IN VITRO-SYNTHESIS OF BARLEY STORAGE PROTEINS

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Summary

Membrane-bound polysomes were isolated from developing endosperms of barley (Hordeum vulgare L.). The messenger RNA associated with the polysomes was separated from the ribosomal components by affinity chromatography on oligo-dT cellulose. The characteristics of the polysomes and of the poly-A^+ RNA fraction are discussed. The poly-A^+ RNA fraction contained components of between 0.55 and 2.5 kilobases in length, and the degree of adenylation is 6.5%. Both polysomes and poly-A^+ RNA were shown, by the use of radioactive precursors, to support the synthesis of trichloroacetic acid (TCA)-insoluble material in a wheat germ cell-free protein-synthesising system. The products of in vitro-protein synthesis resembled hordeins (the prolamin storage proteins of the barley endosperm) in that they were predominantly soluble in 55% propan-2-ol plus 0.05% DTT, contained a low proportion of lysine as compared with leucine, exhibited similar immunological characteristics and had similar, but not identical, electrophoretic properties which were dependent on the barley variety used. Polysome products were the same size as native hordein but poly-A^+ RNA products were approximately 2000 daltons larger.

The larger size of the products of poly-A^+ RNA translation is suggested to be due to presence of an extra leader sequence. The mechanism of processing and transport of the precursor polypeptides was investigated both in vitro, using stripped barley endoplasmic reticulum, and in vivo by injection of poly-A^+ RNA into Xenopus oocytes. It is concluded that hordeins are synthesised as precursors by membrane-bound polysomes, and that the polypeptides are co-translationally shortened and transported into the lumen of the endoplasmic reticulum in a manner consistent with the signal hypothesis. The difference in size between the products of polysome and poly-A^+ RNA translation is discussed in relation to these observations.

The poly-A^+ RNA has been used to prepare clones of Escherichia coli (E. coli), some of which are identified as containing DNA sequences complementary to particular hordein mRNAs.

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Declaration

I declare that this thesis is the result of my own work except where specifically indicated. Some of the results, concerning RNA isolation and identification of in vitro-synthesised products have been reported in:-

Matthews, J. A., Miflin, B. J., 'In vitro synthesis of Barley Storage proteins' Planta (1980) in press.

Miflin, B. J., Matthews, J. A., Burgess, S. R., Faulks, A. J., Shewry, P. R., 'The synthesis of Barley Storage Proteins' in Genome Organisation and Expression in Plants (Ed. C. J.Leaver) Plenum (1980) pp. 233-243.

The remainder of the work has not published previously.

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ABBREVIATIONS

2' + 3' AMP	2' + 3' adenosine monophosphate (mixed isomers)
ATP	adenosine triphosphate
CCMV	cowpea chlorotic mottle virus
cDNA	complementary DNA
CNBr	cyanogen bromide
DBM-paper	diazobenzloxymethyl-paper
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylenediamine-tetracetic acid
E.R.	endoplasmic reticulum
GTP	guanosine triphosphate
Hepes	2-(N-2-hydroxyethylpiperazine-N-yl)ethane sulphonic acid
IEF	isoelectric focusing
IgG	immunoglobulin
kb	kilobases
7-Me GMP	7-methylguanosine monophosphate
Oligo-dT	oligo thymidylic acid
P.b.	protein bodies
PMSF	phenylmethane-sulphonyl fluoride
Poly-A^+ RNA	polyadenylated RNA
Poly-A^- RNA	RNA fraction lacking polyadenylic acid

POPOP	1,4-Di-2-(5-phenyloxazoyl-benzene)
PPO	2,5-diphenyloxazole
RNAase	ribonuclease
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate/polyacrylamide gel electrophoresis
TEMED	NNN'N' tetramethylene ethylene diamine
Tricine	N-Tris hydroxymethyl methyl glycine
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
TMV	tobacco mosaic virus
W	Watts

1. INTRODUCTION

1.1 BACKGROUND

Barley (Hordeum vulgare L.) is the world's fourth most important cereal crop, and the trend, from year to year, is towards increased yields and larger areas of cultivation. It is of particular importance in Europe where approximately 35% of the world's barley is produced (Briggs, 1978). The major uses of barley grain are for malting and animal feeding. Non-ruminant animals require certain essential amino acids from their diet, one of which is lysine. Barley grain is deficient in lysine because the major group of storage proteins (termed the hordeins), which constitute up to 55% of the protein in the endosperm has a very low lysine content (less than 0.5% of the total amino acid residues). Barley cannot, therefore, satisfy the nutritional requirements of non-ruminant animals. This deficiency has generated great interest in the development of the barley grain, particularly in the synthesis of the storage proteins in the endosperm, and in searching for mutants with higher lysine contents.

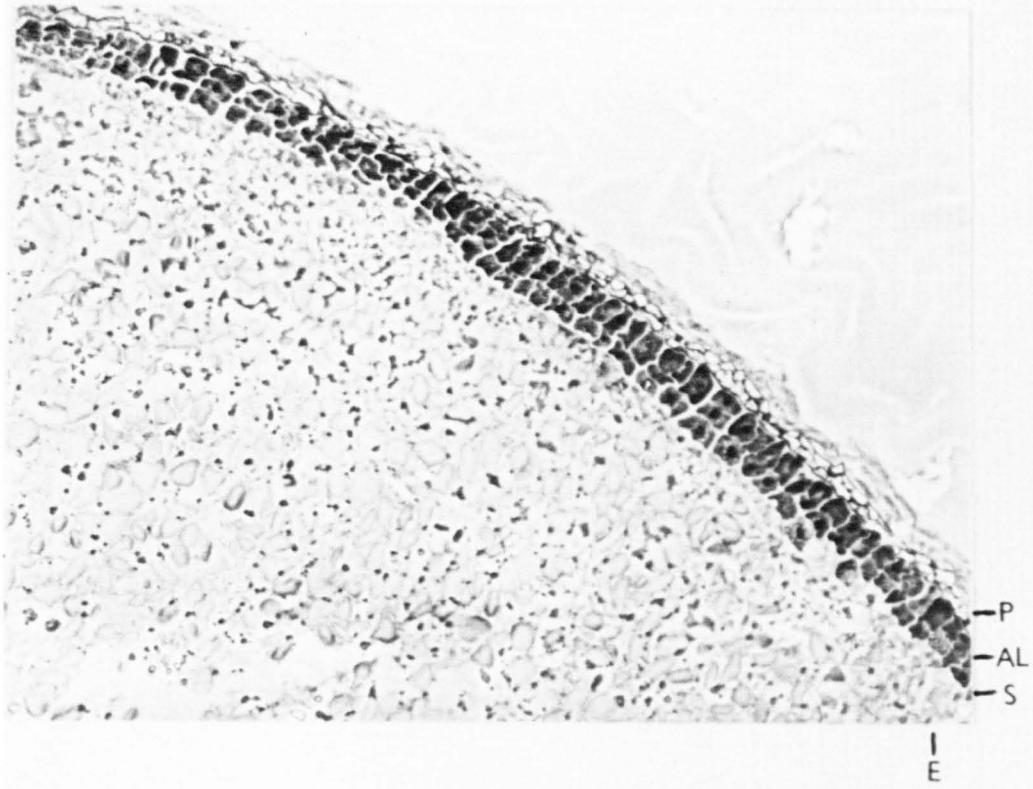
In addition to the importance of seed proteins in live-stock nutrition, the developing seed also provides molecular biologists with a useful system for studying genome expression in higher plants. The rates of storage protein synthesis are very high (sometimes accounting for more than 50% of the total

protein synthesis in the seed) during a defined period in the development of the seed. Although this high rate can alleviate some of the problems of messenger RNA isolation, the chemistry of the proteins themselves is often very complicated, as is the case in barley. The investigation of the molecular mechanisms regulating storage protein biosynthesis may assist in solving the problem of improving the nutritional quality of barley and other seeds of nutritional importance.

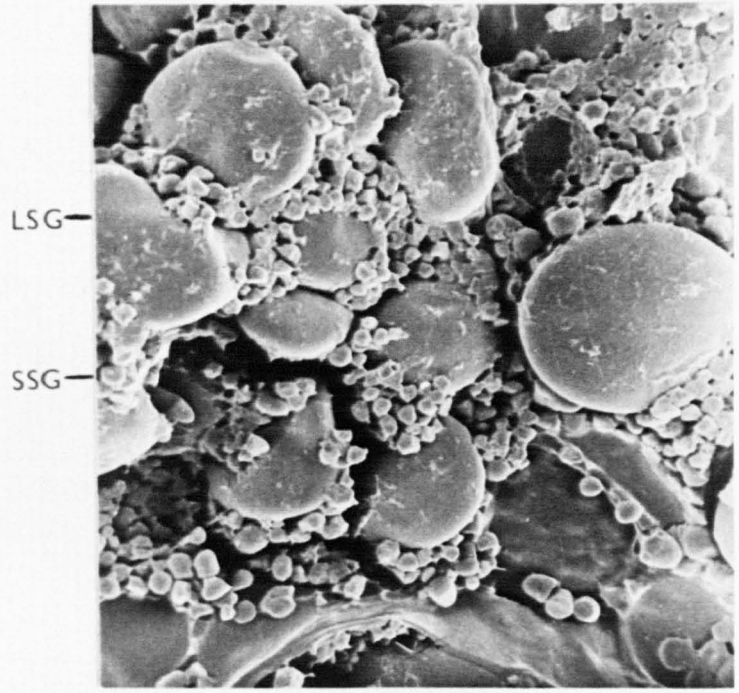
1.2 THE CEREAL ENDOSPERM

1.2.1 Structure and Development

Barley is a monocotyledonous plant which carries the seed in the form of a two or six-rowed spike. In common with other cereals, the nitrogen store for the embryo is laid down in protein deposits in a storage tissue, called the starchy endosperm. The endosperm is a triploid tissue which is formed initially by the fusion of one nucleus from the pollen tube and two nuclei from the embryo sac. Rapid nuclear division occurs (coenocytic phase) followed by cell division, and at this stage the cells lie freely in a 'liquid' endosperm. About two weeks after anthesis, more structure is visible; the aleurone, which is also triploid, forms as a single layer of cells immediately beneath the pericarp, whilst the starchy endosperm proliferates inwards and is filled with deposits of starch and protein. The structure of the mature endosperm is



A



B

Fig. 1 Structure of the barley grain

A. Light micrograph of barley grain stained for protein. The dark structures within the endosperm (E) are protein bodies. The aleurone (AL) and sub-aleurone (S) layers are visible beneath the pericarp (P). Magnification: x 200.

B. Scanning electron micrograph of the starchy endosperm of barley. Both large (LSG) and small (SSG) starch grains are visible.

These photographs were supplied by B. J. Miflin and S. R. Burgess.

shown in Fig. 1A. The aleurone layer is visible, with the sub-aleurone layer beneath, and the aleurone cells are largely filled with protein deposits. The starchy endosperm contains both protein bodies and starch grains. Fig. 1B shows that the starch grains fall into two distinct size classes.

The dry weight of the barley endosperm increases rapidly during the period two to four weeks after anthesis. This increase is paralleled by an increase in the total nitrogen of the seed and in a rapid accumulation of storage proteins (Shewry et al, 1979a). The contributions of individual protein fractions to this accumulation will be discussed in the following sections. Maize and wheat undergo similar developmental sequences to barley in terms of the development of the endosperm and the deposition of starch and protein reserves.

1.2.2 Protein Fractions

The proteins of the cereal endosperm were classified by Osborne (1895) into four groups based on their solubility. The groups were:-

(1) albumins - soluble in water;

(2) globulins - soluble in salt solutions;

(3) prolamins - soluble in aqueous alcohol;

(4) glutelins - soluble in dilute acid or alkali.

In addition, the prolamin fraction is characterised by an unusual amino acid composition, having large amounts of proline

and amide nitrogen, andlittle lysine, arginine or histidine. The techniques of extraction of prolamins have been investigated by a number of workers; it was recognised that hot propyl-alcohols were more efficient than cold alcohols, and that repeated extractions, with shaking, were required for reliable prolamin extraction (Bishop 1928, 29a; Bailey, 1944).

After extraction of the prolamin fraction from barley (termed hordein) with hot alcohol, it was found that a further fraction could be extracted with hot alcohol plus a reducing agent. This fraction was at first termed glutelin but was later shown to be very similar to the first alcohol-soluble hordein fraction (Lontie and Voets, 1959). Maize prolamins (termed zein) are also more soluble in the presence of a reducing agent. Until the importance of reducing agents and repeated extractions was realised, the glutelin fraction was heavily contaminated with prolamins. When prolamin had been removed from the glutelin fraction, it was shown that glutelins are in fact rich in lysine (> 4%) and have a much lower content of glutamine and proline (10-15% and 7-10% respectively). Prolamins have 20-40% glutamine, 10-25% proline and very little lysine (0.1-1%). Barley and wheat prolamins have glutamine and proline contents towards the top end of the range, whereas maize prolamins have lower values (approximately 20% glutamine, 11% proline); for a review of this work see Miflin and Shewry (1977, 1979a).

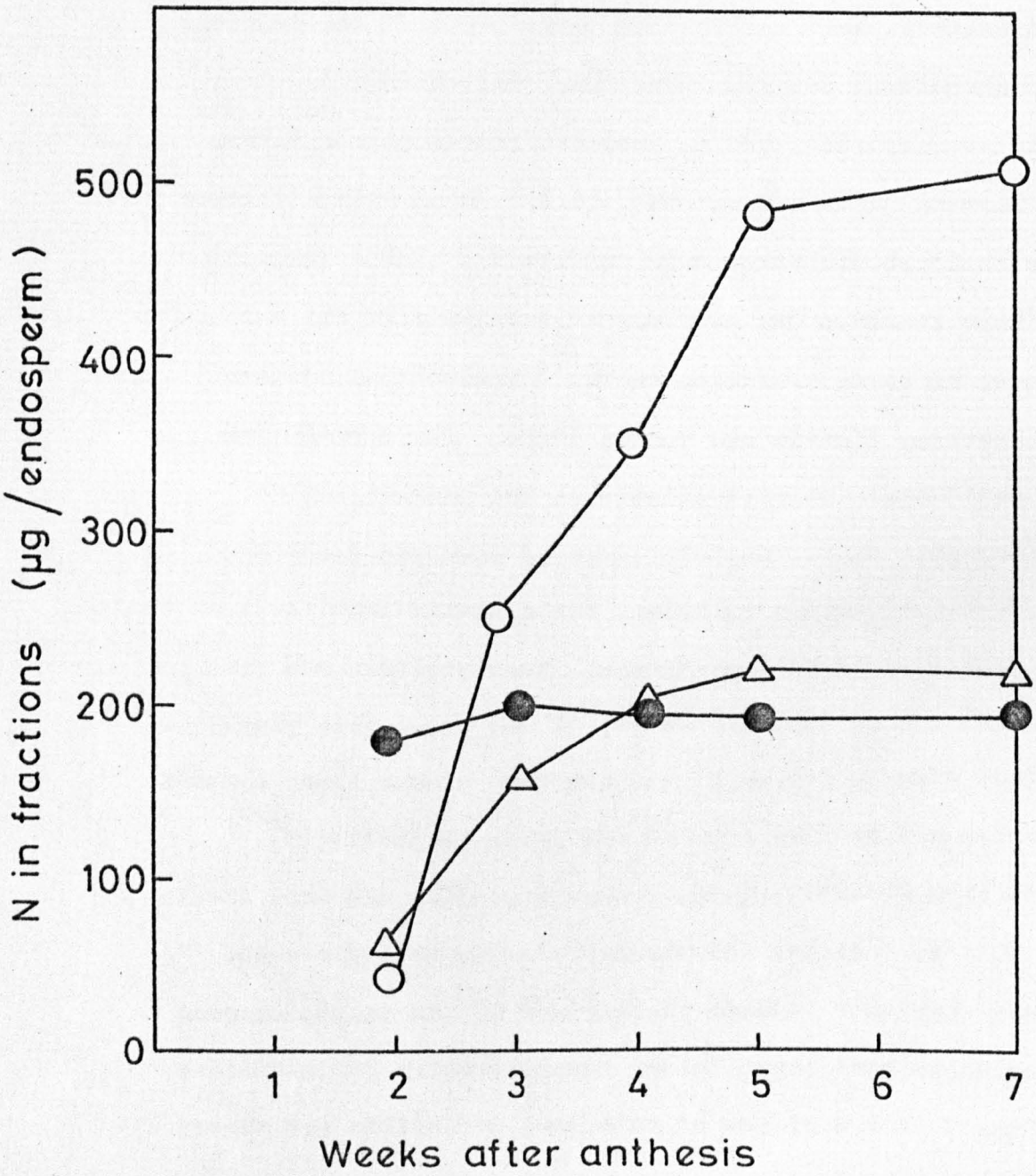

N in fractions (µg / endosperm)
500
400
300
200
100
0
1
2
3
4
5
6
7
Weeks after anthesis

Fig. 2 Synthesis of endosperm proteins

The amount of each of three protein fractions present in the developing barley seed was determined at various times after anthesis:-

●—● Total salt-soluble.

○—○ Hordein; extracted with 55% propan-2-ol, 2% 2-mercaptoethanol at 60°C.

Δ—Δ Glutelin plus residue.

Reproduced, with permission, from Miflin and Shewry (1979a).

There is considerable evidence that the prolamin fraction of the cereal endosperm serves as a nitrogen store for the seed during germination. The evidence is:-

(1) the prolamin fraction is formed fairly late in endosperm development (Fig. 2), and is preferentially increased when high levels of nitrogen are available to the plant (Shewry et al, 1978e; Miflin and Shewry, 1979a);

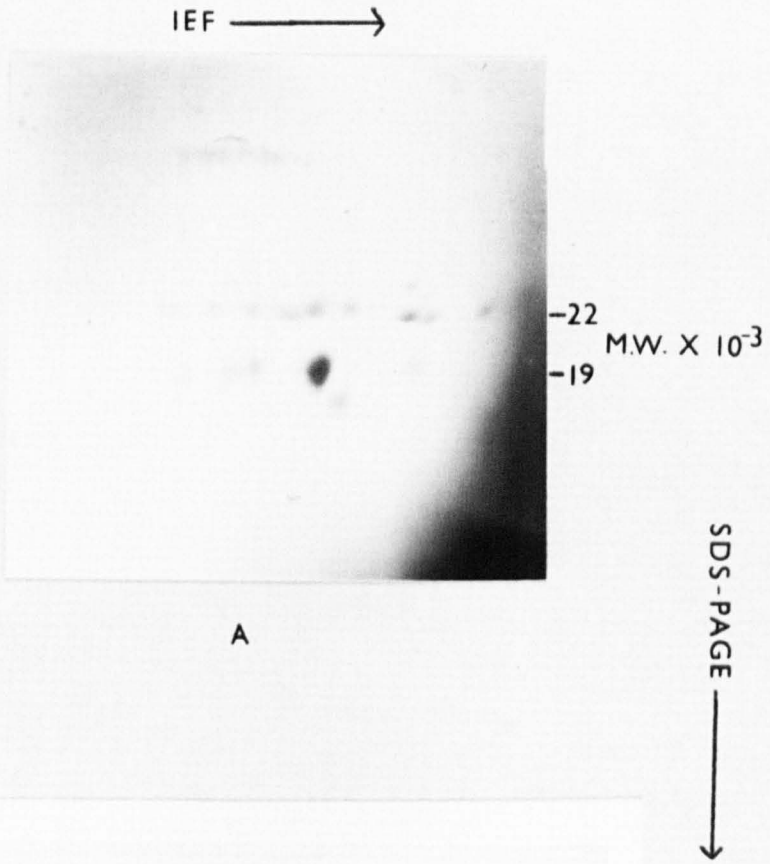
(2) during germination of barley, hordein is degraded preferentially, and this degradation is paralleled by an increase in soluble and protein-nitrogen in the embryo (Bishop, 1929b; Briggs, 1978);

(3) the prolamin fraction has no other apparent metabolic function, although this is negative evidence;

(4) prolamins are packaged into protein bodies in the endosperm (Miflin and Shewry, 1979b; Viotti et al, 1978).

These characteristics are all expected of storage proteins, and the degradation of these proteins during germination provides particularly good evidence of their function as a nitrogen store.

Many workers have, therefore, investigated the nature of the prolamin fractions of various cereal grains by gel electrophoresis in both one and two dimensions. Zein, separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), appears to consist of two major bands of approximately 22000 and 19000 molecular weight and several smaller, minor



A

B

Fig. 3 Two-dimensional gel analysis of wheat and maize prolamins.

Prolamins were extracted from wheat and maize in the presence of a reducing agent, and separated, after alkylation by a two-dimensional technique combining isoelectric focusing (IEF) with SDS-polyacrylamide gel electrophoresis.

A. Maize prolamins - zein

B. Wheat prolamins - gliadin

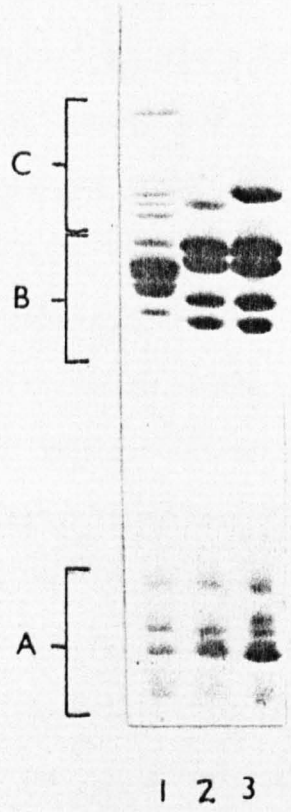
These photographs were supplied by A. J. Faulks.

components. However, two-dimensional analysis (combining iso-electric focusing with SDS-PAGE) shows that zein is made up of more than twenty components; the two major 'SDS-bands' are in fact heterogeneous (Fig. 3A). The wheat prolamin fraction (termed gliadin) consists of an even more complex mixture of polypeptides (Fig. 3B) which is probably related to the fact that wheat is hexaploid whereas maize is diploid. Wheat prolamin polypeptides are considerably larger than those of maize, being as large as 100,000 molecular weight.

The prolamin fraction of barley, termed hordein, constitutes 35-55% of the total protein of the endosperm, the variation being dependent on the nitrogen nutrition and the variety of the barley. After extraction of hordein with 55% (v/v) propan-2-ol plus 2% (v/v) 2-mercaptoethanol, the cysteine residues are usually alkylated with 4-vinyl pyridine prior to further characterisation (Shewry et al, 1977a). Separation of hordein by SDS-PAGE yields three groups of polypeptides (Fig. 4A) which are termed A, B and C (Shewry et al, 1977b). The 'A' fraction is probably not related to fractions 'B' and 'C'; it contains relatively large amounts of lysine. This fraction constitutes only 5% of the total hordein but, due to the high content of lysine, stains more heavily with Coomassie brilliant blue than the 'B' and 'C' fractions.

The 'B' and 'C' fractions have been separated and characterised, and some of their properties are summarized in

A



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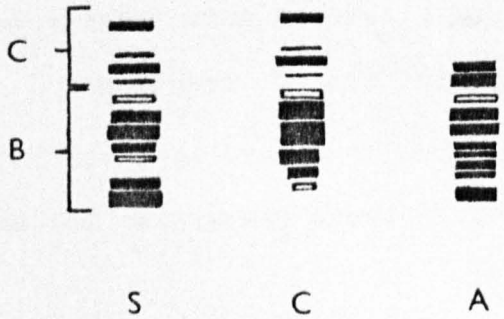


Fig. 4 Analysis of hordein by SDS-PAGE.

The hordein fraction was extracted from a number of varieties of barley grain in the presence of a reducing agent. The polypeptides were separated, after alkylation, by SDS-PAGE.

A. Photograph of gel stained with Coomassie brilliant blue R. The tracks represent barley varieties:-

1. Malta
2. Harka
3. Keg.

B. Diagrammatic representation of hordein patterns from barley varieties:-

S. Sundance
C. Carlsberg II
A. Athos.

The groups of proteins labelled A, B and C are defined in the text (see page 9).

Table 1 (data from Miflin et al, 1980b). A very high proportion of the glutamate and aspartate is amidated (greater than 90%), and the proportion of the amino acids made up by proline plus glutamine is about 55% for the 'B' hordeins and over 70% for the 'C' hordeins. The N-terminal residue of the 'C' hordeins is arginine but that of the 'B' hordeins is blocked. Schmitt and Svendsen (1980) reported that sequence analysis of the 'B' and 'C' hordeins showed considerable homology within the groups. The 'C' hordeins are highly deficient in sulphur-containing amino acids and may not contain any cysteine residues; this is consistent with the observation that alkylation of the cysteine residues of the hordeins does not significantly affect the separation of the 'C' fraction whereas this treatment improves the resolution of the 'B' fraction by SDS-PAGE considerably. There are also slight differences in solubility between the two fractions.

Molecular weight determinations of the hordeins are extremely variable (Table 1) and indicate the unreliability of SDS-PAGE for the estimation of molecular weight. This variability has been reported for other cereal proteins (Hamauzu et al, 1975; Sexson et al, 1978). The molecular weights obtained by equilibrium centrifugation are considered to be the most accurate, that is:- 32000 for the 'B' fraction and 53000 for the 'C' fraction.

Table 1 Comparison of the 'B' and 'C' hordein fractions.

	'B' hordein	'C' hordein	References
Molecular weight (Kd): by SDS-PAGE	45 - 60	68 - 80	(1)
	30 - 51	67 - 86	(2)
by G200 Sephadex chromatography	52 , 66	101	(3)
by equilibrium centrifugation	31.9	52.6	(4)
Amino acid composition in mole %			(4)
Glx	35.4	41.2	
Pro	20.6	30.6	
Lys	0.5	0.2	
Cys	2.5	trace	
Met	0.6	0.2	
Total residues per mole	271	439	

This data is taken from Miflin et al (1980b).

References:- (1) Brandt (1976), (2) Shewry et al (1977b), (3) Mesrob et al (1969), (4) Shewry et al (1980a).

The separation of the hordein fraction by SDS-PAGE reveals a number of distinct 'B' and 'C' patterns occurring in different combinations in the barley varieties examined (Shewry et al, 1979b). The total number of patterns observed so far are seventeen for the 'B' polypeptides and eight for the 'C' polypeptides. Fig. 4B shows that barley varieties Sundance and Carlsberg II have a common 'C' pattern but different 'B' patterns; the variety Athos has different 'B' and 'C' patterns. Hordein is further resolved into approximately twenty polypeptides (depending on variety) when separated by the two-dimensional technique which combines isoelectric focusing with SDS-PAGE. The two-dimensional pattern of the barley variety Sundance is shown in Fig. 5. The structural homology of the 'B' polypeptides has been investigated using two-dimensional mapping and analysis of spots from two-dimensional gels by cleavage with cyanogen bromide (CNBr). By analysing mixtures of hordeins from different barley varieties it was possible to determine which polypeptides were common to the varieties (Fig. 6A). When hordein from the varieties Proctor and Jupiter were mixed and analysed by two-dimensional gel electrophoresis, it was shown that four spots were common to the two varieties, even though the hordein patterns looked very different (Miflin et al, 1980b). Peptide mapping of individual spots from two-dimensional gels, using CNBr, indicated that, although some

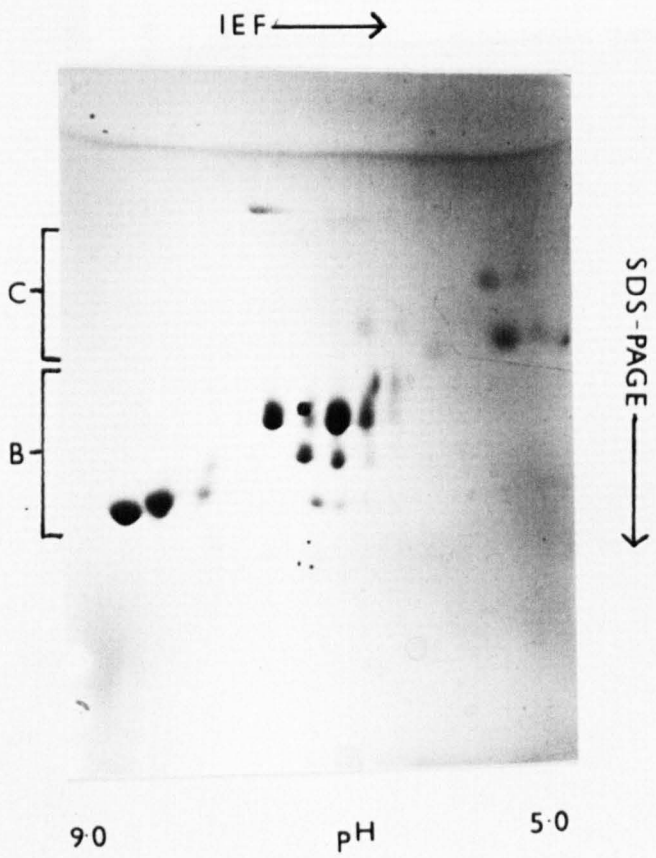
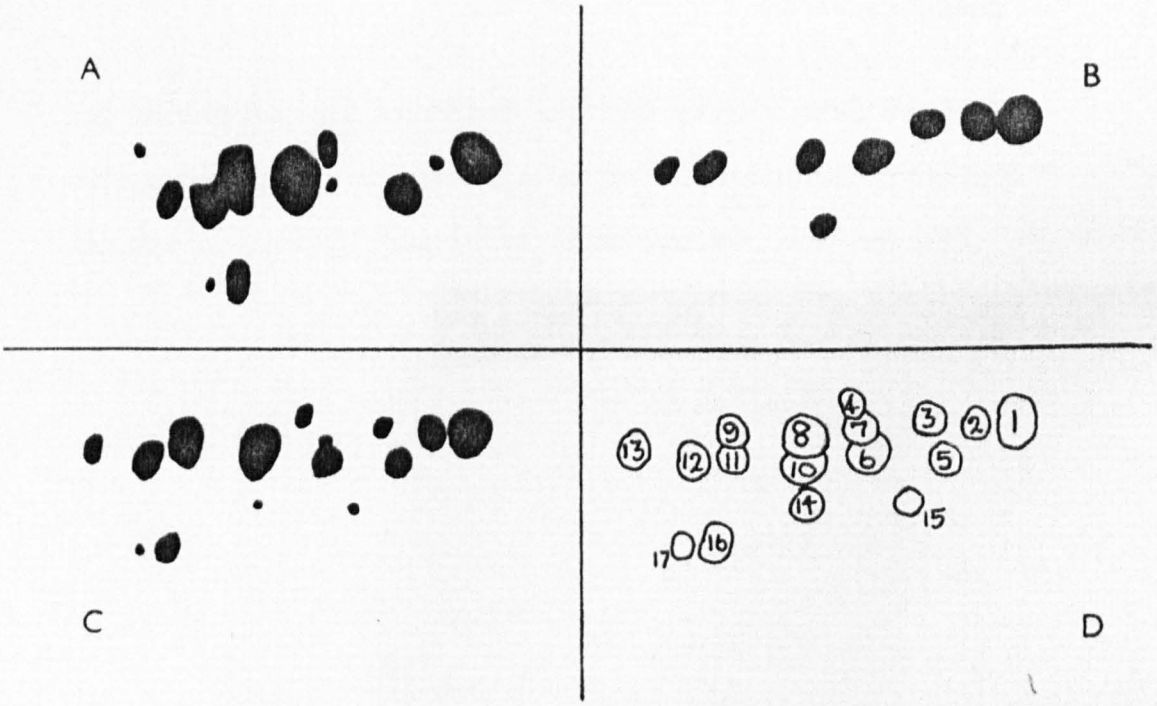

IEF
C
B
SDS-PAGE
9·0
pH
5·0

Fig. 5 Two-dimensional analysis of hordein from barley variety Sundance.

Hordein from barley variety Sundance was separated by a two-dimensional technique combining isoelectric focusing and SDS-PAGE. The gel was stained with Coomassie brilliant blue R.

This photograph was supplied by A. J. Faulks.


A
B
C
D
13
12
9
11
8
10
14
4
7
6
3
5
2
1
15
17
16

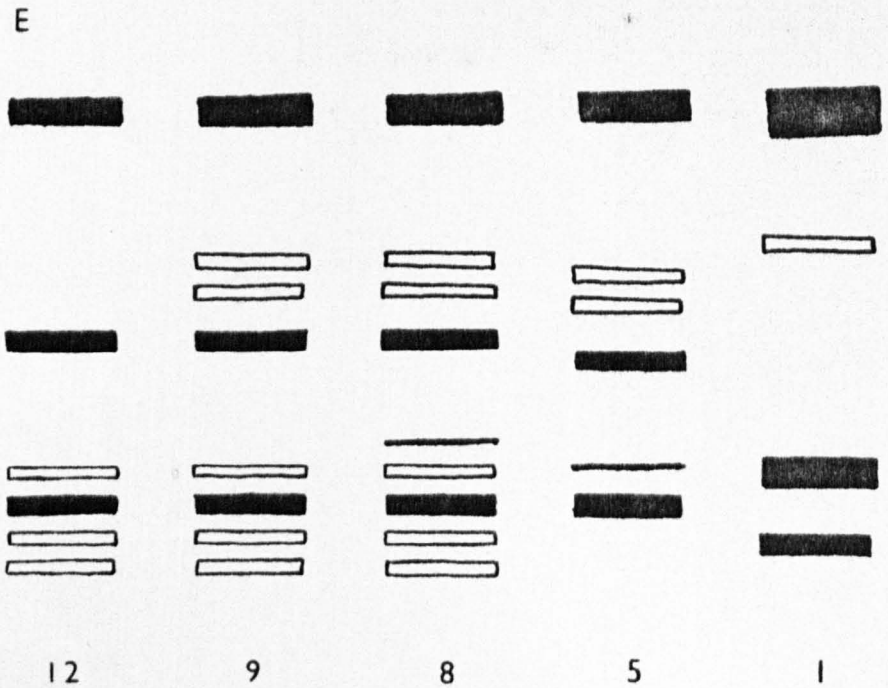

E
12
9
8
5
1

Fig. 6 Structural homology of 'B' hordein polypeptides.

A-C. Two-dimensional analysis of alkylated hordein from barley varieties:-

A. Jupiter

B. Proctor

C. Mixture of Jupiter and Proctor.

D. Diagram of the major 'B' polypeptides present in Proctor and Jupiter. Polypeptides 2, 3, 6, 10, 11, 13-15 occur in Proctor only; 4, 5, 7-9 occur in Jupiter only; 1, 12, 16, 17 occur in both varieties.

E. Gradient SDS-PAGE of peptides produced by cleaving Jupiter polypeptides with CNBr. The number under each track refers to the number of the spot on the two-dimensional map (see D above).

This diagram is redrawn, with permission, from Miflin et al (1980b).

groups of polypeptides have similar maps, differences occur in the intensity and mobility of some of the bands (Fig. 6B; Miflin et al, 1980b) indicating real differences between the components resolved by two-dimensional techniques but suggesting a considerable degree of homology. The 'C' polypeptides are not cleaved by CNBr, which is consistent with their very low content of methionine; Schmitt and Svendsen (1980) reported that the residue next to the 'C' terminus was methionine, so cleavage at this point would not result in an observable difference in size.

Analysis of hordein fractions from different barley varieties by SDS-PAGE has shown that the pattern is specific to the variety (see Fig. 4). It has been shown that the 'B' and 'C' hordeins are controlled by two loci, Hor-2 and Hor-1 respectively, which are approximately 10-15 recombination units apart on chromosome 5. This has been determined by genetic analysis using conventional crosses between varieties (Shewry et al, 1978d; Doll and Brown, 1980; Jensen et al, 1980) and chromosome-doubled monoploids (Shewry et al, 1980b). The analysis of conventional crosses is complicated by the fact that the endosperm is triploid; when scoring for recombination between the 'B' and 'C' patterns it is necessary to take into account that there are two gene doses from the maternal pattern and one from the paternal parent. A map of chromosome 5 is

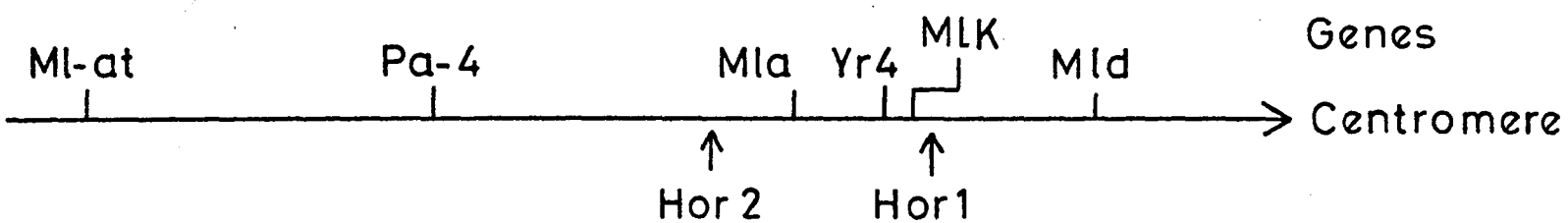

Ml-at
Pa-4
Mla
Yr4
MlK
Mld
Genes
Centromere
Hor 2
Hor1

Fig. 7 Genetic map of part of chromosome 5 of barley.

The genes indicated are:-

Ml-at, a, k, d	-	mildew resistance
Yr 4	-	yellow rust resistance
Pa 4	-	powdery mildew and leaf rust resistance
Hor-1, Hor-2	-	hordein loci

The Hor loci are semi-dominant.

The information in this map is taken from Jensen (1978) and Shewry et al (1980b).

shown in Fig. 7. The two loci are located on either side of the Mla locus with Hor-2 distal from the centromere. Two-dimensional analysis of the hordein fraction from various crosses revealed no crossing over within either locus; however, crossing over within one recombination unit would not have been detected, given the numbers of crosses involved. On the basis of genetic evidence and peptide mapping the hypothesis put forward by Miflin et al (1980b) was that Hor-2 and Hor-1 are complex structural loci based upon two ancestral genes which have duplicated and diverged.

