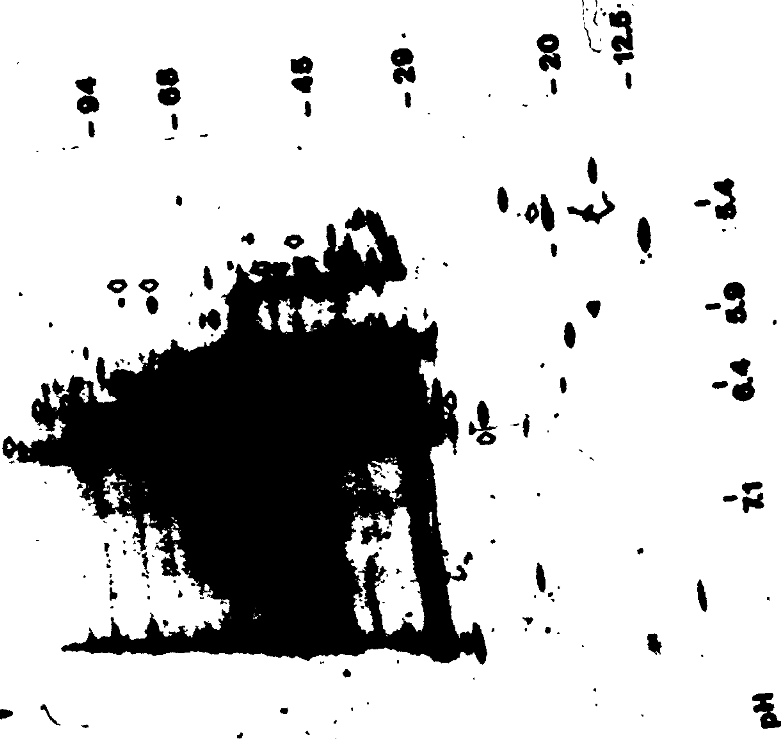


translation products of RNH and RH polysomal mRNA revealed additional differences when analyzed by 2-D PAGE (Fig. 29). The four new polypeptides of high molecular size observed by 1-D PAGE were resolved into at least eight different polypeptides. The polypeptide of 16 kD present on 1-D PAGE, was absent on 2-D gels. This could suggest that this particular polypeptide would be basic and consequently unable to migrate into the first gel.

The distribution of polypeptide spots on gels was distinctly different for translation products for both polysomes and mRNA translated with the S-105 wheat germ fraction and the rabbit reticulocyte respectively.

Finally, these results indicate that both qualitative and quantitative changes in gene expression occurs during growth at low temperature.

Figure 29. Fluorograms of 2-D IEF-SDS-PAGE separations of translation products of RNH and RH polysomal mRNA. The translation was carried out with an optimized rabbit reticulocyte lysate. ➔, new polypeptides; ⇾, polypeptides with increased intensity; ◊, polypeptides with decreased intensity; ◆, polypeptide not any longer detectable. The same protein standards as reported in Figure 25 were used.



5.4 DISCUSSION

Preliminary results showed that when a sample was first solubilized with SDS, the polypeptides showed different pIs than those from untreated samples. Apparently, not all the SDS was dislodged from the polypeptides by the excess of NP-40. These preliminary results agree with those reported by Helenius and Simons (1975) that SDS and NP-40 had different binding sites on polypeptides and once the SDS binds to a polypeptide it is impossible to dislodge it, at least with non-ionic detergent. Similar results on the effect of SDS on pIs of polypeptides have also been observed by Baszczynski (1984) and Dunn and Burghes (1983). Consequently, the pI indicated in the 2-D fluorograms are only an approximation.

A number of reports have shown that gene expression is affected following exposure of plants to low temperature for short period of times varying from few hours up to 18 days (Guy <u>et al</u>., 1986, 1985; Mahopatra <u>et al</u>., 1986; Meza-Basso <u>et al</u>., 1986; Tseng and Li, 1986). Similarly, alteration of gene expression was observed in the translation products of both RH polysomes and RH mRNA. New polypeptides were expressed, some gene products were repressed while the quantity of other polypeptides was increased or decreased. However these results obtained on differential gene expression during growth of rye at low temperature differ in one major respect from those reported

above: both polysomes and mRNA were isolated from plants of comparable developmental stage and translated under carefully optimised conditions.

