In vivo and in vitro synthesis of CM-proteins (A-hordeins) from barley (*Hordeum vulgare* L.)

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Abstract. CM-proteins from barley endosperm (CMa, CMb, CMc, CMd), which are the main components of the A-hordein fraction, are synthesized most actively 10 to 30 d after anthesis (maximum at 15–20 d). They are synthesized by membrane-bound polysomes as precursors of higher apparent molecular weight (13,000–21,000) than the mature proteins (12,000–16,000). The largest in vitro product (21,000) is the putative precursor of protein CMd (16,000), as it is selected with anti-CMd monospecific IgG's, and is coded by an mRNA of greater sedimentation coefficient (9 S) than those encoding the other three proteins (7.5 S). CM-proteins always appear in the soluble fraction, following different homogenization and subcellular fractionation procedures, indicating that these proteins are transferred to the soluble fraction after processing.

Key words: A-hordein – CM-proteins – Endosperm – *Hordeum* (A-hordeins) – Protein biosynthesis.

Introduction

The study of protein synthesis and processing in the developing cereal endosperm is of interest both because of the central role played by this tissue in the nutrition of mankind and because of its value as a plant model system (see Brandt and Ingversen 1978; Burr et al. 1978; Larkins and Hurkman 1978; Mathews and Miflin 1980; Viotti et al. 1979; Wienand and Feix 1978). Barley endosperm is particularly suitable for such a purpose because it can be easily obtained by extrusion from whole ears (O'Dell and Thompson 1982), and important advances have been made in the study of the genetic control of its main proteins (Doll and Brown 1979; Shewry et al. 1980a; Jensen et al. 1980; Munck et al. 1970; Doll et al. 1974).

Up to 60% of the barley endosperm proteins are prolamins (hordeins), which can be extracted with aqueous alcohols. These prolamins were originally classified into A-, B-, and C-hordeins, according to their decreasing mobility in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Køie et al. 1976; Shewry et al. 1977). The B- and C-hordeins have been characterized as storage proteins, located in the protein bodies, and have been found to be controlled by two linked loci, *Hord-2* and *Hord-1*, respectively (Doll and Brown 1979; Shewry et al. 1980a). The function of the A-hordeins is unclear and it has been suggested that they are not located in the protein bodies (Holder and Ingversen 1978; Salcedo et al. 1980). Recent studies from this laboratory have shown that the main components of this fraction are not true prolamins because they are soluble in aqueous buffers and their amino acid compositions are atypical, although, as with the prolamins, they are soluble in aqueous alcohols and in chloroform:methanol mixtures (Salcedo et al. 1980; Aragoncillo et al. 1981; Salcedo et al. 1982). It has also been shown that they are homologous to the wheat and rye CM-proteins, and possibly to similar proteins present in other Gramineae, so it has been proposed that the designation of CM-proteins be extended to the four components purified from barley (CMa, b, c, and d) (Salcedo et al. 1982; Paz-Ares et al., in press). This report deals with the in vivo synthesis and deposition of these proteins and the in vitro synthesis of their putative precursors.

Materials and methods

Plant material. Hordeum vulgare L. cv. Bomi, grown in the greenhouse and in the field, was used throughout the experi-

ments. For RNA purification, endosperms were collected from whole ears by mechanical extrusion into liquid nitrogen (O'Dell and Thompson 1982) 20 d after anthesis. The same procedure was used, when feasible, to collect in vivo labelled endosperms at other stages of development; otherwise the ears were freeze-dried and the endosperms obtained by hand dissection.