The characterisation and genetic analysis of the hordein fraction indicate that it consists of groups of closely related polypeptides. The varietal specificity of the hordein polypeptides has proved valuable in the varietal identification of grain samples (Shewry et al, 1979b). The two-dimensional patterns are highly reproducible and this, coupled with CNBr mapping results, suggests that many of the spots visible on two-dimensional gels are products of different structural genes, and are not simply artefacts of the preparation and separation techniques. A given spot may contain more than one gene product if there is a difference in uncharged amino acids. The heterogeneity of the prolamin fraction is not unexpected as the evolutionary pressures, which operate to maintain the primary sequence of many proteins (for example, enzymes), may

not operate to the same extent on proteins which act as a nitrogen store. The proteins will still function adequately if they are capable of being stored and degraded as required in the developmental sequence.

1.2.3 Deposition of Storage Proteins

Cereal seeds contain two distinct classes of protein body; one class occurs in the aleurone layer and the other in the starchy endosperm. In wheat and barley, the protein bodies of the endosperm are irregular in shape whereas thoseof maize are more spherical. The origin of these protein bodies has been the subject of much discussion. One general view is that the situation is analogous to that in certain animal cells; proteins are synthesised on the rough endoplasmic reticulum, pass into the lumen and from there, into dictysomes before deposition in vacuoles. This series of events has been suggested to explain the deposition of proteins within oilseeds (Diekert and Diekert, 1976) and legume cotyledons (Boulter, 1979). It has been argued, however, that dictysomes may not be involved (Briarty, 1978) but that vesicles bud-off directly from the endoplasmic reticulum for transport to vacuoles. In either case, the site of protein synthesis is separate from the site of deposition of the storage proteins. A number of workers have supported this hypothesis and suggested that protein bodies of the barley endosperm are surrounded by a vacuolar membrane (Ingversen, 1975; Munck and Von Wettstein, 1976; Cameron-Mills *et al*, 1978b).

A second hypothesis is that the storage proteins are deposited directly in the rough endoplasmic reticulum. This was first suggested by Khoo and Wolf (1970) and supported by Larkins and Hurkman (1978) who showed that messenger RNA (mRNA) derived from the rough endoplasmic reticulum of maize directed the synthesis of very similar products to the mRNA from protein bodies. This hypothesis was supported by Miflin and Shewry (1979b) for the formation of protein bodies.

A third hypothesis is that protein bodies are independent organelles, possibly of plastidal origin. However, an early suggestion, that protein bodies contain ribosomes of a lower sedimentation value than those of the cytoplasm (Morton et al 1964), has been contradicted by electron microscopic evidence. Ribosomes have been shown to be on the outside of the protein body membrane of maize (Burr and Burr, 1976; Larkins and Hurkman, 1978; Viotti et al, 1978). In contrast to the disagreement as to the origin of endosperm protein bodies, there is general agreement that cereal aleurone layer protein bodies are of vacuolar origin.

Subcellular fractionation of the endosperm has allowed further investigation of the nature of protein bodies. Miflin et al (1980a) identified the various fractions separated by sucrose gradient centrifugation, in terms of marker enzymes. They found that a large proportion of the endoplasmic reticulum (identified by NADH cytochrome c reductase) was associated with

storage protein in barley, wheat and maize endosperm. In contrast, pea storage proteins did not appear to be associated with endoplasmic reticulum but the protein body peak did contain the vacuolar marker enzyme, N-acetylglucosaminidase. This observation supports the view that pea protein bodies are laid down in a way analogous to that seen in animal cells. There was negligible N-acetylglucosaminidase associated with cereal protein bodies. The evidence presented by Miflin et al (1980a) and Larkins and Hurkman (1978) suggests that cereal protein bodies are formed by the aggregation of storage proteins within the lumen of the endoplasmic reticulum. In wheat and barley the aggregates are irregular in shape and associated with, but not completely surrounded by, endoplasmic reticulum (Fig. 8). It is suggested that the size and nature of the aggregates, possibly due to the large size and hydrophobicity of the proteins, disrupts the membrane. Maize differs in that the deposits are more regular in shape and do appear to be completely surrounded by endoplasmic reticulum. This difference between maize and the other two cereals described here may be correlated with the difference in nature of the prolamins; zein is much smaller in size than the prolamins of barley or wheat and has a significantly lower content of glutamine and proline (Miflin et al, 1980a).

It appears, therefore, that cereal storage proteins cross the endoplasmic reticulum as has been described in legumes

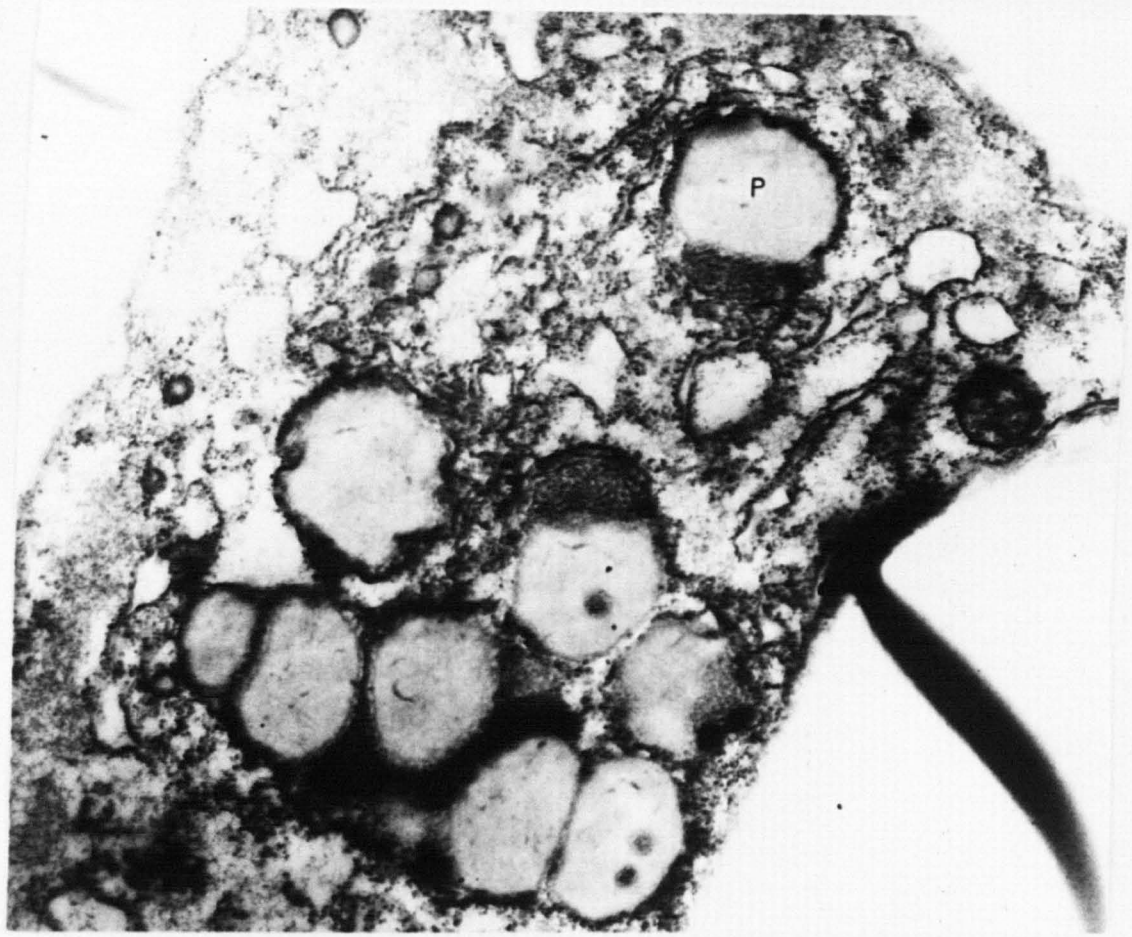

P
1 MICRON

Fig. 8 Protein bodies in the starchy endosperm.

Electron micrograph of a section through developing barley endosperm:- the protein bodies (P) are seen as irregular structures with two distinct regions (termed granular and amorphous). Membranes are associated in part with the protein bodies but they do not appear to completely surround the deposits.

This photograph was supplied by B. J. Miflin.

and for certain proteins in animal cells. However, legume storage proteins and animal secretory proteins arethen transported to other sites. In contrast, cereal storage proteins remain within the lumen of the endoplasmic reticulum and aggregate to form protein bodies, the structure of which may be determined in part by the nature of the proteins.

1.2.4 High-Lysine Barley

One approach to the improvement of the nutritional quality of barley has been to look for and characterise high-lysine lines. The first high-lysine barley recognised was found by screening the world barley collection using the dye-binding capacity technique (Munck et al, 1970). This technique compares the amount of Acilane Orange G dye bound to basic amino acids with the nitrogen content of the seeds. The variety Hiproly was found to have relatively more lysine in the seed than other varieties and also a higher protein content, but the grain weight was lower. Hiproly has been used in breeding programs (for example, Persson and Karlsson, 1977), but to date no line derived from it has been grown commercially.

The same screening procedure was used, at the Danish Atomic Energy Research Station at Risó, to examine large numbers of seeds which had been treated with either chemical or physical mutagens. A number of mutants were identified (Doll et al, 1974) including Risø 1508 (derived by treatment of the variety Bomi with ethylinimine) which has the highest

increase in lysine content yet observed. For a review of high-lysine barley see Miflin and Shewry (1979b).

The high-lysine character of Risø 1508 is due to a reduction in the amount of hordein present in the grain (approximately one-third of the parent variety). The nature of the hordein fraction is also altered (Shewry et al, 1978a). The 'A' polypeptides form a high proportion of the hordein fraction (30% as against less than 5% in the parent variety), the 'C' polypeptides are greatly reduced and the distribution of the 'B' polypeptides is altered. The high molecular weight 'B' polypeptide is increased relative to the other polypeptides; some 'B' polypeptides appear to be missing completely.

The mutation in Risø 1508 does not, however, affect only the hordein fraction; it is a pleiotropic mutation. The effects are widespread throughout the endosperm andinclude the reduction of lipid and carbohydrate levels and an increase in the levels of degradative enzymes such as α-amylase and RNAase. The structure of the endosperm is also altered; there are fewer protein bodies and small starch grains in Risø 1508 than in the parent variety. The protein bodies of Risø 1508 endosperm also have a different structure being more spongy in appearance (Miflin and Shewry, 1979b).

The mutation in Risø 1508 maps as a single gene mutation on chromosome 7 (Karlsson, 1977); it may be a point mutation as the mutagen used, ethylinimine, is considered to produce point

mutations. As the effects are pleiotropic, the mutation is assumed to be in a regulatory function. The only high-lysine barley yet recognised, which has a mutation mapping near to hordein structural loci on chromosome 5, is Risø 56, which was selected in the same way as Risø 1508 from the parent variety Carlsberg II treated with γ-rays. This mutation maps close to the Hor-2 locus but its precise nature is, as yet, unknown (Doll et al, 1980). Risø 56 has an altered hordein fraction; there is less hordein present and the 'B' polypeptides are almost entirely absent. Two minor 'B' polypeptides of Carlsberg II are still present.

Although these two mutant lines have a higher lysine content than their parent varieties making them of better nutritional quality, the grain yield is reduced to 80% of that of their parents. This general phenomenon has been found for all high-lysine lines so far described. Attempts to incorporate the high-lysine character of Risø 1508 into well-filled grain have so far been unsuccessful (Rhodes and Jenkins, 1978). The lower grain yields have so far rendered these lines commercially non-viable but, the production of these lines has stimulated the biochemical investigation of the development of both mutant and normal lines.

1.3 IN VITRO-PROTEIN SYNTHESIS

1.3.1 Introduction

Eukaryotic messenger RNAs (mRNAs) were first purified from tissues or cell types in which protein synthesis is almost exclusively devoted to a particular protein. For example, reticulocytes synthesise large quantities of globulin; polysomes were isolated from reticulocytes and the RNA separated by sucrose density gradient centrifugation. In addition to the ribosomal RNA peaks, a peak was resolved at approximately 9s. The identity of this 9s fraction as globin mRNA was confirmed by translation in an heterologous protein-synthesising system (e.g. Sampson *et al* (1972) using cell-free systems derived from rat or mouse liver; Lane *et al* (1971) by injection of 9s RNA into *Xenopus laevis* oocytes).

The isolation of eukaryotic mRNA was aided by the discovery that many mRNAs contained a polyadenylic acid sequence. One of the first observations of this phenomenon was by Edmonds *et al* (1969, 1971) and has since been confirmed by many workers. The presence of polyadenylic acid (poly-A) sequences enables the purification of mRNAs from other RNA species by chromatography on oligo-dT cellulose or poly-U Sepharose. Aviv and Leder (1972) used oligo-dT cellulose chromatography to purify globin mRNA from crude extracts. The poly-A sequence was shown to be at the 3' end of mRNAs (for example, see Burr and Lingrel, 1971) and is added after transcription is complete. However, not all eukaryotic mRNAs are polyadenylated (e.g. histone mRNAs).

The function of the poly-A sequence is not fully understood but many suggestions have been put forward. It has been suggested that poly-A increases the stability of mRNA; this was demonstrated by Huez et al (1978) who showed that the presence of poly-A sequences increased the functional half-life of mRNA injected into Xenopus oocytes. These workers compared the translation of native and denadenylated globin mRNA, and native and in vitro-adenylated histone mRNA. More recently it has been suggested that poly-A is involved in the processing of the primary transcript of mRNA (Bina et al, 1980). It has been shown that many genes have intervening sequences (introns) between sections of coding sequence (e.g. ovalbumin, Lai et al, 1978); these introns must be cut out of the primary transcript and Bina et al (1980) suggested that poly-A could align the correct splicing sites.

Eukaryotic mRNAs and many viral RNAs also have an unusual feature at the 5' end; this structure has been termed a 'cap' and consists of a methylated guanosine residue linked by a 5'-5' triphosphate bond to the first coded nucleotide of the RNA. The biosynthesis and function of the 'cap' has been reviewed by Shatkin (1976) and Filipowicz (1978). The 'cap' may function to protect the mRNA from degradation by 5'-exonecleases. It has been suggested by others that the 'cap' is involved in the initiation of protein synthesis (Kozak, 1978).

An analogue of the 'cap', 7-methyl guanosine monophosphate, inhibits the translation of capped messengers *in vitro*. However, it does not affect the translation of previously initiated mRNAs. It has been pointed out by many workers that experiments involving this analogue must be interpreted with care as many non-specific interactions can occur (Sonenberg and Shatkin, 1978). The effect of the analogue is also a function of ion concentration (Kemper and Stolarsky, 1977; Chu and Rhodes, 1978).

Although many *in vitro*-translation systems have been described and used, one of the most common ones is that derived from wheat germ. Crude extracts of wheat germ have been shown to be capable of translating mRNA from a variety of sources, for example: TMV RNA and rabbit globin mRNA (Roberts and Paterson, 1973), rat liver albumin mRNA (Sonenstein and Brawerman, 1977), ovalbumin mRNA (Rosen *et al*, 1975), and mRNA from pea leaves (Highfield and Ellis, 1978). When the system was primed with globin, albumin or ovalbumin mRNA, the products of *in vitro*-synthesis resembled the purified proteins in terms of electrophoretic mobility (in SDS-PAGE) and immunological characteristics. Roberts and Paterson (1973) also demonstrated that the products resembled globin in terms of typtic peptide mapping. The results indicate faithful translation.

It was later demonstrated that not all *in vitro*-synthesised proteins were identical to the native protein. For example, the translation of rat pituitary gland mRNA in the

wheat germ system yielded a polypeptide which was larger than prolactin (Maurer et al, 1976). It was shown that this polypeptide shared major typtic peptide fragments with authentic prolactin but had a leucine-rich N-terminal addition of 29 amino acids (Maurer et al, 1977). This phenomenon has been observed for other mRNAs and in vitro-protein synthesising systems, for example: immunoglobulin light chain mRNA translated in the rabbit reticulocyte lysate cell-free system yields a polypeptide larger than the authentic protein (Mach et al, 1973). The small subunit of ribulose bisphosphate carboxylase was also shown to be synthesised in a larger form in the wheat germ cell-free system (Highfield and Ellis, 1978). These larger forms are considered to be precursors and their significance is discussed in section 1.4. These observations indicate that the wheat germ system does not, in general, contain activities capable of modifying precursor proteins to their native size.

The wheat germ cell-free system has, therefore, been shown to faithfully translate mRNAs into primary translation products. This system has certain advantages over other in vitro systems (e.g. rabbit reticulocyte lysate described by Pelham and Jackson, 1976). The extract can be prepared easily and rapidly (approximately 90 min) and the starting material is relatively easy to obtain. However, the source of the wheat germ is important in determining good translational activity (Marcu and Dudock, 1974). The endogenous activity of the

wheat germ cell-free extracts is low resulting in high stimulations of incorporation of radioactivity into trichloroacetic acid (TCA)-insoluble material (20 to 100 fold: Roberts and Paterson, 1973) in response to exogenous mRNA. Some workers have reported that the wheat germ cell-free system does not synthesise large polypeptides efficiently and that considerable premature termination can occur (Prives *et al*, 1974); the reticulocyte lysate system is considered to be superior in this respect. The wheat germ cell-free protein synthesising system is useful in the study of translation of many mRNAs and it has been widely used.

1.3.2 *In vitro*-Synthesis of Cereal Storage Proteins

The wheat germ cell-free system has been used to investigate the synthesis of cereal seed storage proteins, the most widely studied are the storage proteins of maize (zein). Polysomes, detached from either the endoplasmic reticulum or protein bodies of developing maize endosperm, direct the synthesis *in vitro* of products similar to zein in terms of solubility, amino acid composition and electrophoretic mobility (Burr and Burr, 1976). Viotti *et al* (1978) demonstrated that polysome translation products were identical to zein in terms of isoelectric focusing pattern as well as molecular weight. This observation supports the hypothesis that the bands seen on isoelectric focusing gels represent different gene products, and are not due simply to loss of amide groups.

The *in vitro*-translation of zein mRNA derived from membrane-bound polysomes gave rise to products which were larger than native zein by approximately 2000 daltons (Burr *et al*, 1978; Larkins and Hurkman, 1978; Wienand and Feix, 1978). These results suggest that the proteins are synthesised as precursors. Larkins and Hurkman (1978) reported that the products of polysome translation were larger than zein and co-migrated with products of mRNA translation. This observation is contrary to that of Viotti *et al* (1978) but could be explained on the basis that the former authors suspended polysomes in water (in the absence of magnesium); this could cause release of mRNA from the polysomes. Wienand and Feix (1978) electrophoretically separated two size fractions of mRNA from maize endosperm, one coding for the group of large polypeptides (22000 daltons) and the other coding for the smaller major polypeptides (19000 daltons).

Very little work has so far been done on the *in vitro*-synthesis of wheat storage proteins but a situation similar to maize seems to exist for the hordeins of barley. Polysomes, derived from the membrane fraction of barley endosperm, directed the synthesis *in vitro* of products identical to hordein (Fox *et al*, 1977; Brandt and Ingversen, 1978), but *in vitro*-translation of mRNA derived from these polysomes gave rise to larger products. Cameron-Mills and Ingversen (unpublished data

quoted in 1978a) have since stated that polysome translation products are of intermediate size between mRNA products and native hordein.

A possible explanation for the difference in size between mRNA translation products and polysome products is that the majority of the nascent chains have already been shortened (or processed) *in vivo*. Therefore, completion of the nascent chains (polysome 'run-off') gives products identical to the native proteins. It has also been argued (Higgins and Spencer, 1980) that the polysome fractions either have, or are contaminated with, processing activity. This subject is discussed in detail in the general discussion (Section 4).

1.4 TRANSPORT OF PROTEINS ACROSS MEMBRANES

1.4.1 Introduction

Vectorial transport of proteins across membranes is a necessary step in the pathway of many proteins from their site of synthesis to their site of action. In the simplest case, prokaryotic cells have one protein synthetic compartment and are able to secrete proteins across the plasma membrane (for example: β-lactamase) and also to take up proteins from the external environment (for example: certain toxins). The situation is more complex in eukaryotic cells due to the presence of a number of distinct intracellular membranes and sites of protein synthesis. Many proteins are synthesised within the

cytosol and transported across the endoplasmic reticulum for storage or secretion. There is also transport of proteins into chloroplasts, mitochondria, nuclei and peroxisomes. Although chloroplasts and mitochondria have their own genetic and protein synthetic machinery, many of their proteins are encoded in the nuclear genome and synthesised in the cytosol. The demonstration of these processes *in vitro* has led to the formulation of possible mechanisms of transport across the different membranes involved.

1.4.2 Protein Translocation Hypotheses

The demonstration *in vitro* of the transport of a protein across the endoplasmic reticulum (Blöbel and Dobberstein 1975a, b) led to the formulation of the signal hypothesis. This hypothesis was slightly modified by Blöbel *et al* (1979) and is represented diagrammatically in Fig. 9. Blöbel and Dobberstein (1975b) demonstrated that mRNA coding for immunoglobulin (IgG) light chain directed the synthesis of a larger precursor in a heterologous *in vitro*-protein synthesising system. However, when stripped microsomes derived from dog pancreatic rough endoplasmic reticulum were added, the product was transported into the vesicles and was protected from attack by added protease. The products were also cleaved to the native size. These events occurred co-translationally; incubation with microsomes after the completion of translation had no effect.

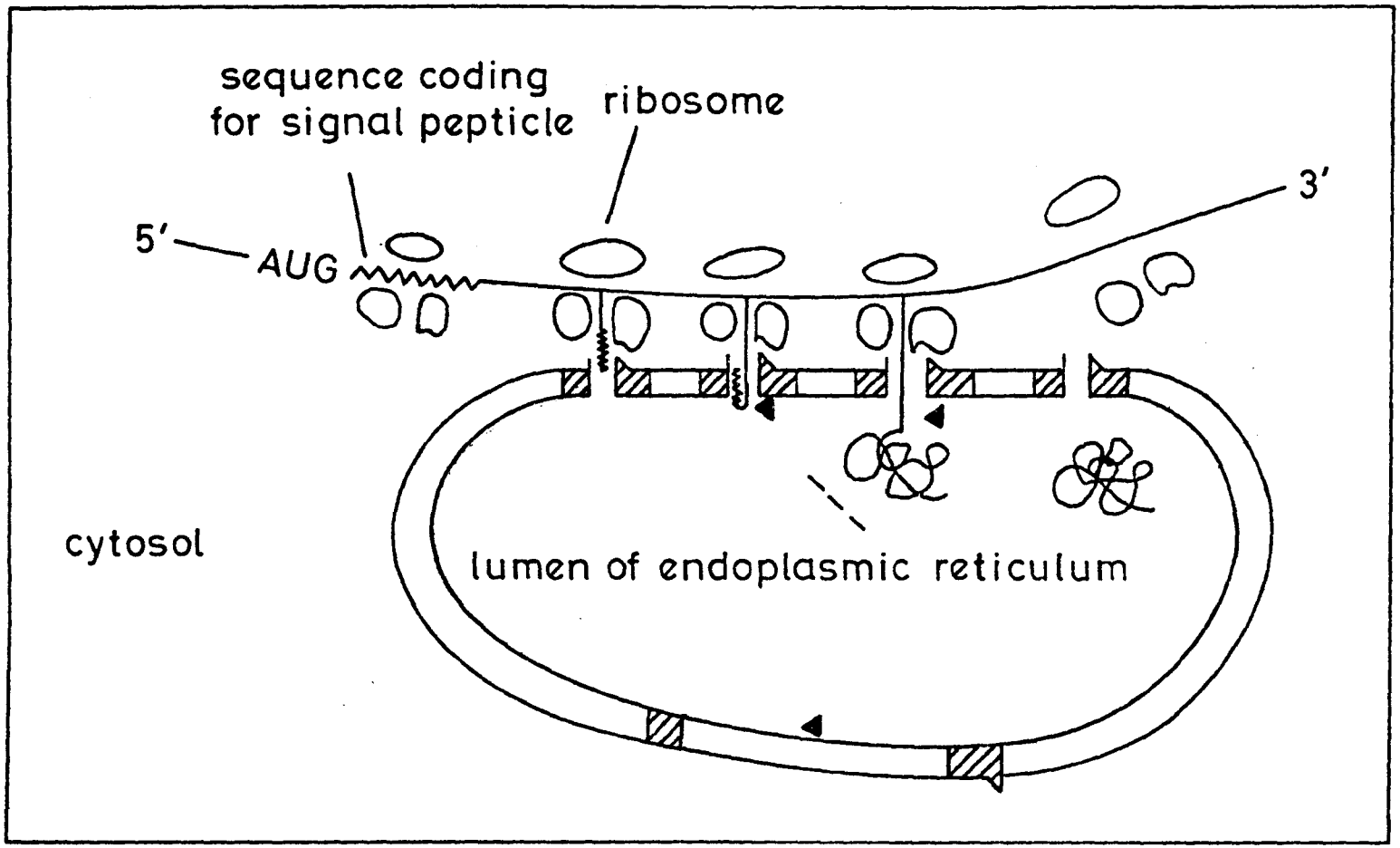

sequence coding
for signal pepticle
ribosome
5'
AUG
3'
cytosol
lumen of endoplasmic reticulum

Fig. 9 Diagram of translocation of proteins into the lumen of the endoplasmic reticulum as suggested by the signal hypothesis (After Blöbel et al, 1979).

After initiation of protein synthesis, the first sequence to be produced is the signal peptide (~~~~). When this peptide emerges from the ribosome, it interacts, together with the ribosome, with membrane receptors (⧄ ⧅). The receptors assemble to form a pore through which the nascent chain passes. The signal sequence is cleaved off by a signal peptidase (◀) and degraded (----), and the remainder of the protein is translocated across the membrane as protein synthesis proceeds. After completion of synthesis and translocation, the ribosome dissociates from the mRNA.

The signal hypothesis suggests that secretory proteins have an extra 'signal' sequence which, together with a site on the ribosome, interacts with a receptor on the membrane. This interaction occurs as soon as the signal sequence has emerged from a protective groove in the ribosome. The groove is probably, but not proven to be, located in the large subunit of the ribosome (Blöbel et al, 1979; Unwin, 1977). The attachment of the ribosome to the membrane causes the assembly, or opening, of a pore through which the nascent peptide begins to pass. A signal peptidase then cleaves off the signal sequence, and the protein continues its passage across the membrane. Following completion of translation and transport, the pore is either closed or disassembled. Walter et al (1979) investigated the nature of the receptors by treating microsomes with trypsin under defined conditions. Part of the translocational activity was destroyed by this treatment, but the signal peptidase activity was unaffected. These workers interpreted the results as indicating that the receptors are proteins embedded in the membrane with cytosol-exposed receptor domains, and that the signal peptidase is on the inside surface of the membranes. Jackson et al (1980) have demonstrated that translocation requires a sulphydryl group on the cytosol-exposed domain; this group is on the peptide released from the membrane by the trypsin treatment described by Walter et al (1979). Recently, Prehn et al (1980) have shown that rough endoplasmic membranes possess

Table 2 Some examples of co-translational translocation of secretory or membrane proteins in a manner consistent with the signal hypothesis.

Organism	Membrane	Protein	Reference
E. coli	Plasma	Lipoprotein	1
E. coli	Plasma	β-lactamase	2,3
Bee	RER	Promelittin	4
Chicken	RER	Ovomucoid	5
Mouse	RER	IgG (heavy chain)	6
Rat	RER	Prolactin	7
Rat	RER	Proinsulin	8
Sheep	RER	Casein	9,10
Cattle	RER	Growth hormone	11
Cattle	RER	Proparathyroid hormone	12
Angler fish	RER	Proinsulin	13

In all of these cases part or all of the signal peptide has been sequenced.

References:- (1) Inouye et al (1977); (2) Ambler and Scott (1978); (3) Sutcliffe (1978); (4) Suchanek et al (1978); (5) Thibodeau et al (1978); (6) Jilka and Pestka (1977); (7) McKean and Maurer (1978); (8) Chan et al (1976); (9) Gaye et al (1977); (10) Mercier et al (1978); (11) Lingappa et al (1977); (12) Habener et al (1978); (13) Shields and Blöbel (1977).

Table 3 Amino acid sequences of N-terminal signal sequences of some secreted proteins

Protein:	Residue 25	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	↓	N-terminal of protein	Ref.
Pre-β-lactamase (*E. coli*)			Met	Ser	Ile	Gln	His	Phe	Arg	Val	Ala	Leu	Ile	Pro	Phe	Phe	Ala	Ala	Phe	Cys	Leu	Pro	Val	Phe	Ala		His	(1)
Pre-lysozyme (hen oviduct)								Met	Arg	Ser	Leu	Leu	Ile	Leu	Val	Leu	Cys	Phe	Leu	Pro	Leu	Ala	Ala	Leu	Gly		Lys	(2)
Pre-immuno globulin light chain (mouse)				Met	Asp	Met	Arg	Ala	Pro	Ala	Gln	Ile	Phe	Gly	Phe	Leu	Leu	Leu	Leu	Phe	Pro	Gly	Thr	Arg	Cys		Asp	(3)
Pre-para-thyroid hormone (cattle)	Met	Met	Ser	Ala	Lys	Asp	Met	Val	Lys	Val	Met	Ile	Val	Met	Leu	Ala	Ile	Cys	Phe	Leu	Ala	Arg	Ser	Asp	Gly		Lys	(4)

Residues of the signal peptide are numbered to the left of the cleavage site ↓ . This table is after Davies & Tsai (1980)

References (1) Sutcliffe (1978); (2) Palmiter *et al.* (1977); (3) Burstein and Schechter (1977); (4) Habener *et al.* (1978)

specific protein-containing receptors on the cytoplasmic side.

A large number of secretory proteins from animals and bacteria have now been shown to be translocated and processed in a manner consistent with the signal hypothesis; some examples are given in Table 2. The primary sequence of the signal peptide has been determined for many (Table 3) but no strict sequence homology has been detected. There are certain similarities between signal sequences, chiefly the presence of a stretch of non-polar amino acids towards the middle of the signal flanked, on either side, by polar or hydrophilic residues. Therefore, it is likely that the secondary structure of the peptide chain is of greater importance. However, for particular proteins, the substitution of a single amino acid in the signal sequence can result in the inhibition of either translocation or cleavage. For example, the substitution of Asp for Gly at position 14 of the 20-residue signal sequence of the outer membrane-lipoprotein of E. coli resulted in the translocation of the protein in the absence of cleavage (Lin et al, 1978). This observation suggests that cleavage is not necessary for translocation to occur.

The transport of many proteins across the endoplasmic reticulum is, therefore, consistent with the signal hypothesis but there are some exceptions. For example, chicken ovalbumin is a secretory protein which crosses the endoplasmic reticulum but it is not synthesised as a larger precursor. It has been

shown that ovalbumin competes with other secretory proteins for the receptor sites on the endoplasmic reticulum (Lingappa et al, 1979). The part of the protein molecule which is involved in this interaction with the receptor has been identified and the primary sequence is similar in some respects to other signal peptides. Ovalbumin appears to have an internal signal sequence which effects its transport across the endoplasmic reticulum but is not cleaved.

A different system, which is available for investigating protein translocation, is the translation of injected mRNAs by Xenopus laevis oocytes. The products of translation of injected mRNAs coding for secretory proteins are processed and transported out of the cell. The specificity of protein secretion by oocytes was demonstrated by Colman and Morser (1979); the secretory proteins interferon, IgG and milk proteins accumulated in the incubation medium, having been secreted at different rates. Newly-synthesised secretory proteins were found in oocytes within the lumen of the endoplasmic reticulum (Zehavi-Willner and Lane, 1977). Globin mRNA was translated by the oocytes, but this non-secretory protein accumulated within the cytosol and was not exported.

These experiments with cell-free systems and oocytes show that translocation of proteins across the plasma membrane of bacteria and the rough endoplasmic reticulum of eukaryotic cells appears to take place by a common mechanism. The conclusion

is that there is enough information encoded in the mRNAs to specify translation, compartmentation and secretion. These observations, however, refer to only two types of cellular membrane, and do not take into account the movement of proteins into other compartments of the cell.

A different mechanism of translocation was found for the uptake of the precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase into the chloroplast (Highfield and Ellis, 1978; Chua and Schmidt, 1978). Translation occurs on free ribosomes and the translocation in this case is post-translational, and has been shown to be energy-dependent (Grossman et al, 1980). The complete precursor is taken up through the chloroplast envelope, and the signal sequence is removed by a peptidase present in the chloroplast stroma (Smith and Ellis, 1979). A diagrammatic representation of this mechanism is shown in Fig. 10. This mechanism has also been described for the import of proteins into the mitochondrial matrix and peroxisomes (Maccecchini et al, 1979; Goldman and Blöbel, 1978) and can proceed across a single or double membrane.

The transport of proteins into the nucleus occurs by another mechanism; Mills et al (1980) demonstrated that an acidic protein of Xenopus laevis, which can assemble nucleosomes in vitro, is taken up efficiently into oocyte nuclei after injection into the cell. The mature protein is translocated

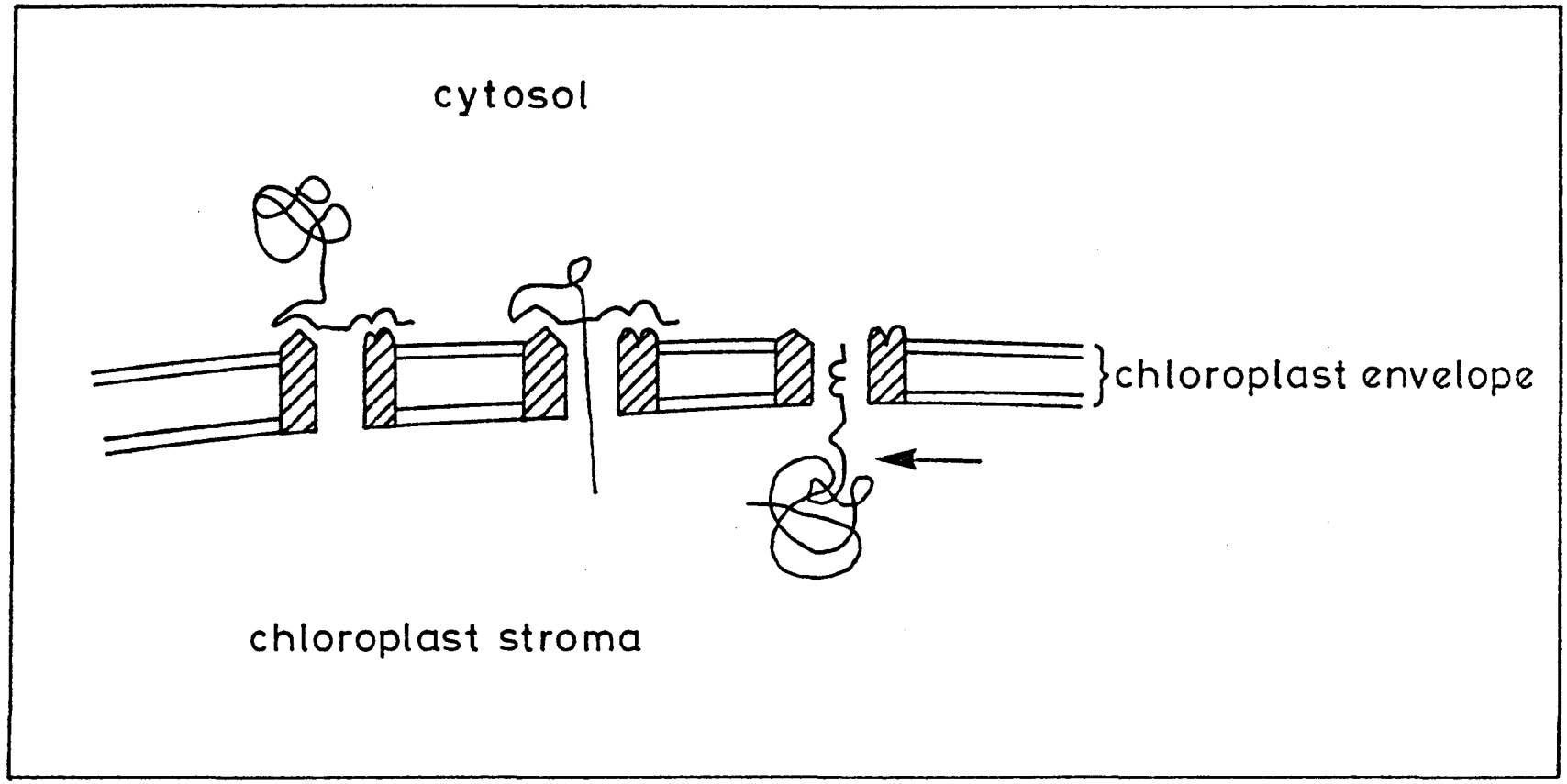

cytosol
chloroplast envelope
chloroplast stroma

Fig. 10 Diagram of the envelope carrier mechanism for the uptake of cytosol-synthesised proteins into the chloroplast (After Blöbel, 1980).

The signal sequence () of the completed pre-protein interacts with the envelope carrier (). This interaction initiates transport across the double membrane of the envelope. After translocation, the signal sequence is cleaved off by a stromal signal peptidase (←), (Smith and Ellis, 1979). The process is energy-dependent (Grossman et al, 1980).

and it is not bound to DNA during the process; a very high nucleoplasmic concentration (5-7mg/ml) of this protein can be achieved.

Thus, both co-translational and post-translational mechanisms, which may or may not involve cleavage of a larger precursor, have been described for the translocation of proteins across membranes. The evidence to date indicates that different membranes have different translocational mechanisms. The insertion of proteins into, rather than through, membranes operates by similar mechanisms but additional refinements have been described which are involved in the correct positioning of the protein (Blöbel, 1980).

1.4.3 Transport of Seed Storage Proteins

Seed storage proteins are synthesised on membrane-bound polysomes (Bollini and Chrispeels, 1979; Fox et al, 1977) and are transported into the lumen of the endoplasmic reticulum prior to deposition in protein bodies. The mechanism of deposition was discussed in section 1.2.5. Transport of the proteins into the endoplasmic reticulum is analogous to that occurring for secretory proteins, but, unlike secretory proteins, storage proteins remain within the cell, either in the endoplasmic reticulum (cereals) or deposited within vacuoles (legumes). The mechanism by which storage proteins cross the membrane has been investigated by a number of workers.

Larkins et al (1979) investigated the processing and transport of pre-zein polypeptides in Xenopus oocytes injected with zein mRNA. Whereas the translation products of mRNA in the wheat germ cell-free system were 2000 daltons larger than native zein, those found within oocytes co-electrophoresed with the native polypeptides. Amino-terminal sequence analysis of the polypeptides synthesised in oocytes confirmed that correct processing of the precursor had taken place. These results are consistent with the signal hypothesis and demonstrate that identical mechanisms can occur in animal and plant systems.

