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TABLE OF CONTENTS

ABSTRACT

The pattern of nitrogen accumulation in seven oat cultivars with different agronomic and biochemical properties was followed during seed development. Nitrogen content and nitrogen percentage were determined at 6 different stages of development from anthesis until maturity. Nitrogen content of oat groats varied significantly among seven cultivars and also among stages of development. However, nitrogen percentage of groats did not change significantly during seed development. This finding may indicate that the protein-carbohydrate ratio of oat groats is probably set at early stage of development and persists throughout development.

Four major classes of storage proteins are found in oat endosperm in different quantities. Salt-soluble proteins (globulins) are the major protein fraction. A new high protein Canadian oat cultivar, Hinoat, was analyzed for these four storage protein fractions. As expected, the major fraction was a salt-soluble globulin comprising about 50% of total oat protein. This fraction was analyzed on SDS-polyacrylamide gel electrophoresis Two major subunits were observed with molecular weights of 21700 and 38000 MW.

To study the biosynthesis of the globulin fraction, total free, and membrane-bound polyribosomes were isolated and translated *in vitro*. All polysome preparations were active in a rabbit reticulocyte lysate cell-free protein synthesizing system. Mg^{2} concentration optimum for the translation of total polysomes was 3 mM.

Activity of polysomes in the cell-free translation system suggested that these polysomes may contain active messenger RNAs. Therefore, the polysomes were used to isolate total poly^{+}A RNA. Total polysomes were dissociated with SDS and deproteinized by phenol-chloroform. Total polysomal RNA was then applied to an oligo d(T)-cellulose column and poly A^{+}RNA fraction was eluted in salt-free conditions. This RNA fraction was very active in both rabbit reticulocyte lysate and wheat germ extract causing the incorporation of (^{3}H)-leucine into TCA precipitable products.

Now that a fairly rapid and efficient method for the isolation of total poly A^{+}RNA with mRNA activity has been developed, this RNA fraction is being analyzed to further purify the mRNA specific for globulin polypeptides. When a fairly purified globulin mRNA is available, a complementary DNA transcript will be synthesized *in vitro* and used to isolate the globulin chromosomal genes by current recombinant DNA techniques. The long term objective of this research is to alter the amino acid composition of globulin and other cereal protein fractions in order to improve the quality of cereal proteins.

بسم الله الرحمن الرحیم

"وقل ربی زدنی علما"

صدق الله العظیم

این رساله به پدر ارجمندم ابراهیم، مادر بزرگ عزیزم میرزاده، و به خاطرهٔ مادر گرامیم، ایران، تقدیم میشود. پشتیبانی و عشق پیوسته ایشان، تحصیلات مرا ممکن ساخته است.

This thesis is dedicated to my dear father, Ebrahim, my grandmother, Mirzadeh, and to the memory of my beloved mother, Iran. Their everlasting support, love, and encouragement have made my studies possible.

ACKNOWLEDGEMENTS

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LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

A_{260}	Absorbance taken at 260nm
A_{280}	Absorbance taken at 280nm
Bis	N,N'-Methylene-bis-acrylamide
DTT	Dithiothreitol
EDTA	Disodium Ethylenediaminotetraacetate
EGTA	Ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetra-acetic acid
GTP	Guanosine triphosphate
K	1000 daltons
mRNA	Messenger ribonucleic acid
O.D.	Optical density
Oligo d(T)	Oligothymidylic acid
PER	Protein Efficiency Ratio - Measurement of weight gain per gram of protein eaten.
Poly A^+ RNA	Polyadenylated ribonucleic acid
Poly A^- RNA	Non-polyadenylated ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonuleic acid
s-d	Sterile-distilled water
SDS	Sodium Dodecyl Sulphate
TCA	Trichloroacetic acid
Temed	N,N,N',N'-tetramethylethylenediamine
Tris	Hydroxymethyl Aminomethane
tRNA	Transfer ribonucleic acid

Chapter I

INTRODUCTION

A. GENERAL INTRODUCTION

More than a third of the world's population suffers from serious malnutrition (1). One of the main nutritional deficiencies affecting populations in developing countries is 'protein-calorie deficiency' (2). This syndrome is developed in humans because of intake of energy and protein lower than minimum requirements (3). Accordingly, protein quantity, and more specifically protein quality, defined as the content of essential amino acids, play an important role in human nutrition. Improving protein quality in food crops is of practical significance in terms of nutrition and health. The efficient utilization of agricultural resources to yield more protein with better nutritional and technological properties may help in this direction. Efforts must continue towards improving the protein quality of food items to produce cereal grains and legumes with a higher essential amino acid content.

The successful productions of Opaque-2 maize (4) and new high protein oat cultivars (5) have demonstrated the potential for improving the quality of cereal grains.

Opaque-2 maize has a much higher lysine content than common maize. Also new oat cultivars such as Hinoat developed by Ottawa Research Station, contain considerably high amounts of protein with good quality, compared to existing agronomic cultivars, such as Sentinel or Dal.

Among cereal grains, oat (Avena sativa. L.) possesses better protein quality. The Protein Efficiency Ratio (PER) value of oatmeal has also been reported to be substantially higher than other cereals (6). A negative correlation of protein quality and quantity has been observed in barley, maize, and sorghum. However, oats retain a good amino acid balance when the protein content is increased (7).

Most cereal grains are deficient in one or more essential amino acids such as lysine and methionine. The reason is that the major group of storage proteins are prolamines (alcohol-soluble proteins), which are very low in lysine and methionine (8). Prolamines constitute about 40-60% of the total proteins in rye, barley, corn, wheat, and sorghum. In contrast, oat has only 12-15% prolamine as a storage protein. The major storage proteins in oats are globulins (salt-soluble proteins) which have a good amino acid balance (9).

Briefly, oat protein has three unique features relative to other cereal grains. First, oat protein has a high biological value as illustrated by feeding trials and by amino acid composition, because of a relatively low

amount of prolamines (4). Secondly, the biological value of this protein does not deteriorate as the protein percentage in the grain increases (10). Thirdly, the protein percentage of oat grain probably can be elevated to very high levels by genetic means (7, 11). This is demonstrated by the production of oat cultivars with protein contents as high as 24%. However, although oat proteins are of high quality, when compared with animal proteins, they are low in lysine and methionine (4).

These unique features have created interest in studying the mechanism of storage protein synthesis in oat endosperm. Developing oat endosperms are very active protein synthesizing systems. More than 50 percent of the total protein synthesized is a storage fraction, globulin (9). Such a high rate of biosynthesis of a single class of storage proteins makes developing oat seeds a useful system for studying genome expression in higher plants. Such research has been conducted extensively in other cereals and legumes. Recent studies on zein in maize (12), phaseolin in french bean (13), hordein in barley (14), and glycinnin in soybean (15) have substantially increased our knowledge of the biosynthesis and deposition of seed storage proteins. More research on the molecular factors regulating storage protein biosynthesis may assist in improving the nutritional quality of cereal grains by manipulating the storage protein genes to alter their amino acid content.

B. STRUCTURE AND DEVELOPMENT OF AVENA STORAGE TISSUE

Aveneae is a tribe in the Graminae family. It is subdivided into several species. Avena Sativa. L. (common oat) is the widely cultivated species.

Oat grain is the product of a developmental process. This process starts with a double fertilization of nuclei in the ovary of the flower. Several weeks later, a large and dry seed with several protective layers is developed. In the double-fertilization event, the stigma (feathery receptor structure) receives a pollen grain on the upper part of the ovary. Two (male) copies of nuclei are released into the ovary through a pollen tube. An embryo is produced by the combination of a male nuclei with the female egg cell. Endosperm is developed by genetic combination of the other male nucleus and a double complement of genetic material (the two polar nuclei). Multiple division of these two new genetic combinations results in the development of embryo and endosperm organs. The pericap layers which surround endosperm and embryo originate initially from part of the maternal ovary. In summary, the mature oat groat (Caryopsis) is comprised of three different organs which originate from three different genetic origins; the embryo, endosperm, and bran (aleurone layers) (16).

1. Bran

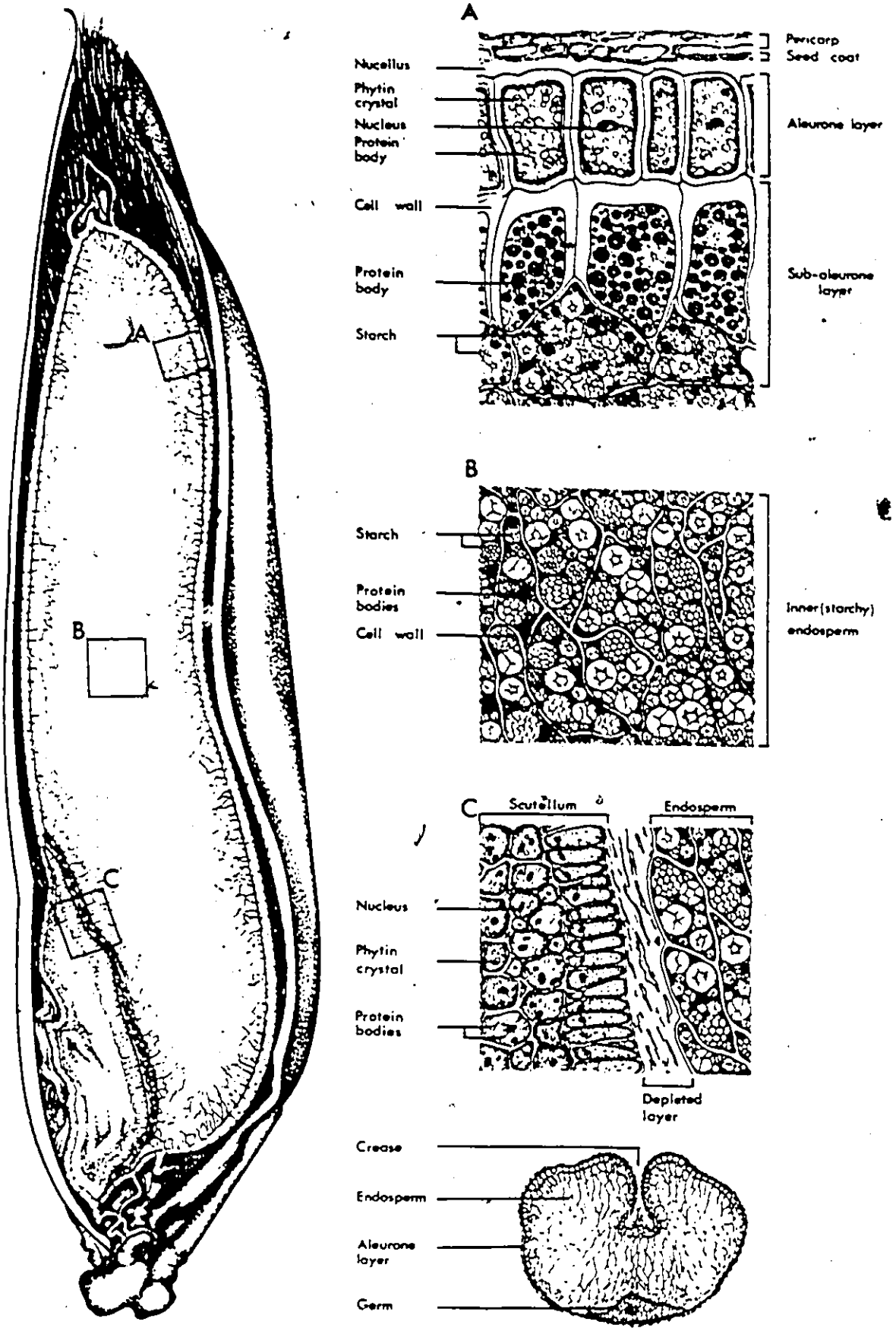
Bran (50-150μm thick) consists of an outermost

pericarp, a double-layered, waxy testa (seed coat) and a thin, compressed layer (nucellus) (Fig 1A). The aleurone layer which is considered as the most important part of the bran, contains large amounts of autofluorescent phenolic compounds (mainly ferulic acid). Inside the aleurone cell, several hundred protein bodies are packed. Two different types of structures have been found in protein bodies; Globoid, a spherical particle containing large amounts of a calcium-magnesium salt of myo-inositol phosphate (phytin), and a so-called 'protein-carbohydrate body' present in aleurone cells, which is apparently composed of periodate-sensitive carbohydrates. It appears that the chemical composition of the proteins present in the aleurone layer protein bodies is different from that of protein bodies in starchy endosperm. The level of lysine seems lower in the former (16).

The aleurone layer has some enzymatic and/or hydrolytic functions necessary for germination. It contains many enzymes such as lipases, proteases, and β-glucanases (17).

2. Starchy Endosperm

The starchy endosperm is a metabolically inactive tissue which comprises up to 80% of the oat groat. Only one type of cell exists in which a huge amount of starch, protein, lipid, and gums are deposited. These cells are packed by particular cell walls which are rich in β-glucans



(Adapted from Fulcher G.1980)

Figure 1. MAJOR STRUCTURAL FEATURES OF THE OAT KERNEL

A. Bran B. Starchy Endosperm C. Embryo

(gums). Endosperm proteins contain relatively high amounts of lysine and other essential amino acids. Endosperm proteins are accumulated in the cells in spherical protein bodies (0.2 to 6 μm in diameter) (16) (Fig 1B).