Fractionation of developing endosperm. Endosperms were homogenized by three different procedures (ground to a powder in liquid N_2, cut with a razor blade at 4° C, or ground with a pestle at 4° C), using two different buffers (50 mM N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine), 100 mM K acetate, 10 mM Mg acetate, 10% sucrose w/w, pH 7.5, or 20 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid (Hepes), 100 mM K acetate, 5 mM Mg acetate, 0.2 M sucrose, pH 7.6), essentially as described by Miflin et al. (1981) and Cameron-Mills (1980). The 500 $g_{av.}$ supernatants were centrifuged over a 30% sucrose (w/w) cushion at 36,000 $g_{av.}$ for 1 h in a SW-27 rotor, and three fractions were collected: supernatant, interphase, and pellet. The last two fractions were washed with water and freeze-dried and the proteins in the supernatant were precipitated with 12.5% trichloroacetic acid (TCA), washed with ethanol, and dried in vacuo. Chloroform: methanol (2:1, v/v) extracts were obtained from the three fractions and the solvents evaporated in vacuo.

In vivo protein synthesis. Proteins synthesized by developing endosperm were labelled with [^{35}S]cysteine (3.5·10^4GBq mmol^{-1}) or [^{35}S] SO_4^{2-} (4.1·10^4 GBq mmol^{-1}), both from New England Nuclear, essentially as described by Donovan and Lee (1977): ears were cut at different stages of development, leaving 5–10 cm stems, and placed in conical tubes with 0.5 ml of a sulphur-free nutrient solution containing 3.8–11.3 MBq radioactive precursor. After the label had been absorbed (~30 min), two successive 0.5 ml aliquots of the same nutrient solution without label were added after which the ear was transferred to a refrigerated (4° C) 25 ml erlenmeyer with nutrient solution.

Purification of mRNA. Free and initially membrane-bound polysomes were prepared from 20-d endosperms as described by Larkins et al. (1976), with minor modifications: 30 g endosperms were ground to a powder in liquid N_2 and mixed with 120 ml 200 mM Tris-HCl, pH 8.5, 50 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol (DTT), 200 mM sucrose. The homogenate was centrifuged for 5 min at 1,000 $g_{av.}$ and the supernatant was then centrifuged at 30,000 $g_{av.}$ for 30 min to separate free polysomes from membrane-bound polysomes. The pellet from this centrifugation was resuspended in the same buffer with 1% Triton X-100 to release initially membrane-bound polysomes and centrifuged again. The two supernatants were separately layered on a cushion of 1.6 M sucrose in 40 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 20 mM KCl, and the polysomes were pelleted at 250,000 $g_{av.}$ for 3 h, using a Beckman 70-Ti rotor.

Polysomes were resuspended in 10 mM 2-amino-2-(hydroxymethyl)-1,3-propandiol(Tris)-HCl, pH 9, 0.1 M NaCl, 0.5% SDS and treated with an equal volume of phenol saturated with the same buffer. After three phenolizations, 1/10 volume of 2 M Na acetate and two volumes of ethanol were added to precipitate RNA. The RNA pellet was washed with 80% ethanol and absolute ethanol, dried, and redissolved in 10 mM Tris-HCl, pH 7.6, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% SDS, to a final concentration of 50 A_{260} units/ml.

Prior to oligo *dT*-cellulose (PL-Biochemical, type 7) chromatography, the RNA solution was heated at 65° C for 5 min and rapidly cooled. The solution was then made 0.5 M in NaCl and gently stirred with oligo *dT*-cellulose in the same buffer (1 mg of oligo *dT*-cellulose for each 2.5–5 A_{260} units of RNA) and allowed to sediment. The supernatant was collected as the poly A^- RNA fraction, and the sediment was washed twice with 10 volumes of the same buffer and packed as a slurry in a sterile column. Poly A^+ mRNA was collected as described by Aviv and Leder (1972), precipitated with 1/10 volume 2 M Na acetate and 2 volumes ethanol and stored at −20° C until required.

Initially membrane-bound poly A^+ mRNA was fractionated in a 10–35% (w/v) linear sucrose gradient: dried RNA (150 µg) was dissolved in 0.5 ml 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5% SDS, heated for 4 min at 80° C, and rapidly cooled on ice. The sample was then loaded in the gradient which was made up in the same buffer. Centrifugation was carried out in a Beckman SW-27 rotor at 100,000 $g_{av.}$ for 26 h at 18° C. Fractions (2 ml each) were collected, precipitated, washed, and stored as described above. RNA's of 5S, 5.8S, 18S, and 26S were centrifuged in parallel to approximately calibrate the RNA in the different fractions.