The situation occurring in the transport of barley storage proteins is not clear. Cameron-Mills and Ingversen (1978a) reported that the translation of barley polysomes in vitro (i.e. the completion of the nascent polypeptides) yields products intermediate in size between the native polypeptides and the mRNA translation products. These workers reconstituted rough microsomes from barley polysomes and stripped endoplasmic reticulum; proteins were transported into the vesicles but no change in size occurred. The polypeptides were taken up into vesicles (and hence, protected from protease attack) whether the membranes were added before or after translation. This report appears to be the first describing alternative mechanisms for the transport of a particular protein into the endoplasmic reticulum. The evidence is not totally consistent with the signal hypothesis in its present form.

Higgins and Spencer (1980) have investigated the processing of precursors to pea storage proteins by pea microsomal membranes and dog pancreatic membranes. Some of the products of mRNA translation *in vitro* were co-translationally modified in the presence of membranes to give bands co-migrating with authentic storage proteins. Roberts *et al* (1980) have found that castor bean storage proteins are processed and translocated by stripped microsomes derived from dog pancreas. These two cases have some features in common with the signal hypothesis.

The mechanism by which storage proteins pass into the lumen of the endoplasmic reticulum is not completely clear. The data for transport of zein (Larkins *et al*, 1979) are consistent with the signal hypothesis. Work on the transport of hordein has not given clear results and this topic requires much more study. The evidence concerning the transport of legume storage proteins is incomplete and, in parts, contradictory; it is complicated by the glycosylation of these proteins. Considerably more work is needed in order to satisfactorily describe the transport of seed storage proteins.

1.5 AIMS AND APPROACHES

The aims of this project were:-

(1) to isolate, from barley endosperm, polysome and mRNA fractions enriched in sequences coding for hordeins by using endosperm which is in the maximum phase of hordein accumulation,

and by isolating membrane-bound polysomes which have previously been shown to synthesise the bulk, if not all, of the hordein (Fox *et al*, 1977). Poly-A^+ RNA will then be separated from polysomal RNA by oligo-dT cellulose chromatography;

(2) to characterise both polysomes and mRNA in terms of their physical characteristics by means of sucrose density gradient centrifugation, polyacrylamide gel electrophoresis and polyadenylic acid content;

(3) to identify and characterise the products of *in vitro*-protein synthesis directed by polysomes and mRNA using a range of different criteria;

(4) to investigate the mechanism by which the primary translation products are processed to the native size and transported into the lumen of the endoplasmic reticulum using both homologous (barley endoplasmic reticulum) and heterologous (*Xenopus laevis* oocytes) processing systems.

The wheat germ cell-free protein synthesising system will be used to investigate the translation of polysomes and mRNA. The products of *in vitro*-protein synthesis will be identified by as many criteria as possible. The size of the products of polysome translation must be investigated particularly carefully in the light of the contradictions present in the literature.

The long term aim of isolating mRNA coding for the hordeins is to prepare cDNA clones and, eventually, to use these

to pick out the genomic DNA sequences. The availability of such clones would enable fine structure mapping and sequence analysis of the two hordein loci. This work has been started in collaboration with other members of my department and is reported in Appendix 1.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Tissues

Ears of field grown barley (Hordeum vulgare L. var Sundance) were harvested two to three weeks after anthesis, and the endosperm squeezed out either by rollers or by hand for freezing in liquid nitrogen (Fig. 11). The endosperm was stored in 50g batches at -80°C for up to 12 months. To provide a fresh supply throughout the winter, plants were grown (six per 9 inch pot) in either a growth room or glasshouse. The growth room had a 16°C/16 hour day and a 12°C/8 hour night and was illuminated by fluorescent lights (26000 lux) and tungsten lights (2000 lux). The relative humidity did not exceed 70%. The glasshouse was maintained at a minimum temperature of 15°C. From July to April, daylight was supplemented with SON/T lamps (16000 lux) to maintain an 18 hour photoperiod. Other varieties of barley (Athos, Carlsberg II) and mutant lines (mutant 1508 from Bomi, mutant 56 from Carlsberg II) were grown in growth rooms.

Tobacco mosaic virus (TMV) was kindly donated by R. White, Plant Pathology Department, Rothamsted. Cowpea chlorotic mottle virus (CCMV) was obtained from cowpeas (Vigna unguiculata L. walp. var Prima) which were inoculated, after expansion of the seed leaves by rubbing with an homogenate of

Fig. 11 Barley ears (var. Athos) showing endosperm squeezed from the developing grains by hand. The endosperms were routinely squeezed directly into liquid nitrogen.

infected leaves. The plants were grown for about three weeks after inoculation in a growth room (20°C/12 hour day, 16°C/12 hour night, light intensity of 23000 lux). The original inoculum was obtained from Dr. R. Trim, John Innes Institute. Young leaves of barley and pea (Pisum sativum L. var Feltham first) were obtained from plants grown in trays of vermiculite in a growth room (conditions as for cowpeas) for about 10 days after germination.

Wheat germ (from Manitoba wheat, Triticum aestivum L.) was a generous gift from Mr. David Marriage, Marriages Mills, Chelmsford, Essex. It was stored desiccated at 4°C. Tobacco (Nicotiana tabacum L. var Xanthi-nc) callus was donated by J. Kueh, Biochemistry Department, Rothamsted. The callus was grown according to the method of Murashige and Skoog (1962) and harvested whilst actively growing. The material was stored at -20°C.

2.1.2 Chemicals and Equipment

Chemicals and equipment were obtained from the following sources:-

Sigma (London) Ltd.: dithiothreitol (DTT), adenosine triphosphate (ATP), creatine phosphate, creatine phophokinase, 2' and 3' adenosine monophosphate (2' + 3' AMP), sucrose (RNAase free), proteinase K, amino acids, guanosine triphosphate (GTP), 7-methyl guanosine monophosphate (7-Me GMP), ribonuclease A, polyadenylic acid, Hepes 2-(N-2-hydroxyethylpiperazine-N-yl) ethanesulphonic acid , Tris(2-amino-2-hydroxymethylpropane-1,3-diol), Tricine (N-Tris hydroxymethyl methyl glycine), myoinostiol, thiamine, urea, glyoxal, ethylenediaminetetra-acetic acid (EDTA,

disodium or ferric salt), gentamycin, Coomassie brilliant blue R, bromophenol blue, toluidine blue, 2-mercaptoethanol, diethyl-pyrocarbonate, ethidium bromide, Staphylococcus aureus cells.

Collaborative Research Ltd.: oligo-dT cellulose.

British Drug Houses: sodium dodecyl sulphate (SDS), Triton X100, glycine, acrylamide, methylene bisacrylamide, NNN'N'-tetramethylene-ethylene diamine (TEMED), Amberlite MB3 resin.

Boehringer Corporation Ltd.: polynucleotide kinase, calf intestinal alkaline phosphatase.

Pharmacia: Sephdex G25 and G75.

LKB: Ampholine pH ranges 7-9, 5-7, 3.5-10.

Whatman: filter and chromatography papers, DEAE-cellulose (DE52), phosphocellulose.

Radiochemical Centre:

L- 4,5-[^{3}H] leucine (130-190 Ci/mmol),

L- 4,5-[^{3}H]lysine monohydrochloride (75-100 Ci/mmol),

L- 2,3,4,5-[^{3}H]proline (100-130 Ci/mmol),

L-[^{35}S]methionine (600 Ci/mmol),

poly 5-[^{3}H]uridylic acid, sodium salt (400-600 mCi/mmol, length 40-140 nucleosides),

adenosine 5- γ -[^{32}P] triphosphate (2000 Ci/mmol).

Fujimex Ltd.: X-ray film (medical grade).

Cuthbert Andrews Ltd.: Protex X-ray cassettes, FT intensifying screens.

All chemicals were of Analar quality or equivalent. For the preparation of wheat germ extracts and solutions for cell-free protein synthesis, double glass-distilled or BDH 'Analar' water was used. Single glass-distilled water was used routinely.

2.2 METHODS

2.2.1 Isolation of Proteins

2.2.1.1 Unlabelled hordein

Hordein was extracted from milled dry seed, essentially according to the method of Shewry *et al* (1977a, 1978a). Meal (1g) was stirred in turn with 10ml of each of the following solvents at 20°C in screw-capped centrifuge tubes:-

1. water-saturated n-butanol; 2 x 30min (to defat)
2. 0.5M NaCl; 3 x 45min (to remove albumins and globulins)
3. 55% (v/v) propan-2-ol, 2% (v/v) 2-mercaptoethanol; 3 x 60min (to extract hordein).

The residue was removed each time by centrifugation at 12000g for 20min. The propan-2-ol extracts were combined, and dialysed for 24 hours against three changes of distilled water at 4°C. Hordein was then collected by centrifugation, and dried under vacuum.

To prepare alkylated hordein two volumes of 0.75M NaCl were added to the propan-2-ol extracts, and the hordein allowed

to precipitate overnight in the cold. The pellets were collected by centrifugation and resuspended in alkylation buffer (8M urea, 1% (v/v) 2-mercaptoethanol, 0.13M Tris-NO_3 pH 7.5, 0.075% (w/v) KCl, 1mM EDTA) and shaken at 20°C overnight. 4-vinyl pyridine was added to a final concentration of 1.5% (v/v), and the reaction allowed to proceed for 2 hours at 20°C with shaking. After alkylation hordein was dialysed and collected as above.

2.2.1.2 Radiolabelled hordein

In vivo labelled hordein was extracted from [^{35}S] labelled grain as described above. Labelling was achieved by feeding [^{35}S] sulphate to barley ears grown in liquid culture using an adaptation of the method of Donovan and Lee (1977). Sulphate-free culture medium was prepared by mixing a number of stock solutions as detailed below.

Solution A:	$CaCl_2$ 80mM
Solution B:	KH_2PO_4 175mM, magnesium acetate 35mM
Iron:	Ferric EDTA 100mM
Solution C:	Thiamine 0.2mM, inositol 110mM
Minor Elements:	As Linsmaier (1965).

These solutions were mixed as follows:-

50ml A, 50ml B, 10ml iron, 10ml minor elements, 25ml [illegible] and 20g sucrose were diluted to 500ml with water and adjusted to pH 5.0 with acetic acid. This medium was autoclaved (30ml in 100ml glass centrifuge tubes sealed with cotton wool plugs) at 15psi for 15min and cooled; an equal volume of a solution

containing 0.1M glutamine, 50µg/ml gentamycin and 10µCi/ml [$^{35}SO_4^{2-}$] was added via a sterile membrane filter. Heads of barley were harvested 7-10 days after anthesis by cutting the stems near the base and standing them in water. The flag leaf and stem were swabbed with ethanol, the stem cut underwater 4cm below the first node, and the flag leaf carefully removed. The stem was again cut underwater just above the first node, and the stem sterilized by standing in 7% (w/v) calcium hypochlorite for 30sec. and sterile distilled water for 30sec. A further 2cm was cut off the stem under sterile water, and the stem was placed in the culture tube at the side of the cotton wool plug (Fig. 12).

The heads in culture were incubated in an illuminated growth cabinet (16h/20°C day, 8h/16°C night, 23000 lux) with the tubes standing in ice to reduce microbial growth; the ice was renewed daily. To test the growth of the grains, one endosperm was collected from the middle of each ear at intervals and weighed. The cultures were maintained until the grain reached maturity, the culture medium being topped up with sterile-filtered medium as necessary. The ears grew well under these conditions, gaining an average of 10mg fresh weight over the first three days. The final dry-grain weight was around 40mg which was slightly less than the normal (approximately 50mg).

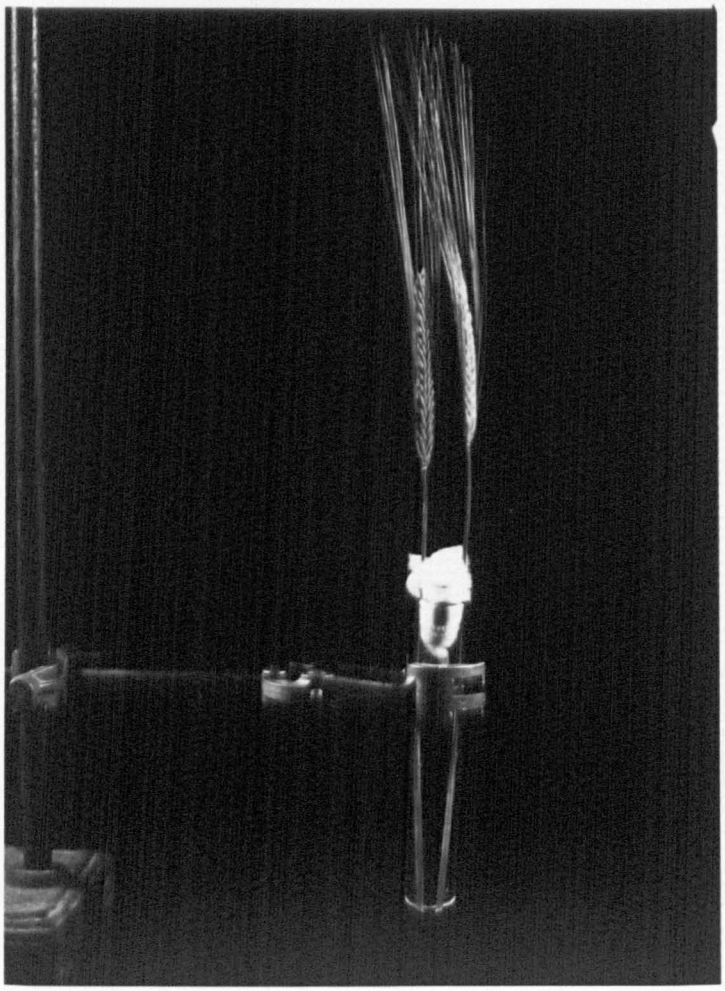


Fig. 12 Barley ears growing in culture. The method used is described in detail in the text (section 2.2.1.2). The tubes normally stood in ice to reduce the growth of any microbial contaminants.

2.2.2 Separation of Proteins by Gel Electrophoresis

2.2.2.1 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The method was modified from Laemmli (1970). The separating gel (16 x 13 x 0.15cm) contained 17.5% (w/v) acrylamide, 0.26% (w/v) bisacrylamide, 0.1% (w/v) sodium dodecylsulphate (SDS), 1mM DTT, 0.375M Tris-HCl pH 8.8, 4M urea and was polymerised by the addition of 0.05% (v/v) NNN N tetramethyleme-ethylene diamine (TEMED) and 0.001% (w/v) ammonium persulphate. There was 1cm of stacking gel (3% (w/v) polyacrylamide) between the separating gel and the bottom of the sample wells; this stacking gel contained 125mM Tris-HCl pH 6.8, 0.1% SDS, 1mM DTT, 4M urea and was polymerised in the same way as the separating gel.

Samples were dissolved in 8M urea, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 65mM Tris-HCl pH 6.8 and heated for 30sec at 60°C; a drop of 0.01% bromophenol blue was then added. The electrode buffer was 25mM Tris pH 8.3, 192mM glycine, 0.1% SDS and electrophoresis took place at 25mA for 16 hours. The gel was fixed for 30min in 10% (w/v) trichloroacetic acid (TCA), 40% (v/v) methanol. Staining was for two to five hours in 0.25% Coomassie brilliant blue R in 45.4% (v/v) methanol, 4.6% (v/v) acetic acid followed by destaining in 25% (v/v) ethanol, 10% (v/v) acetic acid.

2.2.2.2 Iso-electric focusing (IEF).

IEF was carried out using an LKB Multiphor flat bed apparatus as described by Shewry *et al* (1978b). The gel

contained 6M urea, 5% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 1% (w/v) pH 5-7 ampholyte, 1% (w/v) pH 7-9 ampholyte and was polymerised, after degassing, by the addition of ammonium persulphate and TEMED to final concentrations of 0.04% (v/v) and 0.07% (v/v) respectively. The slab was pre-focused at 10W for 1h with 25µl 1% (w/v) pH 3.5-10 ampholyte in each well. The wicks were soaked in 1M phosphoric acid (sample well side) and 1M NaOH (other side). Protein samples were dissolved in alkylation buffer (see section 2.2.1.1) at 10mg/ml and 20µl loaded into the wells. Focusing took place for 3h at 13W and the slab was cooled by the circulation of iced water. The gel was fixed overnight in 10% (w/v) TCA, 5% (w/v) sulphosalicylic acid, washed with three changes of 5% (w/v) TCA over a period of 48h and stained in 0.002% Coomassie BBR in 15% (w/v) TCA.

2.2.2.3 Two-dimensional electrophoresis

Tracks were excised from the unfixed focusing gel with a razor blade, and soaked for 10min in 0.125 Tris-borate pH 8.9, 1% (w/v) SDS. The gel in the buffer was heated to 80°C for 3min, and then the strip was set on top of an 8cm wide SDS gel using the stacking gel (Shewry 1978b). The separating gel was 14cm long and contained 12.5% (w/v) acrylamide, 0.13% (w/v) bisacrylamide. Electrophoresis was carried out at 20mA per slab for 6h. The gels were fixed in 30% (v/v) ethanol, 7% (v/v) acetic acid and stained as in section 2.2.2.1.

2.2.3 Isolation and Analysis of Polysomes

Sterile glassware and media were used in all procedures for the isolation and characterisation of polysomes. Solutions, muslin and plastic materials were autoclaved (15psi, 15min), glassware was heat treated at 180° for 6 hours and unstable solutions were sterile-filtered. Unless otherwise stated the following procedures were carried out at 2°C.

2.2.3.1 Crude separation of free and membrane-bound polysomes.

Frozen endosperm was ground to a powder in a chilled Kenwood blender and then blended for approximately 30sec with 2 volumes polysome isolation buffer (0.2M Tris-acetate pH 9.0, 100mM KCl, 50mM magnesium acetate plus 2mM 2' + 3' AMP, 5mM DTT, 0.2M sucrose) (Larkins et al,1976; Gray,1974). Starch and other debris was removed by centrifugation at 600g for 10min. The supernatant solution was strained through two layers of muslin and then centrifuged at 32000g for 20min to pellet the membrane-bound polysomes whilst leaving free polysomes in the supernatant solution (supernatant 1). The pellets were resuspended in polysome isolation buffer containing 1% (v/v) Triton X100 and then centrifuged at 48000g for 20min; the initially membrane-bound polysomes appear in this supernatant solution (supernatant 2). Both supernatants 1 and 2 were layered on to 54% (w/w) sucrose cushions in 0.2M Tris-acetate, pH 9.0, 100mM KCl, 50mM magnesium acetate and the polysomes pelleted at 200,000gav for 4h in a Beckman Ti70 rotor. The surface of the pellets was rinsed

three times with ice-cold water, then the pellets were stirred carefully into 10mM Hepes-KOH, pH 7.6, 100mM KCl, 4mM magnesium acetate, 1mM DTT and stored at -80°C. For comparative purposes, some polysome preparations and subsequent analyses were carried out in the presence of a lower magnesium concentration (10mM).

2.2.3.2 Separation of free and membrane-bound polysomes by sucrose density gradient centrifugation.

The method used was essentially that of Miflin et al (1980a). Freshly squeezed endosperm (6g) was added to 12ml 50mM Tricine pH 7.6, 100mM KCl, 10mM Mg acetate containing 0.2M sucrose and chopped with a razor blade. The homogenate was squeezed through four layers of muslin and layered on to 2 sucrose gradients prepared in the same buffer; a 10-50% (w/w) linear gradient was poured on to a 5ml cushion of 50% (w/w) sucrose giving a total volume of 31ml. The gradients were centrifuged at 25000rpm for 2.5h in a Beckman SW27 rotor and fractionated using an ISCO gradient fractionator (model 640) at 2ml/min, collecting 1.2ml fractions with monitoring at 254nm or 280nm. Peaks were identified by the position of marker enzymes as described by Miflin et al (1980a). Sucrose content was determined by refractometry. The peak of membrane-bound polysomes was diluted with an equal volume of polysome isolation buffer plus 2% Triton X100, and both this and the free polysomes were pelleted through 54% sucrose cushions at 200,000gav for 4 hours. The pellets were washed and resuspended as described in 2.2.3.1.

2.2.3.3 Analysis of polysome size.

Polysomes were fractionated on 20-50% linear sucrose gradients in 0.2m Tris-acetate pH 9.0, 100mM KCl, 50mM magnesium acetate containing 50µg/ml heparin. The gradients were poured on to a 5ml cushion of 50% (w/w) sucrose giving a total volume of 37ml. Polysomes (6-15 A_{260} units) were diluted to 1ml with the same buffer and layered on to the gradients. Some samples were digested with 20µg RNAase A for 15min at 28°C. The gradients were centrifuged at 25000rpm for 3.3hours in a Beckman SW27 rotor and subsequently fractionated at 3ml/min, using an ISCO gradient fractionator (model 640), collecting 1.2ml fractions with monitoring at 254nm. The sucrose concentration was determined by refractometry.

2.2.4 Isolation of RNA

Glassware and solutions were sterilized as described in 2.2.3. Oligo-dT cellulose was washed with 1% (v/v) diethyl-pyrocarbonate and then extensively washed with sterile distilled water. Unless otherwise stated all procedures were carried out at 2°C.

2.2.4.1 Phenol method for preparing total polysomal poly-A^+ RNA.

Frozen endosperm was homogenized as described for the preparation of polysomes (section 2.2.3.1). Ater removal of debris at 600g for 10min, the membrane-bound polysomes were pelleted at 32000g for 20min. The pellet was resuspended in polysome isolation buffer containing 1% (v/v) Triton X100 and

then centrifuged at 48000g for 20min. The supernatant solution was made 1% (w/v) with respect to SDS before being deproteinized three times with an equal volume of 50% (v/v) phenol (equilibrated with 0.2M Tris-acetate pH 9.0, 10mM EDTA), 49% (v/v) chloroform, 1% (v/v) iso-amyl alcohol. The phases were separated each time by centrifuging at 5000g for 7min.

RNA was precipitated by the addition of 2.5 volumes of absolute ethanol at -20°C overnight; any DNA strands which formed were spooled out with a glass rod. The RNA was collected by centrifugation at 8000g for 30min, washed with 75% ethanol (at 0°C) and then reprecipitated from 0.2M ammonium acetate with 2.5 volumes of ethanol at -20°C overnight. The pellet was collected, washed with ethanol and then dried under vacuum.

Oligo-dT cellulose chromatography was carried out at room temperature using the method of Bantle et al (1976). Total RNA was dissolved in binding buffer (10mM Tris-HCl pH 8.5, 1mM EDTA, 0.4M NaCL, 0.1% SDS) to a concentration of approximately 0.5mg/ml. All RNA concentrations were estimated assuming that 1μg RNA in 1ml has A_{260} = 0.025. Oligo-dT cellulose (1g) was prepared by washing with 0.1M NaOH, then with binding buffer until the pH returned to 8.5. The washed cellulose was gently stirred with the RNA for 20min, poured into a column (1cm diameter) and washed with binding buffer. The fraction not retained by the column was collected as a source of ribosomal RNA (rRNA). The column was washed with a low salt buffer (binding buffer with

the concentration of NaCl reduced to 0.1M) and a fraction collected. Polyadenylated (poly-A^+) RNA was eluted with 10mM Tris-HCl pH 8.5, 1mM EDTA, 0.1% SDS. All fractions were precipitated twice from ethanol in siliconized Corex tubes as described above; poly-A^+ RNA was first made 0.4M with respect to ammonium acetate. After drying, RNA was dissolved in sterile distilled water at a concentration of 1mg/ml and stored at -80°C.

Some samples of poly-A^+ RNA and 'wash' RNA (eluted with low salt buffer) were rechromatographed. RNA (in 100µl) was added to 900µl dimethyl sulphoxide (DMSO) and 100µl 1M LiCl, 50mM EDTA, 2% (w/v) SDS, 10mM Tris-HCl pH 6.5 and heated to 60° for 5min. Binding buffer was then added to reduce the DMSO concentration to 5% (v/v). Oligo-dT cellulose chromatography and ethanol precipitation were carried out as described above.

2.2.4.2 Preparation of poly-A^+ RNA from polysomes using phenol and proteinase K.

Polysomes were prepared as described in 2.2.3.1 and resuspended in 10mM Tris-HCl pH 9, 20mM NaCl, 1mM $MgCl_2$ at approximately 3 A_{260} units/ml. The polysomes were either deproteinized directly or first treated with proteinase K to remove endogenous nucleases. The solution was made to 0.1% SDS and 100µg/ml proteinase K (EC. 3.4.21.14) and incubated at 0°C for 10min; the control was treated in the same way except that proteinase K was omitted. After incubation each solution was made to 1% SDS, 100mM Tris-HCl pH 9.0 and deproteinized with

phenol/chloroform/isoamyl alcohol as described in section 2.2.4.1. Oligo-dT cellulose chromatography was carried out as described in 2.2.4.1.

2.2.4.3 Preparation of poly-A^+ RNA from polysomes using SDS/NaCl.

This method was adapted from Krystosek et al (1975). Polysomes were prepared as described in 2.2.3.1, resuspended in 10mM Hepes-NaOH pH 7.6, 10mM EDTA, 0.5M NaCl, 1% (w/v) SDS and heated to 60°C for 5min. The solution was diluted with two volumes of binding buffer and stirred gently with oligo-dT cellulose. Chromatography was carried out and the RNA fractions precipitated as described in 2.2.4.1. The fraction not retained on the column still contains ribosomal protein and cannot be precipitated directly as a source of rRNA (see following section).

Pea leaf and barley leaf poly-A^+ RNA were prepared using this method.

2.2.4.4 Preparation of ribosomal RNA.

Barley and pea rRNA were prepared from the fraction not retained by oligo-dT cellulose during fractionation of polysomes dissociated with SDS and NaCl. The fraction was made 1% (w/v) with respect to SDS, deproteinized three times, and ethanol-precipitated as described in 2.2.4.1.

2.2.4.5 Preparation of transfer RNA from wheat germ.

Wheat germ tRNA was prepared according to the method of Marcus et al (1974). Wheat germ (5g) was added to 25ml 0.1M Tris-acetate pH 8.0, 10mM magnesium acetate, 5mM EDTA, 50mM NaCl, 10mM

2-mercaptoethanol in the goblet of a Kenwood blender. After settling, the germ was homogenized twice for 30sec each and then centrifuged at 23000g for 10min. The supernatant solution wasdecanted through two layers of muslin into one volume of buffer-saturated phenol and stirred on ice for 15min. The phases were separated by centrifuging at 23000g for 10min. The aqueous phase was re-extracted twice with 0.5 volumes of buffer-saturated phenol for 5min and the final aqueous phase, having been made to 50mM KCl, was precipitated with 2.5 volumes of ethanol at -20°C overnight. The RNA was pelleted at 23000g for 10min, dried under vacuum and resuspended in 0.3M sodium acetate pH 7.0. Propan-2-ol (0.54 volumes) was added dropwise in the cold with vigorous stirring and the temperature was slowly raised to 20°C. The suspension was allowed to stand at room temperature for 5min and centrifuged at 5000g for 6min. The supernatant was collected and a further 0.44 volumes (relative to the original) of propan-2-ol added and stirred for 60min at 2°C. The precipitate of tRNA was collected at 23000g for 10min and dried under vacuum. It was dissolved in sterile distilled water, and dialysed for 5h against water at 2°C before storage in 100µl aliquots at -80°C.

2.2.4.6 Preparation of viral RNA.

Cowpea chlorotic mottle virus (CCMV) was extracted from infected leaves by the method of Bancroft et al (1967). Leaves were homogenized with two volumes 0.2M sodium acetate pH 5.0 for

sixty seconds and the homogenate was squeezed through two layers of muslin. The virus was purified by repeated differential centrifugation (x 3); debris was removed at 12000g for 30min and the virus was then pelleted by centrifugation at 80000g for 3 hours. After pelleting, the virus was resuspended each time in 0.1M sodium acetate pH 5 overnight.

RNA was extracted from CCMV and TMV by the method of Smith *et al* (1977). Virus pellets were resuspended in 20mM NaCl, 10mM Tris-HCl pH 8.0, 2mM $CaCl_2$, 0.5% SDS and then made to 0.5mg/ml proteinase K. The mixtures were incubated at 38° for 30min after which the NaCl concentration was raised to 100mM and 10mM EDTA was added. The RNA was deproteinized three times with an equal volume of buffer-saturated phenol and precipitated twice from ethanol. Viral RNA was dissolved in sterile distilled water and stored at -80°C.

2.2.5 Analysis of RNA

2.2.5.1 Sucrose gradient centrifugation

RNA was analysed on 10-30% (w/w) linear sucrose gradients containing 100mM KCl, 10mM Hepes-KOH pH 7.6, 1mM EDTA, 50µg/ml heparin. Approximately 100µg RNA was dissolved in 200µl of the same buffer, heated to 80°C for 3min and cooled rapidly in ice water before layering on to the gradients (total volume of 5ml). The gradients were centrifuged at 150,000gav for 18h in an SW50.1 Beckman rotor. The gradients were fractionated using an ISCO gradient fractionator (Model 640) at 0.2ml/min, collecting 0.2ml fractions with monitoring at 254nm. Sucrose concentrations were measured by refractometry.

2.2.5.2 Gel electrophoresis

RNA was analysed by gel electrophoresis after glyoxalation using a method modified from McMaster and Carmichael (1977). The slab gel (16 x 16 x 0.15cm) contained 4% (w/v) acrylamide, 0.11% bisacrylamide, 7.5m urea, 0.75% SDS, 36mM Tris-acetate pH 7.6, 30mM sodium acetate, 1mM EDTA. The gel was pre-electrophoresed at 25mA for 1h with 36mM Tris-acetate pH 7.6, 30mM sodium acetate, 1mM EDTA as the electrode buffer. RNA samples were prepared in 50% (v/v) DMSO, 1.0M glyoxal, 10mM sodium phosphate pH 7.0; this solution had been de-ionized three times by shaking with Amberlite MB3 resin for 30min each. The glyoxalation reaction took place at 50°C for 1h, and a drop of 0.02% bromophenol blue was added to each sample. Electro-

phoresis was carried out at 25mA for approximately 16h. The gel was either stained in 100mg/l toluidine blue for 4h and destained in water, or was stained with 1µg/ml ethidium bromide in 0.5M ammonium acetate and viewed under ultra-violet light.

For all RNA gels, acrylamide and bisacrylamide were recrystallised from chloroform (70g/l) and acetone (10g/l) respectively.

2.2.5.3 Determination of poly-A content

Poly-A content was determined by hybridisation to 100nCi [^{3}H] polyuridylic acid in 0.5M NaCl, 10mM Tris-HCl pH 7.0, 1mM magnesium acetate at 28°C for 15min in a volume of 100µl (Brandt et al, 1978). The reaction mixture was diluted to 1ml and single-stranded RNA was digested with 5µg RNAase A (EC.3.1.27.5) for 15min at 28°C. Bovine serum albumin (50µg) was added and the hybrid RNA molecules precipitated by the addition of 2ml 15% (w/v) TCA. After 1 hour at 0°C, the hybrids were collected on Whatman GF/A filters, which were dried with ethanol and diethyl ether, and counted in 5ml toluene-based scintillant (4g PPO (2,5-diphenyloxazole), 0.8g POPOP (1,4-Di-2-(5-phenyloxazoyl-benzene)) in 1l toluene) in a Beckman LS-250 counter. Poly-adenylic acid was used to calibrate the technique.

2.2.6 Wheat Germ Cell-free Protein-synthesising System

2.2.6.1 Preparation of S30 extract

A wheat germ S30 cell-free extract was prepared according to the method of Roberts and Paterson (1973) but

omitting the pre-incubation step. All procedures were carried out using sterile glassware and solutions. Manitoba wheat germ was sieved using test sieves (Endecotts model 410) and 8g taken from the top of the 1.18mm mesh. This was ground in a chilled pestle and mortar with crushed glass and two volumes of grinding buffer (20mM Hepes-KOH pH 7.6, 100mM KCl, 1mM magnesium acetate, 2mM $CaCl_2$, 6mM 2-mercaptoethanol). The homogenate was centrifuged in two 15ml Corex tubes at 30000g for 20min, and the supernatant solution carefully recovered with a pasteur pipette, with care taken not to disturb the soft pellet or floating lipid layer. The supernatant was layered on to a column (1 x 20cm) of Sephadex G25 (coarse), equilibrated and developed with 10mM Hepes-KOH pH 7.6, 100mM KCl, 4mM magnesium acetate, 1mM DTT. As soon as the eluate appeared cloudy a volume equal to that loaded was collected. This eluate was cleared by centrifugation at 30000g for 20min, and frozen into small spheres (approximately 10µl) by dripping into liquid nitrogen through a 21 gauge syringe needle. The spheres were stored at -80°C.

2.2.6.2 Translation and analysis of incorporated radioactivity

The optimum conditions for translation were determined (see section 3.3.1.1). The assay was carried out in reaction mixtures of 40µl volume containing:- 5µl S30 extract, 20mM Hepes-KOH pH 7.6, 1.9-2.5mM magnesium acetate (see 3.3.1.1), 110mM potassium acetate, 300uM spermidine, 1mM ATP, 8mM creatine phosphate, 50µg/ml creatine phosphokinase, 50µM GTP, 2mM DTT,

50µM each unlabelled amino acids, 1-10µCi labelled amino acids ([^{3}H] proline and [^{3}H] leucine, 105-130 Ci/mMol ; [^{3}H] lysine, 107Ci/mMol ; [^{35}S] methionine, 800-1200 Ci/mMol), and either 0.5-10µg RNA or 5-30µg polysomes. Poly-A RNA for translation was usually purified through only one oligo-dT cellulose column. After incubation at 28°C for 40min, 2µl aliquots were removed and spotted on to 1 x 2cm strips of Whatman No. 1 filter paper for analysis of TCA precipitatable radioactivity. The strips were boiled in 10% TCA containing 0.5% cold proline, leucine or methionine and allowed to stand for 20min, after which they were washed in cold 10% TCA, two changes of ethanol and finally in diethylether. The dry strips were placedin 5ml toluene-based scintillant (4g PPO, 0.8g POPOP in 1l toluene) in plastic vials for counting. The samples were counted in a Beckman LS-250 scintillation counter using the ^{3}H (full-range) channel for tritium and the combined ^{3}H/^{14}C channel for ^{35}S.

2.2.6.3 Preparation of in vitro-synthesised products for gel analysis

After incubation incorporation of radioactivity was stopped by the addition of 7µl 1% cold amino acid. The reaction mixture was then made to 55% (v/v) propan-2-ol, 0.05% DTT, and heated to 60°C for 10min to extract hordein. Authentic hordein (20µg) was added as carrier, and the insoluble material pelleted in a microfuge for 5min. The pellets were re-extracted twice with 55% propan-2-ol, 0.05% DTT in 50mM Tris-HCl pH 7.6.

The combined supernatant solutions were sonicated using a Kerry PUL.55 sonic bath for 20min and the proteins alkylated for 2h at room temperature with 1.5% (v/v) 4-vinyl pyridine. The proteins were precipitated with excess 2% (w/v) LiCl, 0.5% (v/v) 2-mercaptoethanol at 2°C for 2h (minimum). The pellets were collected by centrifugation, washed with water and dissolved in the appropriate loading buffer for gel electrophoresis. Aliquots were prepared for scintillation counting as described in 2.2.6.2.

2.2.6.4 Immunological analysis

The antisera were prepared and tested by Dr. Festenstein (unpublished).

After incubation and sampling of the translation reaction mixture, urea and Triton X100 was added to a final concentration of 2M and 1% (v/v) respectively; the mixture was sonicated in a Kerry PUL.55 sonic bath for 15min and centrifuged at 10000g for 2min. Anti-serum (10µl) was added to the supernatant solution and incubated at room temperature for 2h after which 10µl of a suspension of *Staphylococcus aureus* cells was added. The suspension was incubated at room temperature for 15min. The cells were pelleted for 2min in the microfuge, and washed three times with 150mM NaCl, 5mM EDTA, 50mM Tris-HCl pH 7.4, 0.1% Triton X100, 0.1% methionine. The antibody-protein complex was then dissociated and released from the cells by incubation with SDS gel loading buffer at

60°C for 10min, and analysed by SDS-PAGE as described in section 2.2.2.1. Aliquots were prepared for liquid scintillation counting as described in 2.2.6.2.

2.2.6.5 Mapping of products with cyanogen bromide

Protein samples were dissolved in 70% formic acid containing 10mg/ml cyanogen bromide (CNBr) and digested for 24h at room temperature in the dark. The samples were then dried under vacuum and taken up in SDS loading buffer for electrophoresis on linear 5-30% (w/v) polyacrylamide gels. The electrophoresis system was described in section 2.2.2.1.

2.2.7 Processing of Precursors

2.2.7.1 Preparation of stripped microsomes from barley endosperm

Freshly squeezed endosperms (9.5g) were chopped in 19ml isolation buffer (50mM Tricine pH 7.6, 100mM K acetate, 2.5mM Mg acetate) containing 0.2M sucrose and squeezed through four layers of muslin. Sucrose gradients were prepared in the same buffer by pouring a 24ml linear 20-60% (w/w) gradient on to a 5ml 60% (w/w) cushion and overlayering with 2ml 16% (w/w) sucrose. The homogenate was layered on to four gradients and centrifuged at 25000rpm for 2.5h in a Beckman SW27 rotor. The endoplasmic reticulum peak, as determined by NADH cytochrome c reductase (Miflin, 1980) was collected and diluted with an equal volume of 10mM EDTA, sonicated in a Kerry PUL.55 sonic bath for 30sec and stirred for 30min at 2°. This was layered on to a 25-50% (w/w) linear gradient preparedin a magnesium-free

buffer (50mM Tricine pH 7.6, 100mM K acetate, 2.5mM EDTA) and centrifuged at 25000rpm for 2.5h. The gradients were fractionated using an ISCO gradient fractionator (model 640) at 2ml/min, collecting 1.2ml fractions with monitoring at 280nm. The peak coinciding with the position of NADH cytochrome c reductase was diluted to 10% sucrose with the magnesium-free buffer and the membranes pelleted at 150000g for 2h through a 15% sucrose cushion prepared in 20mM Hepes-KOH pH 7.6, 100mM potassium acetate. The pellet was resuspended in the same buffer at a concentration of 40 A_{260} units/ml and stored at -80°C.