In rye new polypeptides of high molecular weights (>110 kD) were observed in addition to the new polypeptides ranging from 18 kD to 110 kD. Similar high molecular weight polypeptides (>110 kD) have been observed in spinach (Guy et al., 1986, 1985) but not in rapeseed, potato or alfalfa (Mahopatra et al., 1986; Meza-Basso et al., 1986; Tseng and Li, 1986).

Whether the translation temperature affected the translation products was a legitimate question since polysomes and mRNA were isolated from leaves grown at low and high temperature. Under the conditions used, the temperature of translation did not significantly affect the complement of translation products.

The differences observed in translation products from both polysomes and mRNA correlate with the anatomical and morphological differences previously reported for rye (Huner et al., 1984; 1981). It is generally accepted that the protein complement changes during plant development. As pointed out earlier, the leaves from RNH and RH seedlings were of comparable developmental stage. Thus, any differences observed in translation products from both polysomes and mRNA probably reflect a cellular response to low temperature. Given the central role of protein metabolism, it is reasonable to expect such changes

along with an increase in freezing tolerance. Changes have also been noted with respect to charge heterogeneity of the polypeptides associated with the large subunit of rubisco although these charge changes were thought to be posttranslational rather than transcriptional (Huner and Hayden, 1982).

Although studies with respect to regulation of gene expression at low temperature are relatively new, high temperature treatments have been used increasingly to study mechanisms of gene expression (Key et al., 1985). These heat shock treatments, which are usually of short duration (0.5 h to 3 h), induce a new set of proteins. Some of these heat shock proteins have been shown to be similar for plant and animal tissues (Baszczynski, 1984; Heikkila et al., 1984; Key et al., 1985). Heat shock treatments differ from growth at low temperature since the time of exposure is relatively shorter in the former. This time of exposure is only few hours for heat shock treatment and days or weeks for cold acclimation. For example, rye was grown for seven to eight weeks at 5°C. Heat shock treatment further differs from development at low temperature by the fact that in the latter case there is growth (Krol et al., 1984) and a full range of polypeptides is synthesized while in the former case, plant growth and synthesis of most of the proteins are suppressed (Baszczynski, 1984; Heikkila et al., 1984; Key et al., 1985). In addition to these differences, the new gene products observed in spinach

leaves during a cold hardening treatment are different from the products induced during heat shock treatment (Guy *et al*., 1986; 1985). The study of induction of new gene products during growth at low temperature is relatively new. Although polypeptides of similar size have been observed in a variety of plant tissues, it is not known whether some of these products are the same proteins. Induction of cold hardening by ABA instead of low temperature has also been reported (Chen *et al*., 1983; Gusta *et al*., 1982; Orr *et al*., 1986; Tseng and Li, 1986) following the observation that the level of ABA increase in leaves upon exposure of plants to low temperature (Chen *et al*., 1983; Kacperska-Palacz, 1978). Following exposure of potato to either low temperature or ABA, a similar complement of polypeptides, especially one of 71 kD, has been observed (Tseng and Li, 1986). It is not known yet whether that polypeptide is similar to the stress polypeptide of 70 kD induced in maize mesocotyls by heat and ABA (Heikkila *et al*., 1984). Unlike potato, no 70 kD polypeptide has been observed in rye. This result suggests once more that rye leaves under active growth at 5°C and mature leaves cold stressed to low temperature for few days up to two weeks represent two completely different systems in which to study cold acclimation. However cDNA libraries are now being made (Guy *et al*., 1985; Mahopatra *et al*., 1986; Meza-Basso *et al*., 1986) and it should soon be

possible to know whether there are common genes which are expressed at low temperature in different plant species.

In addition to the cap structure itself, proteins associated with cap structure have also been reported to affect the translation of mRNA (Kozak, 1983). The differences observed between the translation products obtained from polysomes and phenol-chloroform extracted polysomal mRNA would suggest that differences in initiation of protein synthesis *in vivo* and *in vitro* could be related at least partly to the presence of these cap binding proteins. Differences have also been observed between products obtained with wheat germ system and rabbit reticulocyte lysate (Baszczynski, 1984).

It is not possible at this time to assign the identification, function and localization for the newly expressed and the repressed gene products during growth at low temperature although such information would be necessary in order to ultimately understand the role of altered gene expression in cold acclimation. Not withstanding the above, it is clear from the results of this present study that low temperature regulates gene expression.