In vitro protein synthesis. The wheat germ translation system was prepared and translation carried out as described by Marcu and Dudock (1974), with the following modification: K^+ and Mg^{2+} were added as acetates, 2.5 vol of buffer were used instead of 2 vol., and 180 units/ml of pancreatic ribonuclease inhibitor (RNasin, Biotec Inc) were added to reaction mixtures. Incubations were carried out for 60 min at 29° C and 0.2–0.6 MBq of [^{35}S]cysteine (3.4·10^4 GBq mmol^{-1}, New England Nuclear) were used per 25 µl of reaction mixture. Wheat germ from General Mills (USA) or hand-dissected wheat embryos from tetraploid *Triticum turgidum* cv. Ledesma were used. Each preparation was optimized for K^+, Mg^{2+}, and spermine concentrations (usually, 100 mM K acetate, 2 mM Mg acetate, 70 mM spermine). At saturating mRNA concentrations, 25- to 35-fold stimulations over endogenous activity were obtained.

Immunoprecipitation of in vivo and in vitro products. Details about the preparation of antibodies are given elsewhere (Paz-Ares et al., in press). A mixture of CM-proteins from mature barley endosperm and highly purified CMd were used to separately immunize female rabbits according to Tai and Chey (1978). Specific antibodies were purified by $(NH_4)_2SO_4$ precipitation and affinity chromatography in a protein CMd-Sepharose column, according to Shapiro et al. (1974). The purified antibodies precipitated in-vivo synthesized polypeptides with the same electrophoretic mobility as that of the mature proteins: AH selected CMa, CMb, CMc and CMd and AHd selected CMd (Paz-Ares et al., in press).

Immunoprecipitation of in vivo products was carried out essentially as in Shapiro and Young (1981), modified by Paz-Ares et al. (in press). The same procedure was followed for in vitro products, except that the complete procedure was first performed with pre-immune serum before using specific antibodies, and all washing buffers included 50 µM cysteine.

Electrophoresis and fluorography. Starch gel electrophoresis (SGE) of CM-proteins was carried out in aluminium lactate buffer, pH 3.2, 3 M urea, for 4.5 h at 20 V cm^{-1} and 5° C and stained with 0.05% nigrosine in methanol:water:acetic acid (5:5:1, by vol.) for 16 h and destained with 70% ethanol. When fluorography was required, 10% polyacrylamide gel electrophoresis (PAGE) was performed in the same buffer, in which case proteins CMb and CMc are not well separated from each other. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) and gels stained with Coomassie Brilliant Blue G-250 according to Blakesley and Boezi (1977). Fluorography was