A quicker method was also used; endosperms were chopped in the magnesium-free buffer containing 0.2M sucrose and fractionated on a 20-60% (w/w) linear sucrose gradient in the same buffer. The stripped endoplasmic reticulum peak was collected and diluted to 10% (w/w) sucrose with the same magnesium-free buffer. The membranes were pelleted through a 15% (w/w) sucrose cushion and resuspended in 20mM Hepes-KOH pH 7.6, 100mM potassium acetate at 40 A_{260} units/ml.

2.2.7.2 Assay of microsomes for processing activity

Cell-free protein synthesis was carried out as described in section 2.2.6.2, and microsomes were added at a concentration of 1-10 A_{260} units/ml. After incubation and sampling for analysis of TCA-precipitable counts, the products were treated with proteinase K to test for protease-resistant products. The

reaction mixtures were cooled to 0°C and the volume made up to 65µl by the addition of buffer (20mM Hepes-KOH, pH 7.6, 100mM potassium acetate), 5µg lauric acid, 50µg proteinase K and in some cases Triton X100 to 1% (v/v) final concentration. The digestion proceeded at 0°C for 30min, and was then stopped by the addition of 10µl 35mM phenyl methane-sulphonyl fluoride (PMSF) and incubated for a further 10min at 0°C. An aliquot (2.5µl) was analysed for TCA-precipitable radioactivity as already described, and the remainder was made to 55% (v/v) propan-2-ol, 0.05% DTT. The preparation of products for gel electrophoresis was carried out as described in section 2.2.6.3.

2.2.8 Translation of barley mRNA in Xenopus oocytes

2.2.8.1 Method of translation

Oocytes were prepared for injection exactly as described by Colman and Morser (1979). Barley endosperm poly-A$^+$ RNA (prepared by the SDS/NaCl method and purified by one passage through oligo-dT cellulose) was dissolved in sterile distilled water at a concentration of 1mg/ml. Approximately 20nl was injected into each oocyte. The injected oocytes were either incubated in Barth's solution (Gurdon 1968) for 24h and then incubated in batches of 5 in small wells containing 30ul 2mCi/ml [^{35}S] methionine or were incubated with radioactivity immediately. The oocytes were incubated at 21°C. At the end of the labelling period (3-24h), the oocytes and incubation medium were frozen separately and stored at -80°C prior to analysis.

2.2.8.2 Preparation of products for analysis

Oocytes were homogenized in phosphate-buffered saline containing 1% (v/v) nonidet NP40 and 1mM PMSF (40µl per oocyte), and the yolk removed by centrifugation at 10,000g for 2min. For SDS-PAGE analysis of total products the supernatant solution was added to an equal volume of 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 2mM PMSF, 20mM Tris-HCl pH 6.8, 20% glycerol and 0.004% bromophenol blue; 20µl was loaded per gel track. The incubation medium was treated in the same way, and 10µl loaded per track.

Samples of the total homogenate were extracted with 55% (v/v) propan-2-ol, 0.05% DTT and prepared for analysis in the same way as the products of cell-free translation in the wheat germ system (2.2.6.3). After extraction, the equivalent of 1 oocyte was loaded per gel track and an aliquot taken for scintillation counting.

2.2.9 Autoradiography and Fluorography

Gels were prepared for fluorography according to the method of Laskey and Mills (1975) by impregnation of dimethyl sulphoxide (DMSO) equilibrated gels with diphenyl oxazole (PPO). In later experiments the method described by Chamberlain (1979) was used, in which gels were soaked in water and then in 1M sodium salicylate for 30min each.

SDS and two-dimensional gels were dried down under vacuum on an LKB slab gel drier. Isoelectric focusing and RNA

gels, which contain a much lower percentage acrylamide, were dried down by the method of Mayer (1976). The gels were soaked for 3min in methanol:water:glycerol (70:27:3 by vol.), placed on a clean glass plate, and any excess liquid removed by blotting. The gel was allowed to dry for 5-10min at room temperature and was then removed from the plate on a piece of Whatman 3MM chromatography paper. The paper was then taped to a clean piece of glass, and the drying was accelerated in an oven at 50°C.

The gels were exposed to Fuji medical X-ray film held in cassettes containing Ilford fast tungstate intensifying screens. Exposure was for various times at -80°C. For fluorography the film was pre-exposed for 4sec with an enlarger set at f32 with the lamp covered with a piece of Whatman 3MM paper.

3. RESULTS AND DISCUSSION

3.1 ISOLATION AND ANALYSIS OF POLYSOMES

3.1.1 Comparison of Methods of Preparation

It has been reported that cereal storage proteins (Fox et al,1977; Larkins and Hurkman,1978) and Phaseolus vulgaris storage proteins (Bollini and Chrispeels,1979) are synthesised on membrane-bound polysomes. The isolation and separation of free and membrane-bound polysomes from barley endosperm, which is actively synthesising hordein, is therefore an important step in the isolation of RNA enriched for hordein messages. The first method used (referred to as Method 1, described in section 2.2.3.1) was based on Larkins et al (1976a), with the additional use of the nuclease inhibitors 2' and 3' AMP (Gray 1974), and involved the pelleting of the membrane fraction at 32000g. Polysomes were released from this fraction with Triton X100. High ionic strength and high pH were also used to inhibit plant nucleases. The buffer contained 50mM magnesium ions; since it was possible that this concentration could cause aggregation of the polysomes, this method was also tried at a lower magnesium ion concentration (10mM). Method 2 (section 2.2.3.2) involved separation of polysomes on sucrose density gradients in the presence of 10mM magnesium ions; the profile of a typical gradient is shown in Fig. 13 with the position of the endoplasmic reticulum enzyme, NADH-cytochrome c reductase, marked (Miflin

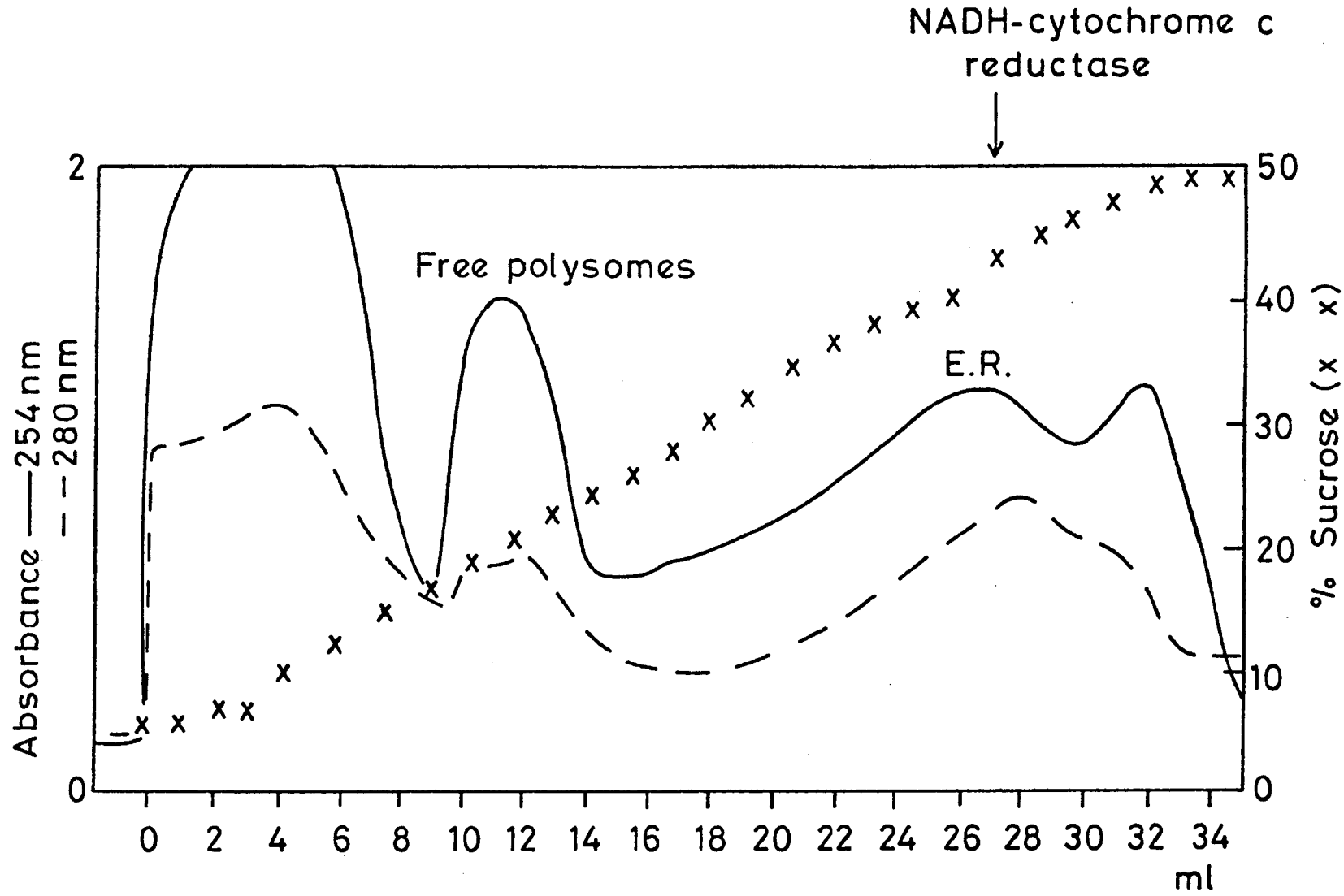

NADH-cytochrome c reductase
Free polysomes
E.R.
Absorbance —254 nm - - 280 nm
% Sucrose (x x)
2
0
50
40
30
20
10
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
ml

Fig. 13 Separation of free and membrane-bound polysomes by sucrose density gradient centrifugation.

An homogenate of developing barley endosperms (3g) was layered on to a linear 10-50% sucrose gradient prepared in 50mM Tricine pH 7.6, 100mM KCl, 10mM magnesium acetate. The gradient was centrifuged for 2.5 hours at 25000rpm in a Beckman SW27 rotor, and was fractionated, using an ISCO automatic fractionator, at a rate of 2ml/min (see section 2.2.3.2). The absorbance was monitored at 254nm (solid line) or 280nm (dashed line), and the sucrose content (x) measured by refractometry. The endoplasmic reticulum (e.r.) fraction was identified by the reported position of the marker enzyme, NADH-cytochrome c reductase (Miflin et al, 1980a).

Table 4 Comparison of yields of polysomes from different methods of preparation

Method		Yield A_{260}/g endosperm	$\frac{A_{260}}{A_{280}}$	Ratio Bound: Free polysomes
1. (50mMMg^{2+})	Free	0.4	1.80	4.5
	Bound	1.8	1.73	
2. (10mMMg^{2+})	Free	0.29	2.16	4.66
	Bound	1.36	1.66	
1. (10mMMg^{2+})	Free	0.27	1.80	4.33
	Bound	1.17	1.76	

The figures in the table represent the mean values of a number of experiments:-

Method 1 (50mMMg^{2+}) - 7 experiments

Method 2 (10mMMg^{2+}) - 2 experiments

Method 1 (10mMMg^{2+}) - 2 experiments

et al 1980b). The yields and absorbance ratios of the different methodsof preparation are summarised in Table 4. The yield of polysomes was slightly higher for the method using 50mM magnesium ions, but the ratio of bound:free polysomes wasnot significantly different for any of the methods. The expected absorbance ratio (A_{260}/A_{280}) for polysomes is 1.75 (Brandt et al 1976) and Method 1 gave values close to this for both concentrations of magnesium ions, indicating that the preparations were relatively pure. The values varied slightly for Method 2; the free polysomes had a higher RNA content whereas the bound polysomeswere contaminated with protein.

Samples of polysomes were analysed by sucrose density gradient centrifugation. Figure 14 shows the profile of bound polysomes prepared by Method 1 with 50mM magnesium ions before andafter nuclease treatment; polysomes containing up to 8 ribosomes are visible, with the most abundant class containing 3-4 ribosomes. Nuclease digestion determined the position of the monoribosome peak, and confirmed that the remaining peaks do represent polysomes. Free polysomes prepared by this method had a higher proportion of monoribosomes present (Fig. 15) but again polysomes containing 8 ribosomes could be seen. Isolation and separation of polysomesin the presence of a low magnesium ion concentration did not significantly alter the profile (Fig. 16).

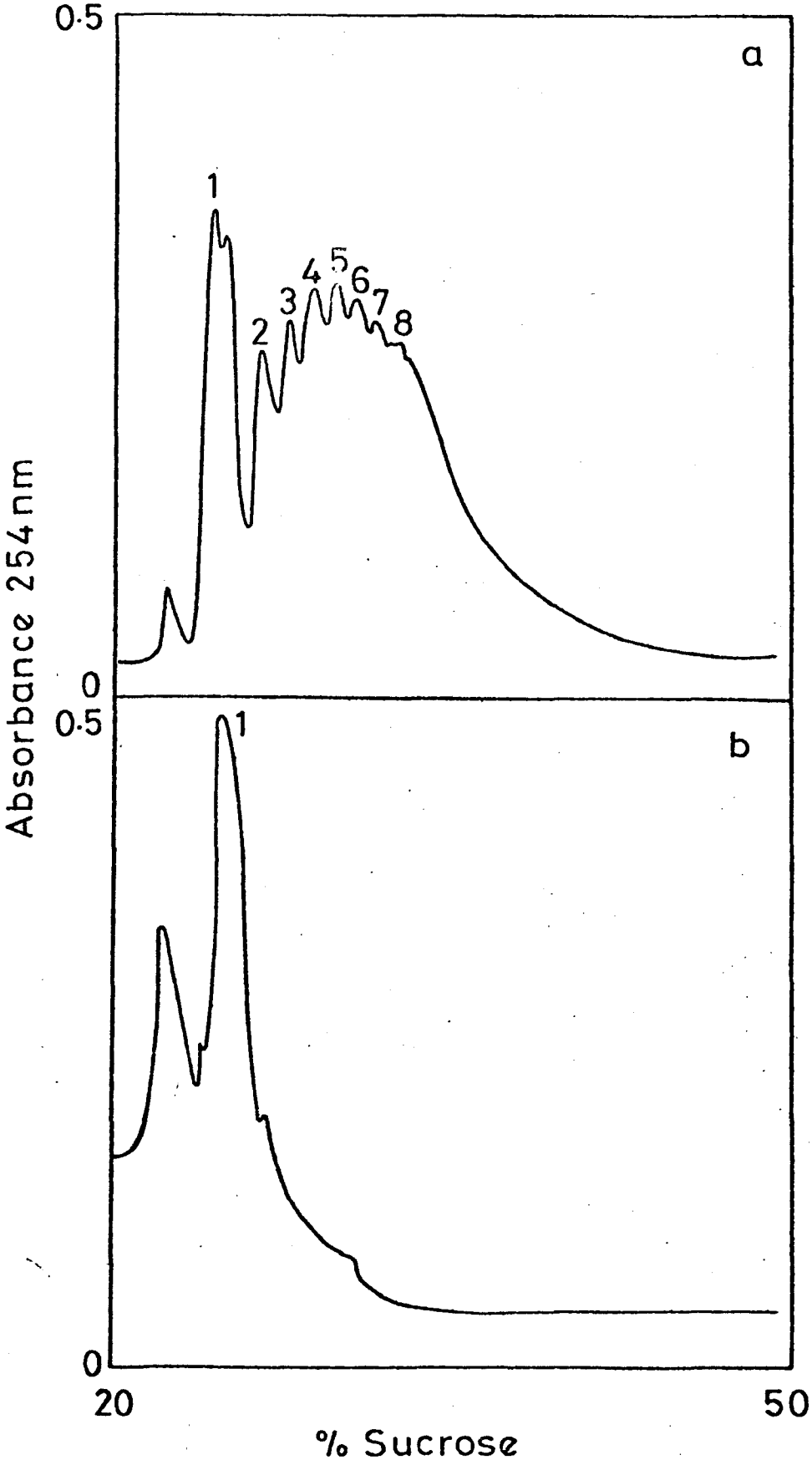

0·5
a
1
2
3
4
5
6
7
8
Absorbance 254 nm
0
0·5
b
1
0
20
50
% Sucrose

Fig. 14 Analysis of membrane-bound polysomes by sucrose density gradient centrifugation

Membrane-bound polysomes, prepared by Method 1 in the presence of 50mM magnesium ions, were suspended in 0.2M Tris pH 9.0, 100mM KCl, 50mM magnesium acetate at a concentration of 6 A_{260} units/ml. Polysomes (1ml) were layered on to linear 20-50% sucrose density gradients (37ml) and centrifuged at 25000rpm for 3.3 hours in a Beckman SW27 rotor (see section 2.2.3.3). The gradients were fractionated at 3ml/min, with monitoring at 254nm. Trace a shows the separation of membrane-bound polysomes; trace b shows separation after digestion of polysomes with 20ug RNAase A for 15min at 28°C. The numbers above the traces indicate the number of ribosomes estimated to be contained in the polysomes in each peak. The monoribosome peak is located by nuclease digestion.

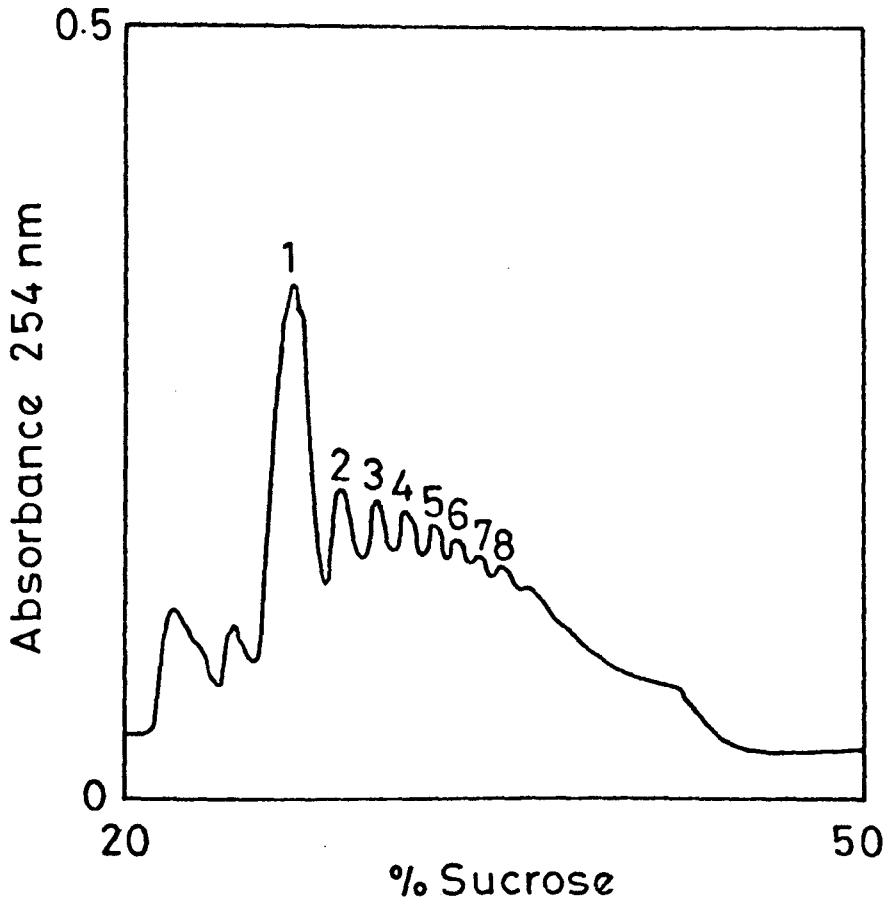

0.5
0
Absorbance 254 nm
1
2
3
4
5
6
7
8
20
50
% Sucrose

Fig. 15 Analysis of free polysomes by sucrose density gradient centrifugation

Free polysomes (Method 1) were suspended in 0.2M Tris pH 9.0, 100mM KCl, 50mM magnesium acetate at a concentration of 6 A_{260} units/ml. Polysomes (1ml) were layered on to a linear 20-50% sucrose gradient (37ml) and centrifuged at 25000rpm for 3.3 hours in a Beckman SW27 rotor (see section 2.2.3.3). The gradients were fractionated at a rate of 3ml/min, with monitoring at 254nm. The numbers above the trace indicate the number of ribosomes estimated to be contained in the polysomes in each peak. The monoribosome peak is identified by comparison with nuclease-treated polysomes (Fig. 14b).

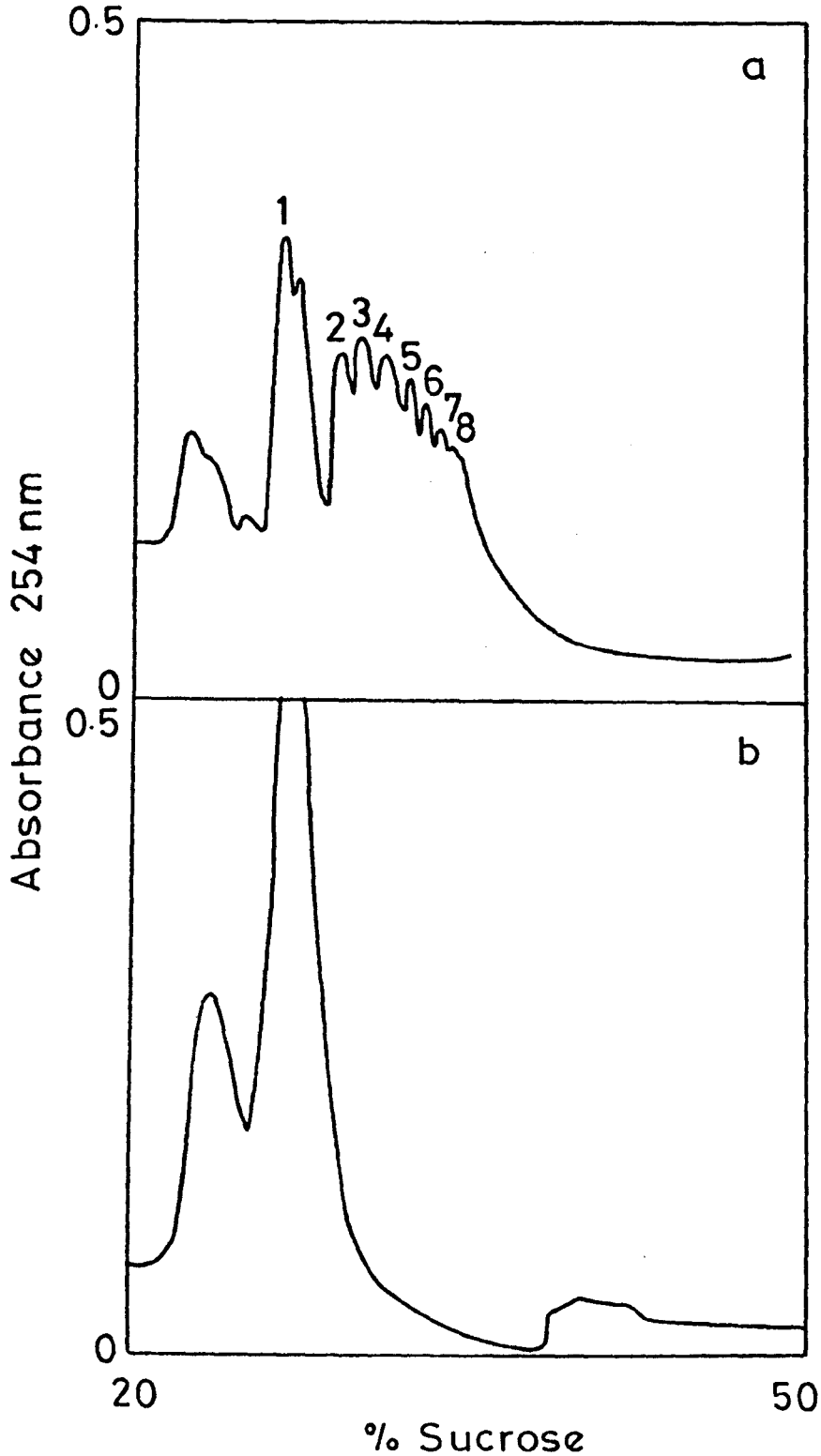

0.5
a
1
2
3
4
5
6
7
8
Absorbance 254 nm
0
0.5
b
0
20
50
% Sucrose

Fig. 16 Analysis of membrane-bound polysomes, prepared by Method 1 in the presence of 10mM magnesium ions, by sucrose density gradient centrifugation.

Membrane-bound polysomes, prepared by Method 1 in the presence of 10mM magnesium ions, were resuspended in 0.2M Tris pH 9.0, 100mM KCl, 10mM magnesium acetate at a concentration of 6 A_{260} units/ml. Polysomes (1ml) were layered on to linear 20-50% sucrose density gradients (37ml) and centrifuged at 25000rpm for 3.3 hours in a Beckman SW27 rotor (see section 2.2.3.3). The gradients were fractionated at a rate of 3ml/min, with monitoring at 254nm. Trace a shows membrane-bound polysomes; trace b shows separation after digestion ofpolysomes with 20ug RNAase A at 28°C for 15min. Thenumbers above the traces indicate the number of ribosomes estimated to be contained in the polysomes in each peak.

3.1.2 Discussion

The different methods of preparation gave polysomes with very similar physical characteristics. Free and membrane-bound polysomes were well separated by sucrose density gradient centrifugation, but this method would be impractical for preparing the large quantities of polysomes necessary for extracting RNA. The template activity was slightly lower (see section 3.3.2). The presence of 50mM magnesium ions during separation did not appear to cause significant aggregation. The ratio of bound to free polysomes was similar for both concentrations of magnesium ions, being approximately 4.5. The true value, however, may be higher than this, as density gradient analysis of polysomes revealed a higher level of monoribosomes in the free polysome preparation.

On the basis of these observations, and also the results discussed in sections 3.3.2 and 3.4.1.1 (which indicate that free polysomes had a similar template activity to bound polysomes but that the proportion of *in vitro*-synthesised products behaving as hordein is very low for free polysomes), it was decided to use membrane-bound polysomes prepared by Method 1 for extraction of mRNA. The higher concentration of magnesium ions was considered preferable as it enhanced the yield of polysomes without significantly affecting their characteristics.

3.2 ISOLATION AND ANALYSIS OF POLY-A^+ RNA

3.2.1 Comparison of Methods of Preparation

In order to prepare an RNA fraction enriched in hordein mRNAs, polyadenylated (poly-A^+) RNA was separated from membrane-bound polysomes by affinity chromatography on oligo-dT cellulose (Bantle *et al*, 1976). Polysomes were either deproteinized with phenol/chloroform or were dissociated with sodium dodecyl sulphate and NaCl (referred to as the SDS/NaCl method; Krystosek *et al*, 1975) prior to chromatography. These methods are described in detail in sections 2.2.4.1 - 2.2.4.3. The trace from a typical column is shown in Fig. 17. The bulk of the RNA passed straight through the column (termed the poly-A^- fraction) and, subsequently, two peaks were eluted. The low-salt washing buffer eluted a fraction (approximately 0.5% of the total RNA) which will be referred to as 'wash' RNA. When this fraction was denatured and rechromatographed on oligo-dT cellulose, the bulk again eluted with the low-salt buffer (Fig. 17, dotted trace). The nature of this fraction is discussed in the following sections. The salt-free buffer eluted a further fraction that accounted for approximately 1.5 to 2.5% of the total RNA; this fraction eluted as a sharp peak and is referred to as poly-A^+ RNA.

The yields of RNA from the different methods are summarised in Table 5. The simplest method was to extract the crude membrane (32000g) pellet with phenol/chloroform prior to oligo-dT cellulose chromatography (section 2.2.4.1). This method

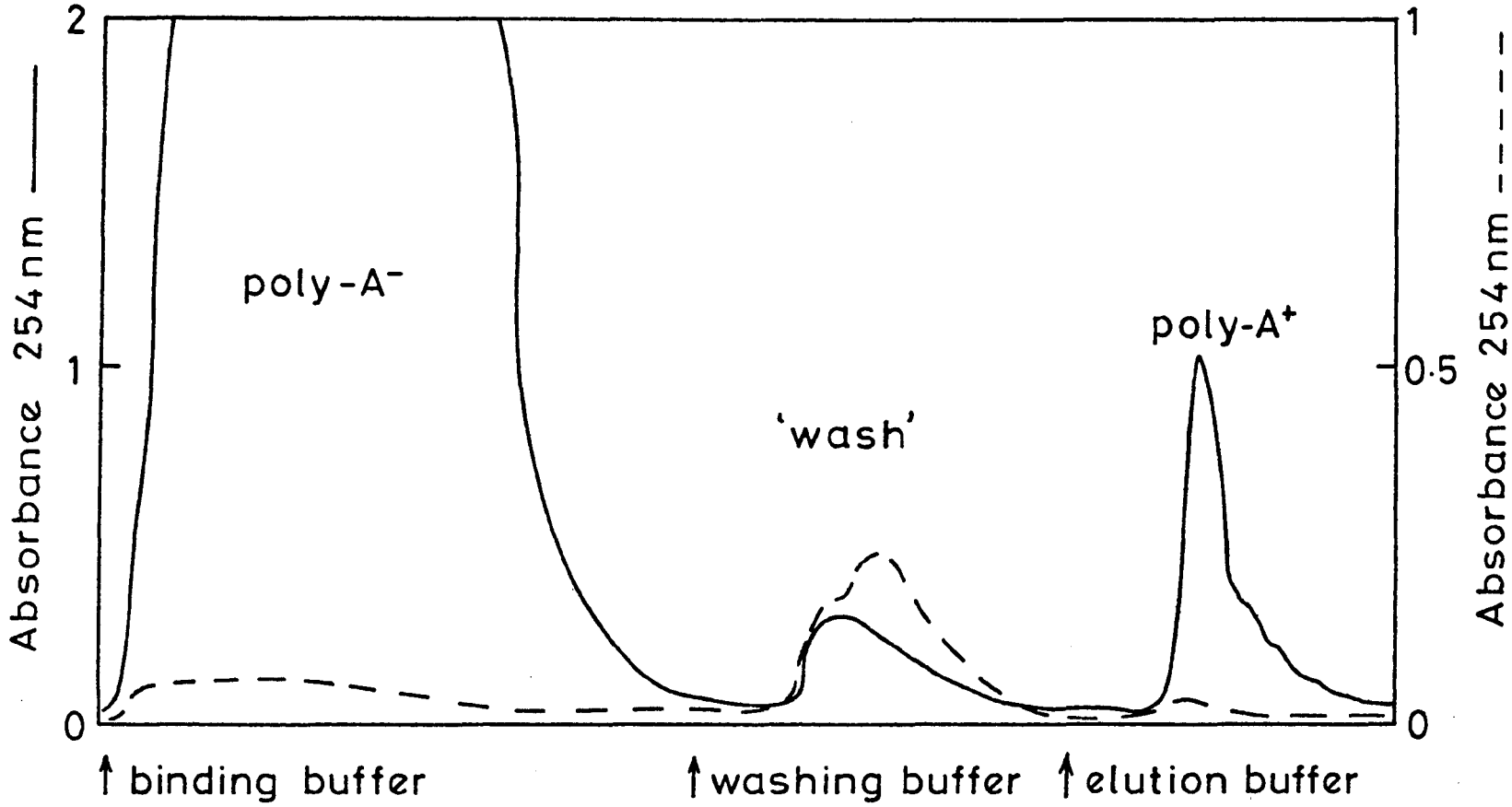

2
1
0
Absorbance 254 nm ——
poly-A⁻
'wash'
poly-A⁺
1
0·5
0
Absorbance 254 nm - - - - -
↑ binding buffer
↑ washing buffer
↑ elution buffer

Fig. 17 Trace from oligo-dT cellulose column

Membrane-bound polysomes were dissociated with 1% SDS and 0.5M NaCl at 60° for 5min. The dissociated material was stirred for 20min at room temperature with 1g oligo-dT cellulose equilibrated with 10mM Tris-HCl pH 8.5, 0.4M NaCl, 0.1% SDS, 1mM EDTA (binding buffer). The slurry was poured into a 1cm diameter column and washed with the same buffer until no more RNA (termed poly-A^-) eluted. The column was monitored continuously at 254nm using a flow cell (solid trace). The column was then washed with the same buffer containing only 0.1M NaCl (washing buffer) and a second fraction eluted ('wash' RNA). The third fraction was eluted with the same buffer minus salt (elution buffer) and was termed poly-A^+ RNA. 'Wash RNA' was ethanol-precipitated and then denatured in 90% DMSO at 60° for 5min before rechromatography (dotted trace). These methods are described in full in section 2.2.4.1.

Table 5 Comparison of methods of preparation of RNA

Method	Yield of poly-A^+RNA[1] (ug per 100g endosperm)	Yield of 'wash' RNA[1] (ug per 100g endosperm)
Phenol[2] (section 2.2.4.1)	25.6 ± 3	6.3 ± 1.2
Proteinasek/polysomes[3] (section 2.2.4.2)	91	61
Phenol/polysomes[3] (section 2.2.4.2)	162	70
SDS/NaCl[2] (section 2.2.4.3)	319 ± 28	65 ± 17

(1) purified once by oligo-dT cellulose chromatography

(2) errors indicate standard error of the mean

(3) figures are the mean of two preparations

The amount of RNA was determined spectrophotometrically at 260nm assuming that 1ug RNA in 1ml water has A_{260} = 0.025 (1cm path length).

however, gave a very poor yield; the major problem was that the membrane pellet was heavily contaminated with starch from the endosperm. This starch formed a viscous solution during phenol extraction, resulting in the loss of solution and RNA. Purification of the polysomes, by centrifugation through a sucrose cushion, increased the yield considerably. However, proteinase K treatment of the polysomes before phenol extraction did not enhance the yield over simple phenol extraction. The highest yields were achieved by dissociation of polysomes with high concentrations of SDS and NaCl (SDS/NaCl method, see section 2.2.4.3). However, as all of the dissociated material was applied to the oligo-dT cellulose column, it was possible that the poly-A^+ fraction could be seriously contaminated with protein. A scan of this fraction in the ultra-violet region is shown in Fig. 18; there is no 'shoulder' at 280nm characteristic of protein, but this does not rule out a small amount of contamination. It was concluded that the SDS/NaCl method was a satisfactory method, and the results reported in the following sections refer to RNA prepared by this method.

When the poly-A^+ RNA fraction was denatured and rechromatographed on oligo-dT cellulose, only about 30% eluted as poly-A^+ RNA. The remainder behaved as poly-A^- RNA and probably represented contaminating rRNA in the first poly-A^+ RNA eluate. Therefore, about 70µg of poly-A^+ RNA was obtained from 100g endosperm after purification by two affinity chromatography

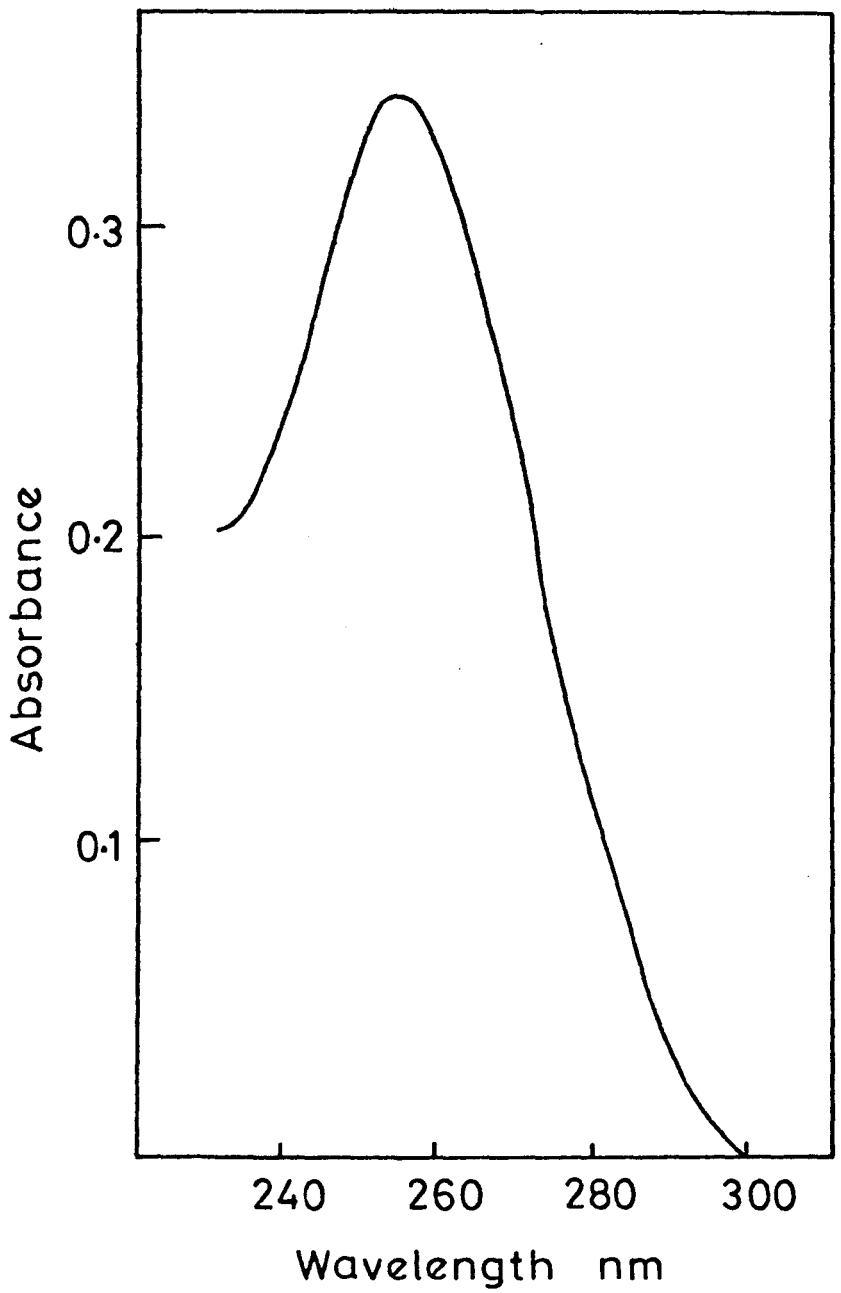

0·3
0·2
0·1
Absorbance
240
260
280
300
Wavelength nm

Fig. 18 Spectrophotometric scan of poly-A^+ RNA prepared by the SDS/NaCl method

Poly-A^+ RNA was prepared by the SDS/NaCl method, ethanol-precipitated and resuspended in sterile distilled water at a concentration between 10 and 15 ug/ml. A sample was scanned in a Cecil model 353 double beam spectrophotometer, with water in the reference cell. Reduced volume (1ml) cells with a 1cm path length were used. The absorbance ratio A_{260}/A_{280} was 3.6 for this particular RNA preparation.

steps. For all translation work, this second chromatography step was omitted, but for synthesis of complementary DNA (see appendix 1) it was included in order to reduce contaminating rRNA.