In high protein cultivars, the sub-aleurone cells are particularly active in protein biosynthesis and store much more protein with very little starch. In low protein varieties, the starch-protein ratio is much higher. In general, the outer-endosperm cells contain more protein than the inner-cells. This gradation exists in all oat cultivars (16).

Proteins, lipids, and gums represent a total of 20 to 30% of the grain's dry weight. Starch is the largest component (70-80%). It exists as starch granules in endosperm cells. It is somewhat similar to wheat and barley starch (18).

3. Embryo

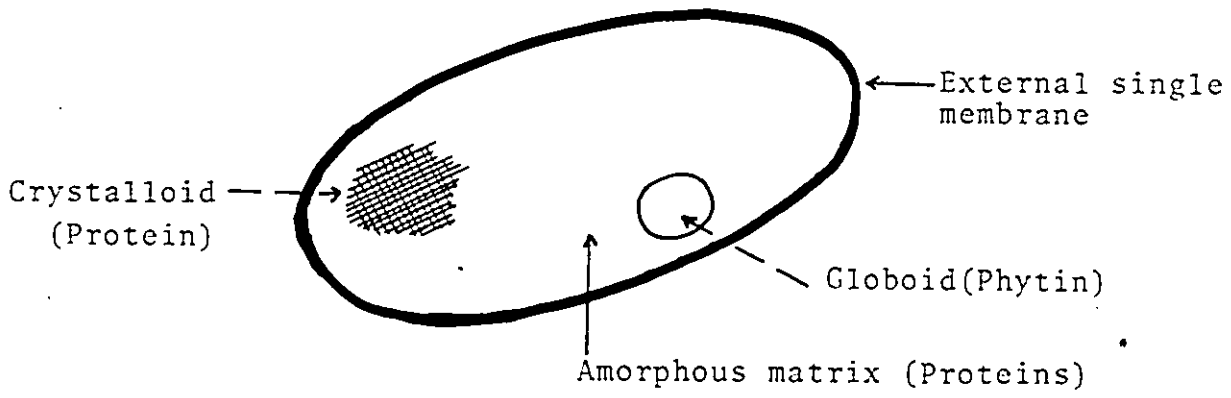
This germ tissue is very similar to the aleurone layer, and is metabolically and enzymatically active. A major part of germ is called 'scutellum' which consists of two distinct tissues, the parenchyma and the epithelium. The parenchyma tissue is made of roughly spherical cells which are used for nutrient storage. It is the major part of the scutellum (80%). The epithelium tissue is constructed of a row of elongated cells which are located on the surface of the parenchyma tissue. They function during germination by absorbing nutrients derived from endosperm

scutellum cells which contain a large number of protein bodies in the form of globoids (16).

4. Protein Bodies

In all plants, protein bodies are cellular organelles containing storage proteins. In monocots (e.g. cereal grains), protein bodies are mainly found in a triploid tissue, endosperm (starchy endosperm and aleurone layers). In dicots (e.g. legumes) no special storage tissues exist; protein bodies are found within cotyledons (part of the embryo). In some seeds, two internal inclusions, the globoid and the crystaloid, are located within protein bodies. A crystaloid is a storage protein deposit but a globoid in not a proteinaceous inclusion (19, 20).

Protein bodies can be isolated by aqueous solvents in cereals and legumes. Density gradient centrifugation is also used successfully for their separation. Average size of protein bodies ranges from 0.1 to 25 μm. In most cases, they are surrounded by a single membrane:



In Graminae grains, two different kinds of protein bodies exist. Those in the aleurone layers have internal inclusions, the globoid and the crystaloid. In the protein bodies of starchy endosperm, no such inclusions are present. Also the protein composition of these two types of protein bodies is different (16).

Generally, protein bodies contain storage proteins (70-80% of dry weight), phytic acid salts (10%), hydrolytic enzymes, cations and ribonucleic acids (21).

5. Development of Protein Bodies

Three hypotheses have been proposed for the formation of protein bodies in the storage tissues of seeds (22). One theory holds that the proteins are synthesized on rough endoplasmic reticulum (RER), pass into the lumen and then are transported to the dictyosomes. Proteins are then cut off in vesicles and these vesicles move to vacuoles where the proteins are stored. This mechanism has been suggested for the deposition of protein bodies in oilseeds and legumes (23, 24). A second hypothesis points out that protein bodies are independent organelles and are able to synthesize and deposit storage proteins. A plastid origin for protein bodies has been assumed in this theory (25). In a third hypothesis, the protein bodies are assumed to be formed by direct synthesis of storage protein on the rough endoplasmic reticulum (RER), with deposition and accumulation of the storage protein in the lumen. This pathway has been proposed for the formation of

maize protein bodies (26).

Miflin et al (1981) have recently taken a fresh look at the synthesis of cereal protein bodies (22). They studied the distribution of various organellar marker enzymes in homogenates from the developing endosperms of barley, wheat and maize. They suggested that the storage proteins in these three cereals are synthesized on RER into which they aggregate. In both barley and wheat, these aggregates separate from the RER to form protein bodies which still contain some RER. However, in maize, the protein is deposited into spherical deposits within RER and these deposits are then released and form protein bodies upon the breakage of the tissue.

C. STORAGE PROTEIN CLASSIFICATION

In 1895, Osborne classified seed proteins on the basis of their solubilities in water and various aqueous solutions. This classification is still widely used:

1. Albumins - Proteins soluble in water.
2. Globulins - Proteins insoluble in water but soluble in dilute salt.
3. Glutelins - Proteins insoluble in water and dilute salt but soluble in weak acid and base.
4. Prolamines - Proteins insoluble in the above solutions but soluble in 70 to 80% ethanol. (27)

Albumins constitute up to 10% of the total storage proteins in oat endosperm. They are relatively rich in essential amino acids such as lysine and

methionine. The major storage protein in oat is globulin which is considered as a high quality protein; the high level of this protein fraction contributes to the oat's good quality. Glutelins are also fairly high in oat storage tissue depending on different cultivars. They comprise about 20-30% of total storage proteins. The alcohol-soluble protein fraction in oat is estimated at 10-15% of total protein (9).

The molecular structures of albumins and glutelins have not yet been characterized. Glutelins seem to be very large polypeptides. A few studies on prolamine structure have been conducted. About 17 polypeptides have been found for the prolamine fraction using starch electrophoresis (28).

D. STRUCTURAL ANALYSIS OF GLOBULIN

A few research groups are currently active in the area of oat protein chemistry. Dr. D.M. Peterson and coworkers at University of Wisconsin, Madison and Dr. Mosse and coworkers at Versailles, France are involved in studying the chemistry of different oat protein fractions. However, only limited information on the structure and physical properties of oat proteins is available.

Salt-soluble cereal proteins, globulins, are the major protein fraction in oat endosperm (29). During development of oat endosperm, globulin concentration in protein bodies increases linearly with time. By the 12th day

(after anthesis), 50% of storage protein is globulin (29). It has been shown that the increase in the total protein of a particular oat cultivar is related to an increase in globulin fraction (30).

Oat globulin is soluble mostly in NaCl solution. The optimum concentration of NaCl for extraction of globulin is 0.8-1 moles at a pH 6.2 (31).

Few studies on the structural analysis of oat globulins have been conducted. In one study (32), the sedimentation constants of native globulin were determined to be 2.6 and 8.1, while in a recent report (31) the $S_{20,w}$ value shown to be 12.1. The latter figure is probably more accurate because it has been obtained in more defined conditions.

Peterson D.M. (1978) determined the molecular weight of the globulin, using sedimentation equilibrium centrifugation, to be 332000 daltons. After electrophoresing this protein component in denaturing conditions (SDS-Polyacrylamide electrophoresis), two major subunits could be observed. These α and β subunits have molecular weights of 21700 and 31700 daltons respectively. In addition to these bands, a few heavier subunits with molecular weights of 56000 are present on the gel. They may be precursors of the α and β subunits. This theory has been supported by the synthesis of these heavy subunits in an in vitro cell-free protein synthesizing system (33). Finally, it has been proposed that the 332K native globulin is composed of 6α and 6β subunits. This 6:6 subunit ratio

also exists for several legume globulins as well (34).

The amino acid composition of oat globulin has also been determined (35). Table I presents the amino acid composition of oat globulin and its major subunits. The proportion of aspartate/asparagine and basic amino acid residues are higher in the α subunit than in the β subunit; but the α subunit has a lower concentration of glutamate/glutamine and glycine compared to the β subunit. The lysine content of the α subunit is much higher(>50%). The total oat globulin fraction has a lower lysine content than legume globulins (34).

It has been postulated that one of the globulin subunits may have a carbohydrate moeity in its structure (31). The structure of this sugar molecule (31) has not been determined.

E. MECHANISM OF PROTEIN BIOSYNTHESIS

1. General

It is not the purpose of this section to explain the whole process of protein biosynthesis in extensive details. Only a brief review of the general mechanism for the synthesis of proteins in eukaryotic systems and a few details on the factors involved in this process occurring in plant cells will be discussed. More detailed reviews on the subject are available (36,37).

The transfer of genetic information from cell genome by transcription and translation results in the

TABLE I

THE AMINO ACID COMPOSITION[a] OF OAT GLOBULIN FRACTION AND ITS MAJOR SUBUNITS[b]

Amino acid	Globulin	α-Subunit	ß-Subunit
Asp/Asn	9.9	12.8	9.1
Thr	3.5	4.0	3.3
Ser	6.2	6.3	7.9
Glu/Gln	21.4	15.3	21.4
Pro	5.3	5.2	4.5
Gly	8.2	7.0	12.1
Ala	6.4	7.2	5.8
Val	5.8	6.2	5.6
Ile	4.4	5.3	3.9
Leu	7.7	7.9	7.7
Tyr	3.5	3.6	3.0
Phe	5.6	4.9	5.7
His	2.0	2.2	1.6
Lys	2.6	3.3	2.1
Arg	6.4	7.2	5.9

a: Half cystine and methionine were not reported because of the partial destruction by HCl hydrolysis, and tryptophan because of complete destruction.

b: Adapted from Peterson D.M.(1978)

biosynthesis of proteins. These processes occur rapidly and substantially in growing cells. Transcription of DNA sequences which contain genes for specific proteins into RNA molecules and subsequent post-transcriptional modification of these new formed RNAs, produces relatively stable messenger RNAs. The newly synthesized mRNAs are transported into cytoplasm. The genetic information of mRNA is then translated into proteins in a protein synthesizing system which includes ribosomes, tRNAs, translation factors, and aminoacyl-tRNA synthetases. Three different sequences of reactions occur in the biosynthetic process, the initiation of a peptide chain, the elongation of that chain, and the termination of its synthesis. A summary of the basic mechanism of protein biosynthesis in the cytoplasm of eukaryotic organisms is presented in Table II.

The mechanism of protein biosynthesis in the cytoplasm of higher plants is basically the same as that operating in other eukaryotic systems (e.g. mammalian cells). However, the mitochondria and the chloroplast organelles of the plant perform the process similar to prokaryotic systems.

2. Ribosomes

At least two distinct classes of ribosomes exist in plant cells. Those found in cytoplasm are usually 80 S ribosomes while the ribosomes localized in the chloroplasts have a sedimentation coefficient of 70 S (38). Table III

TABLE II

MECHANISM OF PROTEIN BIOSYNTHESIS IN EUKARYOTES[a]

INITIATION
Initiator met-tRNA binds to smaller ribosomal subunit. Larger ribosomal subunit and messenger RNA are combined with initiation complex. In this process, initiation factors and GTP are required.
ELONGATION
Elongation factor-1 brings the aminoacyl$_1$-tRNA to ribosome corresponding to second codon on mRNA. Peptide bond is formed on ribosome Elongation factor-2 translocates met-aminoacyl$_1$-tRNA on ribosome(GTP requiring process) Elongation factor-1 brings a second aminoacyl-tRNA corresponding to third codon on mRNA
TERMINATION
Completed peptidyl-tRNA is released from ribosome-mRNA complex corresponding to termination codons on mRNA. Release factors and GTP are required. Ribosomes dissociate into subunits.

a: Adapted from Orio Ciferri(1975)

TABLE III

EUKARYOTIC RIBOSOMAL RNA SPECIES[a]
(SEDIMENTATION COEFFICIENTS)

	Cytoplasm	Mitochondrion	Chloroplast
Higher Plants	80S(25,18,5.8,5)	78S(24,18,5)	70(23,16,5)
Animals	80S(28,18,5.8,5)	55S(17,13,?)	

a: Adapted from Orio Ciferri(1975)

represents the ribosomal RNA species found in cytoplasm and organelles of a typical plant cell.

There are functional differences among cytoplasmic ribosomes (80 S) and organellar (mitochondrion and chloroplast) ribosomes (70 S). Plant cytoplasmic ribosomes respond in in vitro assays to the elongation, initiation and termination factors isolated from prokaryotes. Also, cytoplasmic protein biosynthesis is inhibited by cyclohexamide (a eukaryotic inhibitor) and not by chloramphenicol (a prokaryotic inhibitor). In the case of organellar protein synthesis, the inhibition occurs by chloramphenicol and not by cyclohexamide (38).

The existence of a ribosome cycle in plants has been postulated (39). In this cycle, polysomes dissociate into their subunits (monosomes) immediately after the completion of the peptide chain. For a new round of synthesis, the monosomes and the newly synthesized ribosomal subunits associate.