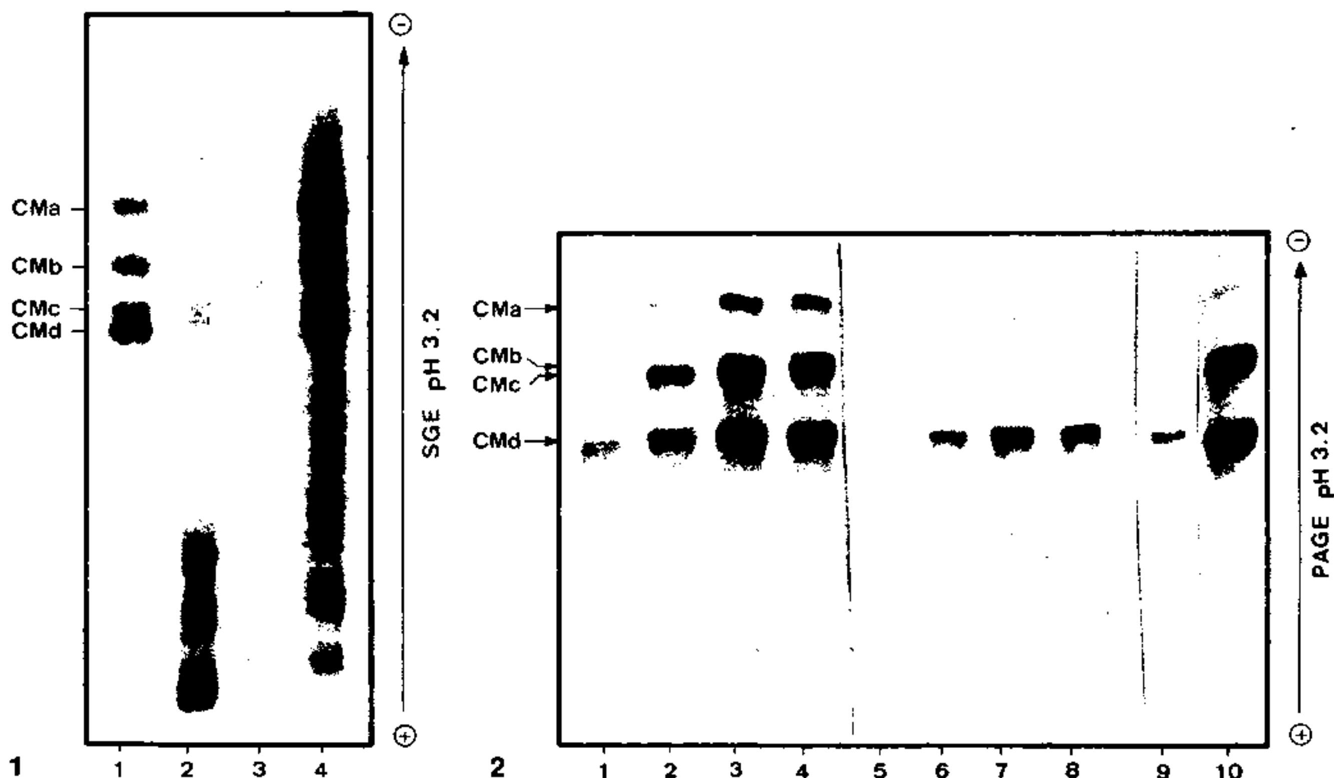

Fig. 1. CM-proteins in subcellular fractions of barley endosperm collected 20 d after anthesis. Endosperm was homogenized with a razor-blade in 50 mM N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine), 100 mM K acetate, 10 mM Mg acetate, pH 7.5, 10% sucrose (w/w), and centrifuged at 36,000 $g_{av.}$ over a 30% (w/w) sucrose cushion. Chloroform:methanol (2:1, v/v) soluble proteins from each of the fractions were separated by pH 3.2 starch gel electrophoresis (SGE). 1) Purified CM-proteins from mature barley endosperm. 2) Pellet. 3) 30% (w/w) sucrose interphase. 4) Supernatant

Fig. 2. Time-course of $^{35}SO_4^{2-}$ incorporation into CM-proteins of 20 d barley endosperm. Samples were collected at 3, 24, 48, and 96 h after label was added and treated separately with A-H and A-Hd antibodies. The immunoprecipitated proteins were separated by pH 3.2 polyacrylamide gel electrophoresis (PAGE) and fluorographed. CM-proteins purified from mature endosperm were inserted in parallel and their positions marked with radioactive ink. Proteins CMb and CMc are practically not resolved from each other in this system. 1–4) Proteins precipitated by A-H at the successive stages. 5–8) Protein precipitated by A-Hd at the successive stages. 9–10) Lanes 5 and 1, respectively, fluorographed a longer time. Incorporation in endosperm at 3 h: 230,000 total cpm mg^{-1}; 50,000 trichloroacetic acid (TCA) insoluble cpm mg^{-1}. At 48–96 h: 360,000 total cpm mg^{-1}; 240,000 TCA insoluble cpm mg^{-1}

performed as described by Laskey and Mills (1975). Protein molecular weight standards, indicated in Legends to the figures, were from Serva AG. Purified proteins from mature endosperm used in electrophoresis were obtained in the course of previous work (Salcedo et al. 1982).