3.2.2 Analysis of RNA by Size

Two different analytical techniques were used for RNA; they were sucrose density gradient centrifugation (2.2.5.1) and polyacrylamide gel electrophoresis (2.2.5.2). The results obtained by sucrose density gradient centrifugation are shown in Fig. 19. Poly-A^+ RNA from barley endosperm was compared with pea leaf RNA which contains four types of rRNA (i.e. that from the cytoplasmic ribosomes and that from chloroplast ribosomes). The four classes of rRNA were well separated, but analysis of the poly-A^+ RNA fraction gave only a broad peak centred around 17s and did not resolve into individual components. For analysis by gel electrophoresis, the RNA was first denatured with glyoxal in the presence of 50% DMSO (dimethyl sulphoxide), as described by McMaster and Carmichael (1977). In this case a number of components were resolved in thepoly-A^+ RNA fraction (Fig. 20). The poly-A and wash RNA fractions were very similar; both had a faint band at the position of the large rRNA, a stronger band at the position of the small rRNA and a number of other bands. The positions of 23s and 16s rRNA from Klebsiella aerogenes are indicated. The sizes of the poly-A^+ RNA bands were estimated using rRNA and CCMV RNA as standards. The bands marked A, B and C were approximately 2.5, 1.35 and 0.55 kilobases (kb) respectively.

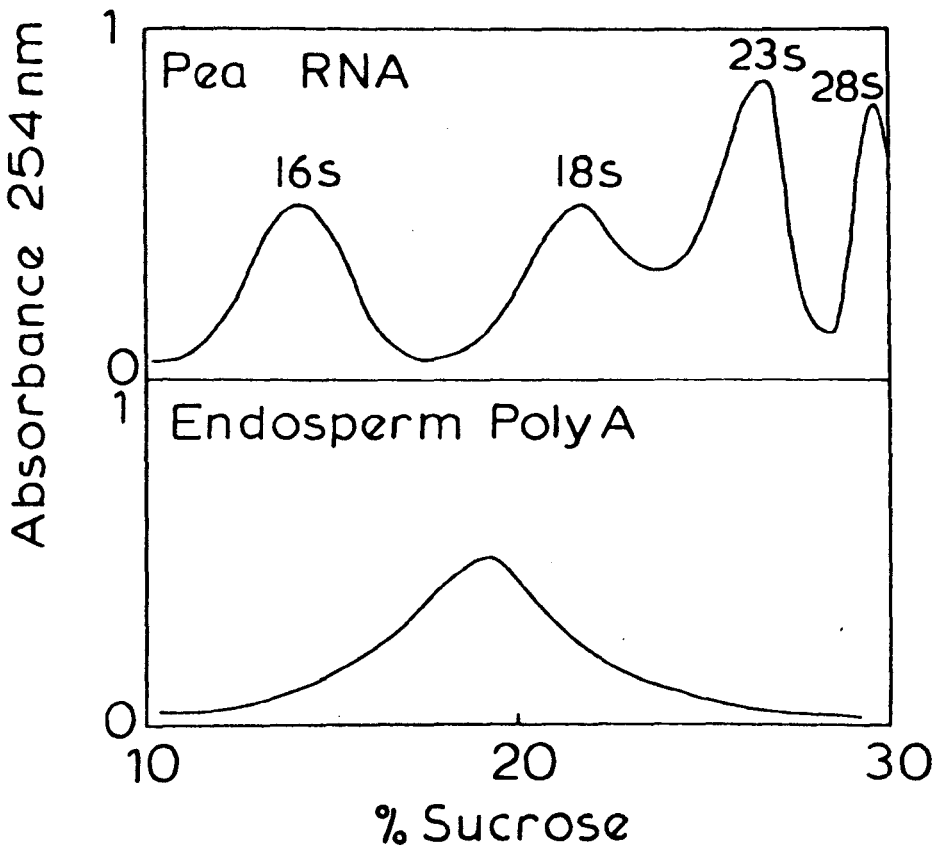

Absorbance 254 nm
1
Pea RNA
16s
18s
23s
28s
0
1
Endosperm Poly A
0
10
20
30
% Sucrose

Fig. 19 Analysis of RNA by sucrose density gradient centrifugation.

RNA was analysedon linear 10-30% sucrose density gradients as described in section 2.2.5.1. The gradients were prepared in 100mM KCl, 10mM Hepes-KOH pH 7.6, 1mM EDTA, 50µg/ml heparin and had a total volume of 5ml. Approximately 100ug RNA was dissolved in 0.2ml of the same buffer, heated to 80°C for 3min, and cooled rapidly in ice-water before layering on to the gradients. The gradients were centrifuged at 150,000gav for 18 hours in a Beckman SW50.1 rotor, and fractionated at 0.2ml/min with monitoring at 254nm.

The upper trace represents total pea leaf RNA and the lower trace represents barley endosperm poly-A^+ RNA.

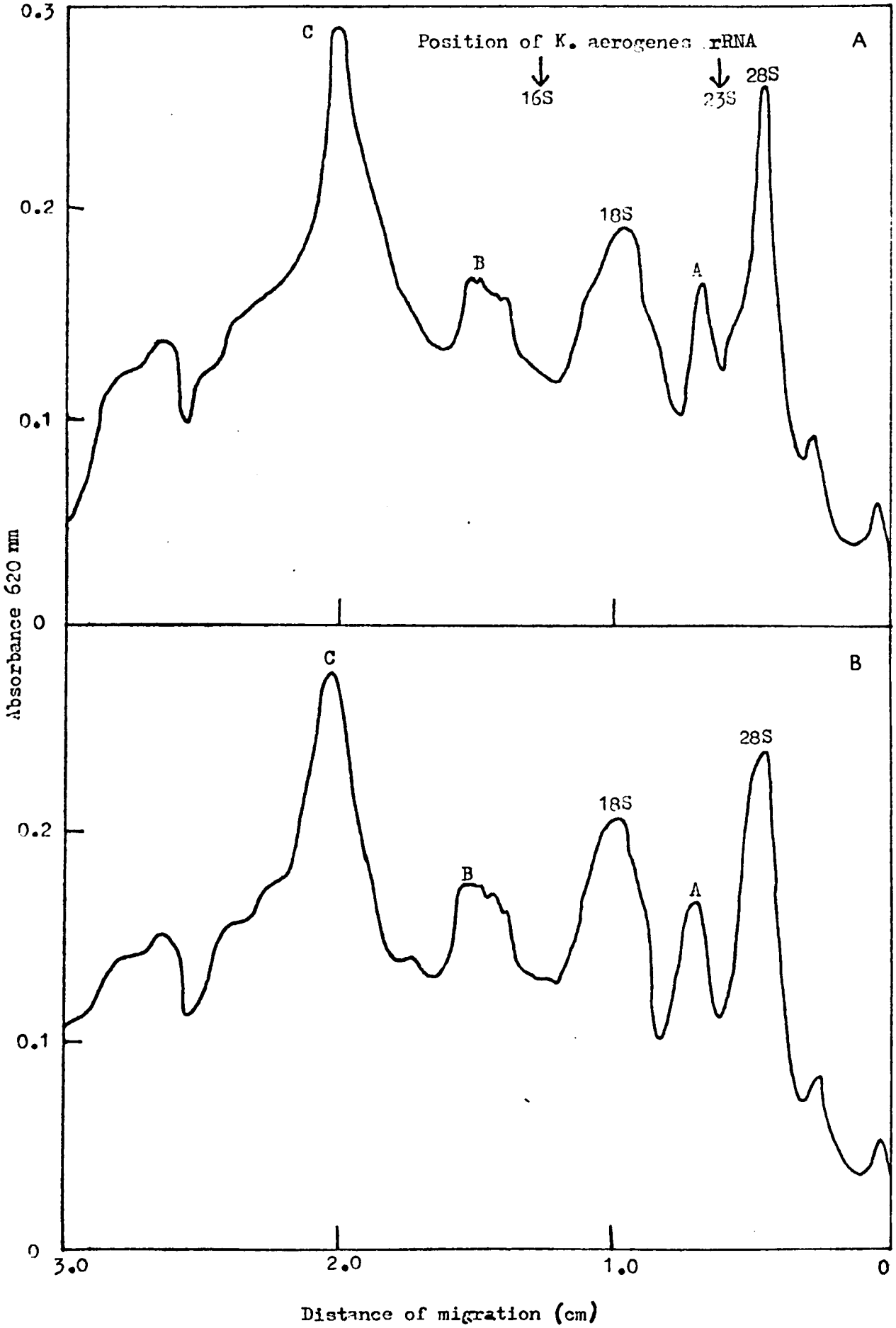

0.3
0.2
0.1
0
Absorbance 620 nm
C
Position of K. aerogenes rRNA
16S
23S
28S
A
18S
B
A
C
B
28S
18S
B
A
0.2
0.1
0
3.0
2.0
1.0
0
Distance of migration (cm)

Fig. 20 Analysis of RNA by polyacrylamide gel electrophoresis.

RNA was denatured with 1M glyoxal, 50% DMSO at 50°C for 1 hour. The samples were then layered on to a 4% polyacrylamide gel containing 7.5M urea and 0.75% SDS. Electrophoresis was carried out at 25mA for 16 hours. This method was modified from McMaster and Carmichael (1977) and is described in full in section 2.2.5.2. The gel was stained in toluidine blue (100mg/l) for 4 hours and destained in water. The tracks were excised with a razor blade and scanned in a Beckman gel scanner with monitoring at 620nm.

Scan A Track containing 5µg poly-A^+ RNA.

The positions of rRNA bands from Klebsiella aerogenes are indicated for reference. In addition to bands co-migrating with barley rRNA, there are three major bands present in poly-A^+ RNA (marked A, B, C) which are estimated to be 2.5, 1.35 and 0.55 kilobases respectively.

Scan B Track containing 5µg 'wash' RNA.

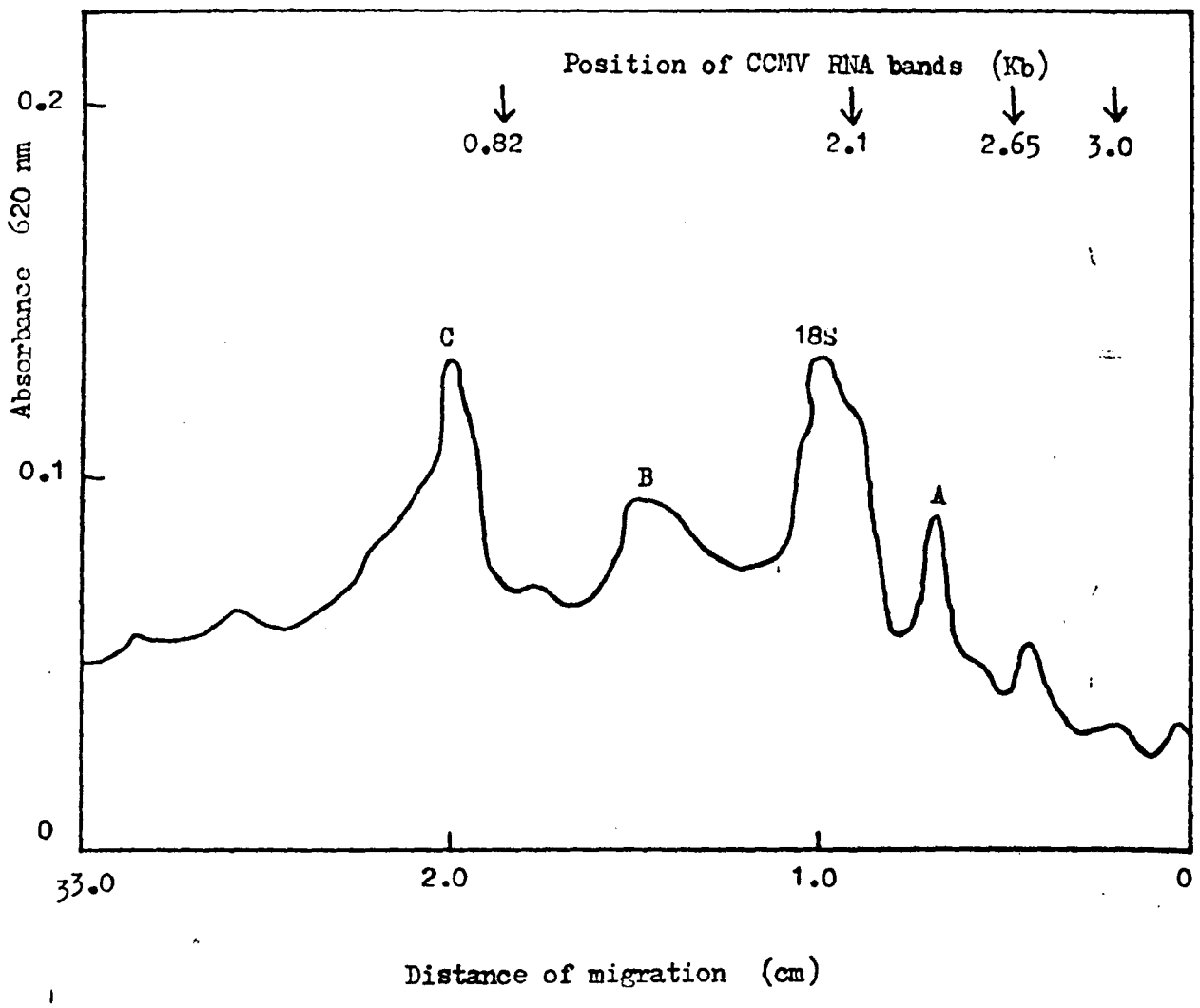

Position of CCMV RNA bands (Kb)
0.82
2.1
2.65
3.0
C
18S
B
A
Absorbance 620 nm
0.2
0.1
0
33.0
2.0
1.0
0
Distance of migration (cm)

Fig. 21 Analysis of RNA by polyacrylamide gel electrophoresis.

Poly-A^+ RNA (2μg) which had been purified twice by oligo-dT cellulose chromatography was separated exactly as described for Fig. 20. The gel was stained and scanned in the same way. The positions of CCMV RNA bands are indicated for reference.

These bands have been observed in a number of poly-A^+ RNA preparations from different varieties of barley. When poly-A^+ RNA has been purified twice by affinity chromatography, the 28s band disappears but the 18s band remains as a major component. The bands termed A, B and C are present but fainter (Fig. 21).

3.2.3 Polyadenylic Acid Content

The proportion of each RNA fraction present in the form of polyadenylic acid was determined by hybridisation to tritiated polyuridylic acid (see section 2.2.5.3; Brandt et al, 1978). A standard graph was constructed using varying concentrations of polyadenylic acid (poly-A). For each RNA fraction, the amount of polyuridylic acid was determined for three different RNA concentrations and compared with values in the standard graph. The results for one preparation of poly-A^+ RNA from Sundance barley are shown in Fig. 22; the proportion of the sequence present as poly-A was 5.8%. The results for other preparations are summarised in Table 6. The average poly-A content of poly-A^+ RNA from Sundance barley was 6.5% but Mutant 56 gave a higher value of 9%. The poly-A^- RNA fraction contained negligible poly-A, but the 'wash' RNA gave a value of 2.6%, lower than that for poly-A^+ RNA.

3.2.4 Discussion

Poly-A^+ RNA was separated from polysomes using affinity chromatography on oligo-dT cellulose; the polysomes were prepared for chromatography in a number of different ways. The

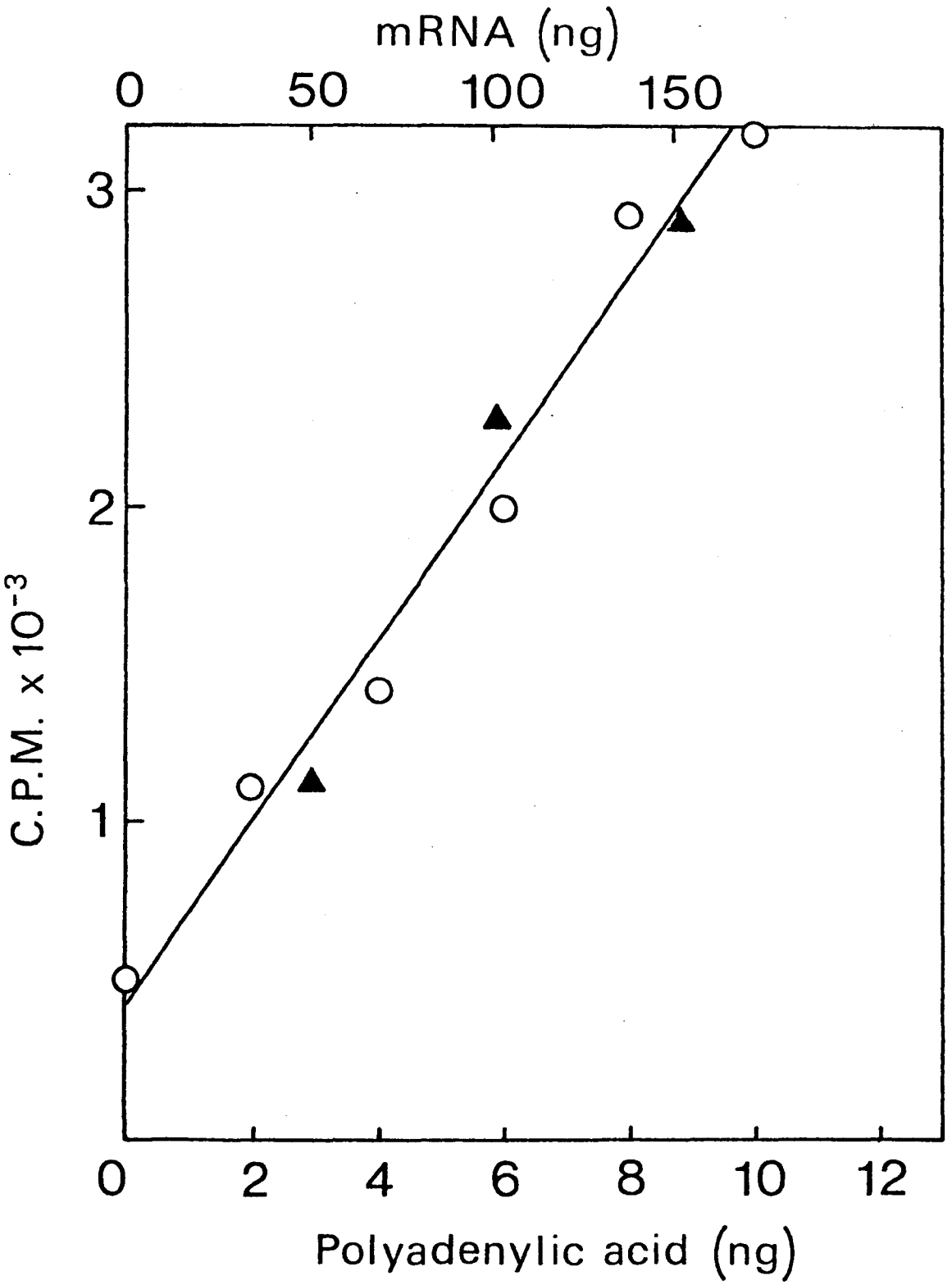

mRNA (ng)
0
50
100
150
3
2
1
C.P.M. x 10⁻³
0
2
4
6
8
10
12
Polyadenylic acid (ng)

Fig. 22 Determination of polyadenylic acid content.

RNA was hybridised to 100nCi [^{3}H] polyuridylic acid at 28°C for 15min (Brandt et al, 1978; see section 2.2.5.3). Single stranded RNA was digested with 5µg RNAase A, and the hybrid RNA molecules recovered by TCA precipitation for quantitation by liquid scintillation counting. A standard graph was constructed using polyadenylic acid (O). Three different concentrations of poly-A^+ RNA from Sundance barley (▲) were used in order to determine the amount of polyadenylic acid present.

Table 6 Table to show the polyadenylic acid content of various RNA fractions

Variety of Barley	RNA fraction	% polyadenylic acid present in RNA fraction
Sundance	poly-A^+	6.8, 7.1, 6.3, 5.8(1)
Sundance	wash	2.6
Sundance	poly-A^-	0.5
Mutant 56	poly-A^+	9.0, 8.3(1)

(1) values are for different preparations of RNA.

The polyadenylic acid content was determined by hybridisation to [^{3}H]polyuridylic acid. Single-stranded RNA was digested with 5ug RNAase A, and the hybrid RNA molecules collected and quantitated by scintillation counting. The technique was calibrated with polyadenylic acid (see section 2.2.5.3).

highest yields were obtained by dissociation of the polysomes with high concentrations of SDS and NaCl. Analysis of the poly-A^+ RNA by sucrose density gradient centrifugation failed to resolve individual components; this result was probably due to incomplete denaturation of the RNA and the inherent low resolution of the technique. Polyacrylamide gel electrophoresis of glyoxalated poly-A^+ RNA showed two bands comigrating with barley rRNA and a number of other components. When poly-A^+ RNA was purified a second time by oligo-dT cellulose chromatography, the band comigrating with 18s rRNA remained. There are two possible explanations of this observation:-

(1) 18s rRNA was not completely removed by two chromatography steps;

(2) the 18s band contained an mRNA component.

A combination of these two explanations is likely. The 18s band was broad compared with the 28s band and 18s band of poly-A^- RNA, indicating a degree of heterogeneity; this phenomenon may be due to the presence of a number of different sequences or variation in the length of poly-A tails. This band hybridised very weakly to cloned barley ribosomal DNA under conditions in which barley rRNA showed strong hybridisation (M. B. Bahramian, unpublished). RNA recovered from the same-sized band on urea gels exhibited translational activity (M. Kreis, unpublished).

If the 18s band (approximately 1.9kb) is included as an mRNA component, 4 major bands can be resolved in the poly-A^+ RNA

fraction. Their sizes are 2.5, 1.9, 1.35 and 0.55kb. The width of the bands suggests some degree of heterogeneity, again possibly due to variation in the length of poly-A tails. In order to calculate the maximum potential coding sequence of the components, it is necessary to subtract the length of the poly-A tails. The degree of adenylation was estimated as 6.5% for poly-A^+ RNA from Sundance barley; the significance of the higher value of 9% for Mutant 56 is not understood. The method of determining poly-A content also detects short lengths of poly-A within the sequence, and therefore, does not prove that there is a continuous tract at the 3' end. Assuming that this is the case, the average lengths of the poly-A tails are 150, 125, 90 and 38 on the four major bands. However, the length of poly-A is not necessarily proportional to the length of the RNA molecule. The maximum potential coding sequences are 2.35, 1.77, 1.26 and 0.51kb. The 'C' and 'B' hordeins contain about 270 and 460 amino acid residues respectively (Shewry *et al*, 1980a), requiring coding sequences of 1.4 and 0.8kb. The larger of the major RNA species isolated from barley polysomes are, therefore, large enough to code for hordeins with some extra sequences remaining. The smaller species of RNA could represent other messages or, possibly, degradation products.

The properties of the fraction of RNA eluted from oligo-dT cellulose by low-salt buffer ('wash' RNA) have been investigated. The fraction has components of a very similar size to poly-A^+ RNA.

The degree of adenylation is, however, much lower, only 2.6%. It would appear that this fraction is probably polyadenylated RNA which has only short poly-A tails. Its interaction with oligo-dT is, therefore, weaker and it can be eluted with low-salt buffer. The translation activity of 'wash' RNA supports this hypothesis and is discussed in sections 3.3.2.2 and 3.4.3.

3.3 ASSAY OF TEMPLATE ACTIVITY OF POLYSOMES AND RNA

3.3.1 Characterisation of the Wheat Germ Cell-free System

The conditions of assay were based on those described by Roberts and Paterson (1973), but the optimum concentrations of various components were determined for the translation of both polysomes and RNA from barley endosperm. The dependence on magnesium ion concentration was the most critical; the optimum concentration differed between polysomes and RNA, and also between wheat germ preparations. The results for one wheat germ preparation (termed WG9) are described in Fig. 23. The optimum concentration of magnesium ions for polysome translation was 3.5mM, and for poly-A^+ RNA translation 2.5mM. These values take into account the magnesium ions present in the wheat germ S30 extract (approximately 4mM). The optimal value for poly-A^+ RNA translation varied from 1.7 to 2.6mM depending on the wheat germ preparation. The optimum concentrations of potassium ions and spermidine ions were 110mM and 300μM respectively; these varied little between polysomes and RNA, or between different wheat germ preparations.

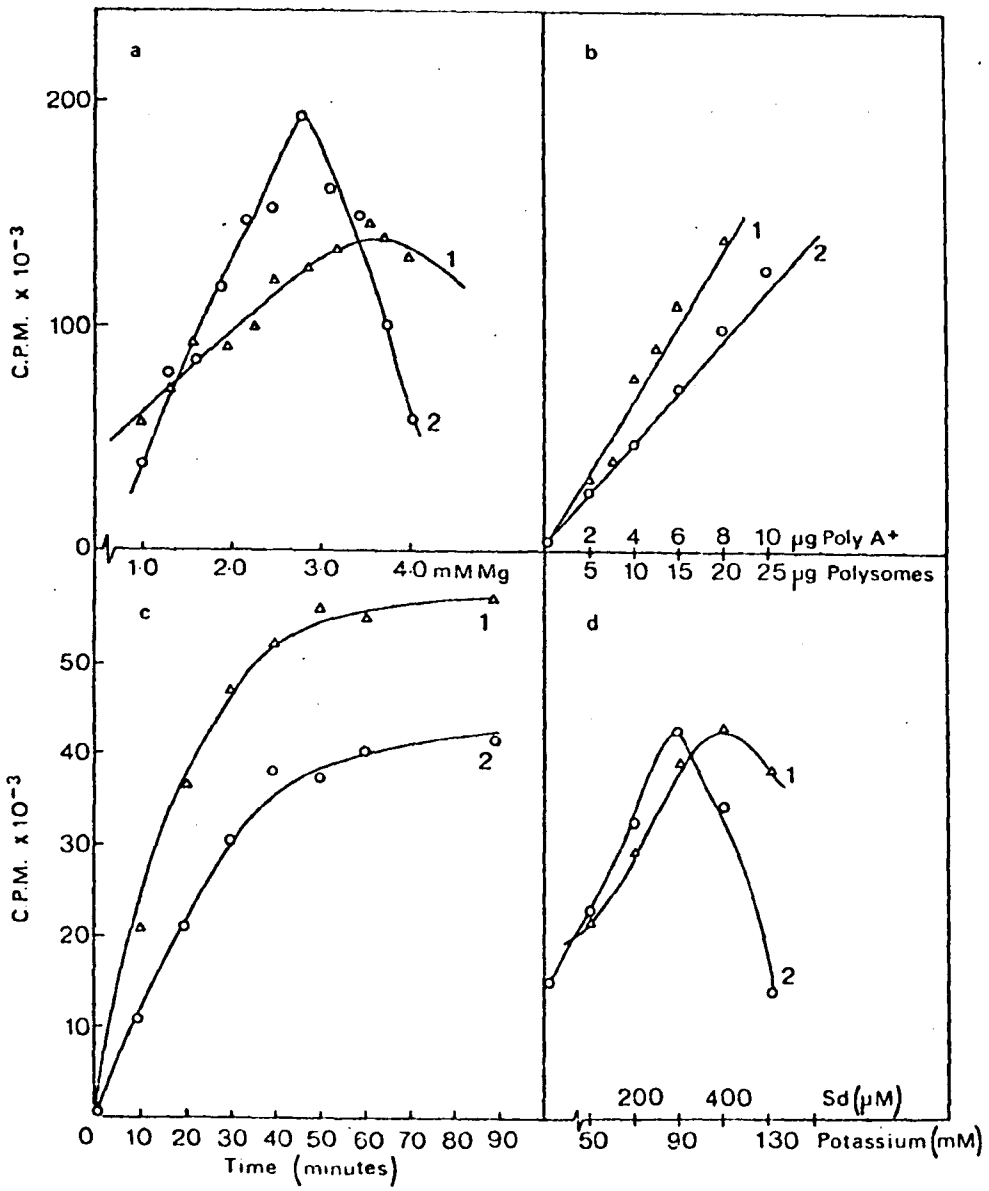

a
b
c
d
C.P.M. x 10⁻³
200
100
0
1·0
2·0
3·0
4·0 mM Mg
2 4 6 8 10 µg Poly A+
5 10 15 20 25 µg Polysomes
50
40
30
20
10
0 10 20 30 40 50 60 70 80 90
Time (minutes)
200 400 Sd(µM)
50 90 130 Potassium (mM)
1
2

Fig. 23 Determination of optimum conditions for in vitro-protein synthesis.

Poly-A RNA and membrane-bound polysomes were used to direct in vitro-protein synthesis in the wheat germ cell-free system. The method of assay is describedin full in section 2.2.6.2. The wheat germ preparation used in all these experiments is termed WG9.

(a) The effect of magnesium ion concentration on the translation of polysomes (1) and poly-A^+ RNA (2):- the amounts of template added were 5µg poly-A^+ RNA and 8µg polysomes, and the translation was carried out in the presence of 10µCi [^{3}H]proline, 110mM potassium acetate and 300µM spermidine ions.

(b) The effect of polysome (1) and poly-A^+ RNA (2) concentration on the incorporation of radioactivity into TCA-precipitable material:- the assays were carriedout in the presence of optimum concentrations of magnesium, potassium and spermidine ions with 5µCi [^{3}H]proline added.

(c) Time course of polysome (1) and poly-A^+ RNA (2) translation:- the assays were carried out in the presence of optimum concentrations of magnesium, potassium and spermidine ions with 5µCi [^{3}H]proline added. The amounts of template added were 5µg poly-A^+ RNA and 10µg polysomes.

(d) The effect of potassium ion (1) and spermidine ion (Sd, 2) concentration on the translation of poly-A^+ RNA:- the assays were carried out in the presence of optimum magnesium ion concentrations with 5μCi $[^3H]$ proline added. The amount of template added was 5μg poly-A^+ RNA.

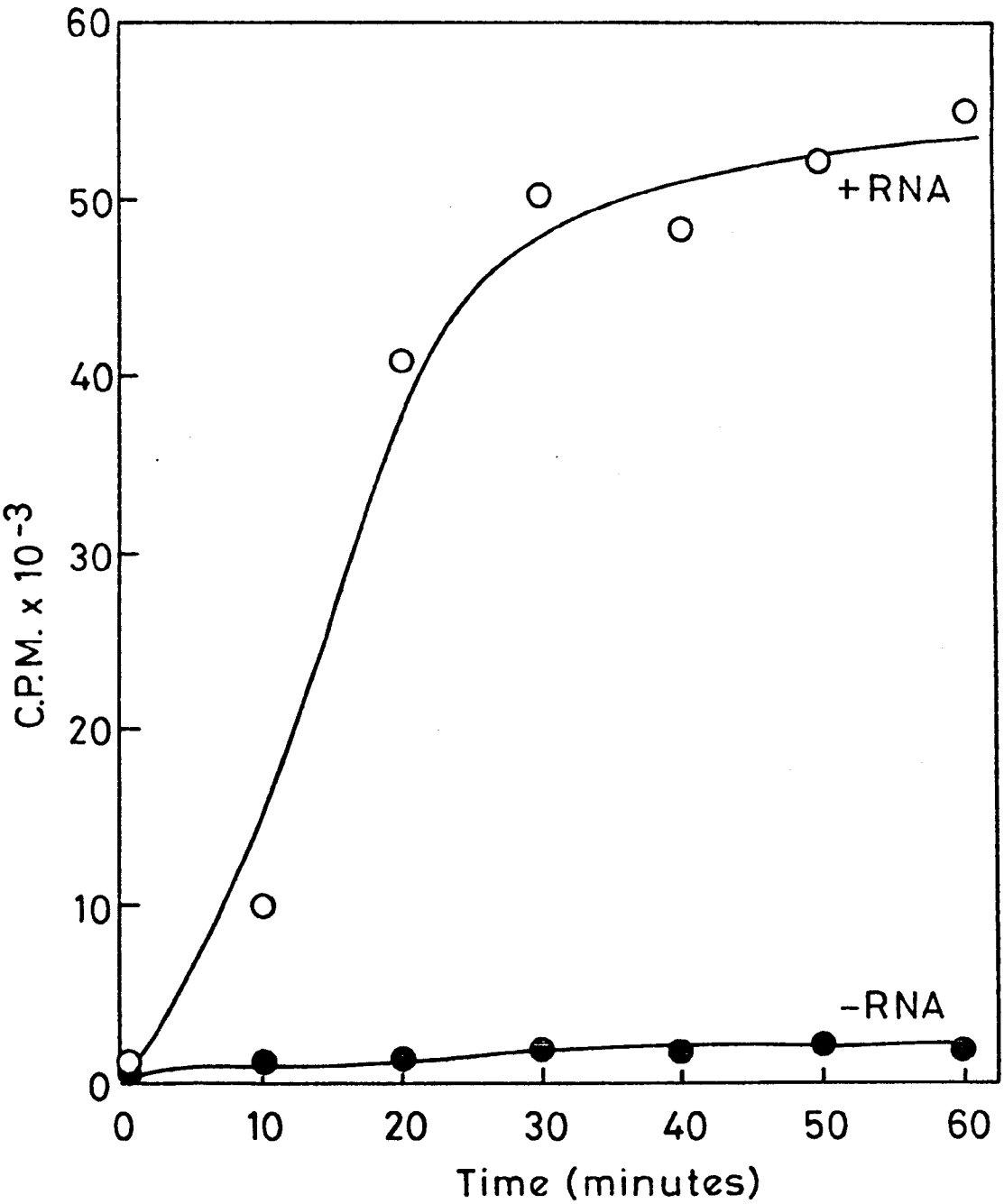

60
50
40
30
20
10
0
C.P.M. x 10-3
+RNA
-RNA
0
10
20
30
40
50
60
Time (minutes)

Fig. 24 Time course of *in vitro*-protein synthesis.

Wheat germ cell-free protein synthesis was carried out in the absence (●) and presence (O) of added RNA; 2µl aliquots were removed for analysis of incorporated radioactivity at 10min intervals. The assays were carried out under optimum conditions (see Fig. 23) with 5µCi $[^3H]$proline added, according to the method described in section 2.2.6.2. The RNA added was 5µg poly-A^+ RNA prepared by the SDS/NaCl method (section 2.2.4.3).

The dependence of translation on the amount of polysomes or RNA added was approximately linear within the range tested. For wheat germ preparation 9, the incorporation of radioactivity stopped after 40min for polysomes and 35min for RNA. The time course in Fig. 24 compares the incorporation of radioactivity into TCA-precipitable material in the presence and absence of RNA. Many wheat germ preparations translated polysomes for a shorter period of time, approximately 20min; the phenomenon is discussed in section 3.4.2. Assay solutions were normally incubated for 40min.

Varying the concentrations of GTP, ATP and creatine phosphate had little effect on translation. Creatine phosphokinase was necessary for efficient incorporation of radioactivity into TCA-insoluble material. In the absence of creatine phosphokinase the incorporation fell, in both the presence and absence of added RNA, to about 10% of normally observed levels.

3.3.2 Template Activity of Polysomes and RNA

Polysomes prepared by different methods were translated in the wheat germ cell-free system (Table 7). Membrane-bound polysomes, prepared by the crude separation method (Method 1) in the presence of 50mM magnesium ions, were slightly more active than either free polysomes or membrane-bound polysomes prepared in 10mM magnesium ions. The effect was small. Polysomes prepared by the sucrose density gradient method (Method 2) were very much less active, which may possibly be due to degradation during

Table 7 The template activities of different polysome preparations

Method of preparation of polysomes	Type of Polysomes	TCA-precipitable radioactivity (C.P.M. x 10^{-3}/A_{260} unit polysomes) Experiment 1	2	3
1 (50mM Mg^{2+})	Membrane-bound	40.8	33.1	38.1
1 (50mM Mg^{2+})	Free	33.5	28.1	31.3
1 (10mM Mg^{2+})	Membrane-bound	31.9	-	-
2 (10mM Mg^{2+})	Membrane-bound	17.3	-	-
2 (10mM Mg^{2+})	Free	11.8	-	-

All assays were carried out under optimum conditions with 1μCi [^{35}S] methionine added. The endogenous incorporation under these conditions was approximately 1.2 x 10^3 C.P. M., and has been subtracted from each value. The extraction methods are defined in section 2.2.3. Experiments 1, 2 and 3 were carried out with different preparations of polysomes.

Between 0·3 and 0·6 A_{260} units of polysomes were added to each assay.

Table 8 The template activities of different RNA fractions.

RNA fraction	TCA-precipitable radioactivity (C.P.M. x 10^{-3}/ug RNA)
Poly-A^+	23.6 ± 5.3
Poly-A^-	0.7 ± 0.1
Wash	8.4 ± 1.1

All assays were carried out under optimum conditions with 1μCi [^{35}S]methionine added. The endogenous incorporation under these conditions was approximately 1 x 10^3 C.P. M., and has been subtracted from the values. The values given represent the mean of five experiments, and error margins are the standard errors of the means. The RNA fractions were derived from oligo-dT cellulose chromatography of polysomal material dissociated by the SDS/NaCl method. Poly-A^- RNA was deproteinized with phenol before translation (see sections 2.2.4.3 and 2.2.4.4). The amount of RNA added to the assays was between two and five ug for the poly-A^+ and wash RNA fractions, and between 20 and 50 ug for the poly-A^- RNA fraction.

the longer procedure for preparation. Three different preparations of free and bound polysomes gave similar results indicating that the system was reproducible. When [^{35}S] methionine was used as the precursor, the stimulation of incorporation above the endogenous amount was usually between 25 and 35-fold. Higher stimulations were found when tritiated proline and leucine were used (80-110-fold), probably due to the very high proline and leucine content of the hordeins compared to methionine (see Table 1, section 1.2.3).

Translation of RNA gave more variable results, depending on the preparation of RNA used. Poly-A$^+$RNA derived from membrane-bound polysomes was usually two to three times as active as 'wash' RNA (Table 8). Poly-A$^-$ RNA had negligible messenger activity (approximately 2% of poly-A RNA). Stimulation of incorporation of radioactivity was usually between 10 and 25-fold when [^{35}S]-methionine was used as the precursor for poly-A$^+$ RNA translation, but as for polysomes, stimulation was much higher for tritiated proline and leucine (60-80-fold).