3. Plant Protein Biosynthesis

a) Initiation

In the cytoplasm of higher plants, peptide chains are initiated with non-formylated methionine. No transformylase is thought to exist in cytoplasm. Two methionyl-tRNAs can be isolated from cytoplasm of higher plants (e.g. wheat embryo) (40). One of the two met-tRNAs transfers non-formylated methionine to the N-terminal

position of the peptide chain. This tRNA also binds to 40 S ribosomal subunits in low Mg^{+2} concentration and in the presence of the initiator codon AUG. The second met-tRNA transfers methionine to the internal positions of the peptide chain. Initiation factors have also been located on the ribosomes (41).

There are distinct sets of tRNAs for cytoplasmic, chloroplastic, and mitochondrial protein synthesis in higher plants. Plant cells contain more than 20 aminoacyl-tRNA synthetases. Some aminoacylate only cytoplasmic tRNAs while others are able to aminoacylate both cytoplasmic and organellar tRNAs (42). A single amino acid may be specified by different complements of synthetases and tRNAs (38).

Plant tRNAs have been shown to possess cytokinin activities (43). This property of tRNAs and the high concentration of these molecules in rapid growing tissues may indicate a possible role of tRNA in the control of protein synthesis.

b) Elongation

Two protein factors, EF-1 and EF-2 are required for the elongation of the peptide chain in the plant cytoplasmic systems (44). EF-1 (70 K) induces the binding of the aminoacyl-tRNA to the ribosomes in the presence of GTP. EF-2 (60 K) translocates the peptidyl-tRNA from the ribosomal acceptor site to the donor site. It has been postulated that a complex form of EF-1 and

EF-2 is the actual active form of the elongation factors (45).

c) Termination

The termination codons UAG, UAA, and UGA are deemed to act as chain terminators in the cytoplasm of plants (36). Very little is known about this stage of protein biosynthesis in plants (36).

4. In Vitro Synthesis of Storage Proteins

Storage proteins are the main products of cytoplasmic protein biosynthesis in legumes and cereal seeds. A number of storage proteins from these plants have been synthesized in vitro in cell-free protein synthesizing systems. This has become possible by the isolation of polysomes from developing seeds and translating them in a cell-free system. In recent years, because of the discovery that most eukaryotic messenger RNAs have a poly A tail at the 3' end (46), the isolation and purification of mRNAs for certain storage protein fractions has also become possible. The poly A sequence of a particular mRNA enables one to separate these molecules from other RNA species using oligo d(T) cellulose chromatography (47) or poly-U Sepharose (48).

The in vitro-translation systems commonly used for translation are rabbit-reticulocyte lysate and wheat germ extract. Both systems have been shown to faithfully translate specific plant mRNAs

products (49, 50). These in vitro-translation systems have been widely used to study seed storage protein synthesis during development and growth.

One of the well-studied storage proteins in terms of its synthesis and storage is zein (the alcohol-soluble protein fraction of maize). Burr et al (12,25) have extensively studied the synthesis of zein on the ribosomes bound to the outer membrane of the zein protein bodies. They have proposed an interesting mechanism for the biosynthesis and deposition of zein in protein bodies during development of maize endosperm. Their theory is an adaptation of the earlier 'signal hypothesis' proposed by Blobel and Dobberstein (1975) (51). Figure 2 illustrates this hypothesis.

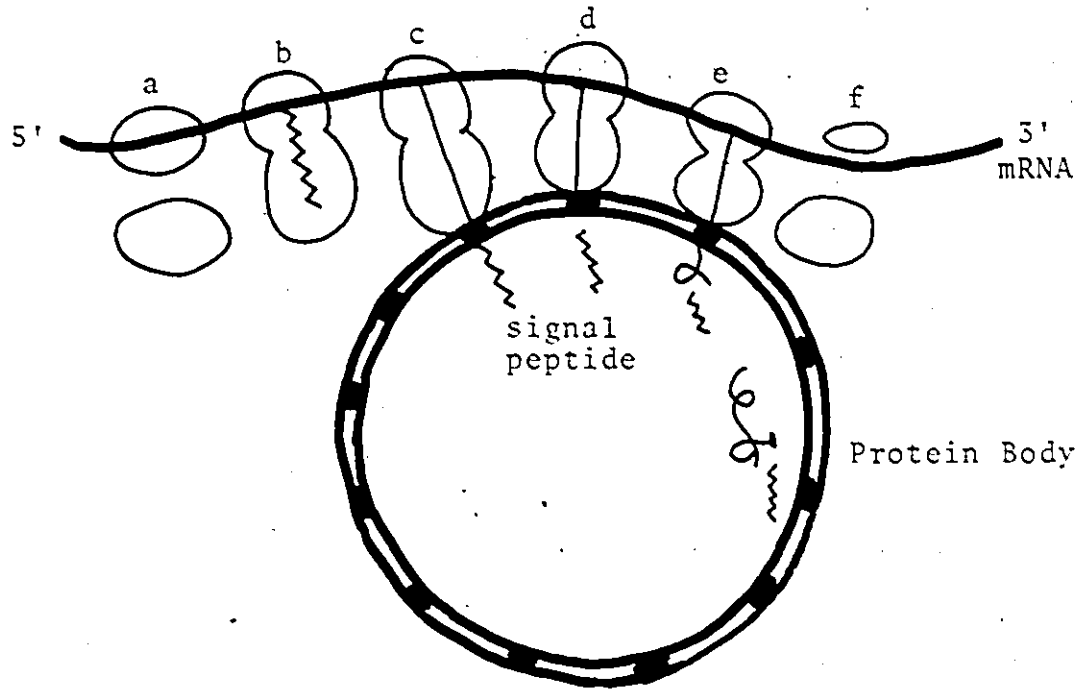


Figure 2. Biosynthesis and Processing of Zein Polypeptides on a Protein Body. Stages of the Process:

a. The small ribosomal subunit binds to the initiation site close to the 5' end of the zein mRNA.

b. Translation begins after the association of both small and large subunits; the signal peptide is formed.

c. The signal peptide is recognized by the membrane of the protein body and moves through the membrane.

d. A specific peptidase cleaves the signal peptide in the membrane or within protein body.

e. A glucosyl-transferase associated with protein body transfers a glucose molecule to each zein polypeptide later in translation.

f. When the translation is completed the zein polypeptide is released in the protein body and stored. The release of the ribosomal subunits prepares the system for a new round of synthesis.

(Adapted from Burr F.A.,1979)

Several other storage proteins of legumes and cereals have been synthesized *in vitro* using cell-free systems. Sun et al (1975) (52) reported the synthesis of G1 protein of *Phaseolus vulgaris in vitro* in wheat germ extract. Recently, the mRNA for this protein from french bean was isolated and shown to efficiently translate in cell-free system producing actual G1 globulin polypeptides (13). Similarly, the polysomes and the mRNAs for vicillin and legumin proteins have been purified by immunoprecipitation. These mRNAs are able to conduct the synthesis of vicillin and legumin polypeptides in cell-free systems (53). More recently, the mRNA for soybean storage protein, legumin, has been purified which directs the synthesis of legumin polypeptides *in vitro* (15).

A number of cereal storage proteins have also been synthesized *in vitro*. Burr and Burr (1976) isolated polysomes from developing maize endosperms which directed the synthesis of very similar zein polypeptides (25). Recently, the zein mRNA was isolated from membrane-bound polysomes and shown to synthesize *in vitro* primary polypeptides 1100 and 2000 daltons larger than authentic zein polypeptides (12,54). They concluded that the products of cell-free translation system are the precursors of zein which are later modified in post-translational modification process. Membrane-bound polysomes have also been isolated from barley developing endosperm and shown

to synthesize hordein (the major protein fraction in barley) polypeptides in the wheat germ protein synthesizing system (14, 55). Lately, J.A. Mathews working in Dr. B.J. Miflin's laboratory at Rothamsted Experimental Station in England, has purified the mRNA associated with membrane-bound polysomes. These molecules are between 0.55 and 2.55 kilobases in length with about 6% poly A. They could be translated in wheat germ extract with products similar to the native hordeins.

Free and membrane-bound polysomes have been isolated from developing oat groats (33). The membrane-bound polysomes are translated more efficiently than free polysomes in wheat germ extract and produce polypeptides which co-electrophorese with authentic globulin on polyacrylamide-SDS gels.

F. RESEARCH OBJECTIVES

The objectives of this research were:

1. To investigate the nitrogen accumulation of oat storage tissue during development and to explore possible varietal and developmental differences.

2. To isolate the storage protein fractions which are synthesized during nitrogen accumulation and to characterize the major storage protein fraction in a newly developed high protein oat cultivar.

3. To investigate the amount of RNA in oat storage tissue during seed development.

4. To isolate polyribosomes from oat endosperm and investigate their ability to direct the synthesis of oat proteins in a cell-free protein synthesizing system.

5. To isolate poly A^+-enriched RNA from oat polyribosomes and investigate the translation of this RNA fraction in a cell-free translation system.

First, these investigations have resulted in some basic understanding of the physiology and biochemistry of oat storage tissue. More detailed experiments are in progress to understand the mechanism for the biosynthesis and deposition of oat storage proteins and to investigate which hypothesis holds true in the biosynthesis of oat storage proteins.

Secondly, a rather efficient method for the isolation of total oat poly A^+RNA from oat endosperm has been developed. Such RNA preparations are currently being used to further purify poly A^+ RNA sequences coding for globulin polypeptides.

The long term objectives of purifying the poly A+ RNA for globulin are to prepare a complementary DNA sequence to this RNA fraction and use this cDNA to locate the genomic sequences (genes) coding for globulins. This will enable us to isolate and elucidate the structure and organization of globulin gene(s).

Chapter II

MATERIALS AND METHODS

A. MATERIALS

1. Plant Tissues

Seven oat cultivars (Avena sativa L.) with a wide range of grain protein content (Table IV) were chosen and grown at the Ottawa Research Station, Agriculture Canada. Four row plots (3m long and 30.5cm between rows) were grown in triplicate in a randomized block design. Oat panicles were harvested at 6 weekly intervals after anthesis. The panicles were stored at -20°C for studies on the development of oat storage tissue.

The high protein cultivar, Hinoat, was chosen for the purpose of protein and RNA extractions. In order to have a constant access to fresh developing oat endosperm throughout the winter, oat plants (Hinoat) were grown in a glasshouse under defined conditions (18 hr. day, 24°C day, 16°C night) in the Department of Biology greenhouse, University of Ottawa.

2. Chemicals

Sigma Chemical Co.: Lauryl sulfate, Hepes buffer, Magnesium acetate, EDTA, EGTA

TABLE IV

THE CHARACTERISTICS OF SEVEN OAT CULTIVARS

Cultivar[a]	Protein Content(%)[b]	Grain Yield
Hinoat	very high (22)	low
Dal	high (18)	moderate
Sentinel	high (18)	high
Elgin	medium (17)	moderate-high
OA 424-1	low-intermediate	high
Harmon	low (15)	high
OT 215[c]	low (15)	moderate

a: All cultivars were grown at Ottawa Research Station in 1980 from 1979 Ottawa seed.

b: Values were obtained by multiplying average nitrogen percentage by a factor of 6.25, the common factor for oats.

c: A high oil cultivar.

Baker Analyzed Reagents: ammonium persulfate, lithium chloride, phenol, formamide, formaldehyde, 2-mercaptoethanol, potassium acetate, tris base, ethanol, potassium sulfate, mercuric oxide, sulfuric acid, sodium hydroxide, hydrochloric acid, tricholoroacetic acid, 2-propanol, urea, chloroform, glycerol, ether.

Bio-Rad Laboratories: chemicals for SDS- acrylamide gel electrophoresis, acrylamide, temed (N, N, N', N'-tetramethylethylenediamine), Coomassie brilliant blue.

Fisher Scientific Company: ammonium sulfate, potassium chloride.

Bethesda Research Labs: oligo-d(T) cellulose, RNase-free sucrose, rabbit reticulocyte lysate

Boehringer Corporation Ltd: E.coli RNA standard (23S, 18S, 5S) viral RNA standard.

BDH Lab. Reagent: sodium azide.

Difco Laboratories: sodium deoxycholate.

New England Nuclear: rabbit reticulocyte lysate translation kit, (^{3}H)-leucine.

B. METHODS

1. Moisture Content

Ten to twenty kernels from each plot were carefully dehulled manually using a tweezer. Fresh weights were

recorded and dry weights of the groats were determined after drying in an oven at 105°C for 24 hours. This was repeated for all seven cultivars. Moisture content of oat groats was determined using the following standard formula.

$$\text{Moisture content percent fresh weight} = \frac{\text{F.W.} - \text{D.W.}}{\text{F.W.}} \times 100$$

2. Total Nitrogen determination

The dried groats were ground finely into a flour using liquid nitrogen in a mortar and pestle. The nitrogen content of the flour was determined by the micro-Kjeldahl procedure. Two different instruments were used, the semi-automatic Kjeltec apparatus (Tecator) and the automatic Kjel-Foss analyzer (Foss Electric). The nitrogen content measurements of the groats from each plot were conducted in triplicate making a total of 9 determinations for each cultivar at each harvest.

Micro-Kjeldahl Procedure:

The method used was basically the same as that described by Triebold and Aurand (1963)(56).This method is a modification of the original micro-Kjeldahl procedure and follows the same principles.

Dried oat flour (45 mg) was placed in a Kjeldahl flask followed by 50mg of mercuric oxide, 2.0 grams of potassium sulfate and 5ml of concentrated sulfuric acid.