Results

In a preliminary survey, endosperms were collected and freeze-dried at 5 d intervals, starting 15 d after anthesis, and the CM-proteins were fractionated by electrophoresis (not shown). Protein bands corresponding to those found in mature barley were barely detected at 15 d, but were detected more clearly in subsequent samples.

In order to ascertain that CM-proteins are not associated with protein bodies, 20-d endosperms were homogenized by three different methods, using two different buffers (Miflin et al. 1981; Cameron-Mills 1980), and fractionated by ultracentrifugation. In all cases, the CM-proteins appeared in the 35,000 *g* supernatant, as exemplified in Fig. 1.

The time course of in vivo CM-protein synthesis was followed in 20-d ears for a period of 4 d, pulse-labelling with $^{35}SO_4^{2-}$ (Fig. 2). The first sample, which was collected 3 h after initiation of labelling, already showed the CM-protein patterns of mature barley. At that time, only 15% of the total cpm incorporated into the endosperm was insoluble in 10% trichloroacetic acid (TCA). At 48 h a stable

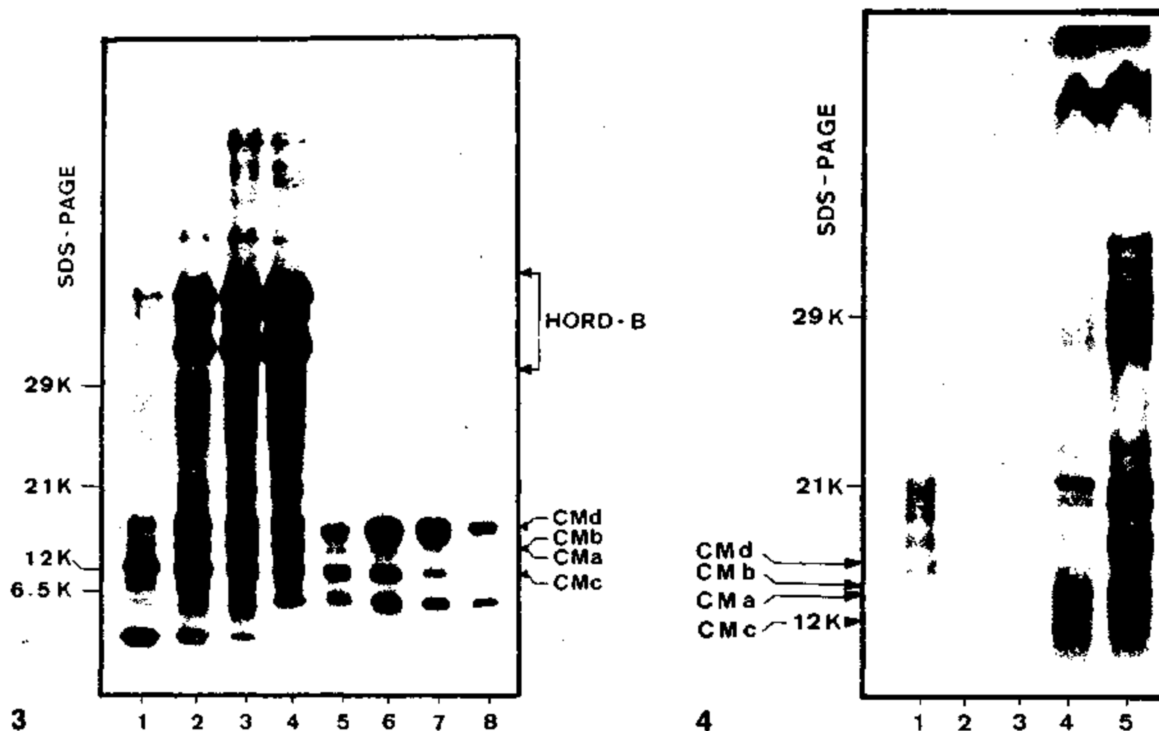

Fig. 3. Synthesis of CM-proteins at different stages during endosperm development. Ears collected at 10, 15, 20 and 30 d after anthesis were labelled for 48 h as in Fig. 2. CM-proteins were either directly extracted with chloroform:methanol (2:1, v/v) (10, 15, 20, and 30 d: lanes 1–4) or immunoprecipitated with an excess of A-Hd (10, 15, 20, and 30 d; lanes 5–8). Separation of protein was by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified proteins and molecular weight standards (carbonic anhydrase, 29,000; soybean trypsin inhibitor, 21,000; cytochrome C, 12,000; lung trypsin inhibitor, 6,500) were run in parallel and their positions marked with radioactive ink. (K = × 1,000)

Fig. 4. SDS-PAGE of translation products directed by poly A^+ RNA's purified from free and initially membrane-bound polysomes from 20 d endosperm in a wheat germ cell-free system. 1) Products selected by A-H from the bound poly A^+ mRNA reaction. 2) Idem from the free poly A^+ mRNA reaction. 3) Products of wheat-germ endogenous activity. 4) Total free poly A^+ mRNA translation products. 5) Total bound poly A^+ mRNA translation products. Bound versus free translation activity was about 3:1 on a per endosperm weight basis. (K = × 1,000)