Poly-A$^+$ RNA was translated in the presence of 7-methyl guanosine monophosphate (7-Me GMP), which is an analogue of the 'cap' structure found at the 5' end of many eukaryotic mRNAs. This compound competitively inhibits the initiation of capped mRNAs. When increasing concentrations of 7-Me GMP were added to the poly-A$^+$ RNA, translation was inhibited (Fig. 25). At 0.5mM 7-Me GMP, incorporation of radioactivity into TCA-precipitable material was inhibited by 92%. The inhibition was

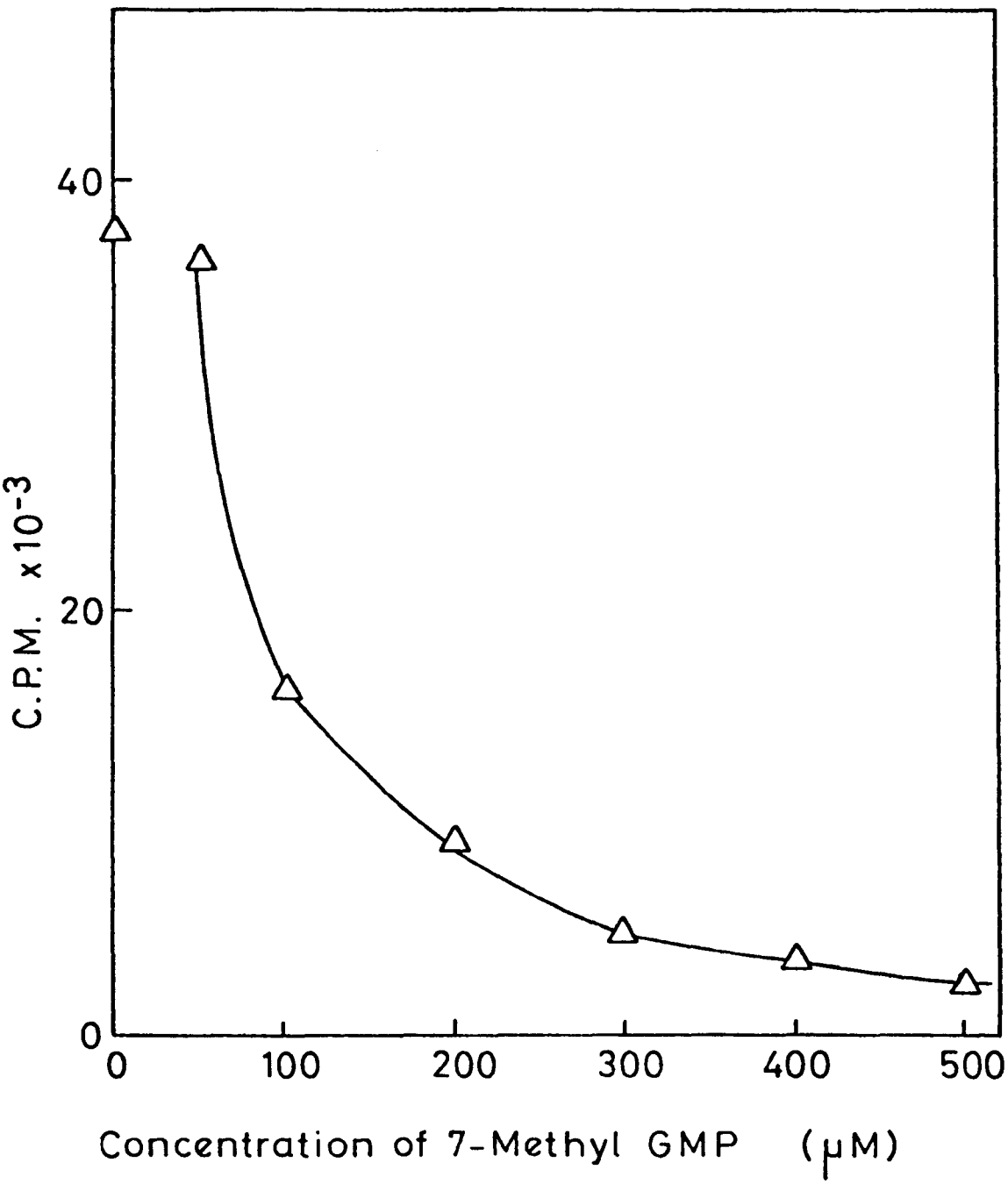

40
20
0
C.P.M. x10-3
0
100
200
300
400
500
Concentration of 7-Methyl GMP (μM)

Fig. 25 The effect of 7-methyl guanosine monophosphate on the translation of barley endosperm poly-A^+ RNA.

In vitro-protein synthesis was carried out as described in section 2.2.6.2; 2µl aliquots were removed for analysis of incorporated radioactivity. Tritiated proline (5µCi) and 5µg poly-A^+ RNA were added to each assay, together with increasing amounts of 7-methyl GMP.

partly dependent on potassium ion concentration; the effect was greatest at 100mM potassium acetate. Concentrations of GMP and GTP similar to the inhibitory concentrations of 7-Me GMP had no effect on translation.

3.3.3 Discussion

The optimum conditions determined for wheat germ cell-free protein synthesis are in general agreement with figures reported in the literature (for example; see Marcu and Dudock, 1974). The wheat germ system reliably translated polysomes and poly-A^+ RNA, giving stimulations of incorporation of radioactivity into TCA-precipitable material as high as 110-fold when [^{3}H]-proline or leucine were used as precursors. Some variation occurred between different RNA preparations and different wheat germ preparations. One of the most noticeable differences between wheat germ preparations, for example, was the time course of polysome translation; wheat germ preparation 9 translated polysomes over a longer period of time, and was slightly more active than other preparations. The results for the translation of polysomes prepared by different methods support the conclusions presented in section 3.1.2. The use of 50mM magnesium ions in the isolation procedures ensures that polysomes have a high template activity as well as appearing in high yield.

Poly-A^+ RNA had a high template activity whereas the activity of poly-A^- RNA was very low. This observation suggests that the bulk of the messenger RNA activity is polyadenylated.

The fraction termed 'wash' RNA also contained messenger activity, but was less active than poly-A^+ RNA. The physical similarity between the two fractions is consistent with the presence of messenger activity in the 'wash' RNA. This fraction is discussed further in section 3.4.1.2 in relation to the nature of the in vitro-synthesised products.

Results obtained using the initiation inhibitor 7-Me GMP suggest that most of the poly-A^+ RNA, which is being translated, is capped. These results are in agreement with those of Brandt and Ingversen (1978). Experiments with 7-Me GMP must be interpreted with care as many factors (e.g. salt concentration; Kemper and Stolarsky, 1977) affect the inhibition. Many non-specific interactions can also occur (Sonenberg and Shatkin, 1978). The specificity of the effect was checked by substituting similar compounds, GMP and GTP; these had no inhibitory effect. Unfortunately, no uncapped mRNA was available as a control. The controls carried out suggest that the effect is specific and that those RNA sequences being translated are capped.

3.4 CHARACTERISATION OF IN VITRO SYNTHESISED PRODUCTS

3.4.1 Comparison with Authentic Hordein

3.4.1.1 Solubility and amino acid composition

The nature of the products of in vitro protein synthesis was investigated. Hordein is soluble in 55% propan-2-ol (plus a reducing agent, 2% 2-mercaptoethanol or 0.05% DTT) whereas most proteins are not. The proportion of TCA-precipitable

radioactivity soluble in this solvent was determined for the products a number of sources of template RNA (Table 9). A small proportion was soluble in all cases but when polysomes or poly-A^+ RNA, derived from the membrane fraction of barley endosperm, were used as templates more than half of the products were soluble in 55% propan-2-ol, 0.05% DTT. This proportion is, to a certain extent, a function of the amount of RNA added to the *in vitro* system (Fig. 26). The amount of propan-2-ol-soluble radioactivity did not increase if more than 6µg poly-A^+ RNA was added to the assay solution. Possible explanations for this are that either the increased RNA concentrations resulted in a greater population of prematurely terminated fragments, or that sequences were translated with different efficiencies.

Hordein is low in lysine compared with other amino acids, for example leucine; therefore, the ability of endosperm poly-A^+ RNA to support the incorporation of these two amino acids was tested. The results are summarised in Table 10. Pea leaf poly-A^+ RNA supported the incorporation of 2.3 times as much leucine as lysine, which compares well with the ratio of 2.1 determined from the amino acid composition of total legume leaf protein (Byers, 1971). In contrast, endosperm poly-A^+ RNA directed the incorporation of 5.2 times more leucine than lysine into total TCA-precipitable material. The ratio of these amino acids, on a molar basis, in total endosperm protein three weeks after anthesis is 2.3:1 but in the crude hordein fraction is

Table 9 Propanol-soluble material synthesised *in vitro* on different RNA templates.

Source of RNA	% propanol-soluble radioactivity using polysomes as template	% propanol-soluble radioactivity using RNA as template
TMV	-	20 ± 3.5
Barley endosperm (soluble fraction)	33 ± 2.1	29
Barley endosperm (membrane fraction)	57 ± 4.7	61 ± 2.7
Barley leaves	-	28 ± 2.3

After translation of polysomes or RNA in the wheat germ cell-free system in the presence of [^{35}S] methionine as precursor, the *in vitro*-synthesised products were extracted with 55% propan-2-ol, 0.05% DTT. Samples were taken from the propanol extracts and the TCA-insoluble radioactivity present in them determined (see sections 2.2.6.2, 2.2.6.3). This value is expressed as a percentage of the total TCA-insoluble material synthesised in response to RNA or polysomes. Error margins indicate the standard error of the mean.

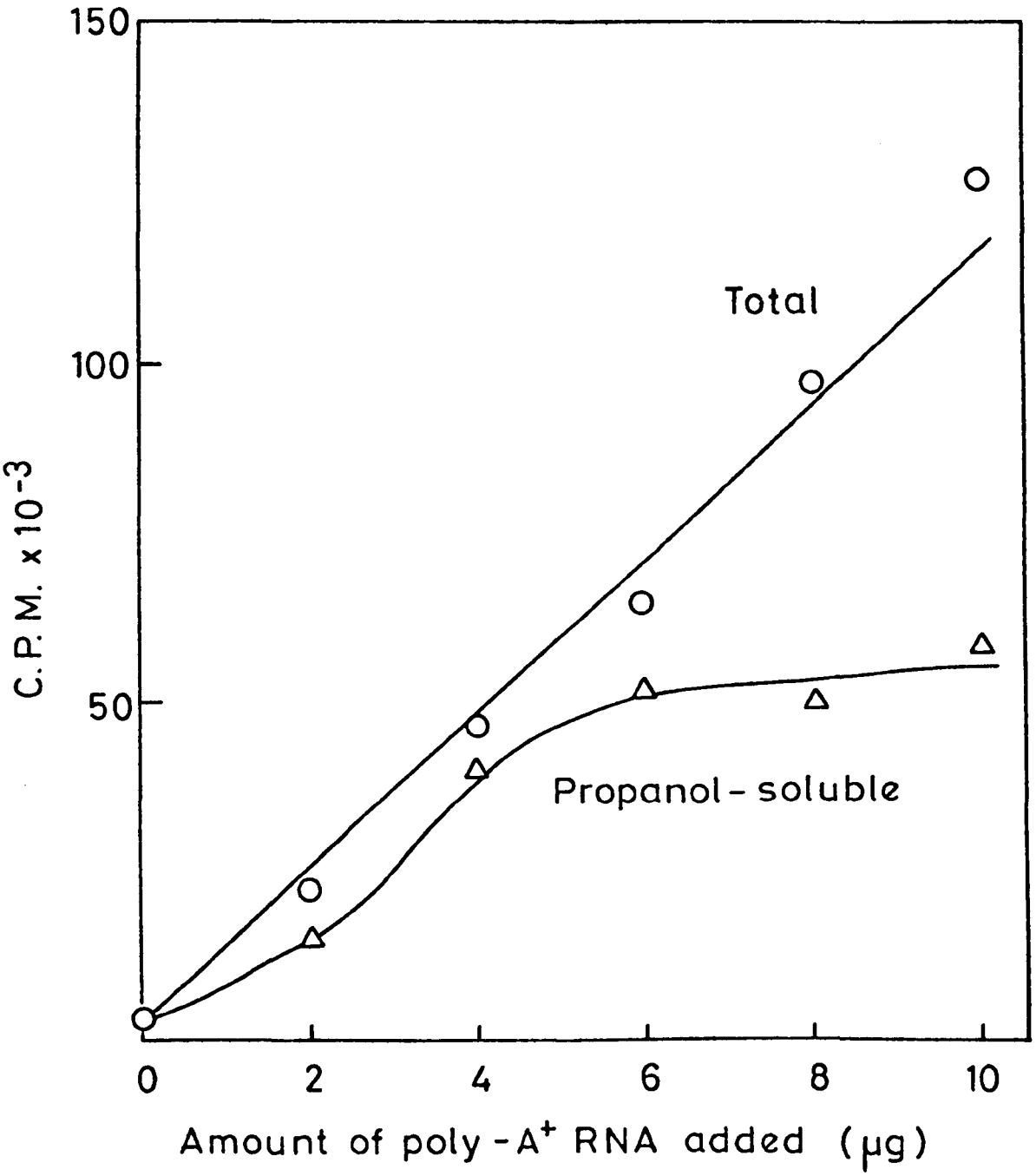

150
100
50
C.P.M. x 10⁻³
Total
Propanol - soluble
0
2
4
6
8
10
Amount of poly - A⁺ RNA added (µg)

Fig. 26 The effect of poly-A^+ RNA concentration on the synthesis of total TCA-insoluble material and on the amount of that material soluble in 55% propan-2-ol, 0.05% DTT.

In vitro-protein synthesis was carried out under optimum conditions in the presence of 5uCi [^{3}H]proline and increasing amounts of barley endosperm poly-A^+ RNA. The amount of TCA-insoluble radioactivity was determined as described in section 2.2.6.2, and the amount of propanol-soluble, TCA-insoluble radioactivity in an equivalent volume was determined as described in section 2.2.6.3.

Table 10 Incorporation of leucine and lysine into material synthesised *in vitro* on different RNA templates

Source of RNA	*In vitro* incorporation Leu:Lys
TMV	2.6:1
Pea leaf poly-A^+ RNA	2.3:1
Barley endosperm poly-A^+ RNA (membrane fraction)	5.2:1

In vitro-protein synthesis was carried out in the presence of either [^{3}H]Leucine or [^{3}H]Lysine, and the relative incorporations into TCA-insoluble material was determined (see section 2.2.6.2). The figures take into account differences in specific activity of the tritiated amino acids.

10:1. This indicates that a high proportion of the products behave similarly to hordein.

3.4.1.2 Analysis of *in vitro* translation products by polyacrylamide gel electrophoresis.

The products of *in vitro*-protein synthesis were further characterised by gel electrophoresis followed by autoradiography. When the total products were run on sodium dodecylsulphate polyacrylamide gels (SDS-PAGE) a complex mixture of polypeptides was found; therefore, in order to simplify the pattern, the solubility of hordein in 55% propan-2-ol, 0.05% DTT was exploited (Fig. 27). A pattern of bands is visible in the products of polysome translation exactly co-migrating with authentic hordein. The standard hordein used for comparison was that extracted from cultured ears fed [^{35}S] sulphate (section 2.2.1.2). The Coomassie blue-stained hordein pattern of the standard was exactly as expected, but the autoradiograph shows that there is little or no label present at the position of the 'C' polypeptides (Fig. 27). However, the 'C' polypeptides are heavily labelled in the products of polysome translation. This apparent anomaly is discussed in section 3.4.3.

The propanol-soluble products of poly-A$^+$ RNA translation are shown in Fig. 28, and have a lower mobility than the *in vivo* labelled proteins on SDS-PAGE although the spatial relationship remains the same. The size difference is estimated at approximately 2000 daltons although this cannot be determined accurately

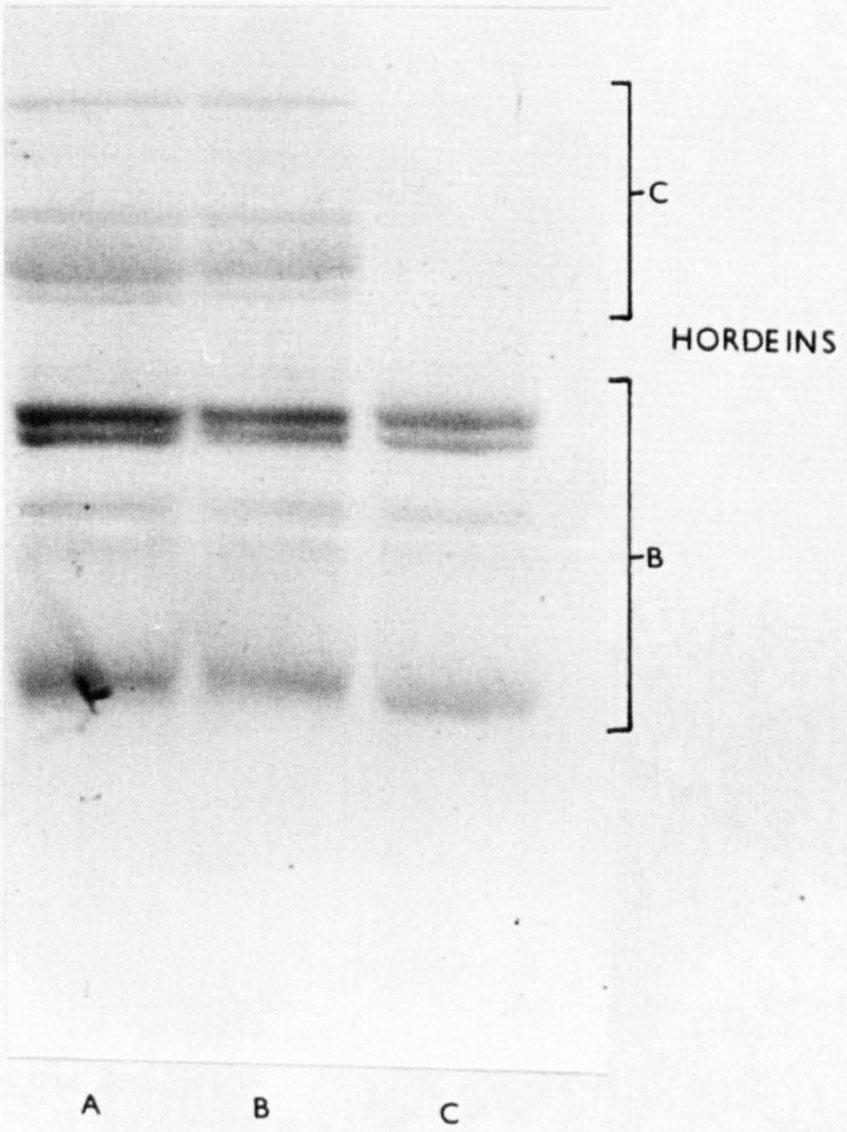

C
HORDEINS
B
A
B
C

Fig. 27 Analysis of polysome products by SDS-PAGE

Membrane-bound polysomes were translated in the wheat germ cell-free protein synthesising system in the presence of [^{35}S]methionine. The products were analysed by SDS-PAGE, and the gel was subsequently dried and autoradiographed at -80°C for 10 days (see sections 2.2.6.2, 2.2.2.1, 2.9). Tracks A and B represent polysome translation products which have been extracted with 55% propan-2-ol, 0.05% DTT and precipitated with 2% LiCl after alkylation (see section 2.2.6.3). Approximately 2.5 x 10^5 C.P.M. were loaded in each track. Track C represents authentic *in vivo*-[^{35}S]labelled hordein (0.6 x 10^5 C.P.M. loaded). The 'B' and 'C' hordein polypeptides are indicated.

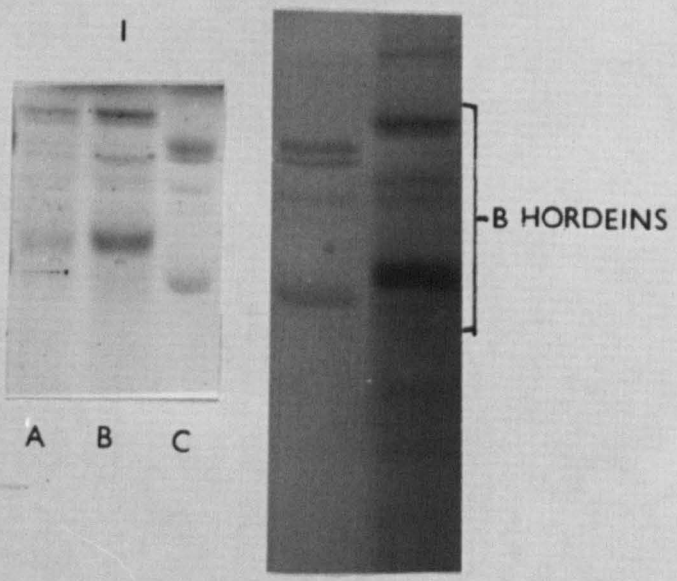
as the mobility of prolamins on SDS-PAGE does not appear to be a strict function of molecular weight (Hamauzu et al, 1975; Shewry et al, 1980a). The poly-A^+ RNA and 'wash' RNA gave apparently identical products (Fig. 28(1) tracks A and B) but the 'wash' RNA had a lower template activity (see also section 3.3.2.2). Poly-A^- RNA had negligible messenger activity and no bands were visible on an autoradiograph of propanol-soluble products.

The in vitro-translation products were characterised further by isoelectric focusing (Fig. 28(2) A,B). The products of poly-A^+ RNA translation gave a similar isoelectric focusing pattern to authentic hordein indicating that many polypeptides had identical isoelectric points. Two dimensional analysis of the poly-A^+ RNA translation products confirms this (Fig. 29), as the pattern of spots obtained on the autoradiograph is very similar to that given by authentic hordein. Careful comparison of the autoradiograph with the stained gel shows that the labelled spots have a slightly lower mobility than the stained spots (see also Fig. 28(1)), but the spatial relationship of the polypeptides remains the same.

3.4.1.3 Digestion with cyanogen bromide

In vitro-synthesised proteins directed by poly-A^+ RNA, labelled with either $[^{35}S]$ methionine or with $[^{3}H]$ proline and $[^{3}H]$ leucine, were digested with CNBr for analysis by electrophoresis and autoradiography. The pattern of tritiated fragments

1



2

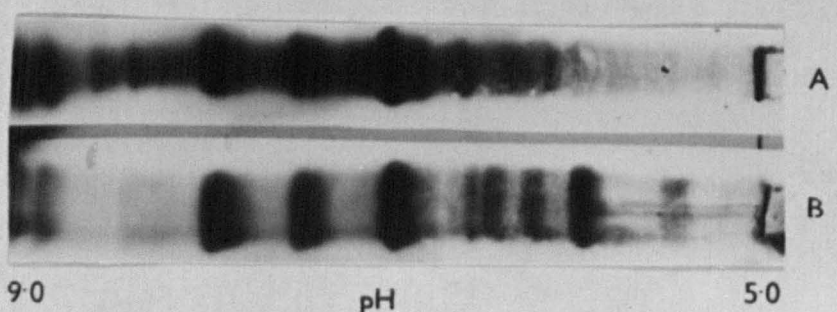


Fig. 28 Analysis of poly-A^+ RNA and 'wash' RNA translation products by SDS-PAGE and isoelectric focusing.

Poly-A^+ RNA and 'wash' RNA were translated in the wheat germ cell-free protein synthesising system in the presence of [^{35}S]methionine. The products were extracted with 55% propan-2-ol, 0.05% DTT, alkylated and precipitated with 2% LiCl prior to resuspension in the appropriate gel loading buffer (see sections 2.2.6.2/3).

(1) SDS-PAGE (see 2.2.2.1)

The translation products were analysed by SDS-PAGE and the gel was dried and autoradiographed at -80°C for 14 days. The autoradiograph is shown in the diagram. Only the 'B' hordeins are shown.

Track A: 'wash' RNA translation products (2.1×10^5 C.P.M.)

Track B: poly-A^+ RNA translation products (4×10^5 C.P.M.)

Tracks C, D: *in vivo*-[^{35}S] labelled hordein (approx. 0.5×10^5 C.P.M.)

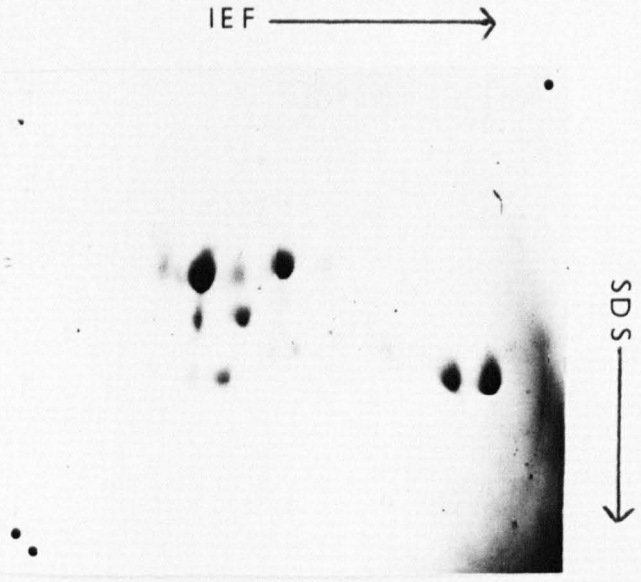
Track E: poly-A^+ RNA translation products (5×10^5 C.P.M.)

(2) Isoelectric focusing (see 2.2.2.2)

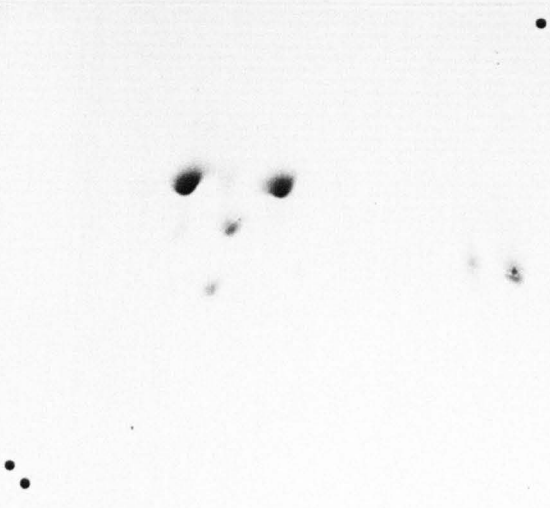
The translation products were analysed by isoelectric focusing and the gel was dried and autoradiographed at -80°C for 14 days.

Track A: poly-A^+ RNA translation products (6.4×10^5 C.P.M.)

Track B: *in vivo*-[^{35}S] labelled hordein (2.2×10^5 C.P.M.)



A



B

Fig. 29 Two dimensional analysis of poly-A^+ RNA translation products.

Poly-A^+ RNA was translated in the wheat germ cell-free protein synthesising system in the presence of [^{35}S]methionine; a 100µl assay mixture was used. The products were extracted with 55% propan-2-ol, 0.05% DTT, alkylated and precipiated with 2% LiCl prior to resuspension in the gel loading buffer (see sections 2.2.6.2/3). For two dimensional analysis 200µg carrier hordein was added. Two dimensional analysis, combining IEF and SDS-PAGE, was carried out as described in section 2.2.2.3. The gel was dried and fluorographed at -80°C for 14 days as described in section 2.9. The stained gel is shown in Fig. A and the fluorograph of the gel is shown in Fig. B; approximately 1.4×10^6 C.P.M. was loaded. The small spots at the corners of the gels are radioactive marker ink.

is similar to the stained pattern of digested hordein (Fig. 30); the 'B' polypeptides are cleaved into smaller fragments but the 'C' polypeptides are still present at the same position. All labelled methionine is lost from the ^{35}S labelled products, indicating that digestion is complete, as CNBr digests methionine residues. This observation would suggest that a methionine residue is close to the end of the 'C' polypeptides as digestion does not appreciably alter the molecular weight.

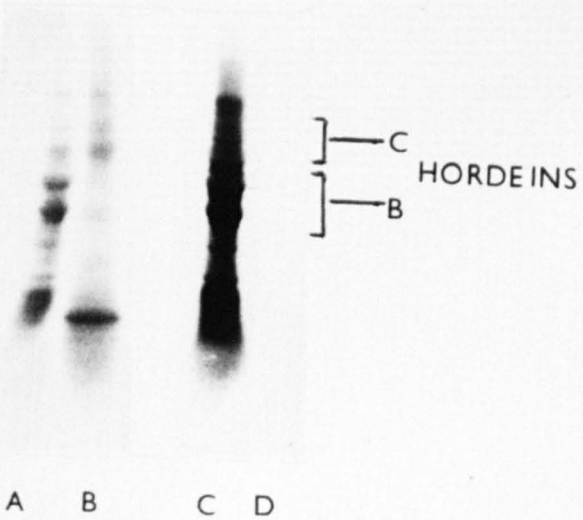
3.4.1.4 Varietal comparison

The results reported so far on the characterisation of in vitro-synthesised products have referred to RNA or polysomes extracted from the barley variety 'Sundance'. Barley varieties can be classified according to hordein pattern (Shewry et al, 1978c); therefore, some other varieties and mutants were investigated. The products were separated by SDS-PAGE and isoelectric focusing (Fig. 31). The polysome products were, in all cases, the same as authentic hordein from the same variety. The products of poly-A^+ translation were shown to be of lower mobility than authentic hordein for Riso mutant 1508, and for the 'C' bands of mutant 56. Mutant 56 is deficient in 'B' polypeptides and these are absent from the polysome products. Some polypeptides were present in this region of the gel in the products synthesised using mutant 56 polysomal poly-A^+ RNA; these did not appear to exactly comigrate with those from the parent variety, Carlsberg II. Further experiments on these mutants were not possible due to shortage of endosperm.

1

A B C D

2



A B C D

Fig. 30 Analysis of poly-A^+ RNA translation products by SDS-PAGE of CNBr cleavage fragments

Poly-A^+ RNA was translated in the wheat germ cell-free protein synthesising system in the presence of $[^{35}S]$methionine or $[^{3}H]$proline plus $[^{3}H]$leucine. The products were extracted with 55% propan-2-ol, 0.05% DTT, alkylated and precipitated with 2% LiCl (see sections 2.2.6.2/3). Carrier hordein (20µg) was added. The extracted products were cleaved with CNBr (10mg/ml in 70% formic acid) overnight and dried under vacuum. Digested and undigested samples were resuspended in SDS loading buffer for analysis on linear 5-30% SDS-polyacrylamide gels (see section 2.2.6.5). After staining, the gel was cut in half, and the tracks containing tritium were fluorographed and the tracks containing $[^{35}S]$ were autoradiographed at -80°C for 8 days.

(1) Coomassie blue-stained gel.

Tracks A, C. Undigested hordein

Tracks B, D. Digested hordein

(2) Radiolabelled polypeptides.

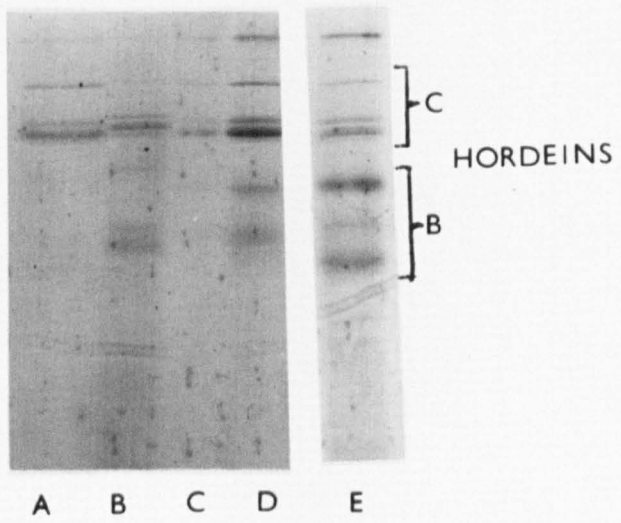
Track A: Undigested poly-A^+ RNA translation products, labelled with tritium (5×10^5 C.P.M.).

Track B: Digested poly-A^+ RNA translation products, labelled with tritium (4.5×10^5 C.P.M., before digestion)

Track C: Undigested poly-A^+ RNA translation products, labelled with [^{35}S]methionine (6.7×10^5 C.P.M.).

Track D: Digested poly-A^+ RNA translation products, labelled with [^{35}S]methionine (6.5×10^5 C.P.M., before digestion).

1



2

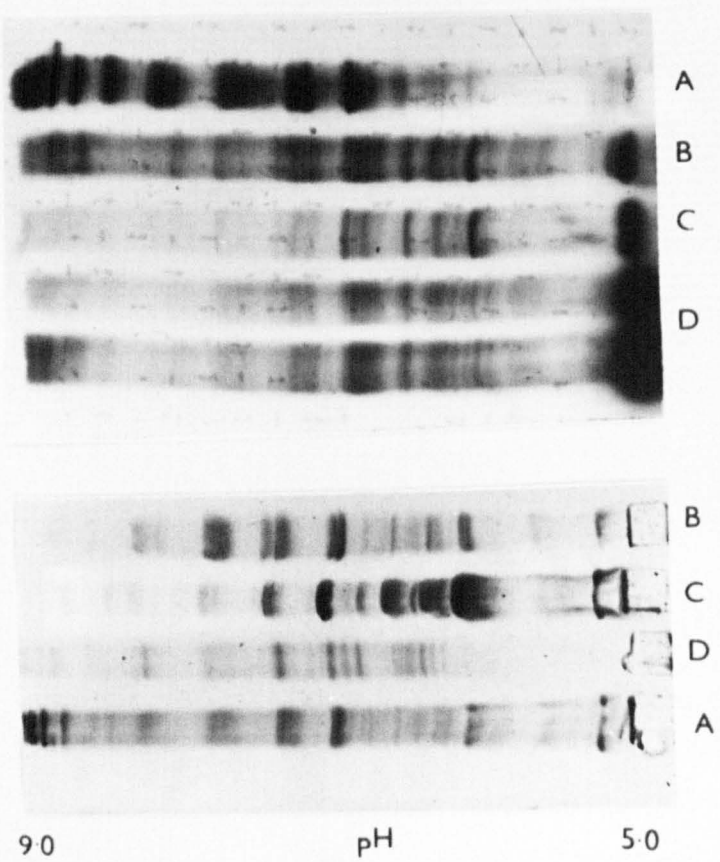


Fig. 31 Comparison of *in vitro*-synthesised products directed by polysomes or RNA from different barley varieties.

Polysomes and poly-A^+ RNA were isolated from different varieties of barley, and translated in the wheat germ cell-free protein synthesising system in the presence of ^{35}S methionine. The products were extracted with 55% propan-2-ol, 0.05% DTT, alkylated and precipitated with 2% LiCl (see sections 2.2.6.2/3) prior to analysis by SDS-PAGE (2.2.2.1) or isoelectric focusing (2.2.2.2). The gels were dried and autoradiographed at -80° for 5 days.

(1) SDS-PAGE analysis of translation products of:-

Track A. Mutant 56 polysomes (1.6×10^5 C.P.M.)

Track B. Mutant 56 poly-A^+ RNA (2.9×10^5 C.P.M.)

Track C. Carlsberg II polysomes (3.1×10^5 C.P.M.)

Track D. 1508 polysomes $^+$(2.7×10^5 C.P.M.)

Track E. Sundance poly-A^+ RNA (3.6×10^5 C.P.M.)

Carlsberg II polysome products are the same size as authentic hordein. Since all these varieties have the same 'C' polypeptide pattern, Carlsberg II polysomes act as a size standard for the 'C' polypeptides.

(2) IEF analysis of polysome translation products.

The upper tracks show the autoradiograph of the translation products, and the lower tracks represent Coomassie blue-stained protein samples of the four varieties.

Tracks A. Sundance ($7{\cdot}5 \times 10^5$ C.P.M.)

Tracks B. 1508 ($3{\cdot}9 \times 10^5$ C.P.M.)

Tracks C. Mutant 56 ($2{\cdot}1 \times 10^5$ C.P.M.)

Tracks D. Carlsberg II ($4{\cdot}1 \times 10^5$ C.P.M. & $4{\cdot}9 \times 10^5$ C.P.M.)

3.4.1.5 Immunological analysis

In vitro-synthesised products were reacted with antisera raised against purified 'B' and 'C' hordein polypeptides, and the antibody-antigen complexes precipitated with Staphylococcus aureus cells. The radioactivity bound to the antibody was determined, and the results are summarised in Table 11. There is some cross reactivity between the antibodies. These results indicate that a high proportion of the in vitro-synthesised products are antigenically similar to hordein. Gel analysis of the immuno-precipitated products was not achieved due to the difficulty of dissociating the insoluble hordein from the antibody-S.aureus complex.

3.4.2 Reinitiation during Polysome Translation

One wheat germ S30 preparation (termed WG9) consistently yielded polysome translation products with a more complex pattern of polypeptides in the propanol-soluble fraction. Bands comigrating with authentic hordein and with the larger products, characteristic of poly-A^+ RNA translation, were visible (Fig. 32, tracks a-c). This led to the investigation of the possibility that reinitiation could occur during polysome translation.

MDMP (2-(4-methyl-2,6-dinitroanilino)-N-methyl propionamide) has been reported to inhibit initiation, by interfering with the attachment of mRNA to the ribosomes (Baxter and McGowan, 1975), but does not affect the translation of previously initiated RNA. 7-methyl guanosine monophosphate (7-MeGMP) is

Table 11 Immunological analysis of *in vitro*-synthesised products

Antibody	C.P.M. retained on *S. aureus* cells	% total TCA-insoluble C.P.M. retained on *S. aureus* cells
Control (non-immune)	3000	6.9
Anti 'B'	35000	68.6
Anti 'C'	40000	83.0

Polysomes from mutant 1508 endosperm were used to direct *in vitro*-protein synthesis. The products were reacted with the antisera in the presence of 2M urea and 1% Triton X100, and then precipitated with *S. aureus* cells. The amount of radioactivity retained on *S. aureus* cells, after washing to minimize non specific binding, was determined. The method is described in full in section 2.2.6.4.