The flask was heated to boiling under a fumehood until the solution became clear (1-1.5 hrs.). The flask was left to cool and the precipitate was dissolved by the minimum volume of distilled water. The digest solution was transfered to a distillation apparatus (Kjeltec) after neutralization with 20 ml of 50% NaOH/25% $Na_2S_2O_3$. The distilled ammonia was collected in an Erlenmeyer flask containing 5 ml of saturated boric acid and a few drops of methyl red-methyl blue (2/1) until the total volume was 25 ml. The distilled ammonia was then titrated with 0.05 hydrochloric acid to a gray end point. The following formula was used to calculate the total nitrogen content of oat flour based on dry weight. Mean and standard deviations were determined and the data was treated with analysis of variance.

$$N\ \% = \frac{(\text{sample titre} - \text{blank titre})\ X\ \text{Normality of HCL}\ X\ 14.007}{\text{milligrams of dry flour}(45)\ X\ 100}$$

3. Extraction of Oat Protein Fractions

Like other storage proteins, oat protein is generally classified into four major fractions based on their solubility in various solvents. The extractions of globulins, albumins, prolamines, and glutelins are described respectively.

a) Extraction of salt-soluble globulins

Developing and mature oat kernels (Hinoat) were dehulled and ground finely using a mortar and pestle. The flour (3g) was mixed with 200 ml of 1 M NaCl, 0.05 M

Tris (pH 8.5) for 3 hours at room temperature. The homogenate was centrifuged for 20 minutes at 3,000 X g. The supernatant was removed (the pellet was kept for the prolamine extraction) and dialyzed against distilled water for 48 hours. After centrifugation at 13,000 X g for 20 min. the precipitate was suspended in distilled water (the supernatant was preserved for albumin "extraction") and freeze-dried overnight. This procedure is a modification of that of Peterson et al (1976) (29).

b) Extraction of water-soluble albumins

The supernatant obtained after dialysis and centrifugation during globulin extraction was used for albumin recovery. The albumin fraction was precipitated with 0.25 volume of 50% (W/V) TCA, centrifuged, and suspended in water for lyophilization (29).

c) Extraction of alcohol-soluble prolamines

The flour pellet obtained after the isolation of globulins was suspended in 60 ml of 55% 2-propanol, 43% H_2O, 2% 2-mercaptoethanol and stirred for 2 hours at 60° C. The mixture was centrifuged for 25 min. at 13,000 X g. The supernatant was made 4% NaCl to precipitate prolamines and left overnight. After centrifuging at 20,000 X g for 25 min , the pellet (prolamine) was suspended in 100 ml of H_2O and dialyzed against distilled water for 24 hours. The dialyzed solution was then centrifuged and freeze-dried. The above procedure was a modification of

that of Shewry, P.R. et al (1978)(57).

d) Extraction of base-soluble glutelins

The pellet from prolamine extraction was extracted with 0.1N NaOH for 2 hours at room termperature. After centrifugation for 30 minutes at 13,000 X g, the supernatant was dialyzed for 24 hours and centrifuged again. The precipitate was suspended in distilled water and freeze-dried (29).

4. Analysis of Globulins on SDS-acrylamide Gel Electrophoresis

Oat globulin was electrophoresed on a 12% SDS-polyacrylamide gel in denaturing conditions. The procedure was a modification of that of Laemmli (58). Resolving gel contained: 12% acrylamide/Bis, 4M urea, 0.37 M Tris/HCl (pH 8.8), Temed (20 µl),0.05% ammonium persulfate. Oat globulin sample was dissolved in sample buffer containing 8M urea, 1% SDS, 1% 2-mercaptoethanol and 0.65 M Tris/HCl (pH 6.8), and applied to slab gels (0.75mm X 10cm X 14cm) which were cast between two glass plates. The electrophoresis was performed at 40 volts, constant voltage for 16 hours. After electrophoresis the gel was stained with Coomassie Brilliant Blue R-250 for one hour and destained overnight.

5. Isolation of Total Large Molecular RNAs from Oat Storage Tissue

The total RNA was isolated essentially as described by Cuming et al (59). Young oat kernels (Hinoat-milky stage) were collected on dry ice, ground in liquid nitrogen and mixed with 180 ml of phenol-chloroform (1:1 v/v) and 180 ml of 0.1 M Tris/HCl (pH 8.5) for 15 minutes. The homogenate was centrifuged at 500 X g for 30 min. and 13,000 X g for 20 min. The aqueous phase was removed and the organic phase was extracted again with one volume of 0.1 M Tris buffer. The aqueous phases were pooled and extracted once with half-volume of phenol-chloroform. The final aqueous phase was mixed with 0.1 volume of 20% potassium acetate and 2 volumes of ethanol. The total RNA was precipitated at -20°C for 24 hours. After centrifugation at 13,000 X g for 30 min. the RNA pellet was dissolved in sterile distilled H_2O, made 2.5 M with respect to NaCl and left at 0°C for 36 hours. Large molecular RNAs were precipitated at these conditions and collected by centrifugation. The RNA pellet was dissolved in H_2O and A_{260} and A_{280} were measured to estimate the amount of RNA recovered.

All glasswares and buffers were autoclaved at 121°C for 15 min. Diethyl carbonate (a RNase inhibitor) was added to all buffers at a concentration of 0.001%.

6. Isolation of Total Polyribosomes

The procedure for the isolation of polysomes was modified from that of Davies et al (60). Oat kernels

were ground in liquid nitrogen and homogenized in homogenization buffer containing 250 mM sucrose, 200 mM Tris/HCl (pH 8.5), 60 mM KCl, 30 mM Mg acetate, and 5 mM 2-mercaptoethanol. After filtering through two layers of cheesecloth the homogenate was made 3% (w/v) sodium deoxycholate and layered on a sucrose cushion (1.5 M sucrose, 40 mM KCl, 10 mM Mg acetate in 40 mM Tris/HCl (pH 8.5). After centrifugation at 13,000 X g for 2 hours 4°C in a Ti-60 rotor of a L2-65B ultracentrifuge (Beckman), the polysome pellet was washed with sterile distilled water and suspended in washing buffer containing 40 mM Tris/HCl (pH 8.5), 40 mM KCl, 10 mM Mg acetate, and 20% (w/v) glycerol. This suspension was centrifuged on a similar sucrose cushion for 2 hours. The washed polysomes were stored at -80°C. An aliquot of polysomes was suspended in H_2O and A_{260}/A_{280} was measured.

All buffers were autoclaved but in the case of buffers containing sucrose, the buffers were autoclaved without sucrose and RNase-free sucrose was added after.

Isolation of free and membrane-bound polyribosomes: Polyribosomes were isolated from developing oat groats by a modification of the method of Matthews and Miflin (1980) (61). Oat groats were frozen in liquid N_2 and ground in a coffee grinder. The flour was mixed with extraction buffer containing 0.2M Tris (pH 7.5), 100 mM KCl, 10 mM MgAc, 0.25M sucrose, 10 mM DTT; and homogenized in a blender for 2 minutes. After 2 cycles of centrifugation

at 6,000 X g for 5 minutes, the supernatant (containing free polysomes) was layered on 4 ml of 1.5M sucrose cushion in the same buffer. On the other hand, the pellet was re-extracted by the same extraction buffer containing 1% Triton X-100. The mixture was centrifuged once at 6,000 X g for 5 min and then at 17,000 X g for 15 min. The supernatant (containing membrane-bound polysomes) was also layered on an identical sucrose cushion. All solutions were centrifuged in a Ti-60 rotor in a L2-65B Beckman centrifuge at 130,000 X g for 4 hours.

Polysome pellets were carefully washed twice with sterile-distilled water (s-d), suspended in s-d H_2O and stored at -80°C.

7. Isolation of Polysomal RNA and Poly A^+ RNA

Isolated polysomes were dissociated with SDS and polysomal RNA was extracted with phenol-chloroform as described by (62). Polysomes were suspended in dissociation buffer (0.2 M Tris pH9, 0.1M NaCl, 10 mM EDTA, 0.5% SDS) mixed with one volume of phenol-chloroform (v/v-1:1) and centrifuged at 13,000 X g for 15 min. The aqueous phase was removed and the organic phase was re-extracted with one volume of buffer. The aqueous phases were pooled and extracted with half-volume of phenol-chloroform. The aqueous phase was removed and the organic phase was extracted with half-volume of buffer. All aqueous phases were again pooled, made 0.2M ammonium

acetate and mixed with 2.5 volumes of ethanol and left at -20°C overnight. Polysomal RNA was recovered by centrifuging at 10000 rpm for 30 min.

Oligo d(T)-Cellulose Chromatography:

The column was run as described by Aviv and Leder (47).

Oligo d(T)-cellulose (1 gram) was suspended in high-salt binding buffer (20 mM Tris/HCl-pH 7.4, 0.4 M NaCl, 0.2% SDS), and poured into a column (1cm X 6cm). The column was then washed extensively with high-salt binding buffer and stored at 4°C in 0.02% sodium azide. The chromatography was performed by the following procedure.

The column was first washed with s-d H_2O and then with high-salt buffer. Total RNA was dissolved in high-salt buffer and applied to the column. The first column eluate was passed through the column a second time to maximize poly A^+ RNA adsorption. Then, the column was washed with 10 volumes of high salt buffer and 5 volumes of low salt buffer (20 mM Tris/HCl-pH 7.4, 0.2 M NaCl) respectively. Poly A^+ RNA was eluted with 1 volume of no-salt buffer (20 mM Tris/HCl-pH 7.4). The elutant was made 0.3 M NaCl, mixed with 2.5 volumes of 99% ethanol and left at -20°C for 24 hours. The poly A^+ RNA was recovered by centrifugation and stored at -80°C.

The column was monitored by a Dual path (254, 280 nm) UV-2 monitor (Pharmacia Fine Chemicals).

8. Cell-free Translation of Oat Polysomes and RNA

The polysomes and RNA preparations were translated in the rabbit reticulocyte lysate system(49). The standard translational incubations contained, in a final volume of 25 µl: 10 µl lysate(3 X concentrated, 3.5 mM MgCl, 0.05 mM EDTA, 25 mM potassium chloride, 0.5 mM dithiothreitol, 25µM hemin, 50 µg/ml creatine kinase, 1 mM calcium chloride, 2 mM EGTA, 70 mM sodium chloride),and 13 µl of premix containing: 8.5 µl L-(^{3}H)-leucine(1mCi/ml),2.0 µl cocktail*, 2.0 µl 1M potassium acetate and 0.5 µl of varying concentrations of magnesium acetate; and 2µl of sample. Rabbit globin mRNA was used as standard. After mixing all the components, the mixture was incubated at 37° C for 1 hour. An aliquot of the translation mixture was removed and placed on a strip of filter paper. The filter papers were left in a 10% TCA solution for 15 min and then boiled in 5% TCA for 10 min. The strips were washed twice with cold 5% TCA, once with 95% ethanol and finally with ether. After drying, the papers were put in scintillation vials containing 10 ml of Econofluor pre-mixed solution. Radioactivity was determined in a liquid scintillation counter(on the tritium channel).

*Cocktail contained: spermidine, creatine phosphate, and guanosine triphosphate in HEPES buffer. The concentrations were not mentioned in the New England Nuclear Translation kit.

CHAPTER III

RESULTS

A. The variation in fresh and dry weights, and nitrogen content of oat groats during development

Seven oat cultivars with different agronomic and biochemical properties were chosen. The pattern of nitrogen accumulation was followed. To express nitrogen content on a dry weight basis, the changes in moisture content were first determined during seed development. Table V represents the variations in fresh weights of oat groats in seven different oat cultivars during growth. Groats were dissected from the whole grain and the measurements were all performed on isolated groats. Some previous investigators (29) have followed the nitrogen accumulation of oat grains with hulls on. However, more accurate results may be obtained by removing the hulls. At maturity, OA 424-1 groats weigh the most. Hinoat, although a very high protein cultivar, has a relatively moderate yield. Dry weight of oat cultivars also increased linearly from anthesis until maturity(Table VI).

Nitrogen contents of oat cultivars were determined and expressed in both nitrogen percentage(Table VII) and nitrogen content per 100 seeds(Table VIII), because the pattern of nitrogen accumulation was to be followed in individual seeds. The nitrogen content of all seven cultivars increased significantly during development as determined by the nitrogen content per 100 seeds

TABLE V

GROAT FRESH WEIGHT DURING DEVELOPMENT (mg per 100 seeds)[a]

	Harvest Date (weeks after anthesis)					
Cultivar	1	2	3	4	5	6
Hinoat	398	3185	4650	3700	3460	3233
Dal	264	2000	3670	3850	3500	3300
Elgin	253	2850	4865	5200	3720	3646
Sentinel	145	1760	3440	5000	3670	3395
Harmon	180	2290	4285	5400	3830	3373
OA 424-1	281	2413	5070	5600	5130	4306
OT 213	225	2140	4185	4400	3240	2886

a: Values calculated from fresh weight measurements of 10-20 oat groats.