value for TCA-insoluble cpm was reached; a labelling period at 48 h was therefore adopted to investigate CM-protein synthesis at different time periods after anthesis (Fig. 3). The CM-proteins were obtained from each sample by two different procedures: i) direct chloroform:methanol (2:1, v/v) extraction; and ii) 0.5 M NaCl extraction, followed by immunoprecipitation with an excess (3×) of A-Hd monospecific antibodies. Under the latter conditions the four CM-proteins are precipitated (Paz-Ares et al. unpublished data). It should be pointed out that an additional component with apparent molecular weight just under 5,000 is also detected by fluorography after fractionation by SDS-PAGE. This component is not detected when the separation is by pH 3.2-PAGE (Fig. 2) or when $[^{35}S]$cysteine is used to label endosperm proteins (Fig. 5). CM-protein synthesis is already detected at 10 d, reaches a maximum around 15–20 d and is still proceeding at a reduced rate 30 d after anthesis, while the B-hordeins, which are also included in the chloroform:methanol (2:1, v/v) extract, are still synthesized at top rate at this stage. Synthesis of the four CM-proteins (CMa, b, c, and d) does not seem to be completely synchronous.

Poly A^+ mRNA's were obtained from free and initially membrane-bound polysomes from 20-d endosperms and translated in a wheat-germ cell-free system. Translation products were immunoprecipitated with A-H antiserum, which has been shown to select the four in vivo-synthesized CM-proteins (Paz-Ares et al., in press). Only the poly A^+ RNA from initially membrane-bound polysomes yielded products that could be precipitated with A-H (Fig. 4). These products were in the 13,000–21,000 size range, which is higher than the size range of the proteins purified from mature barley (12,000–16,000). In order to check this