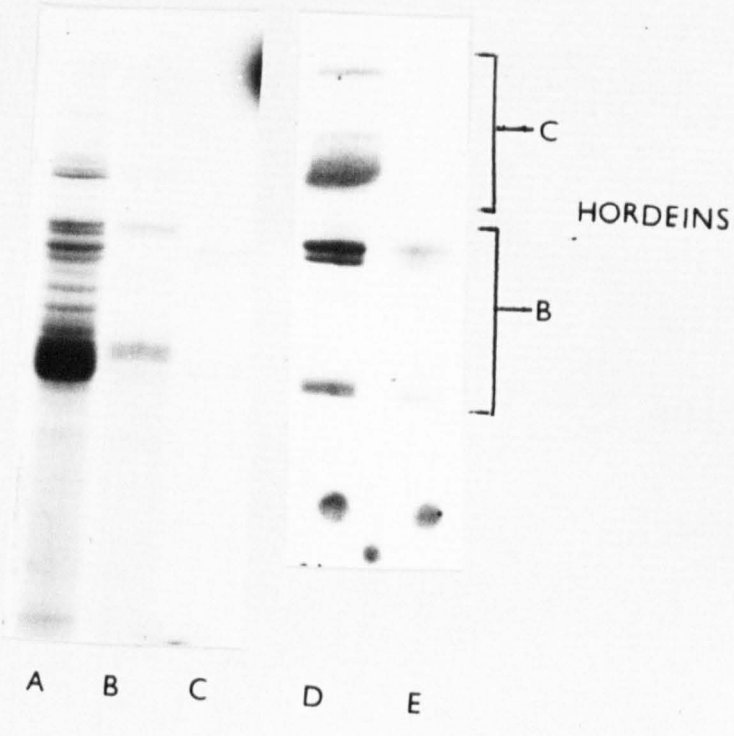

C
HORDEINS
B
A
B
C
D
E

Fig. 32 Reinitiation during polysome translation.

To test for reinitiation of polysomal RNA in wheat germ preparation 9, polysomes were translated in the absence and presence of the initiation inhibitor MDMP. The products were compared with native hordein and poly-A$^+$ RNA translation products. The products were extracted with 55% propan-2-ol plus 0.05% DTT, alkylated and precipitated with 2% LiCl (see sections 2.2.6.2/3). The extracted products were separated by SDS-PAGE and the gel was autoradiographed at -80°C for 6 days. The autoradiograph is shown in the diagram.

Track A. Polysome translation products (9.4×10^5 C.P.M.)

Track B. Poly-A$^+$ RNA translation products (4.4×10^5 C.P.M.)

Tracks C, E. *In vivo*-[^{35}S]labelled hordein (Approx. 1×10^5 C.P.M.)

Track D. Polysomes translated in the presence of 50μM MDMP (4.2×10^5 C.P.M.)

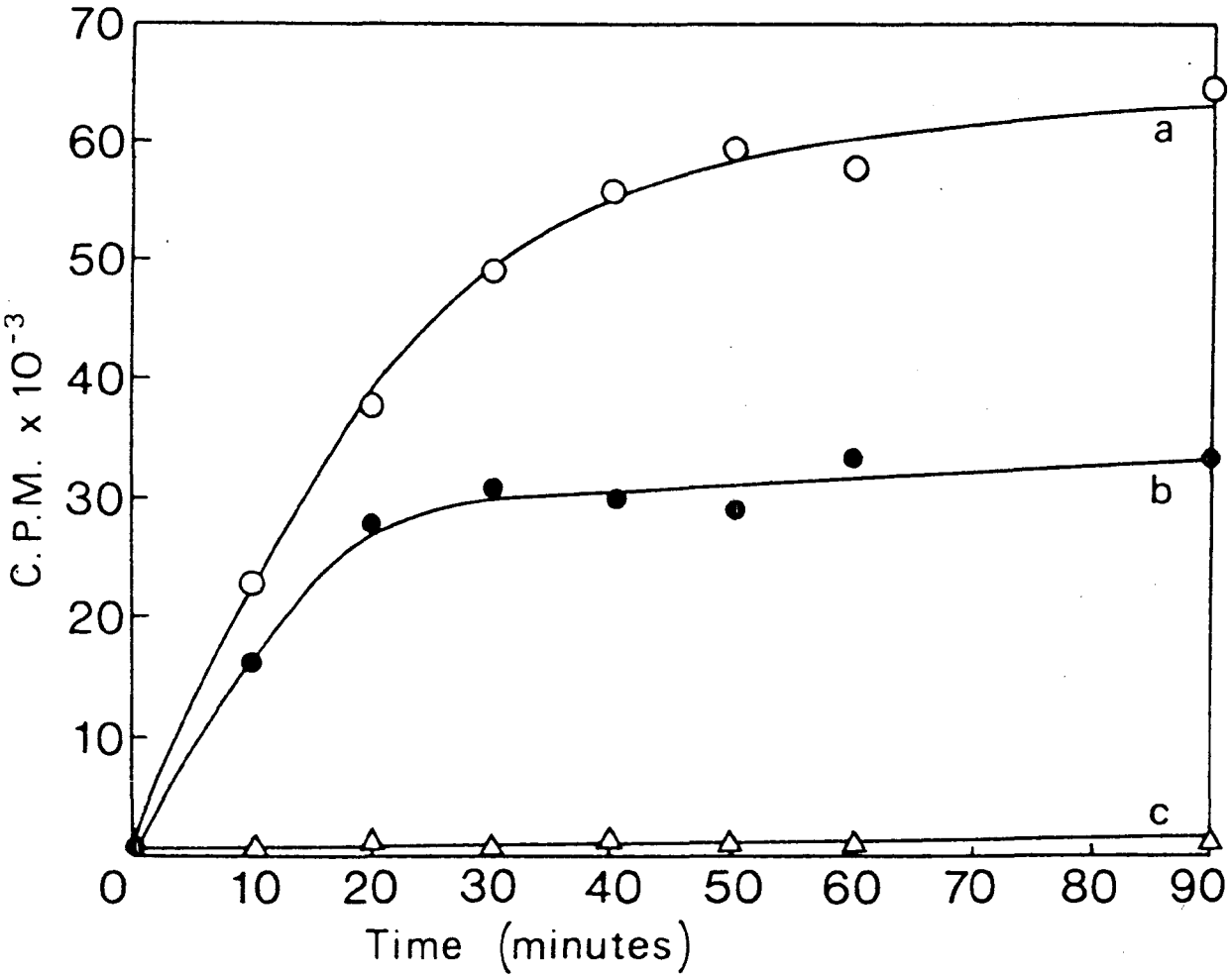

70
60
50
40
30
20
10
C.P.M. x 10-3
a
b
c
0
10
20
30
40
50
60
70
80
90
Time (minutes)

Fig. 33 Time course of polysome translation.

Polysomes were translated in the wheat germ cell-free protein synthesising system with 5uCi [^{3}H]proline and an aliquot was removed every 10min for analysis of incorporated radioactivity (see section 2.2.6.2). Curve a represents the translation of 10µg polysomes. Curve b represents the translation of 10µg polysomes in the presence of 50µM MDMP. These are compared with the incorporation when no template is added (Curve c).

an analogue of the cap structure at the 5' end of many eukaryotic mRNAs and behaves similarly, competing for the mRNA binding site on the ribosomes. The translation of poly-A^+ RNA from barley endosperm is inhibited by 92% by 0.5mM 7-Me GMP (Fig. 25, section 3.3.2.3) and by 96% by 50μM MDMP. These inhibitors inhibited polysome translation in WG9 after a lag time (Fig. 33); translation stopped after 20min instead of the usual 40min. For the first 20min, the incorporation of radioactivity was similar but after 40min the final level of incorporation was lower in the presence of the inhibitor. These results suggested that considerable reinitiation of polysomal RNA could occur although the amount varied from 23 to 40% of the total amount of translation depending on the preparation of polysomes. The SDS-PAGE pattern of the translation products in the presence of MDMP is shown in Fig. 32, track e; the larger products characteristic of poly-A^+ RNA translation are no longer present, supporting the hypothesis of reinitiation.

3.4.3 Discussion

A number of criteria have been used to identify the products of *in vitro*-protein synthesis directed by polysomes or poly-A^+ RNA from the membrane fraction of developing barley endosperm. These products are judged to be hordeins according to the following criteria:-

(1) They have the same solubility characteristics as the native proteins, being soluble in 55% propan-2-ol, 0.05% DTT and precipitated by 2% salt solutions.

(2) The incorporation of lysine into them is very low in comparison with that of leucine.

(3) They react with antisera raised against purified authentic hordein.

(4) The products of polysome translation have identical mobility on SDS-PAGE, and identical isoelectric points on IEF to authentic hordein. The poly-A^+ RNA translation products have the same isoelectric points but a lower mobility on SDS-PAGE than the authentic proteins. Two dimensional analysis shows the same spatial relationship of spots, all having a lower mobility.

(5) The products are dependent on the hor loci present in the variety of barley used as a source of RNA or polysomes.

The mRNA used in this work was purified by affinity chromatography on oligo-dT cellulose. The fraction not bound to the column (poly-A^- RNA) showed negligible messenger activity, and no polypeptides were visible on an autoradiograph of propanol-soluble products. The 'wash' RNA directed the synthesis of identical products to poly-A^+ RNA. Evidence was discussed in section 3.2.5 indicating that this RNA was a similar population to poly-A^+ RNA, but had shorter poly-A tails; these results support this hypothesis. It is therefore likely that all hordein mRNA is polyadenylated.

An apparent anomaly in the results is that 'C' polypeptides are labelled with [^{35}S]methionine in the translation products whereas they are not labelled by [$^{35}SO_4$] fed *in vivo* (Figs. 27 and 32; Shewry *et al*, 1980a). This cannot be explained by methionine in a leader sequence as polysome products, which are the native size ('run-off' products) are very heavily labelled. Part of the C-terminal amino acid sequence of hordeins has recently been published (Schmitt and Svendsen, 1980) and there appears to be a methionine residue next to the C-terminal amino acid of the 'C' polypeptides. The heavy labelling of the polysome products is due to the fact that all chains which are completed will have a methionine residue present. The 'C' polypeptides do not appear labelled after *in vivo* feeding because, at the specific activity achieved (2000 c.p.m./μg), the amount of radioactivity in the 'C' polypeptides is below the level of sensitivity of the analytical technique, as there is only one methionine residue and probably no cysteine present.

The identification of the *in vitro* synthesised products as hordeins is further reinforced by the varietal specificity of pattern visualised by autoradiography. One difference is that extra bands are visible in the 'B' region of the products of mutant 56 poly-A$^+$ RNA translation. It is interesting that these extra bands only arise in the products of the RNA derived from polysomes and not in the polysome 'run-off' products. This may simply be due to differences in the level of labelling,

as the intensity of these bands is close to the limit of detection. The bands do not appear to exactly comigrate with the parent variety, Carlsberg II, and their identity cannot be firmly established from these experiments. Mutant 56 contains a mutation mapping near the 'B' locus resulting in drastically reduced amounts of 'B' polypeptides (Doll, 1980). Some mRNAs coding for some 'B' type polypeptides appear to be present. This variety would be an interesting one to follow up as the very low level of 'B' mRNA makes it potentially useful for screening cDNA clones (see appendix 1); mutant 56 mRNA should hybridise strongly only to cloned DNA complementary to 'C' mRNAs.

The evidence provided by the translation of polysomes in the presence and absence of initiation inhibitors is consistent with the hypothesis that, in some wheat germ preparations, re-initiation and retranslation of polysomal RNA can occur. To prove this beyond doubt would require the detection of initiation complexes but the lack of larger products in the presence of MDMP, coupled with the observation that synthesis stops earlier under these conditions, strongly supports the hypothesis that, after 'run-off', the polysomal RNA can be retranslated.

The products of mRNA translation, and of reinitiation of polysomal RNA are larger than authentic hordeins but their exact molecular weight cannot be determined due to the anomalous mobility of prolamins on SDS-PAGE (3.4.1.2). It is suggested that the larger products represent precursors containing an

extra sequence which is removed during transport of the proteins across the endoplasmic reticulum. The extra sequence is largely unchanged as the precursors have similar isoelectric points to the authentic proteins. Since the polysome 'run-off' products are an identical size to native hordein, shortening (or processing) must have occurred before the polysomes were isolated from the endosperm. It is therefore suggested that processing and transport are co-translational as suggested by the signal hypothesis of Blöbel and Dobberstein (1975). The observation that polysome products are identical to the native proteins is discussed more fully in section 4 (General Discussion). The signal hypothesis predicts that the extra sequence will be N-terminal; whether this is the case has not yet been determined.

Similar results and conclusions have been presented for maize storage protein (zein) synthesis (Burr et al, 1978; Larkins and Hurkman, 1978), and evidence presented that the zein mRNA product can be correctly processed by a heterologous system i.e. Xenopus oocytes (Larkins et al, 1979). Different conclusions have been presented previously for barley storage proteins in which intermediate-sized polypeptides occur, the processing being both co and post-translational (Cameron-Mills et al, 1978a, b; see also 1.4.3). Experiments were therefore undertaken to examine the processing mechanism for barley storage proteins, and these are described in the next section.

3.5 PROCESSING OF mRNA TRANSLATION PRODUCTS

3.5.1 Processing by Plant Membranes

3.5.1.1 Proteinase K digestion after *in vitro*-protein synthesis

The processing of mRNA translation products was investigated using stripped endoplasmic reticulum fractions prepared from developing endosperms of barley in two different ways (see section 2.2.7.1). Miflin *et al* (1980a) demonstrated that hordein is associated with endoplasmic reticulum in the endosperm. One method of preparation yielded a stripped membrane fraction which still had hordein associated with it (termed stripped e.r., type 1); this method involved the isolation of endoplasmic reticulum, without sonication, in the presence of EDTA. A typical profile of a preparative sucrose density gradient run in the presence of EDTA is shown in Fig. 34 with the position of the marker enzyme indicated. Miflin *et al* (1980a) demonstrated that endoplasmic reticulum isolated in this way was free of RNA but not of hordein.

The alternative method involved isolating endoplasmic reticulum in the presence of magnesium ions and then stripping off the ribosomes by sonicating in the presence of 10mM EDTA. The fraction was then further purified by a second sucrose density gradient step (Fig. 35). These membranes were shown, by SDS-PAGE, to be free of hordein. When added to the *in vitro*-protein synthesising system neither membrane preparation had any template activity, whereas rough endoplasmic reticulum

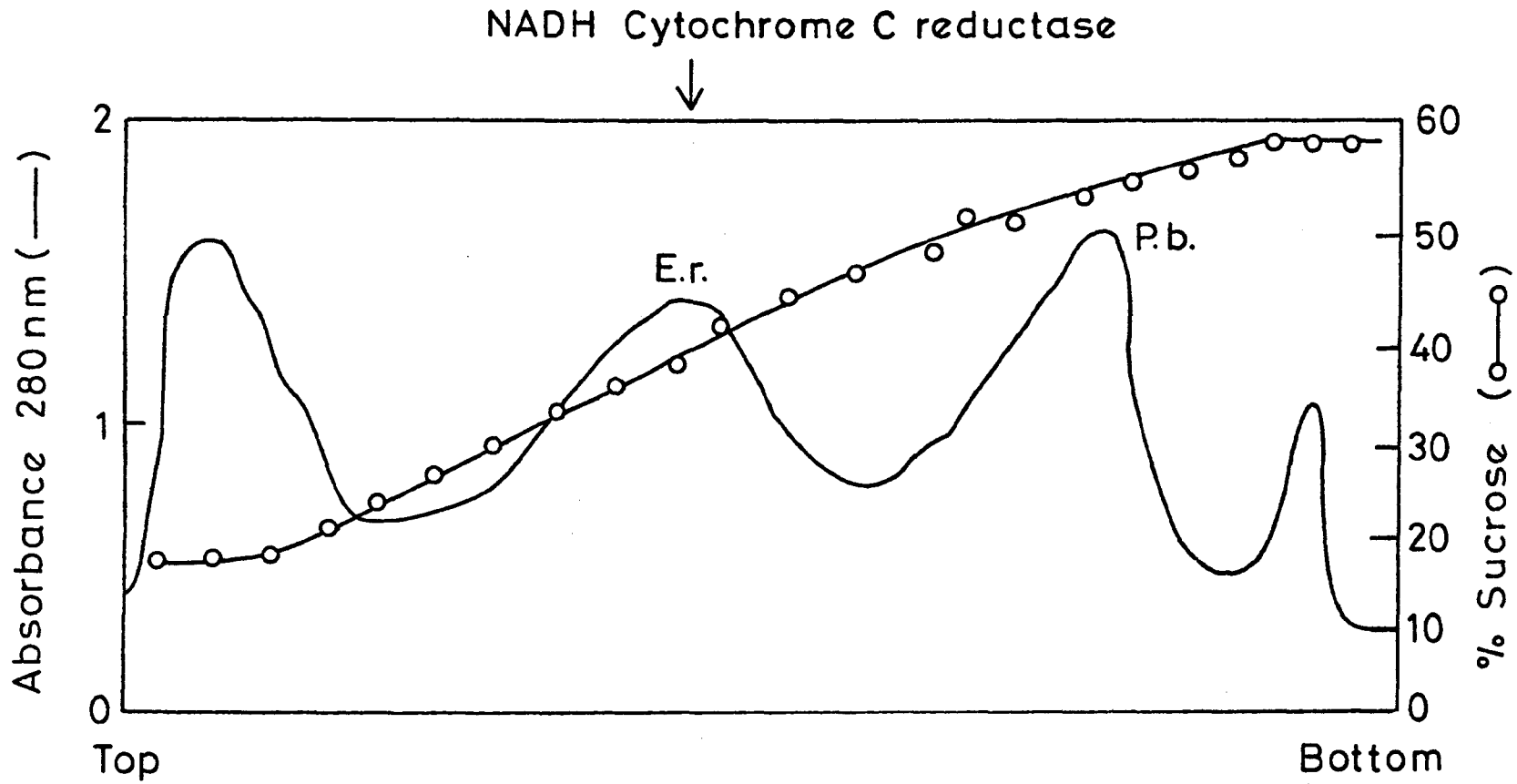

NADH Cytochrome C reductase
Absorbance 280nm (—)
% Sucrose (o—o)
E.r.
P.b.
2
1
0
60
50
40
30
20
10
0
Top
Bottom

Fig. 34 Isolation of barley endoplasmic reticulum in the presence of EDTA.

An homogenate of developing barley endosperms (3g) in 50mM Tricine pH 7.6, 100mM potassium acetate, 2.5mM EDTA, 0.2M sucrose was separated on a 20-60% linear sucrose density gradient. The gradients were centrifuged at 25000rpm for 2.5h in a Beckman SW27 rotor and fractionated at 2ml/min, using an ISCO gradient fractionator, with monitoring at 280nm. The profile of the gradient is shown in the figure with the sucrose concentrations superimposed. The position of the endoplasmic reticulum (E.r.) fraction and the protein body (P.b.) fraction was determined according to Miflin et al (1980a). The position of the major peak of the endoplasmic reticulum enzyme, NADH cytochrome c reductase, is marked (see section 2.2.7.1). The endoplasmic reticulum peak was dilute to 10% sucrose, and pelleted through a 15% sucrose cushion in 20mM Hepes-KOH pH 7.6, 100mM potassium acetate at 150000g for 2h. The pellet was resuspended in the same buffer at 40 A_{260} units/ml; this fraction is termed stripped endoplasmic reticulum, type 1.

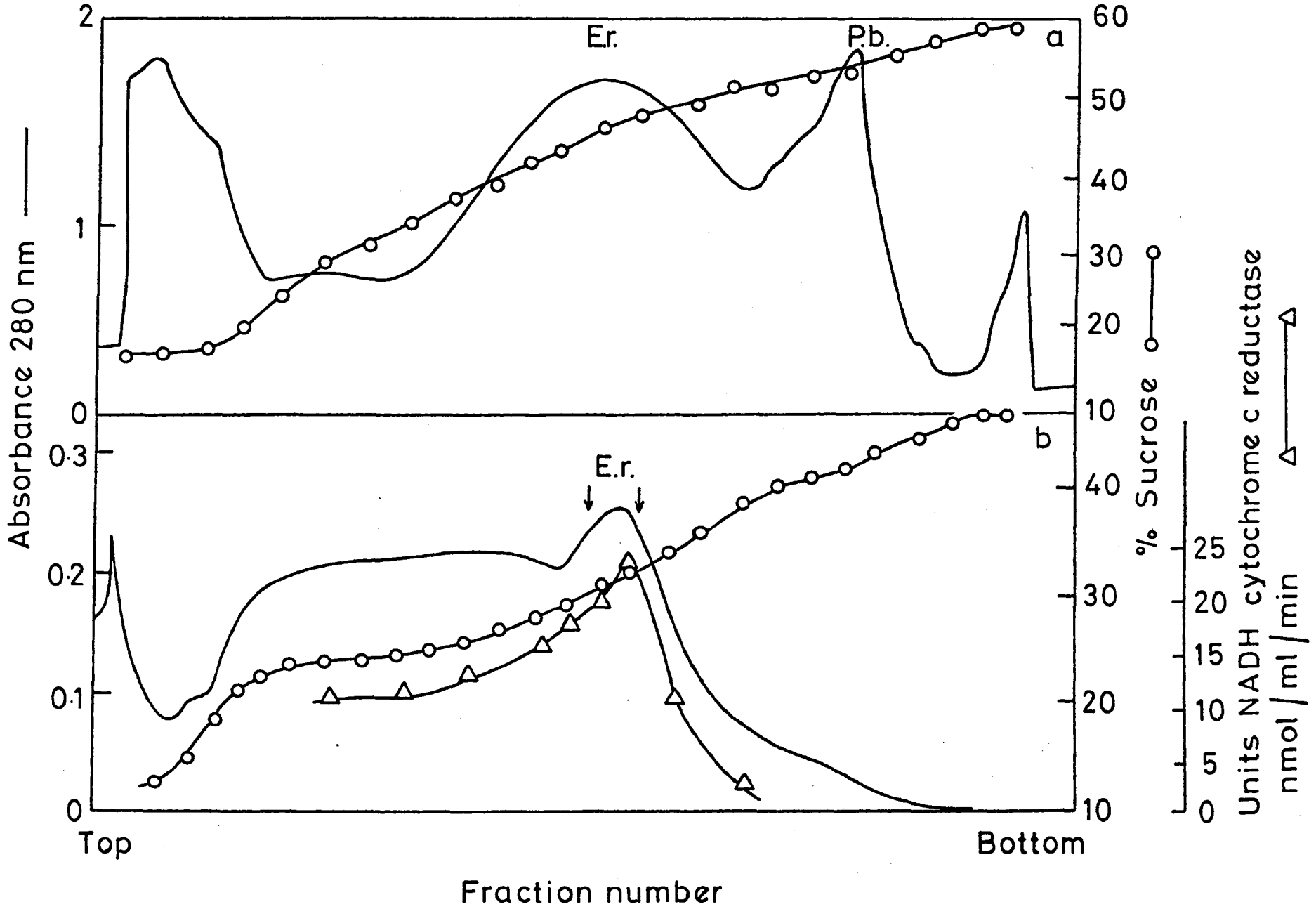

Er.
P.b.
a
b
E.r.
Absorbance 280 nm
% Sucrose
Units NADH cytochrome c reductase
nmol / ml / min
Top
Bottom
Fraction number

Fig. 35 Isolation of barley endoplasmic reticulum in the presence of magnesium ions and subsequent stripping with EDTA.

An homogenate of developing barley endosperms (3g) in 50mM Tricine pH 7.6, 100mM potassium acetate, 2.5mM magnesium acetate, 0.2M sucrose was separated on a 20-60% linear sucrose density gradient (graph a). The gradients were centrifuged at 25000rpm for 2.5h in a Beckman SW27 rotor and fractionated at 2ml/min, using an ISCO gradient fractionator, with monitoring at 280nm. The position of the endoplasmic reticulum (E.r.) fraction and the protein body (P.b.) fraction was determined according to Miflin et al (1980a). The endoplasmic reticulum fraction was diluted with an equal volume of 10mM EDTA and stirred at 2°C for 30min with 30sec sonication in a Kerry PUL55 sonic bath. This fraction was layered on to a linear 25-50% sucrose gradient prepared in the absence of magnesium ions, and centrifuged for 2.5h at 25000rpm in a Beckman SW27 rotor. The gradients were fractionated as above. Graph b shows this second density gradient; fractions were assayed for NADH cytochrome c reductase (Miflin et al, 1980a). The fraction marked E.r. was collected and diluted to 10% sucrose, and pelleted through at 15% sucrose cushion in 20mM Hepes-KOH pH 7.6, 100mM potassium acetate at 150000g for 2h. The pellet was resuspended in the same buffer at 40 A_{260} units/ml; this fraction is termed stripped endoplasmic reticulum, type 2.

isolated in the presence of magnesium stimulated the incorporation of radioactive precursors into TCA-precipitable material. The addition of membranes had no effect on polysome translation but, on some occasions, slightly stimulated the translation of poly-A^+ RNA in terms of total TCA-precipitable radioactivity (Table 12).

Vectorial transport of *in vitro*-synthesised polypeptides across membranes should result in their deposition inside vesicles and thus they should be protected from protease attack. Therefore, after *in vitro*-synthesis in the presence of membranes, the products were treated with proteinase K and the proportion of TCA-precipitable radioactivity protected from attack was determined (Table 13). Theonly products protected were those synthesised using mRNA as a template in the presence of hordein-free membranes, and to a certain extent those synthesised on rough endoplasmic reticulum. The protection was abolished by digestion in the presence of Triton X100. When membranes were added after translation, protection of the products did not occur. Furthermore, polysome 'run-off' products were not protected by the addition of membranes at any stage. All of these experiments were carried out using a wheat germ preparation which did not support reinitiation of polysomal RNA. Membranes which still had hordein associated with them did not protect the products of mRNA translation; this phenomenon is discussed in more detail below (section 3.5.3).

Table 12 The effect of membranes on cell-free protein synthesis

Template	TCA-precipitable radioactivity (C.P.M. x 10^{-3}/A260 unit polysomes or membranes or per ug RNA)	Stimulation (Fold above endogenous level)		
	Experiment 1	Experiment 2	3	4
Polysomes	425.3	24.9	24.0	28.9
Stripped e.r. type 1	1.7	1.03	1.0	0.88
Stripped e.r. type 2	2.3	1.06	-	0.98
Polysomes + stripped e.r. type 1	367.9	21.6	21.0	-
Polysomes + stripped e.r. type 2	424.4	24.8	-	24.9
Rough e.r.	129.6	8.1	10.4	9.3
Poly-A^+ RNA	93.4	17.8	36.2	33.4
Poly-A^+ RNA + stripped e.r. type 1	103.5	18.3	-	31.5
Poly-A^+ RNA + stripped e.r. type 2	96.2	19.6	36.6	-

In vitro-protein synthesis was carried out in the presence of stripped endoplasmic reticulum as described in sections 2.2.6.2 and 2.2.7.2. Experiments 1 to 4 were carried out using different polysome and RNA preparations but the same membrane preparations. In experiment 1, 7.6 µCi [^{35}S]methionine was added as precursor;

the endogenous incorporation was approximately 10^4 C.P.M. and this has been subtracted from the figures. The results of experiments 2 to 4 are expressed as the stimulation of incorporation of radioactivity above the endogenous level. The two types of stripped endoplasmic reticulum are defined in section 3.5.1.

Table 13 The effect of the addition of membranes to the cell-free translation system on the protection of the products from protease attack.

Template	Type of stripped e.r. added	Time of addition	Proteinase K digestion	%TCA-precipitable radioactivity remaining after digestion
Poly-A^+RNA	-	-	Yes	21.5
	2	Beginning	No	99.7
	2	Beginning	Yes	76.5
	2	Beginning	Yes Triton X100	22.9
	2	End	Yes	19.0
	1	Beginning	Yes	27.4
Rough e.r.	-	-	No	97.1
	-	-	Yes	52.4
Polysomes	-	-	Yes	25.7
	2	Beginning	Yes	27.7
	2	Beginning	Yes Triton X100	19.0
	2	End	Yes	20.8

In vitro-protein synthesis was carried out in the presence of stripped endoplasmic reticulum as described in sections 2.2.6.2 and 2.2.7.2 and the amount of TCA-precipitable material which was resistant to

protease attack was determined. The values represent the mean of between two and four experiments. The two types of stripped endoplasmic reticulum are defined in section 3.5.1. When membranes were added at the end of translation, the solutions were incubated for a further 30min at 28°C before digestion with proteinase K.

3.5.1.2 Analysis of products by SDS-PAGE

The products synthesised in vitro in the presence of membranes were analysed by separation on SDS-PAGE and subsequent autoradiography. Stripped endoplasmic reticulum alone did not direct the synthesis of any propanol-soluble polypeptides confirming that they had no messenger activity. The products of mRNA translation in the presence of hordein-free membrane vesicles were shown to co-migrate with polysome products and to be protected from protease attack except in the presence of Triton X100 (Fig. 36, tracks A, B, E). Products of mRNA translation in the presence of membranes containing hordein, although not protected from protease attack were also shortened to the native size (Fig. 36, tracks B, D). When membranes were added after translation, to test for post-translational modification, no shortening of the polypeptides occurred (Fig. 36, track G). Polysome products were also unaffected by the presence of membranes (Fig. 36, track C).

3.5.2 Translation Studies using Xenopus Oocytes

The processing of hordein precursors was further investigated using a heterologous processing system, Xenopus laevis oocytes (Colman and Morser, 1979). Preliminary experiments, using the standard technique of a 24h pre-incubation before a 24h incubation with radioactive amino acids, gave inconclusive results. When the total products were run on SDS-PAGE and autoradiographed, the tracks containing control

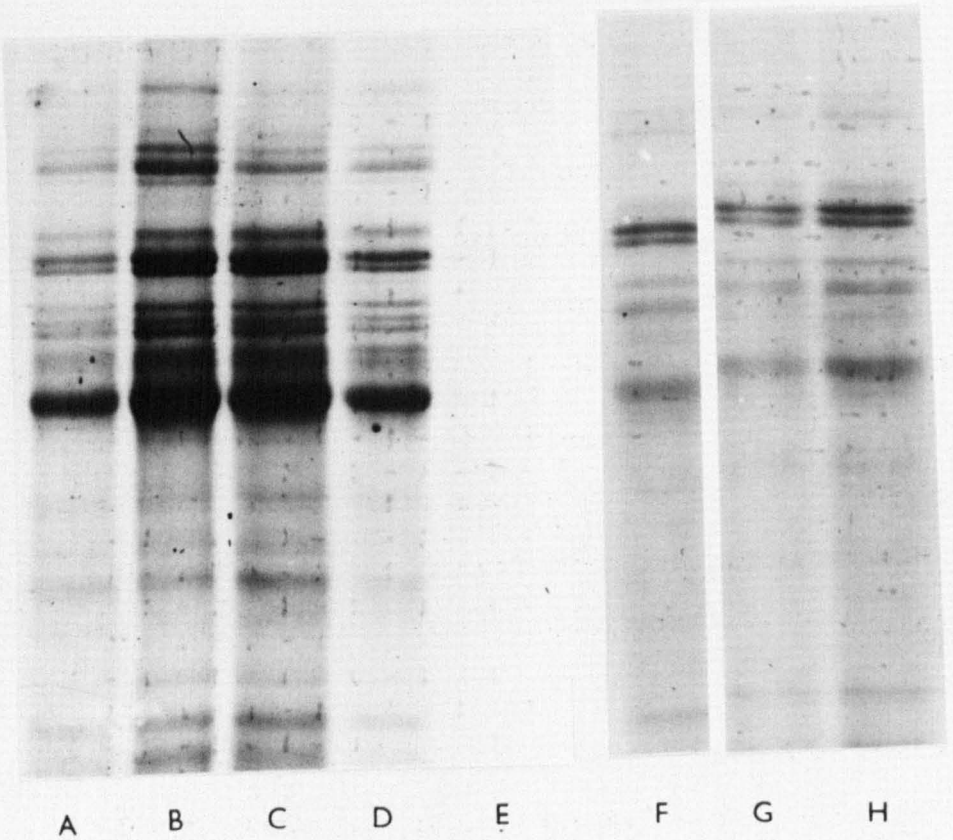

A
B
C
D
E
F
G
H

Fig. 36 The effect of membranes on in vitro-translation products

Polysomes and poly-A^+ RNA were translated in the presence and absence of membrane fractions. Some samples were treated with proteinase K after translation (see section 2.2.7.2). The products were extracted with 55% propan-2-ol plus 0.05% DTT, alkylated and precipitated with 2% LiCl prior to analysis by SDS-PAGE (2.2.6.3). The gels were dried and autoradiographed at -80°C for 7-10 days. The tracks on the autoradiographs represent translation products of:-

A. Poly-A^+ RNA plus hordein-free (type 2) membranes, digested with proteinase K;

B. Polysomes;

C. Polysomes plus hordein-free (type 2) membranes;

D. Poly-A^+ RNA plus hordein-containing (type 1) membranes;

E. Poly-A^+ RNA plus hordein-free (type 2) membranes, digested with proteinase K in the presence of Triton X100;

F. Polysomes;

G. Poly-A^+ RNA translation products incubated for 30min with hordein-free (type 2) membranes after translation;

H. Poly-A^+ RNA.

Tracks A-E represent the products of poly-A^+ RNA and polysomes prepared from a mixture of endosperms from barley varieties Sundance and Athos. Tracks F-G represent the products of poly-A^+ RNA and polysomes prepared from Sundance endosperm.

and injected oocytes were very similar, with little evidence of bands characteristic of hordein (Fig. 37, tracks A, B). The incubation medium from injected oocytes contained a number of radioactive polypeptides, mostly of low molecular weight, but again none characteristic of hordein (Fig. 37, tracks K, L). When the oocytes were extracted with 55% propan-2-ol, 0.05% DTT no polypeptides were visible on an autoradiograph.

Further experiments involved incubating injected oocytes for different periods of time both with and without pre-incubation. The total products were again separated by SDS-PAGE and autoradiographed, and on this occasion differences were visible (Fig. 37, tracks C-F). In oocytes which were incubated with [^{35}S]methionine for 3.5h directly after injection, extra bands were visible at apparent molecular weights similar to hordein. These oocytes were also extracted with 55% propan-2-ol, 0.05% DTT and the products separated by SDS-PAGE and autoradiographed (Fig. 37, tracks G-J). Bands co-migrating with authentic hordein were present in oocytes which had been incubated for short time periods.

3.5.3 Discussion

The evidence presented in the previous sections is consistent with hordein precursors being processed and transported across the endoplasmic reticulum in a single co-translational step, as suggested by the signal hypothesis

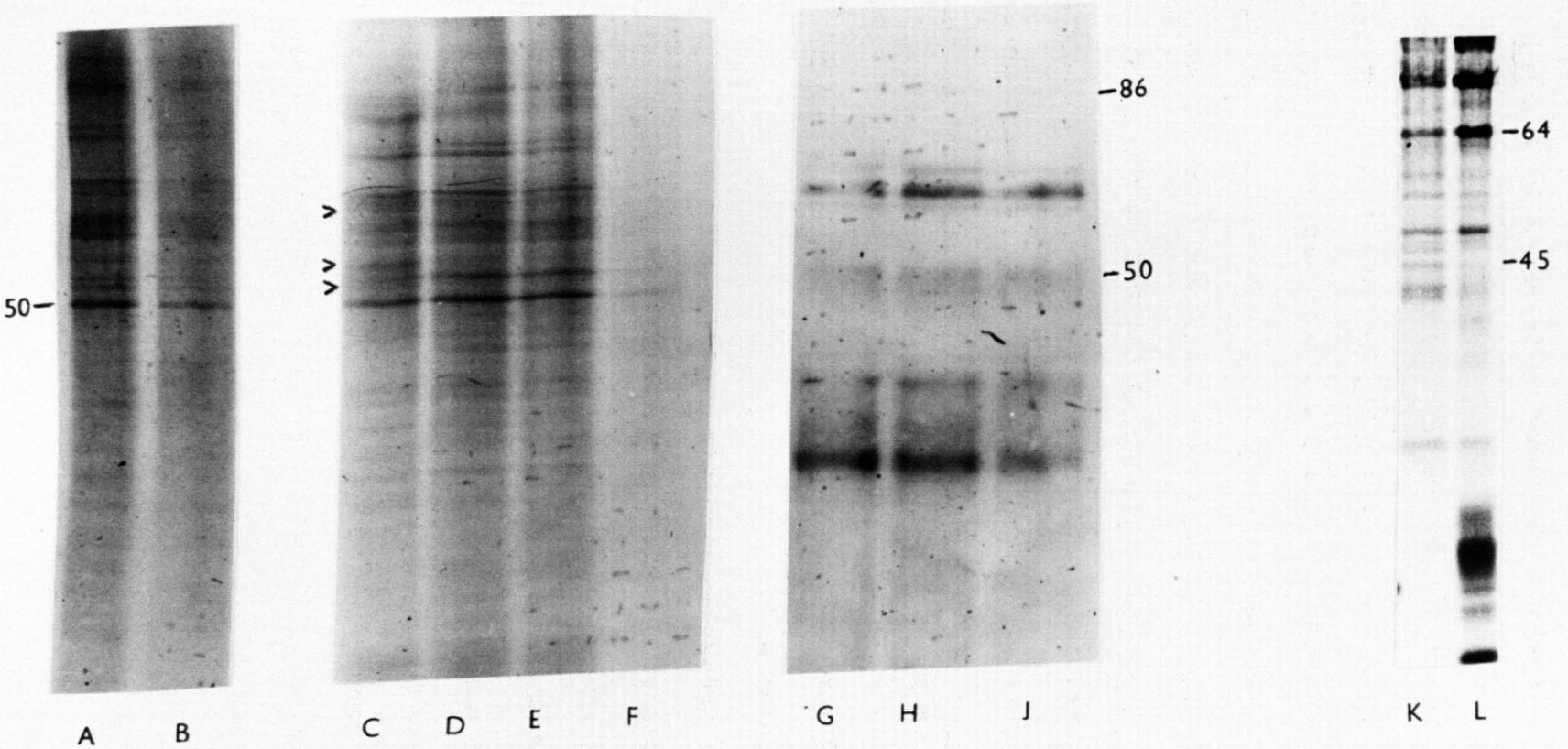

50
86
50
64
45
A
B
C
D
E
F
G
H
J
K
L

Fig. 37 Analysis of products synthesised in Xenopus oocytes

Poly-A$^+$ RNA was injected into Xenopus oocytes, and the oocytes were incubated with [^{35}S]methionine for varying periods of time (see 2.2.8.1). Samples of the oocytes and incubation media were prepared for analysis by SDS-PAGE as described in section 2.2.8.2. The gels were dried and autoradiographed at -80°C for 3 days (total products) or 10 days (propanol-soluble products). The tracks in the autoradiographs represent:-

A. Total products: oocytes were injected with endosperm poly-A$^+$ RNA and incubated for 24h prior to 24h incubation with [^{35}S]methionine.