TABLE VI

GROAT DRY WEIGHT DURING DEVELOPMENT (mg per 100 seeds)

	Harvest Date(weeks after anthesis)					
Cultivar	1	2	3	4	5	6
Hinoat	88	1165	2092	2300	2877	2768
Dal	84	610	1530	2590	2807	2753
Elgin	61	910	2150	2600	2900	3066
Sentinel	69	780	1605	2700	3025	2846
Harmon	72	670	1715	2400	2700	2840
OA 424-1	92	566	2130	2100	3930	3653
OT 215	72	656	1725	2150	2480	2440

TABLE VII

GROAT NITROGEN PERCENTAGE[a] DURING DEVELOPMENT

(based on dry weight of the groat)

CULTIVAR	Harvest Dates (weeks after anthesis)[b]				
	2	3	4	5	6
Hinoat	3.17±0.23	3.60±0.10	3.73±0.00	3.37±0.26	3.50±0.13
Dal	2.68±0.06	2.53±0.14	2.66±0.00	2.75±0.05	2.80±0.10
Elgin	2.30±0.22	2.83±0.13	2.86±0.10	2.66±0.05	2.77±0.11
Sentinel	2.81±0.17	2.97±0.16	2.90±0.01	2.88±0.18	2.96±0.10
Harmon	2.68±0.09	2.72±0.03	2.71±0.13	2.32±0.03	2.47±0.08
OA 424-1	2.57±0.07	2.62±0.04	2.70±0.10	2.62±0.21	2.64±0.07
OT 213	2.64±0.06	2.60±0.11	2.58±0.08	2.50±0.08	2.46±0.07

a: Each value is an average of 9 measurements with the exception of harvest 4 where each value is an average of 3 measurements.

b: Harvest 1 was omitted because N% was determined based on fresh weight for this harvest date.

TABLE VIII

GROAT NITROGEN CONTENT DURING DEVELOPMENT[a,b]

(mg per hundred seeds)

	Harvest Dates (weeks after anthesis)[c]				
CULTIVAR	2	3	4	5	6
Hinoat	36.9	75.3	85.7	96.9	96.8
Dal	16.5	40.3	68.8	78.5	77.9
Elgin	21.5	62.3	76.1	77.4	88.3
Sentinel	21.9	47.7	50.8	90.7	86.5
Harmon	18.2	47.1	66.9	63.4	70.4
OA 424-1	14.5	56.4	57.9	107	96.4
OT 213	17.3	35.7	34.3	63.4	60.5

a: Mean nitrogen content per 100 seeds were determined for these harvests based on groat dry weight.

b: Standard deviations were illustrated in Table VII.

c: Harvest 1 was omitted because the nitrogen percentage was determined based on fresh weight.

based on the dry weight of the groat. Hinoat and OA 424-1 showed the highest values at maturity. A drastic increase in the nitrogen content of OA 424-1 groats was observed late in maturation due to the significant increase in the groat biomass.

However, the nitrogen percentage of oat groats (based on dry weight) did not change significantly during development(Table VII). Appendix 1 presents the statistical treatment and a graph of the data. The nitrogen content and percentage both varied among the seven cultivars. Hinoat groats possessed the greatest nitrogen percentage throughout development. In contrast, OT 213 had the lowest values.

The interaction between protein content and the mature grain yield revealed that Hinoat, although high in protein, has a relatively lower yield(Figure 3). Based on these observations, Sentinel appears to be a suitable oat cultivar both in term of its protein content and its yield.

B. OAT PROTEIN FRACTIONS:

Oat(Avena sativa L., cultivar Hinoat) protein fractions were extracted using various solvents as described in Materials and Mehtods(section c). Table IX represents the extractability of these fractions. Globulins(salt-soluble fraction) were the major protein fraction comprising about 50% of total seed protein. This major fraction was analyzed on SDS-polyacrylamide gel electrophoresis using marker standards:

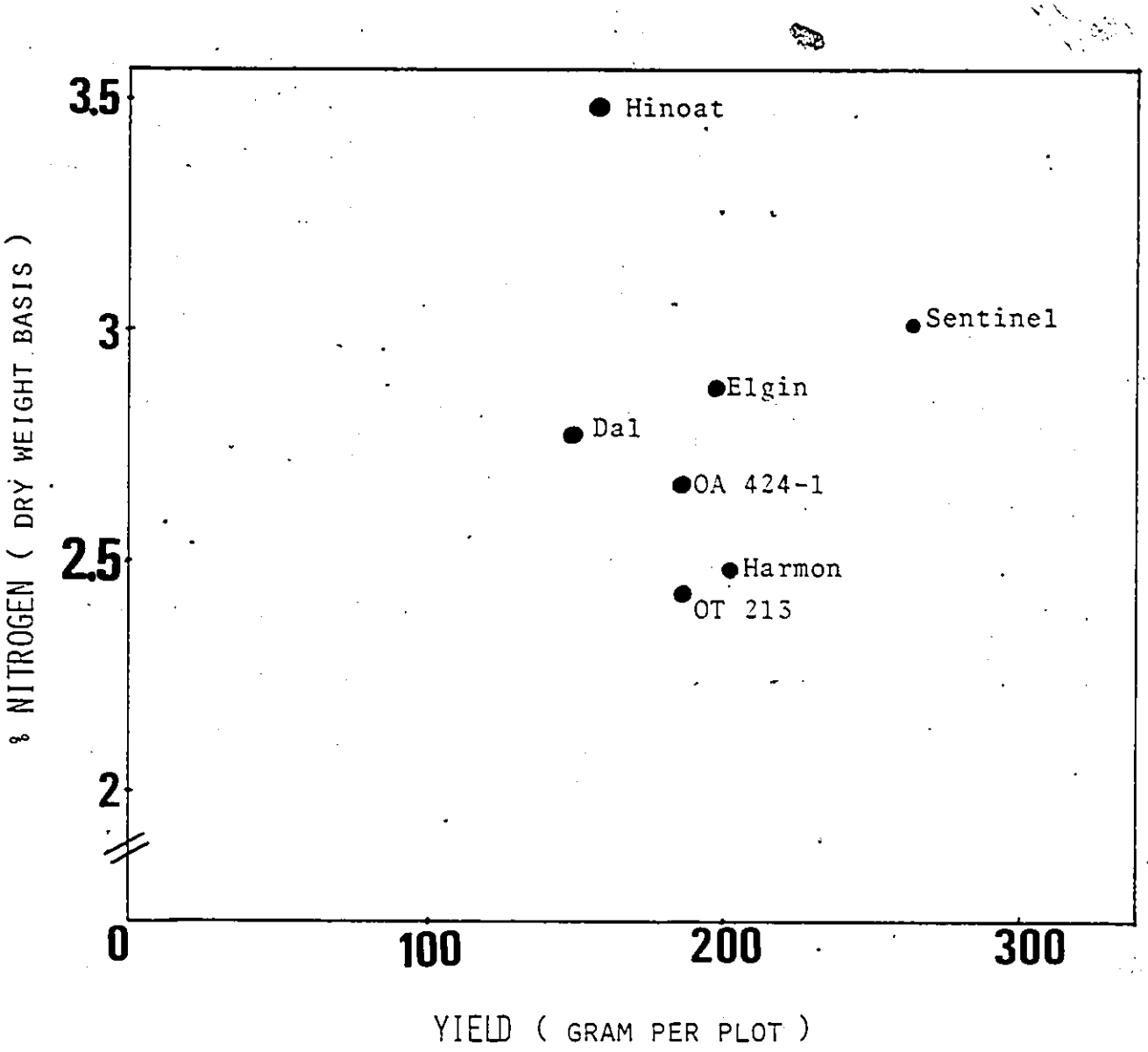


Figure 3. Interaction between nitrogen percentage and the yield in seven oat cultivars

TABLE IX

SOLUBILITY FRACTIONS OF OAT STORAGE PROTEIN

Protein Fraction	Solvent used	Total protein recovered (%)[a]
Albumins	Water ——	9 ± 2.5
Globulins	1M NaCl	47 ± 5
Glutelins	0.1M NaOH	21 ± 3
Prolamines	2-propanol	10 ± 4
Residue	—	13 ± 1.5
Total	—	100 %

Note: Total protein percentage of the oat groat was 20% as determined by the micro-kjeldahl procedure. The common factor of 6.25 was used to convert N% to protein%.

a: Extraction was performed three times and the values are the average of three measurements.

Phosphorylase b, Albumin, Ovalbumin, Carbonic Anydrase, Trypsin inhibitor(20100-94000 MW). Figure 4 shows the electrophoretic pattern of globulin polypeptides. Two major classes of polypeptides could be observed with molecular weights of 21700 and 38000 (Figure 4). Several other minor bands are also present (Figure 4).

C. Changes in RNA Content During Seed Development

It was a logical step in studying the biosynthesis of oat storage proteins to follow the pattern of RNA content during development. The method of Cumming et al who studied the RNA content of developing and mature wheat groats, was used. High molecular RNAs(including heterogenous RNAs, ribosomal RNAs, and messenger RNAs) were isolated from developing endosperms of the cultivar Hinoat at four different stages of development. The yields of RNA were measured based on the assumption that 25 O.D. units is equal to 1 mg/ml RNA(Table X). Young embryos contain a higher percentage of RNA(based on dry weight) compared with mature groats. A constant decrease in RNA percentage was observed as the oat seed developed.(Figure 5).

D. Isolation and Cell-Free Translation of Total, Free and Membrane-Bound Polyribosomes

Free and membrane-bound polysomes were isolated from oat endosperms(Hinoat, milky stage). The yields were determined by suspending the polysomes in sterile-distilled H_2O, centrifuging and measuring A_{260} of the supernatant. It was assumed that 13 O.D. units represents 1 mg/ml of polysomes.

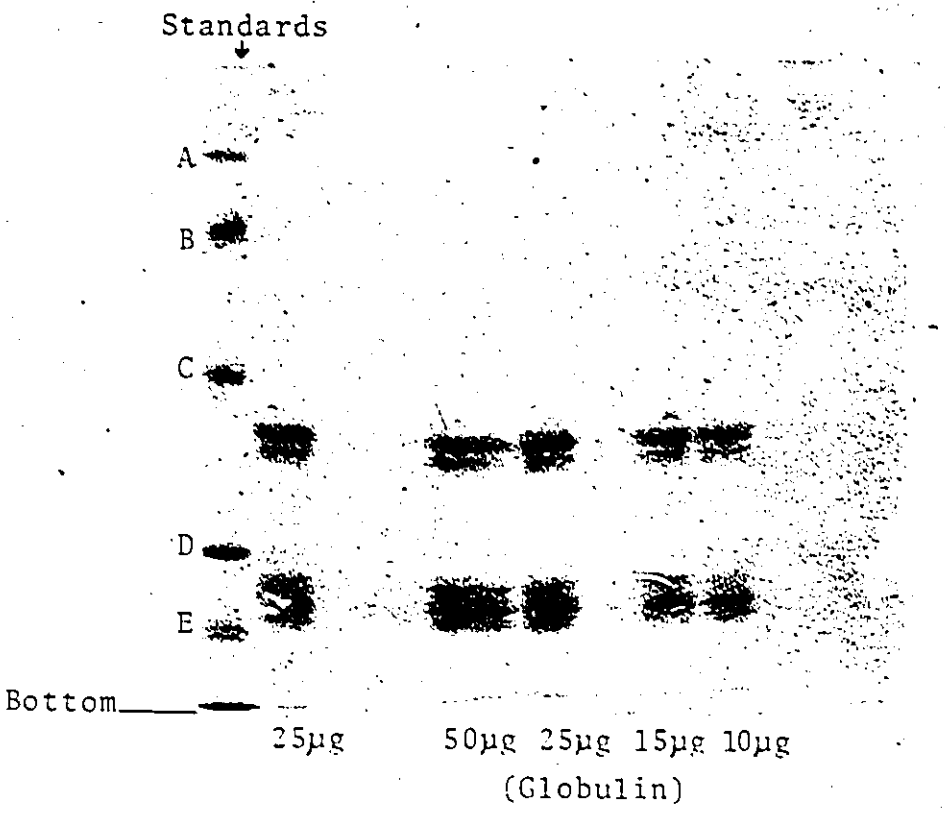


Figure 4. SDS-polyacrylamide gel electrophoresis of oat globulin fraction. Standards were:

A. Phosphorylase b(94000 MW)
B. Albumin(67000 MW)
C. Ovalbumin(43000 MW)
D. Carbonic Anhydrase(30000 MW)
E. Trypsin inhibitor (20100 MW)

Two major classes of polypeptides are observed at the areas of 21700 and 38000 MW. Other minor bands are also present.

TABLE X

VARIATIONS IN RNA CONTENT OF OAT GROATS DURING DEVELOPMENT

	Stage of development (mg/100gr.)			
Hinoat	1[a]	2[b]	3[c]	4[d]
Large Molecular RNA Content	1374[e]	623	358	257

a: One week after anthesis, green embryo

b: Two-three weeks after anthesis, milky stage.

c: Four weeks after anthesis, mid-maturation stage.

d: Mature groat .

e: Each value is an average of three measurements.