CHAPTER 6

GENERAL DISCUSSION

In order to examine the effect of growth at low temperature on polysome metabolism in mature rye leaves, it was first necessary to isolate intact polysomes. Although systematic studies have been reported with respect to the isolation of intact polysomes from young and etiolated tissues, there are no comparable studies available for mature tissues. This may be due at least in part to the well documented fact that RNase activity is higher in older tissues and the assumption that higher RNAse level would necessarily interfere with polysome isolation. The degree of preservation of isolated polysomes is usually judged by the ratio of large polysomes to small polysomes. However, this study has shown that <u>in vitro</u> translation may be used effectively in conjunction with LP/SP ratios to evaluate polysome integrity. At the same time, it was also necessary to establish the optimal conditions for translation. In this regard, it is clearly important to establish appropriate concentrations of magnesium ion and methionine. Potassium ion has a small effect but it is not that critical. Both polysome concentration and the effect of temperature must be carefully monitored. There was a strong correlation between LP/SP ratio and translation. Polysomes with higher LP/SP ratios had both a higher translation rate and a higher proportion of large

translational products. The results obtained by these two independent but complementary methods show that, in contrast to some supposition, it is clearly possible to isolate structurally intact and functionally effective polysomes from mature leaves tissues.

In electron micrographs of tissues from leaves grown at low temperature, an increase in both size and quantity of polysomes has been observed. This observation is not artefactual since data confirm that cold grown tissues yield larger quantities of polysomes and that those polysomes are more highly polymerized. These results are in substantial agreement with those previously reported for barley and potato leaves. The increased quantity of polysomes in rye can be attributed to a parallel increase in the cytoplasmic volume of those cells rather than a direct consequence of growth at low temperature on polysome metabolism. On the other hand, the larger polysomes suggest that translational activity of polysomes could be altered due to an effect of low temperature on initiation and/or termination rate.

RNH and RH polysomes were similar to the extent that neither rRNA nor core ribosomal proteins appeared to be significantly affected by low temperature. These observations are not surprising because of the high degree of ribosome conservation through evolution. On the other hand, these polysomes are different in a substantial number of points. Modifications such as a decrease in the melting

point and differences in the complement of peripheral proteins and sensitivity to detergent were found. Collectively these alterations in the RNA-RNA and/or RNA-protein association, ribosome subunit association and the complement of peripheral proteins would suggest a change in ribosome conformation. Whether RH polysomes had a different conformation or require a different conformation in order to translate effectively RH mRNA is not clear. However there is clearly a conformational difference since the data showed a different optimal ion requirement and, more particularly, a sharp difference in the magnesium requirement. A difference in conformation was further supported by the reduced sensitivity of RH-polysomes to excess of magnesium during translation.

It is generally agreed that ribosome conformation affects ribosome function and differences in the function of RNH and RH ribosomes were observed. RH polysome translational capacity was twice as high as for RNH polysomes regardless of translation temperature. This enhanced translational capacity could reflect either or both a higher rate of elongation and/or initiation. A higher rate of initiation of protein synthesis would explain the occurrence of larger polysomes. The data on the effectiveness of mRNA to direct protein synthesis showed that alteration in translational capacity was due solely to the polysomes themselves.

Transcripts are also different at low temperature. First, translation products characterized by 1-D and 2-D PAGE are different. This indicates that new mRNA is expressed and that others are repressed at low temperature. These results on differential gene expression are consistent with the notion that cells with different anatomy and morphology contain a different complement of polypeptides. Second, a higher level of magnesium is required to translate RH mRNA, suggesting a change in the secondary structure of mRNA.

The rye system used in this study involves seedlings which have undergone extensive development at either low or high temperatures. As previously pointed out, this system differs from others in which plants exposed to low temperature are apparently undergoing senescence. These results show clearly that assembly of ribosomes at low temperature (1) results in ribosomes with a different conformation, (2) the translational activity of polysomes is more efficient regardless of the temperature of translation and (3) new genes are expressed while other are repressed. While it is not possible to assess cause or effect at this time, given the central role of transcription and translation in development these changes in polysome activity and gene expression are clearly significant in the cold hardening process.

Two interesting points should be investigated in order to further understand the molecular mechanism of growth and

development at low temperature. First, the variation in the proportion of paired G-C bases at low temperature should be examined in order to understand the nature of the changes in the polysome organization. Second, work on the identification and regulation of genes newly expressed and repressed at low temperature should be pursued.

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