B. Total products: control (non-injected) oocytes were incubated as A.

C. Total products: injected oocytes were incubated immediately with [^{35}S]methionine for 3.5h.

D. Total products: injected oocytes were incubated immediately with [^{35}S]methionine for 17h.

E. Total products: injected oocytes were incubated immediately with [^{35}S]methionine for 24h.

F. Total products: control oocytes were incubated as C.

G. Propanol-soluble products: injected oocytes were incubated as D.

H. Propanol-soluble products: polysomes were translated in the wheat germ cell-free protein synthesising system.

J. Propanol-soluble products: injected oocytes were incubated as C.

K. Incubation medium: control oocytes were incubated as A.

L. Incubation medium: injected oocytes were incubated as A.

The figures at the sides of the autoradiograph give an indication of the apparent molecular weights ($\times 10^{-3}$).

(Blöbel and Dobberstein, 1975). The evidence is:-

(1) The mRNA translation products are deposited within membrane vesicles during translation.

(2) The mRNA translation products are shortened to the native size when translation takes place in the presence of stripped endoplasmic reticulum.

(3) No post-translational processing occurred.

(4) Translation of rough endoplasmic reticulum yields native sized products, a proportion of which are protected from protease attack.

(5) Translation of mRNA (shown by other criteria to specify hordein polypeptides) in oocytes, over short time periods, yields polypeptides soluble in 55% propan-2-ol, 0.05% DTT and of a similar size to authentic hordein.

In all of this work there is no evidence for any in vitro synthesised polypeptides intermediate in size between the native proteins and the primary mRNA translation products.

The translation of mRNA in the wheat germ cell-free system was not significantly inhibited by barley membrane vesicles, which is in contrast to what is found for dog pancreas membranes (Shields and Blöbel, 1978). There are a number of other points also requiring further discussion. Hordein-contaminated membranes did not protect processed products from

attack by protease and, during RNA translation in oocytes, the products disappeared after long incubations. This suggests that the proteins were being degraded in the oocytes. On the basis of studies of protein body structure and formation, Miflin et al (1980a, b) proposed that hordein aggregates within the lumen of the endoplasmic reticulum to form clumps of protein which disrupt the membrane. These aggregates or protein bodies are, therefore, associated with endoplasmic reticulum but not completely surrounded by it. The in vitro-processing work supports this hypothesis. Hordein-contaminated membranes processed the mRNA products but did not protect them from protease attack, whereas hordein-free membranes did offer protection. The actual amount of protein synthesised in vitro would not be enough to disrupt the membranes, but, when the membranes are already associated with hordein, the aggregates are large enough to disrupt the membranes, and the transported, processed products are not protected. Xenopus oocytes synthesise more protein than the wheat germ cell-free system; in this case, large enough aggregates of the newly synthesised hordein might be formed and the endoplasmic reticulum disrupted. Since proteolytic activity has been reported in oocytes and shown to degrade foreign proteins within the cytoplasm (Lane et al, 1979), the hordein products could thus be broken down.

The results with Xenopus oocytes also suggest that the mRNA is being degraded as no new protein is being synthesised

after long time periods. Alternatively, newly synthesised protein could, at this stage, be being degraded as it is synthesised if the endoplasmic reticulum has been severely disrupted. It is known that polyadenylated RNA is usually stable within oocytes (Gurdon et al, 1973; Huez et al, 1978), therefore hordein mRNA would be expected to be stable.

In contrast to these results with hordein, Larkins et al (1979) have reported that the maize storage proteins (zein) were synthesised and processing in Xenopus oocytes in response to injected mRNA over a long period of time (48h). However, these differences between maize and barley are also in agreement with the results of Miflin et al (1980a, b) who demonstrated that maize protein bodies differ from those of barley in that they are completely surrounded by endoplasmic reticulum and resistant to proteolytic degradation. The results of Larkins et al (1979) are also consistent with the signal hypothesis; the pre-zein polypeptides were processed correctly by the oocytes as determined by amino-terminal sequence analysis of zein synthesised in oocytes. No sequence data is available for the barley products synthesised in oocytes to confirm correct processing.

Previous work on barley storage proteins suggested that transport of hordein into microsomes could occur either co or post-translationally and did not require the presence of the

signal sequence found on mRNA translation products (Cameron-Mills *et al*, 1978b). These workers claimed that polysome products were of an intermediate size between mRNA translation products and native hordein and that the transport of polysome products into microsomes involved no change in size. No evidence is presented here to support such a mechanism; no transport of polysome products was detected. The polysome 'run-off' products were clearly identical to native hordein in terms of isoelectric point and mobility on SDS-PAGE; This observation is discussed in more detail in the general discussion. The products of mRNA translation were processed to an identical size to polysome products both by barley stripped endoplasmic reticulum and by *Xenopus* oocytes, suggesting a simpler mechanism involving a single co-translational step. This is consistent with results reported for both animal secretory proteins crossing the endoplasmic reticulum (Blöbel *et al*, 1979) and for maize storage proteins (Larkins *et al*, 1979).

4. GENERAL DISCUSSION

A rapid and efficient method is described in this thesis for the preparation of polyadenylated RNA from the membrane fraction of developing barley endosperm. This RNA fraction is highly enriched in sequences coding for hordein, as demonstrated by characterisation of the products of *in vitro*-translation. The enrichment is largely due to the age of the endosperm (see section 1.2.2, Fig. 5) and to the separation of membrane-bound polysomes from free polysomes during the isolation procedure (Fox *et al*, 1977). The products of *in vitro*-translation behave similarly to authentic hordein in terms of solubility, amino acid composition, iso-electric focusing pattern and immunological characteristics, but they have lower mobilities on SDS-PAGE. Two-dimensional analysis yields very similar patterns for the *in vitro*-synthesised products and the authentic proteins. This observation supports the hypothesis that many of the polypeptides observed on two-dimensional gels are products of different genes and are not simply artefacts of preparation and separation techniques. The proportion of the *in vitro*-synthesised products behaving as hordein varies from 50-80% depending on RNA preparation. This RNA fraction is considered to be suitable starting material for the synthesis of complementary DNA (cDNA) which can be cloned in bacterial plasmids; these experiments are outlined in Appendix 1.

The bulk of the mRNA fraction is polyadenylated but it is contaminated with some rRNA. The degree of adenylation is approximately 6.5% which is similar to other reported values. In vitro-translation studies suggest that all hordein mRNAs are polyadenylated, and also provide circumstantial evidence that the hordein mRNAs are capped. Sonenberg and Shatkin (1978) pointed out that many non-specific interactions can occur when testing for a cap with the initiation inhibitor 7-methyl GMP. However, the inhibition appears to be specific in this case as GMP did not interfere with initiation; the potassium ion concentration-dependence of the inhibition caused by 7-methyl GMP also closely followed that described by Kemper and Stolarsky (1977).

The sizes of the major components of the hordein-enriched mRNA fraction are large enough to code for the hordeins, even when polyadenylation and the presence of other extra sequences are taken into account. Brandt and Ingwersen (1978) reported that hordein-enriched mRNA contained components of approximately 1.3, 1.0 and 0.8kb. These are not large enough to code for the 'C' hordeins; however, these workers did not take into account the possibility that messenger activity may co-migrate with the rRNA bands during electrophoresis. It has been clearly demonstrated (B. Bahramian, M. Kreis; unpublished) that messenger activity does coincide with the 18s rRNA band.

The primary translation products directed by hordein mRNA *in vitro* are larger than the authentic proteins. The larger size is interpreted as indicating the presence of a signal sequence involved in the translocation of the polypeptides into the lumen of the endoplasmic reticulum. The results presented in section 3.5 are consistent with the translocation occurring co-translationally as suggested by the modified signal hypothesis (Blöbel *et al*, 1979). The evidence is based both on *in vitro* studies using barley stripped endoplasmic reticulum and on the injection of mRNA into *Xenopus* oocytes. The same mechanism appears to be occurring in plant and animal cells. This situation is similar to that occurring for zein (Larkins *et al*, 1979) but contradicts the results reported by Cameron-Mills *et al*,(1979b) for hordein. These latter workers investigated the transport of polysome translation products, which were smaller than mRNA translation products. They claimed that a change in size was not necessary for translocation and that translocation could occur either coincident with or after translation. This is not totally consistent with the modified signal hypothesis; Blöbel (1980) indicated that a protein, and in fact a membrane system, would be expected to show either co- or post-translational transport but not both.

The products formed when polysomes of developing barley endosperm are translated *in vitro* are identical in size to

authentic hordein. This phenomenon has been reported in other systems where a precursor is synthesised in response to mRNA, for example:- maize storage proteins (Larkins *et al*, 1976a, Violti *et al*, 1978), french bean storage proteins (Sun *et al*, 1975; Hall *et al*, 1978), pea vicilin (Higgins and Spencer, 1980). Cameron-Mills *et al* (1978a), working with barley, stated that polysome products were of an intermediate size, whereas Evans *et al* (1979) and Croy *et al* (1980), working with pea, indicated that vicilin is synthesised *in vitro* at the native size using either polysomes or mRNA as a template. It is expected from the signal hypothesis that isolated polysomes would synthesise a mixture of the native and precursor forms; many nascent chains would already have been processed *in vivo* but some, which had recently initiated, would still have the signal peptide attached. Okita *et al* (1979) demonstrated that this was the case for the *in vitro*-synthesis of α-amylase directed by polysomes derived from wheat aleurone cells; both precursor and native forms were visualised by autoradiography of *in vitro*-synthesised products.

In some experiments barley polysomes yield a mixture of products (see Fig. 32) but this is due to reinitiation of the polysomal RNA, as shown by the effect of initiation inhibitors. The presence of precursor forms in the polysome translation products is dependent on the wheat germ extract used, and not

on the polysome preparation; this has been confirmed by M. Kreis (unpublished). The question remains, therefore, as to why, in many cases, only native-size products are formed by the completion of nascent chains on polysomes. The phenomenon cannot be explained by processing activity in the wheat germ extract because mRNA directs the synthesis of larger products, except in the results for pea reported by Evans et al (1979) and Croy et al (1980). Higgins and Spencer (1980) suggested that their polysome preparations were contaminated with processing activity, but this is also unlikely as the reinitiating system described here for polysomes still synthesises precursors. A possible explanation is that the precursors synthesised on polysomes are not observed on quantitative grounds. For the 'C' band hordeins less that 10% of the ribosomes would be expected to be associated with a nascent chain containing the signal sequence at any one time, assuming an even distribution of the length of nascent chains and that at least 40 amino acids must be polymerised before cleavage can take place. These ribosomes will either not be attached to or be associated loosely with the membrane, and hence are more likely to be lost from the membrane during the isolation procedure. The proportion of precursor-size products expected is, therefore, very low, possibly below the limits of detection. A higher proportion of precursor-size products would be expected to be synthesised by the free polysome fraction;

this has not yet been observed but is being further investigated. These results are not considered to contradict the evidence that hordein precursors are processed in a manner consistent with the signal hypothesis. The results do not support a more complex mechanism suggested by the results of Cameron-Mills *et al* (1978b). The signal mechanism appears to occur in both plant and animal systems.

The main aims of this project have, therefore, been achieved. The mRNA fraction enriched in sequences coding for hordein have been used successfully for the production of cDNA clones. As described in Appendix 1, five clones have so far been identified and all appear to be complementary to mRNA coding for the largest 'C' hordein. Work is in progress to produce more clones complementary to other hordein mRNAs. These clones will be a useful tool for the isolation of genomic DNA and the analysis of the *hor* loci, both in normal and some high-lysine lines (for example:- mutant 56 from Carlsberg II, in which the mutation is close to the *hor*-2 locus). Whether it is feasible to manipulate the storage protein genes in order to manipulate the storage protein genes, in order to improve the nutritional quality of barley, remains to be seen. Such manipulation may alter the properties of the genes and proteins to such an extent as to interfere with the development of the endosperm. However, the mechanisms of synthesis, translocation and deposition of the proteins are now better understood, and this provides a groundwork for further investigations along these lines.

Appendix 1 The Cloning of Double-stranded DNA Sequences Complementary to Hordein mRNA

Acknowledgements

This work was carried out in collaboration with other workers. Initial cloning experiments were carried out by Dr. R. Thompson at the Plant Breeding Institute, Cambridge. The E. coli clones described here were produced by Dr. B. Forde in the laboratory of Dr. J. Bishop, Edinburgh. The hybridisation work involved in clone analysis was carried out by Dr. B. Bahramian. I isolated the RNA fractions and performed translations. The use of facilities in other laboratories was necessary in order to comply with biological containment (category 2) regulations.

1. Introduction

The cloning of eukaryotic structural gene sequences by insertion of double-strnaded DNA synthesised from purified mRNA into bacterial plasmids has led to a greater understanding of the structure of eukaryotic genes, and provided a good method of sequencing the proteins for which they code. Cloned sequences of this type can subsequently be used to isolate genomic DNA in order to compare mRNA and gene sequences. The first complementary DNA (cDNA) to be cloned in this way was synthesised from rabbit globin mRNA (Higuchi et al, 1976; Maniatis et al, 1976; Rabbitts, 1976; Rougeon et al, 1975).

Subsequently many other mRNAs have been treated in the same way, e.g. ovalbumin (McReynolds et al, 1977), vitellogenin (Smith, Searle and Williams, 1979). Comparison of cloned cDNA and genomic DNA have revealed that the structural sequences of many eukaryotic genes contain several non-coding regions e.g. ovalbumin (Breathnach et al, 1977; Lai et al, 1978). These types of studies have also led to the investigation of the sequences surrounding structural genes which may be involved in the control of gene expression, e.g. globin (Jeffreys and Flavell, 1977).

A general methos for cloning cDNA was described by Higuchi et al, 1976; the method is shown diagrammatically in Fig. 38. A review of the enzymes involved in this procedure and of types of useful plasmid vectors can be found in Malcolm (1979) and Sherratt (1979). Plasmids have been constructed with the intention of simplifying clone identification by enabling selection of those clones which contain a recombinant plasmid. For example, one widely used plasmid of E. coli, pBR322, contains two drug resistance genes for ampicillin and tetracycline; insertion of a sequence at the single Pst 1 site results in the destruction of the ampicillin resistance gene. Therefore, colonies of bacteria which have been transformed by a recombinant plasmid will be tetracycline-resistant but ampicillin-sensitive.

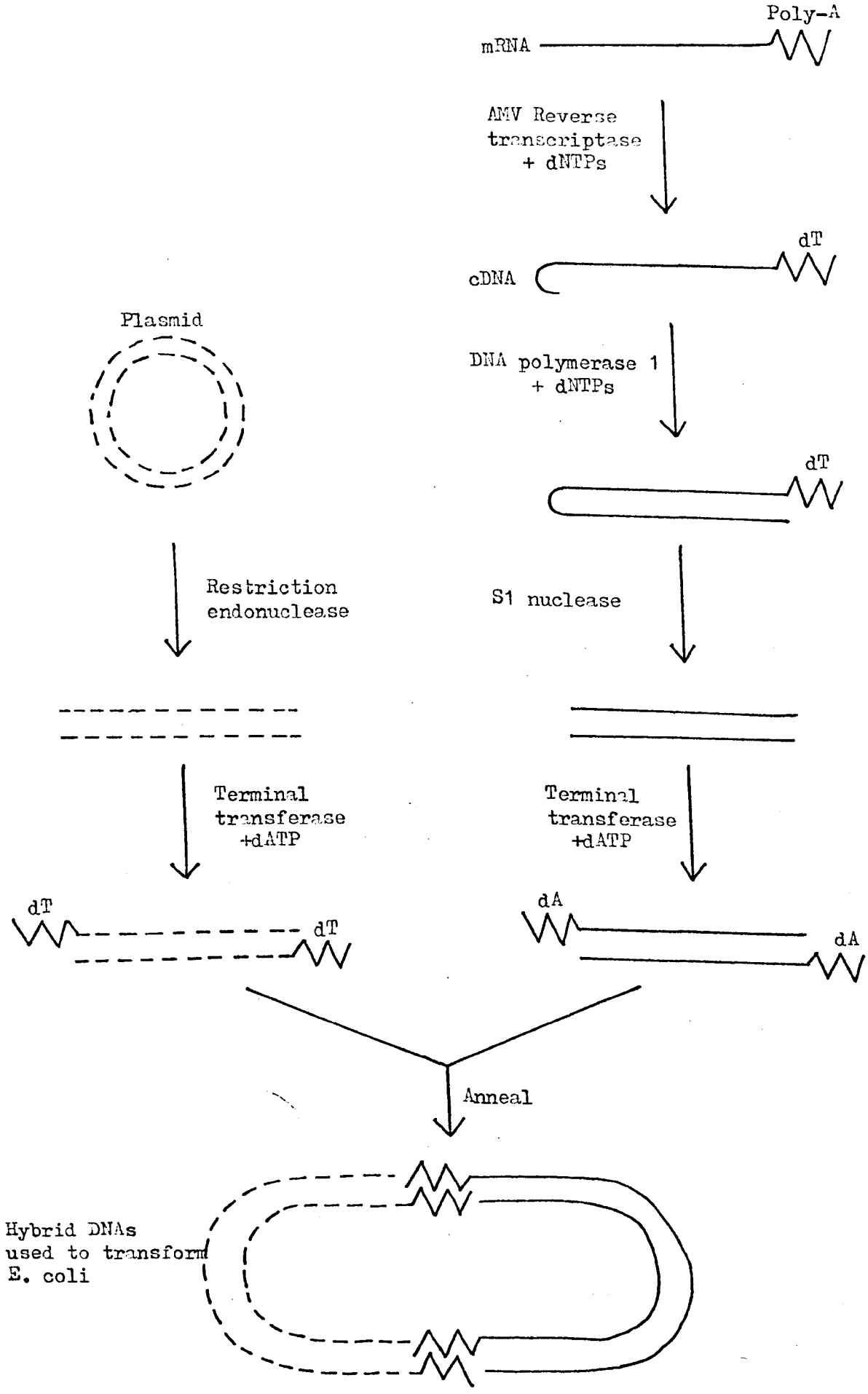

Poly-A
mRNA
AMV Reverse transcriptase + dNTPs
dT
cDNA
Plasmid
DNA polymerase 1 + dNTPs
dT
Restriction endonuclease
S1 nuclease
Terminal transferase +dATP
Terminal transferase +dATP
dT
dT
dA
dA
Anneal
Hybrid DNAs used to transform E. coli

Fig. 38 Principles of cloning.

Diagram of the enzymatic steps leading to the creation of duplex DNA and its insertion into a bacterial plasmid.

After this first selection procedure, colonies can again be selected by hybridising the plasmids to radiolabelled RNA or cDNA. This procedure was described by Grunstein and Hogness (1975) and involves lysis of colonies on Millipore filters. The filters are then incubated with radiolabelled RNA or cDNA under hybridising conditions and subsequently washed, dried and autoradiographed. Colonies which are radiolabelled probably contain a sequence complementary to components in the RNA population although strict controls must be used, for example: hybridisation of poly-A sequences is possible.

Clones can be further characterised by translation assays. Patterson et al, 1977 described a method in which the recombinant plasmids were hybridised to an mRNA population; this mixture was placed in a cell-free translation system but any sequence involved in hybridisation would be unavailable for translation. Hence, the sequence in the recombinant plasmid would code for the polypeptide which was missing from the translation products. This assay was termed hybrid-arrested translation. A second type of translation assay is to immobilize the plasmid on filter paper and hybridise the mRNA population to it. The mRNA can then be eluted from the hybrid and translated; the mRNA selected in this way should code for the single polypeptide encoded in the cloned DNA. This method was described by Alwine et al, 1980.

Brandt (1979) has reported the cloning of cDNA synthesised from hordein mRNA. The cloning was carried out essentially as

described in Fig. 38 using the plasmid, pBR322. The cDNA was inserted at the Hind III site which is in the tetracycline resistance gene. Colonies were screened for drug resistance and then by the procedure of Grunstein and Hogness, 1975. Final identification was made by hybrid-arrested translation. One clone was identified which contained an 850 base pair insert which hybridized to mRNAs coding for two 'B' hordein polypeptides.

The aim of the present work is to produce E. coli clones containing cDNA sequences complementary to particular hordein mRNAs. The long term aim is to use these clones to isolate genomic DNA in order to investigate the structure of the Hor loci.

2. Materials and Methods

2.1 Materials

Materials were obtained from the sources described in section 2.1 of this thesis. In addition, avian myeloblastosis virus reverse transcriptase was obtained from Dr. J. Beard, Life Sciences Inc., Florida. Other enzymes were obtained from the Boehringer Corporation Ltd.

The RNA used as the starting material was poly-A^+ RNA prepared from barley endosperm by the SDS/NaCl method described in section 2.2.4.3. The poly-A^+ RNA was purified twice by oligo-dT cellulose chromatography.

2.2 Methods

2.2.1 Synthesis and characterisation of complementary DNA (cDNA).

The method used was that described by Efstratiadis et al (1976). The poly-A^+ RNA fraction was copied into single-stranded cDNA using reverse transcriptase and the second strand was then synthesised using E. coli DNA polymerase 1. Radiolabelling was achieved by the inclusion of either deoxy-cytosine 5'- γ -$[^{32}P]$ triphosphate or $[^{3}H]$ deoxy-cytosine triphosphate as precursor. The double-strnaded DNA prepared in this was possessed a hairpin loop at the 3' end of the first strand; this hairpin was digested with S1 nuclease. Some samples of double-stranded cDNA were cleaved with the restriction nuclease, Hae III.

Samples of cDNA were analysed by polyacrylamide gel electrophoresis. The 6% polyacrylamide slab gel (16 x 18 x 0.15cm) contained 25% glycerol, 90mM Tris-borate buffer pH 8.0, 2.5mM EDTA. Samples were loaded in the same buffer containing 50% (v/v) glycerol, 0.004% (w/v) bromophenol blue. Electrophoresis took place at 16mA for approximately 16 hours. The gel was stained in 1μg/ml ethidium bromide, destained in water and viewed under ultra-violet light; the gel was then dried down for autoradiography (see 2.2.9 in main thesis).

2.2.2 Cloning of double-stranded cDNA.

Double-stranded cDNA, which had been treated with S1 nuclease to remove the hairpin loop, was inserted into a plasmid of E. coli by dA-dT tailing (Wensink et al, 1974; Higuchi et al,

1976). A homopolymer of poly-dA was added to the double-stranded cDNA using terminal transferase. The bacterial plasmid pPH207* was nicked with Hind III, and a homopolymer of poly-dT was added to thr linearised molecule. This plasmid contains two drug resistance genes for chloramphenicol and tetracycline; the single Hind III site occurs within the tetracycline resistance gene. The tailed cDNA and plasmid were annealed together and this DNA was used to transform E. coli HB101 cells. The cells were grown on LB agar plates containing chloramphenicol. Colonies which grew under these conditions were then tested on tetracycline plates. Those colonies which were chloramphenicol-resistant and tetracycline-sensitive were assumed to contain cDNA inserts in the plasmid, and were investigated further

2.2.3 Identification of clones.

The size of the plasmid present in each E. coli clone, which had been selected by drug resistance, was determined by analysis of single colony lysates on polyacrylamide gels (Barnes, 1977). Those plasmids containing inserted sequences were then isolated from the cells and purified (Zasloff et al, 1978), and the sizes of the inserts determined more accurately by restriction enzyme mapping.

To investigate the relationship of the cloned sequences to the original mRNA population, the 'Northern-blot' technique was used. The poly-A RNA was separated by agarose gel electro-

* Dr. J. Bishop, personal communication. This plasmid is derived from pMB9 (see Sherratt, 1979).

phoresis in the presence of Methyl-mercury hydroxide as described by Bailey and Davidson (1976). The RNA was then transferred from the gel to diazobenzloxymethyl-paper (DBM-paper) and hybridised to the plasmid which had been radiolabelled by nick-translation (Alwine et al, 1977; Rigby et al, 1977; Wahl et al, 1979). The DBM paper was then autoradiographed to locate bands which had hybridised to nick-translated plasmid DNA. Plasmid DNA containing no inserted sequences was used as a control.

For further characterisation, plasmid DNA was nicked, denatured and immobilized on DBM-paper, and hybridised to the poly-A^+ RNA fraction. RNA sequences hybridising to the DNA were then eluted from the paper (Alwine et al, 1980). The eluted RNA was ethanol-precipitated and then translated in the wheat germ cell-free translation system. Translation products were analysed by SDS-PAGE. The translation methods are described in full in sections 2.2.6.2/3.

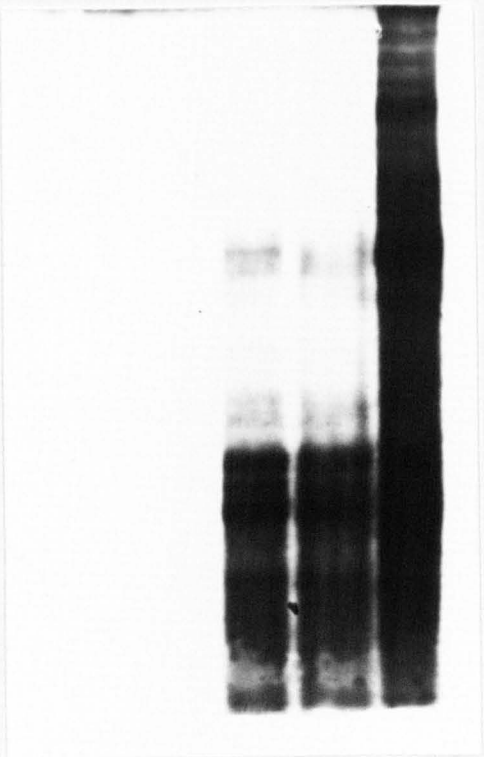
3. Results

The synthesis of cDNA from poly-A RNA was dependent on the addition of oligo-dT as primer. The products were heterodisperse with an average molecular weight of approximately 2.5×10^5. After second strand synthesis and digestion of the hairpin loop with S1 nuclease to give sDNA, the average molecular weight was approximately 1×10^5. Although the product was

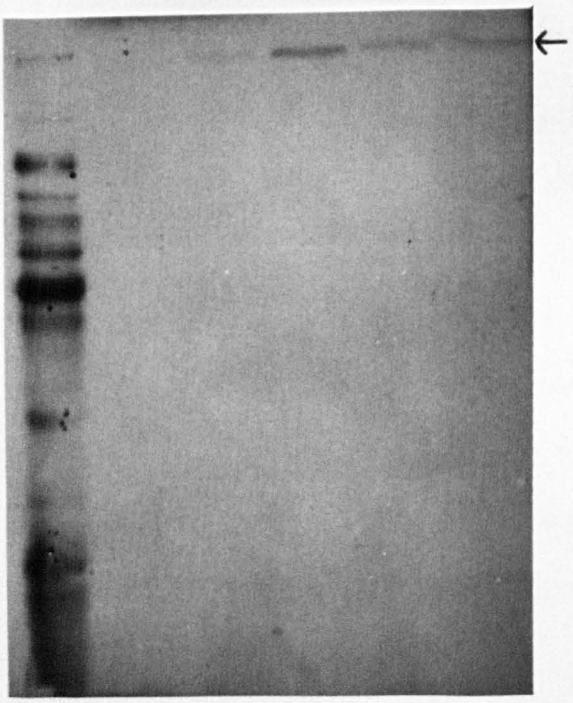
heterodisperse, digestion of the sDNA with HaeIII gave a characteristic banding pattern after fractionation by gel electrophoresis. The pattern differed when poly-A^+ RNA derived from different varieties of barley (Athos and Sundance) were used as template. Two bands appeared to be common to the two varieties, but other bands were also visible in both. These two varieties have different hordein patterns.

Initial attempts to amplify these sequences by insertion into E. coli plasmids failed. The first successful attempt yielded six E. coli clones which were resistant to chloramphenicol and sensitive to tetracycline. Analysis of the DNA in single colony lysates by gel electrophoresis indicated that the plasmid in one clone contained a deletion but the plasmids in the remaining five were larger than the normal plasmid. The inserts were small, ranging from 180-280 base pairs. The five clones will be referred to subsequently as HOR 1 to HOR 5.

The plasmids were isolated, nick-translated and hybridised to fractionated poly-A^+ RNA. HOR 1 and HOR 2 appear to hybridise to the same components of poly-A^+ RNA; the molecular weight range was $2.6\text{-}2.8 \times 10^5$. HOR 3 hybridised strongly to components in the molecular weight range $4.6\text{-}5.1 \times 10^5$ and HOR 4 hybridised to a broad band in the molecular weight range $2.4\text{-}3.2 \times 10^5$. These sequences range in length from 650kb to 1400kb. HOR 5 failed to hybridise to poly-A^+ RNA.



A B C D E



L K J H G F

Fig. 39 Analysis of translation products of mRNA selected by hybridisation to cDNA inserted into bacterial plasmids.

Poly-A^+ RNA was hybridised to plasmids HOR 1 - HOR 5 and the sequences retained in the hybrids were then eluted and translated in the wheat germ cell-free system with [^{35}S]methionine as precursor (see Appendix 1, 2.2.3; 2.2.6.3). The total translation products were analysed by SDS-PAGE (see legend to Fig. 27). When the mRNA was ethanol-precipitated prior to translation, 5µg tRNA was added as carrier; tRNA was therefore used as a control. The gel was fluorographed at -80°C for 14 days.

A. Translation products in the absence of added RNA (1.2×10^4 C.P.M.)

B. Translation products with 5ug tRNA added (1.6×10^4 C.P.M.)

C. Poly-A^+ RNA translation products (1.8×10^5 C.P.M.)

D. Poly-A^+ RNA + 5ug tRNA translation products (1.9×10^5 C.P.M.)

E. Polysome translation products (4.5×10^5 C.P.M.)

F. mRNA which had been selected by HOR 1 (2.6×10^4 C.P.M:)

G. by HOR 2 (3.0×10^4 C.P.M.)

H. by HOR 3 (2.7×10^4 C.P.M.)

J. by HOR 4 (2.5×10^4 C.P.M.)

K. by HOR 5 (2.0×10^4 C.P.M.)

L. Propanol-extracted translation products (1×10^5 C.P.M.)

For tracks A-E, half of the total products were analysed. For tracks F-K, all of the total products were analysed. The arrow indicates the band visible in tracks F-J.

The plasmids were used to select complementary mRNAs as described by Alwine et al (1980). The mRNAs were then translated in the wheat germ cell-free protein-synthesising system, and total products were analysed by SDS-PAGE and subsequent fluorography (Fig. 39). HOR 5 gave no visible polypeptides; HOR 1-4 each gave a single polypeptide; the four were of similar but not identical molecular weight. These polypeptides appeared to co-migrate with the largest 'C' hordein band, which contains a number of related polypeptides. This band is not visible in the tRNA control, but is present in the propanol-extracted poly-A^+ RNA products. In the total poly-A^+ RNA translation products this component is very close to the limits of detection and is not easily visible.

4. Discussion

Although sDNA was synthesised efficiently from barley endosperm poly-A RNA, a number of attempts to insert this sDNA into E. coli plasmids failed. When this procedure was carried out successfully for the first time only five clones were produced which contained recombinant plasmids. It was, therefore, unnecessary to carry out colony hybridization to radiolabelled RNA (Grunstein and Hogness, 1975; see introduction to this appendix) to identify plasmids carrying inserts complementary to hordein mRNA. All five clones were characterised by the 'Northern blot' procedure (Alwine et al, 1977) to determine

to which size classes, if any, of RNA the recombinant plasmids hybridized. All five clones were also used to select individual complementary mRNAs.

Four of the clones appear to carry sequences complementary to mRNA coding for the largest 'C' hordeins. The molecular weight of these polypeptides is estimated to be 50000 and so they require a coding sequence of approximately 1400 bases. In the 'Northern blot' procedure the recombinant plasmids hybridise to RNA sequences ranging from 650-1400 bases. There are possible explanations of this anomaly; the first is that the RNA size estimates are incorrect but the results have been reproduced a number of times on completely denaturing methyl-mercury gels. The second is that the poly-A^+ RNA is degraded by the methyl-mercury gel system and the plasmids are hybridising to degradation products. The third is that the selection of mRNAs for translation is giving artefactual results. The hybridisation cannot simply be due to a sequence in the plasmid because HOR 5 does not behave as HOR 1 - HOR 4. If there were a lot of cross-hybridisation between the different hordein mRNAs, it would be expected that more than one polypeptide would be seen in the translation products. The third explanation does not, therefore, seem likely. It is, however, an unexpected result that the clones appear to be complementary to mRNAs coding for the same group of polypeptides.

There is clearly considerably more work to be done on cloning hordein cDNA sequences. We appear to have cloned some short pieces of cDNA coding for 'C' hordeins. Brandt (1979) reported the cloning of an 850 base pair DNA sequence complementary to mRNAs coding for two 'B' hordein polypeptides. When a bank of such clones has been produced, it should be possible to isolate chromosomal DNA in order to analyse the Hor-1 and Hor-2 loci. This would also lead to a greater understanding of the nature of the heterogeneity of the proteins.

APPENDIX 2 End-labelling of mRNA after enzymatic decapping.

1. Introduction

The use of polynucleotide kinase to label nucleic acids is inefficient when the substrate is a capped messenger RNA. The cap must first be removed; Efstratiadis et al (1977) reported a method involving two enzymatic steps which were 1. removal of the cap using tobacco acid pyrophosphatase, 2. removal of remaining phosphates with alkaline phosphatase. This method was used successfully to end-label mRNA enriched for sequences coding for hordein. This labelled RNA could then be used as a probe for screening cDNA clones. The method used is detailed below.

2.1 Preparation of tobacco acid pyrophosphatase (EC.3.6.1.1)

Tobacco callus was harvested whilst still actively growing, and stored at -80°C until enzyme extraction. All operations were carried out at 2-5°C. Cells (10g) were homogenized using a Polytron at speed setting 2 for approximately 30sec in 15ml 0.1M sodium acetate pH 5.0, 0.2M NaCl, 10mM 2-mercaptoethanol, 1mM EDTA, and debris removed by centrifugation at 2500g for 20min. The supernatant solution was centrifuged a second time at 30000g for 20min, and ammonium sulphate (52g per 100ml) was stirred into the second supernatant solution over a period of 30min. After stirring for a further 30min, the pellet was collected at 30000g for 20min and

resuspended in 1ml buffer A (10mM Tris-HCl pH 7.5, 10mM 2-mercaptoethanol, 0.01% Triton X100). This suspension was dialysed overnight against 2l buffer A at 2°C and centrifuged again at 30000g for 20min. The supernatant solution was passed over a 1ml column of DEAE cellulose (Whatman DE52) which was equilibrated and developed with buffer A. The effluent was collected and passed over a 1ml column of phosphocellulose (Whatman) equilibrated with buffer A. After washing the column with buffer A plus 0.04M NaCl, the enzyme was eluted with buffer A plus 0.12M NaCl, 50% glycerol; 3 x 1ml fractions were collected and stored at -20°C.

2.2 Method of assay of tobacco acid pyrophosphatase

The activity of the enzyme was checked by assay of its ATPase activity. The 10µl reaction mixture contained 50mM sodium acetate pH 6.0, 10mM 2-mercaptoethanol, 1µl enzyme and 40nCi γ-$[^{32}P]$ ATP, and was incubated at 37° for 30min. The amount of $[^{32}PO_4^{2-}]$ released was determined using a method adapted from Berenblum and Chain (1938). The reaction was stopped by addition of 0.1ml ice-cold 10% (w/v) TCA followed by 0.2ml 1.25mM KH_2PO_4 in 0.5M H_2SO_4, 0.1ml 5% ammonium molybdate and 0.5ml isobutanol:toluene (1:1 v/v). Phosphate is extracted into the organic phase which was sampled and 100µl counted in 3ml toluene-based scintillant.

2.3 Purification of calf intestinal phosphatase (EC.3.1.3.1)

The enzyme was obtained from the Boehringer Corporation Ltd. and further purified to remove RNAase. The enzyme (0.5mg) was pelleted from ammonium sulphate suspension in a Beckman microfuge for 2min and dissolved in 0.1ml sterile distilled water. This solution was applied to a Sephadex G75 column (1 x 15cm), equilibrated and developed with 20mM Tris-HCl pH 8.4, 100mM KCl. The fraction eluting in the void volume was collected andmixed with an equal volume of glycerol for storage at -20°C. The activity was checked in the same way as described for tobacco acid pyrophosphatase, but in 10mM Tris-HCl pH 8.0.

2.4 Labelling of RNA with $[^{32}P]$ using polynucleotide kinase (EC.2.7.1.28)

The method described by Efstratiadis et al (1977) was used to enzymatically decap mRNA prior to end-labelling. The first step was to remove the cap structure using tobacco acid pyrophosphatase. The 10µl reaction mixture contained 50mM sodium acetate pH 6.0, 10mM 2-mercaptoethanol, 2µl enzyme, 2-4µg RNA and was incubated at 37°C for 30min. The second step was to remove the remaining 5' phosphate groups using calf intestinal alkaline phosphatase. After decapping, 2µl 0.5M Tris-HCl pH 8.3, 2µl enzyme and 6µl H_2O were added to the reaction mixture and this reaction was incubated at 37°C for 30min. The reaction was then inhibited by the addition of

1µl 250mM potassium phosphate pH 9.5; this addition does not affect the subsequent polynucleotide kinase reaction. The reaction mixture was transferred to a tube containing 0.5µCi γ-[^{32}P]ATP (2000Ci/mMol, dried down under vacuum); 1µl 250mM magnesium acetate, 2µl 50mM DTT and 1µl (4 units) polynucleotide kinase were added and the mixture incubated at 37°C for 30min. The labelled RNA was precipitated by addition of 100µl 2M ammonium acetate, 50µg tRNA and 300µl ethanol with rapid chilling and held at -20°C for 3h. The RNA was pelleted by centrifugation at 10000g for 10min and then dissolved in glyoxalation buffer prior to gel electrophoresis (see section 2.2.5.2).

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