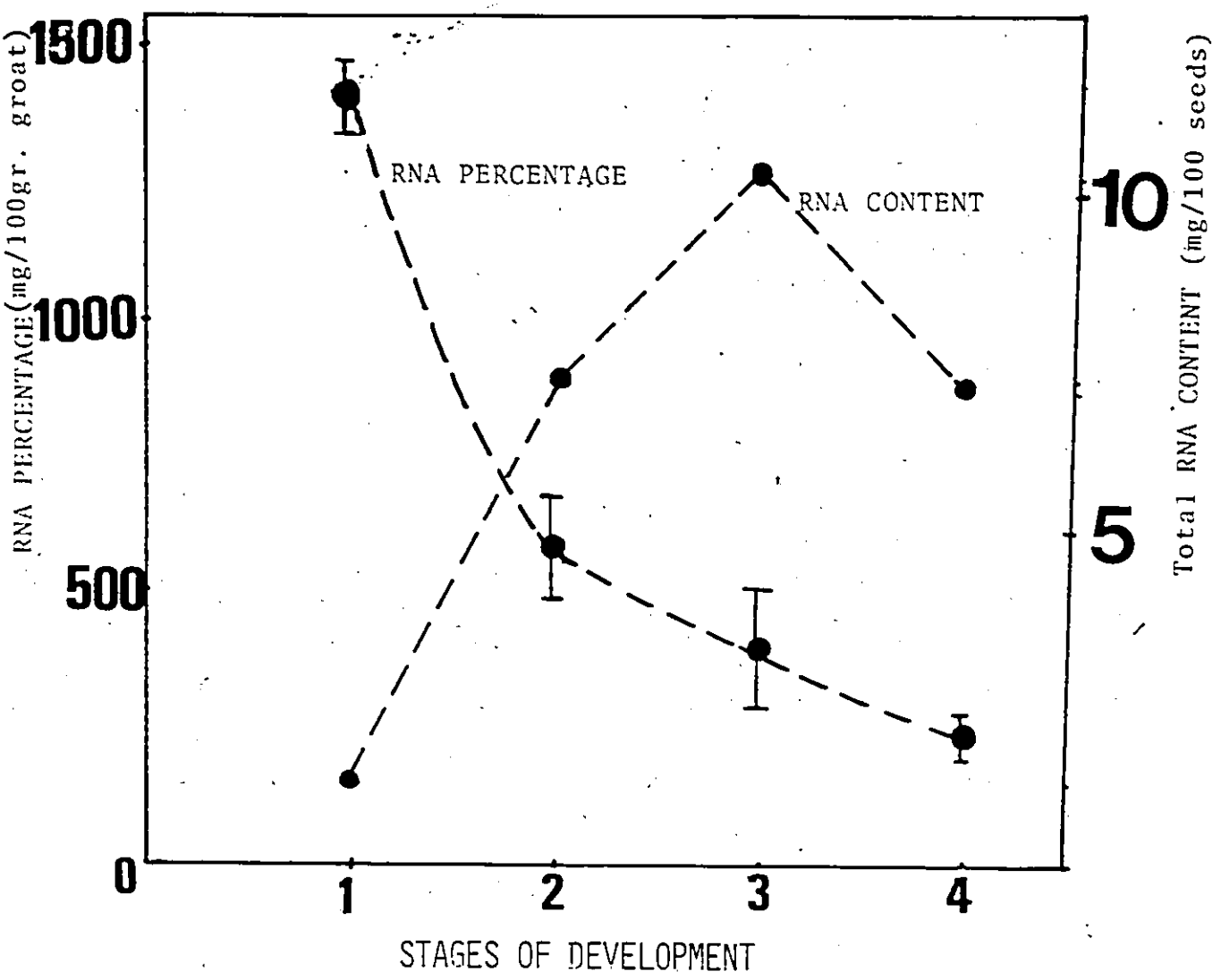


Figure 5. Changes in RNA content of oat groats during development. Total large molecular weight RNA content of oat groats were determined by the method of Cuming et al at four different stages of development.

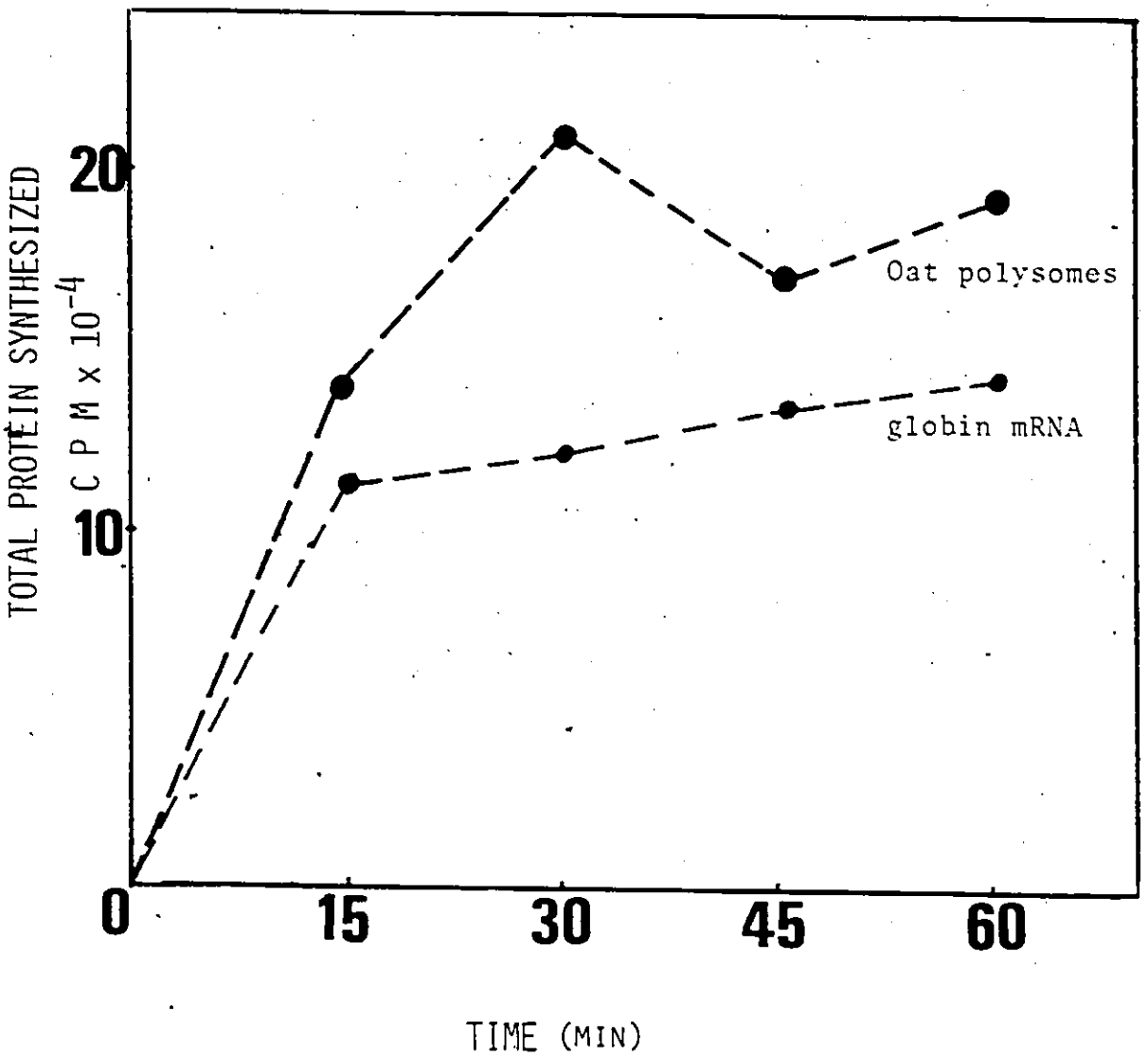


Figure 6. Time course of the translation of total polysomes from developing oat groats. Mg^{+2} concentration was 3mM. Blank values were subtracted from total counts of oat polysomes and rabbit globin mRNA.

Table XI represents the yield of different classes of polysomes from developing oat endosperms. Membrane-bound polysomes showed a higher yield than free polysomes.

Total polysomes were added to a rabbit reticulocyte lysate cell-free translation system, causing the efficient incorporation of L-(^{3}H)-leucine into TCA-precipitable proteins (Figure 6). The optimal concentration of Mg^{+2} for the translation of polysomes in the cell-free translation system was determined to be 3 mM(Figure 7).

Figure 8 shows the in vitro synthesis of oat proteins by polysomes at different concentrations. Excess amounts of polysomes inhibited the translation efficiency of the cell-free protein synthesizing system.

E. Isolation and Cell-Free translation of Total Oat Poly A^+ RNA

To obtain a template active messenger RNA fraction from oat endosperms, total translatable polysomes were dissociated by SDS and polysomal RNA was extracted by the phenol-chloroform method(62). The yield of polysomal RNA from 100 gr. of endosperm was 275 O.D. units or 11.01 mg.

Total polysomal RNA was fractionated by affinity chromatography on an oligo d(T)-cellulose column. The poly A^+ RNA fraction was eluted under salt-free conditions(Figure 9).

Poly A^+ RNA was successfully translated in a rabbit reticulocyte lysate cell-free protein synthesizing system and could direct the incorporation of amino acids into TCA-precipitable products(Figure 10). The optimal concentration of Mg^{+2}

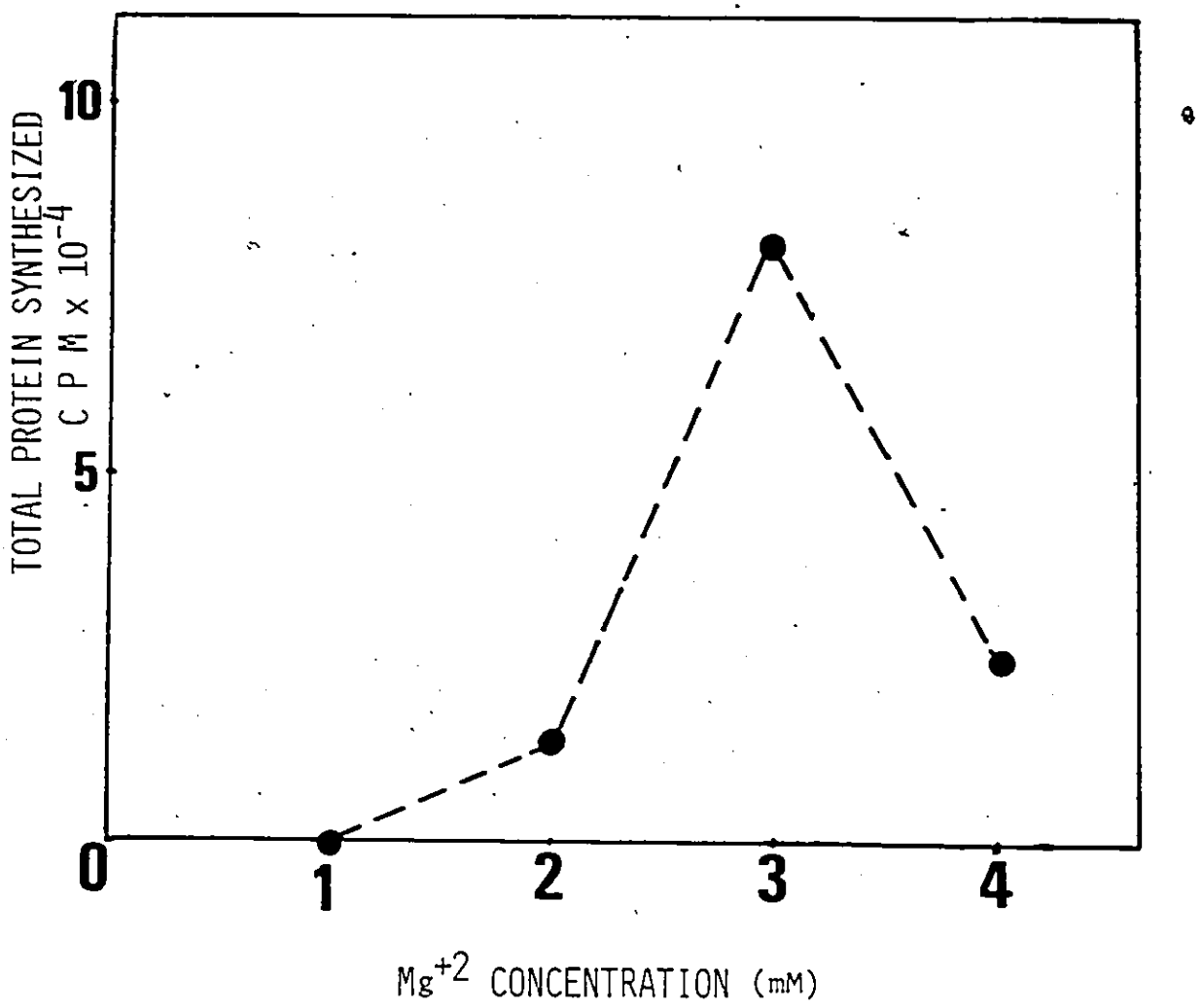


Figure 7. Effect of magnesium concentration variability of the reticulocyte lysate cell-free protein synthesizing system on the translation efficiency of total oat polysomes. Translation was performed at 37°C for 60 min.

Total incorporation of radioactivity in control was 12359 cpm at 3 mM Mg^{+2} concentration.