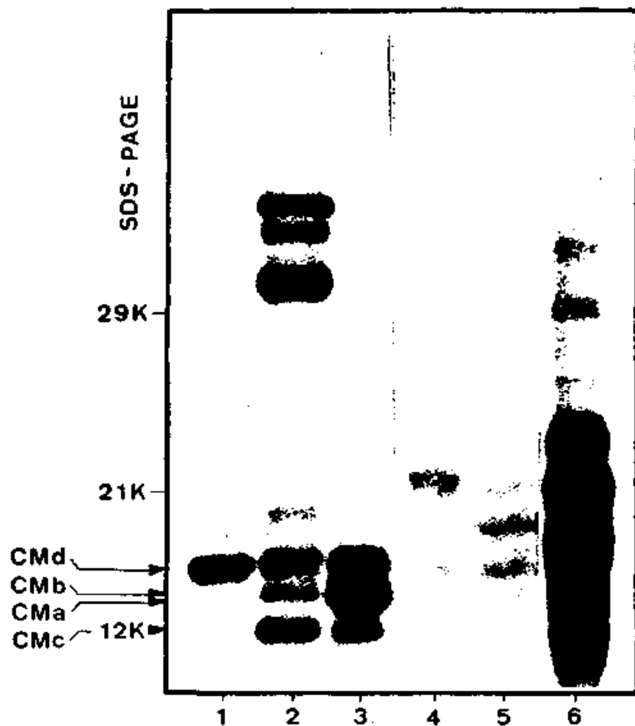

Fig. 5. Comparison of in vivo and in vitro products by SDS-PAGE, selected using two antibody preparations (A-H and A-Hd). [^{35}S]cysteine was used to label 20 d endosperms. 1) In vivo, products selected by A-Hd. 2) Total chloroform:methanol (2:1, v/v) extract from labelled endosperm. 3) In vivo, products selected by A-H. 4) In vitro, products selected by A-Hd. 5) In vitro, products selected by A-H. 6) In vitro, total products. Other details as in Fig. 3. (K = × 1,000)

result, in vivo and in vitro products precipitated by A-H and by monospecific A-Hd were compared in the same gel (Fig. 5). Under the conditions used, A-Hd selectively precipitated CMd (16,000) from the endosperm and a 21,000 product, with a small proportion of a 15,000 product from the in vitro translation mixture; whereas A-H precipitates four products from the same mixture (21,000, 17,000, 15,000, and 13,000). Fractionation by sucrose-gradient centrifugation of poly A$^+$ mRNA from initially membrane-bound polysomes separated the mRNA for the 21,000 product from those for the other CM-products and from those for B-hordeins (Fig. 6).

Discussion

The above results seem to indicate that barley CM-proteins are synthesized by membrane-bound polysomes as larger precursors, which are probably co-translationally processed and eventually transferred to the soluble fraction. However, this interpretation needs some qualifications. Even the gentler homogenization procedures (see Miflin et al. 1981) rendered about 90% of the CM-proteins in the soluble fraction, but the possibility that these proteins, which have low polarity and are soluble both in organic and in aqueous solvents, could be externally associated in a labile manner with membranes in vivo can not be excluded. If these proteins are indeed in the soluble fraction in vivo, their synthesis by membrane-bound polysomes could be related to a possible requirement for processing in the membrane fraction. Evidence that these proteins are glycosilated is inconclusive at present, because, although some preparations of these proteins yielded 3–5% neutral sugars by the phenol-sulphuric method of Ashwell (1966), none of the components stained as a glycoprotein by using Racusen's method (1979), when electrophoretic gels were treated under conditions in which standard glycoproteins were stained (Aragoncillo et al. 1981).

The in vitro translation products are larger than those detected in vivo with the same specific antibodies. The largest translation product (21,000) which corresponds to the mRNA with the higher sedimentation coefficient is probably the precursor of CMd (16,000). A minor product also selected with anti-CMd monospecific IgG (A-Hd) is smaller (15,000) than the mature protein and is probably the precursor of some of the other CM-proteins which possibly cross-react with A-Hd. The apparent difference in molecular weights between the mature CMd and its putative precursor is about 5,000, but it should be pointed out that hydrophobic peptides, e.g., those studied here, are known to deviate from their expected mobility in SDS-PAGE (Hamauzu et al. 1975; Shewry et al. 1980b). In this context, the 5,000 component, which is also precipitated by A-Hd from $^{35}SO_4^{2-}$-labelled protein extracts (Fig. 3), could represent the excised peptide, which would have remained non-covalently attached to the mature protein. However, alternative identities for this component, e.g., a sulphur-containing prosthetic group or a non-specific ligand, are equally possible and can not be eliminated with the present evidence.

No in vitro processing experiments have been carried out yet with these proteins, so the hypothesis that there are no temporal precursors for the CM-proteins and that they are co-translationally processed is based solely on in vivo observations: As early as 3 h after initiation of labelling (Fig. 2), when only a small fraction of the label has been incorporated into protein, the electrophoretic pattern of CM-proteins is that of the mature endosperm and no possible precursors are detected.