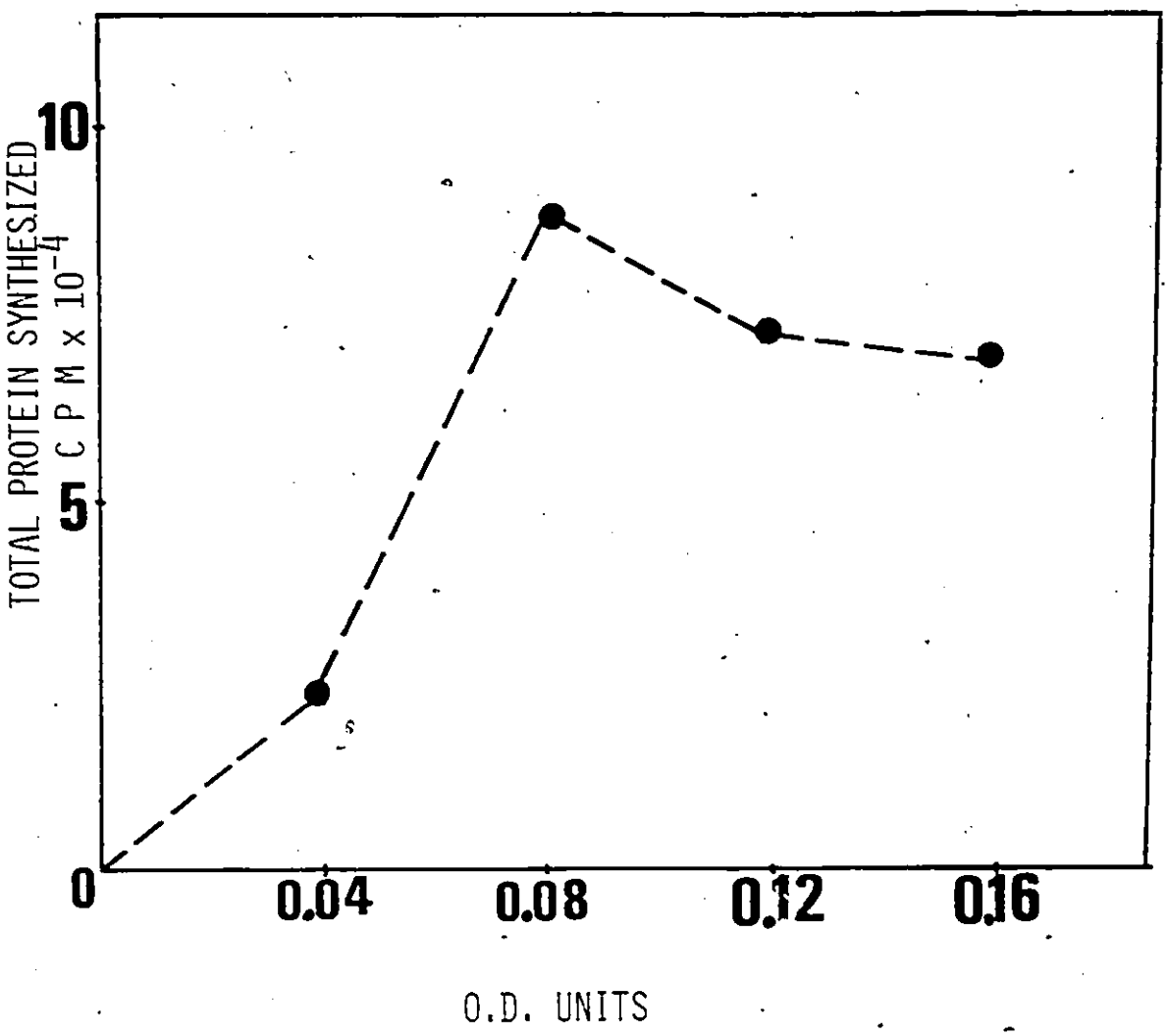


Figure 8. Saturation curve for the translation of total polysomes in the reticulocyte lysate cell-free translation system. Reaction was performed at 37°C for 60 min.
Total incorporation of radioactivity in control was 37470 cpm.

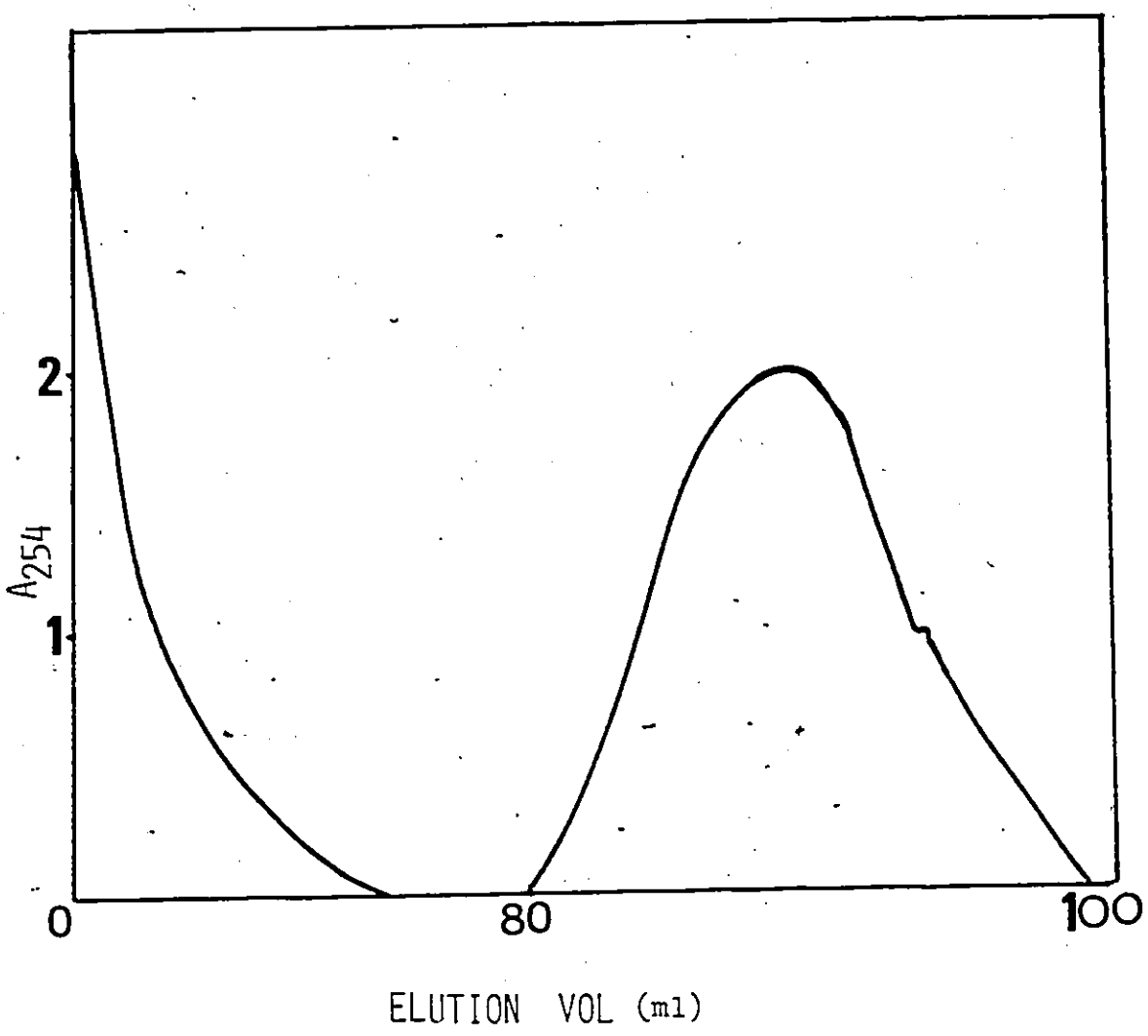


Figure 9. Elution of poly A^+ RNA from oligo d(T)-cellulose column. The chromatography was performed essentially as described by Aviv and Leder(47). Poly A^+ RNA was eluted by salt-free buffer.

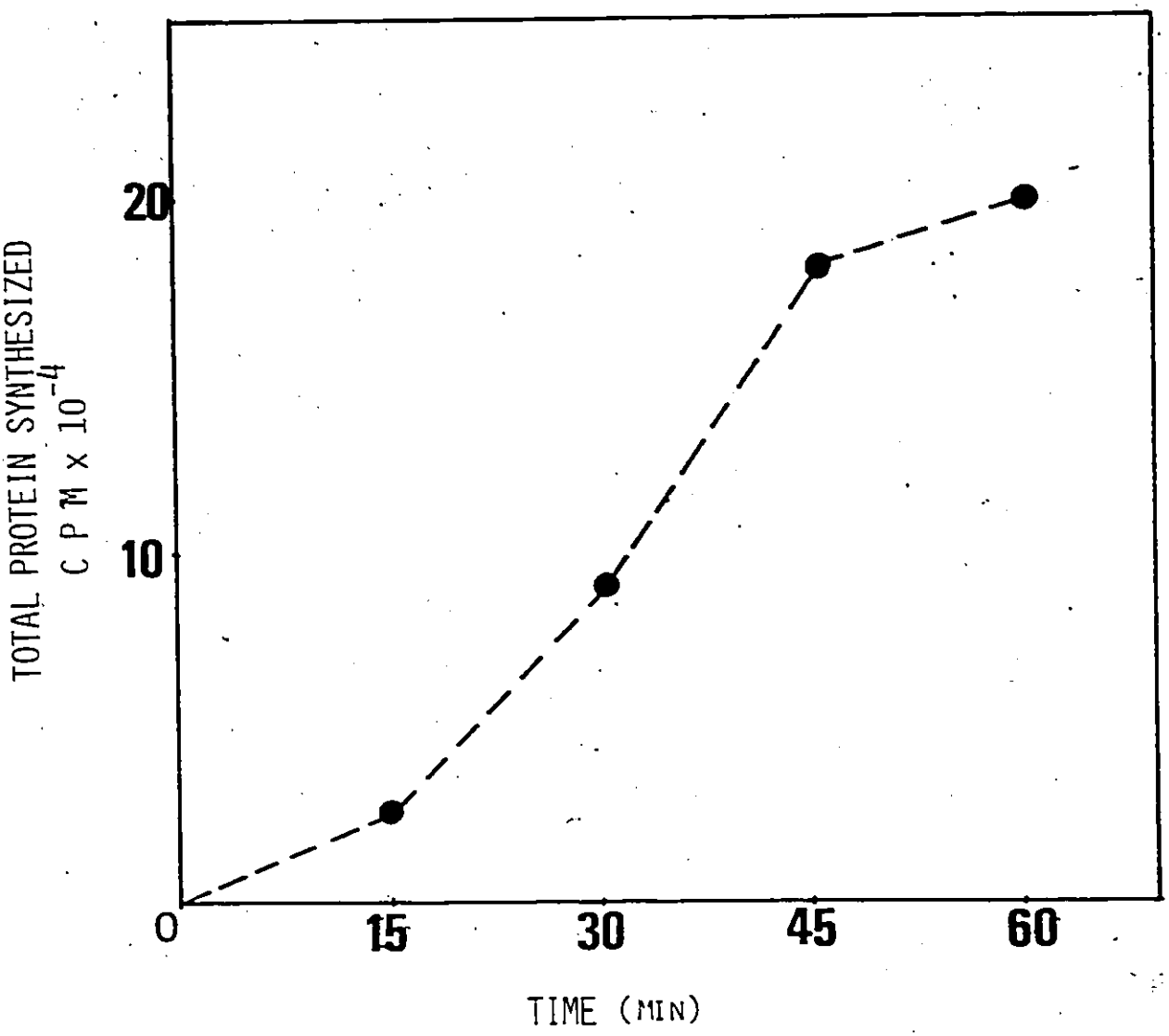


Figure 10. Time course of the translation of total poly A^+ RNA from developing oat endosperm. RNA preparations were translated in the rabbit reticulocyte lysate at Mg^{+2} concentration of 2.4 mM.

Total incorporation of radioactivity in control was $3.7 \times 10^{+4}$ cpm at 60 minutes.

for poly A^+ RNA translation was 2.4mM (Figure 11). At this Mg^{+2} concentration, poly A^+ RNA was translated up to 32 times the background.

Poly A^- RNA and total polysomal RNA were both not very active when translated in the same system under the same conditions. Table XII shows that poly A^+ RNA has a higher specific activity than total RNA and poly A^-RNA.