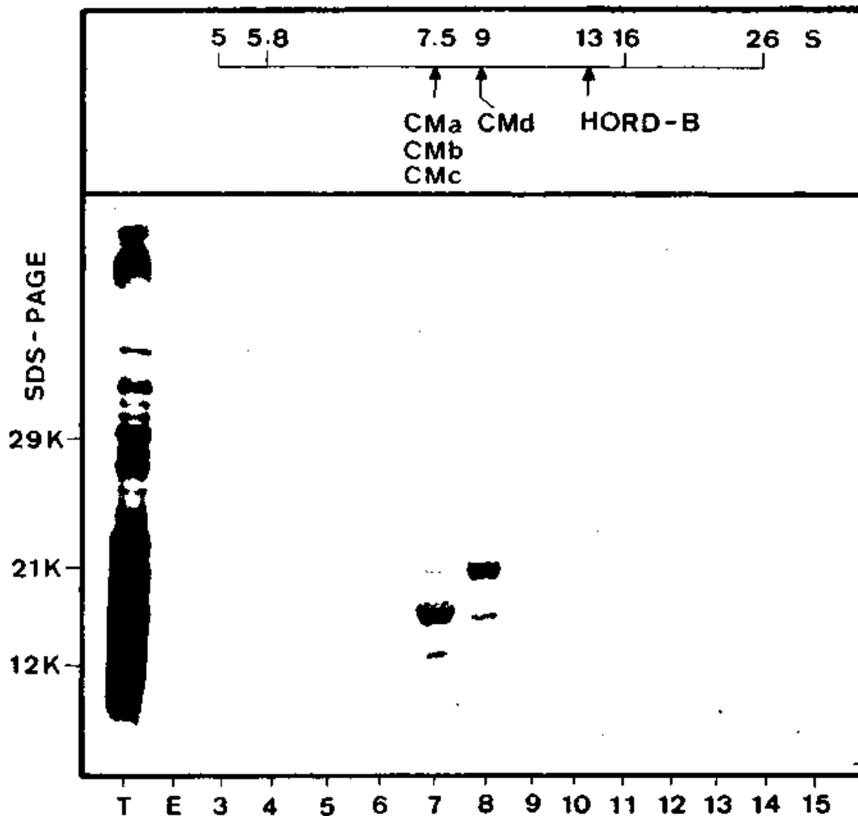

Fig. 6. Sucrose-gradient (10–35%, w/v) centrifugation of poly A$^+$ mRNA (150 μg) of initially membrane bound polysomes. Centrifugation was carried out for 26 h at 100,000 $g_{av.}$ (18° C) in a Beckman SW-27 rotor and 18 fractions of 2 ml each were collected. An aliquot of 4% from each fraction was translated and products reacting with A-H were precipitated, separated by SDS-PAGE and fluorographed. The position of B-hordein messengers in the gradient was determined by analysing these proteins in the translation mixtures as described by Mathews and Miflin (1980) and SDS-PAGE (not shown). *T*, Translation products of poly A$^+$ mRNA applied to gradient. *E*, Wheat germ endogenous activity. 3–15) Gradient fractions. Other details as in previous figures. (K = × 1,000)

With hordothionin in vivo synthesis under the same conditions, only a temporal precursor was detected at 3 h, which was still detected at 24 h together with the mature protein (unpublished).

Active synthesis of CM-proteins (A-hordeins) starts somewhat earlier than that of B- and C-hordeins and significantly declines beyond 20 d after anthesis, while the other two groups of proteins are synthesized throughout endosperm development. In this respect, the CM-proteins would correlate to the time course of the deposition of salt-soluble proteins as reported by Shewry et al. (1979).

This work was supported by a grant from the Comisión Asesora de Investigación Científica y Técnica.

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Received 8 August; accepted 1 October 1982