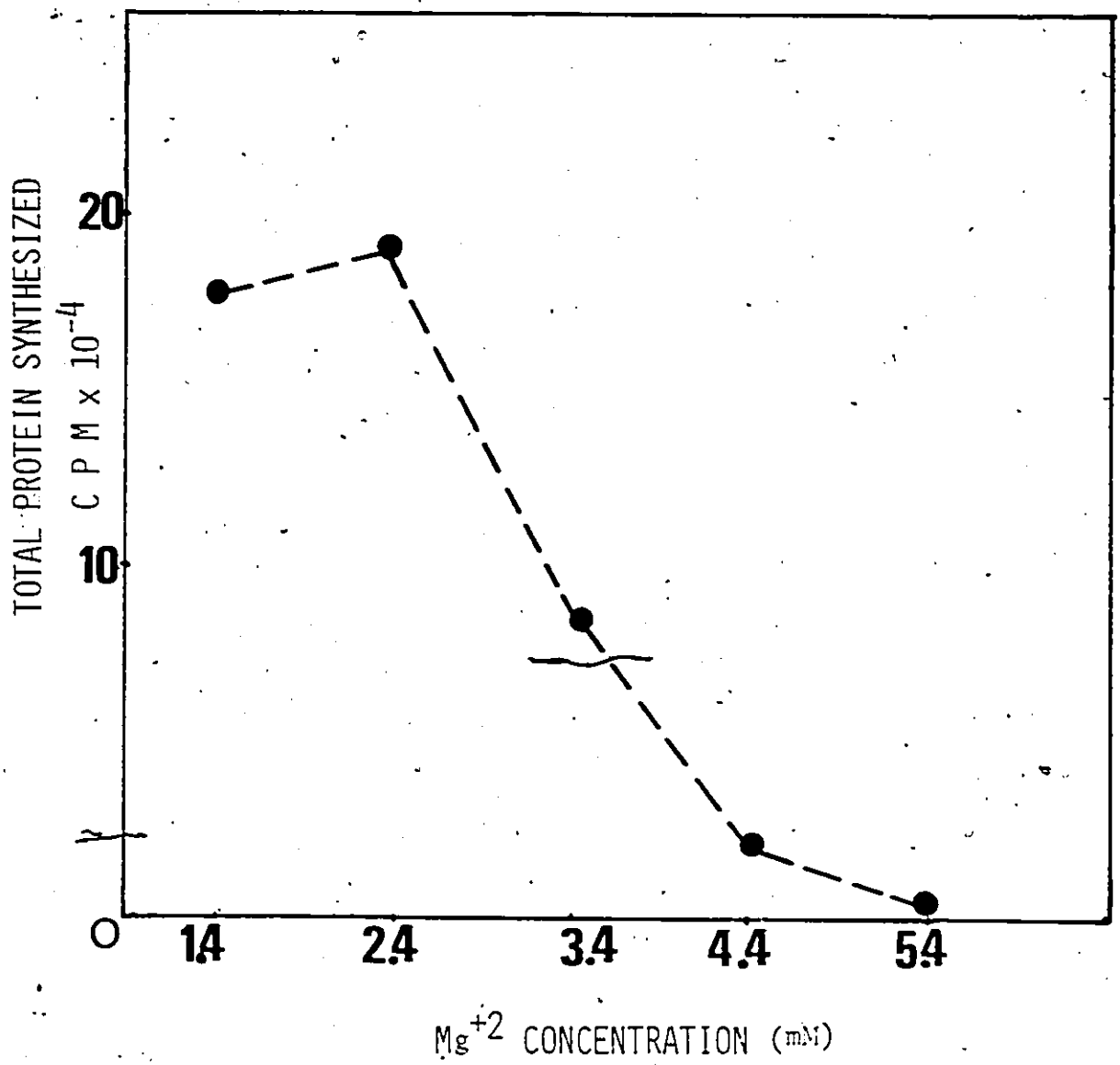


Figure 11. Effect of the variation in magnesium concentration of the reticulocyte lysate cell-free translation system on translation efficiency of total oat poly A^+ RNA. Translation was at 37° C for 60 min. Total incorporation of radioactivity in control was 37470 cpm. at 3mM Mg^{+2} concentration.

TABLE XI

THE YIELD OF POLYSOME AND RNA FRACTIONS FROM OAT ENDOSPERM

FRACTION	YIELD (mg/100gr. developing endosperm)
Free polysomes[a]	15.4
Membrane-bound polysomes[a]	34.6
Total polysomal RNA[b]	11.0
Poly A^- RNA[c]	10.1
Poly A^+ RNA[d]	0.36

a: Free and membrane-bound polysomes were prepared according to method of Matthews(14).

b: Total RNA was isolated by the phenol-chloroform method of (62).

c: Poly A^- RNA is the RNA fraction which could not bind to oligo d(T)-cellulose column.

d: Poly A^+ RNA is the RNA fraction which could bind to oligo d(T)-cellulose column.

The amount of polysomes was determined with the assumption that at A_{260}, 13 O.D. units represent 1mg/ml polysomes(68).

The amount of RNA was determined with the assumption that at A_{260}, 25 O.D. units represent 1mg/ml RNA(68).

TABLE XII

TRANSLATION EFFICIENCY OF DIFFERENT RNA POPULATIONS FROM OAT ENDOSPERM[a,b]

RNA POPULATION	TOTAL TCA PRECIPITABLE PRODUCTS(CPM/µg RNA)
Total polysomal RNA	63387
Poly A^- RNA	43525
Poly A^+ RNA	223312

a: Total incorporation of radioactivity in control(no RNA added) was 31225 cpm.

b: All translation mixtures were incubated at 37° C for 60 min.

Chapter IV

DISCUSSION

A. THE PATTERN OF NITROGEN ACCUMULATION IN DEVELOPING OAT ENDOSPERM

A typical increase in fresh and dry weights of all seven oat cultivars was observed during development(Tables V,VI). A rapid dehydration of oat groats occurred at late maturation stages in the course of seed development. Certain cultivars such as OA 424-1 showed a greater increase in total biomass.

Total nitrogen content based on dry weight of oat groats also increased during their growth and development(Table VIII). Similar results have been reported for other oat cultivars(29,63). However, a rather different result was obtained for nitrogen percentage of oat groats during development(Table VII). Statistical analysis of the data showed no significant and logical variations among nitrogen percentage of oat groats at different stages of development(Appendix 1). Earlier reports by Peterson et al(29) and Welch et al.(63) indicated an increase in nitrogen percentage of developimg oat groats. In comparison, the present report may be more valid due to the higher number of measurements for each harvest and the higher number of cultivars tested. Nevertheless, more experiments on the nitrogen assimilation and metabolism of oat or other cereal grains are required

to confirm this finding. According to the present results, one may assume that the nitrogen-carbohydrate ratio of oat grains is set early in development and persists throughout. In other words, protein and starch are synthesized in relation to each other and this rather strict relationship is kept constant throughout the seed development.

In general, nitrogen content and nitrogen percentage of oat groats varied significantly among seven different cultivars. Hinoat showed the highest increase in nitrogen content throughout development. OA 424-1, although it had a relatively low nitrogen percentage, possessed very high nitrogen content due to a higher yield.

A negative relationship between nitrogen percentage and grain yield was observed only for some of the cultivars. Hinoat, although a high nitrogen cultivar, has a lower yield. Sentinel seems a suitable cultivar both in terms of nitrogen content and yield. Recently, Welch et al reported a similar negative correlation too. This is an undesirable factor and a natural barrier to obtaining a desirable cultivar having both high protein content and high yield.

B. STORAGE PROTEIN BIOSYNTHESIS

If a factor of 6.25 is assumed for the conversion of nitrogen percentage to protein percentage in oat, an identical pattern for protein accumulation is observed during seed development. Peterson et al (1976) showed that various oat protein fractions in 5 American cultivars are synthesized at different rates in the course of seed

development. Globulins are synthesized rapidly at about 12 days after anthesis and comprise more than 50% of oat proteins at this stage. Albumins and glutelins are actively synthesized early in development but their percentage declines later in maturity. In contrast, prolamine fraction synthesis is very low at early stages of development and highest at mid-maturity.

To see if these findings also hold true for a new Canadian high protein oat cultivar (Hinoat), analyses of four protein fractions were conducted. A similar pattern for the distribution of four different protein fractions was observed in Hinoat. As expected, globulins are the major protein fraction and comprise up to 50% of total storage proteins in mature grains.

To characterize the globulin subunits in Hinoat grains, the globulin fraction was analyzed on SDS-polyacrylamide gel electrophoresis in denaturing and reducing conditions. Two major subunits with the molecular weights of 21000 and 38000 were present. Each subunit consists of two to three components. Other minor polypeptides are also present specially at the area of 56000 MW. Somewhat similar results were reported earlier by Peterson et al for an American cultivar (Frocker). Recent analyses of globulin polypeptides extracted from a number of different cultivars showed that two major α and β subunits are present in all cases and the difference only lies among the minor bands (Robert, 1981, unpublished data).

Glutelins represent the second highest fraction in oat storage tissue. The glutelin polypeptides are currently being characterized (Cudjoe, in progress). Albumins comprise only a small portion of total oat protein (11%) and their structure and compositions are not known. The alcohol-soluble protein fraction, prolamines, comprise about 15% of total protein. A few studies on the structure and composition of this fraction are available. Recent analysis of this fraction on starch gel electrophoresis revealed about 17 different bands (28).

In comparison with other cereals such as wheat, rye, barley and maize, the proportion of oat protein fractions are very different. The major protein fraction in these cereals is prolamine (50-60%). Globulins which are the major fraction in oats are very low in these cereals. Because of this major difference, oat is nutritionally superior to other cereals due to the high level of essential amino acids in globulins compared to prolamines. This has been proven by feeding trials in rats and chickens (64). Table XIII summarises these comparisons.

Oat globulin appears to be a suitable system for studying the protein biosynthesis of cereal storage protein. It comprises up to 60% of total oat protein and it separates into two major bands on SDS-polyacrylamide gel electrophoresis which represent roughly 80% of total globulin. A primary and important step in studying protein biosynthesis is to isolate and characterize the

TABLE XIII

THE COMPARISON OF GLOBULIN AND PROLAMINE CONTENT OAT WITH OTHER CEREALS AND THEIR EFFECT ON CEREAL PROTEIN QUALITY

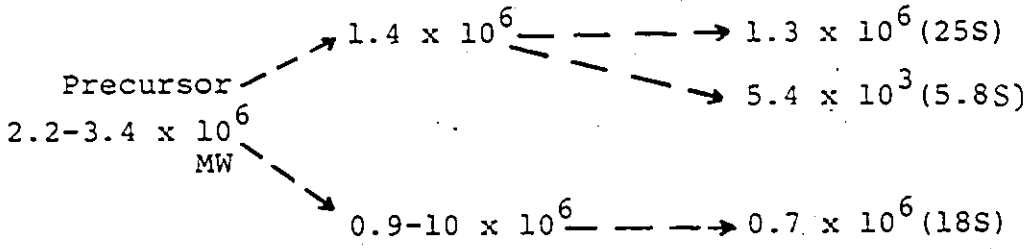
	PROTEIN FRACTION		
CEREAL	% GLOBULIN	% PROLAMINE	PER
Wheat	.6-10	40-50	1.7
Rye	5-10	30-50	1.3
Barley	10-20	35-40	2.0
Maize	5-6	50-55	1.6
Oat	55-60	10-15	2.2

a. The values for wheat, Rye, Barley, and maize have been taken from reference (8).

elements of this process.

C. RNA SYNTHESIS DURING DEVELOPMENT

In the course of seed development, large molecular weight RNA molecules are synthesized in the nucleous as precursors. About 10% of these precursors are processed in the nucleolus and transported into the cytoplasm as messenger RNAs (65). Also large precursors of ribosomal RNA exist in the nucleolus of developing plant cells (65). These precursors are thought to be processed and yield the 25, 18, and 5.8 S ribosomal RNAs. A scheme for post-transcriptional modification of plant ribosomal RNAs has been proposed (65).



The rate of RNA synthesis in developing cereal endosperms is not clearly known. A basic study on the content of large molecular weight RNAs at four different stages of seed development was conducted. A significant decline in RNA content of oat groats during development was observed. Young embryos contained higher percentage of RNA (based on dry weight) than mature grains. These results may be explained by the fact that young seeds are very active in RNA and protein biosynthesis whereas

mature grains are not.

D. ISOLATION AND CELL-FREE TRANSLATION OF POLYSOMES

Certain cereal storage proteins such as zein in maize and hordein in barley are mainly synthesized on membrane-bound polysomes (12, 25, 26). Oat storage proteins are thought to be synthesized on both free and membrane-bound polyribosomes (33). Therefore, the isolation and separation of oat polysomes is a necessary step in the isolation of messenger RNAs for oat storage proteins. A modification of the method of Larkins et al (1976) was used to isolate and separate free and membrane-bound polysomes. Membrane-bound polysomes were extracted from the cell debris using Triton X-100 or sodium deoxycholate. The former detergent proved to be more efficient. The polysomes isolated by this method were not as active as those obtained by the method of Davies et al (60).In this method, polysomes were washed by a second cycle of centrifugation through a sucrose cushion. This obviously improved the translation activity of the polysomes by removing the possible inhibitors. Plant nucleases were inhibited during extraction by the high ionic strength, high pH (8.5), and the addition of 2' and 3' AMP (66).

The polysome preparations were relatively pure because the A_{260}/A_{280} ratio was higher than 1.75, the expected ratio for polysomes (67). The ratio for free and membrane-bound polysomes were 2.04 and 1.87 respectively.

The ratio of membrane-bound to free polysomes was

approximately 2.25. This value is lower than those obtained for other cereal grains. Matthews (68) reported a ratio of 4.5 for barley polysomes. These differences may suggest that oat endosperms contain a higher concentration of free polysomes than other cereals.

Both free and membrane-bound polysomes were efficiently translated in a rabbit reticulocyte cell-free translation system. The incorporation of radioactivity was linear for the first 30 min for both classes of polysomes. Total polysomes were translated at maximum efficiency with a magnesium concentration of 3mM. Luthe and Peterson (1977) reported the isolation of oat polysomes. My results are essentially similar. However, Luthe and Peterson used wheat germ extract as a cell-free translation system.

Based on the above-mentioned observations, both free and membrane-bound polysomes were assumed to contain functional messenger RNAs and thus used to isolate oat messenger RNAs.

E. ISOLATION AND CELL-FREE TRANSLATION OF POLY A^+ RNA

Total poly A^+ RNA was isolated from free and membrane-bound polysomes by deproteinization with phenol-chloroform and affinity chromatography on oligo d(T) cellulose. This method was more efficient than direct isolation of poly A^+ RNA from endosperm without isolating polysomes. The methods of Lane et al (Phenol-NaCl extraction), Hall et al (proteinase K-SDS method) and

Bowden et al (Phenol-chloroform extraction) were all tried but did not yield translatable poly A^+ RNAs. The yield of total RNA in these methods was very high but such RNA preparations did not have template activity. One possible explanation may be the loss of the poly A tail of messenger RNAs by the action of phenol and proteinase K. This prevents one from isolating poly A^+ RNA by affinity chromatography on oligo d(T) cellulose. Also RNase contamination might have occurred.

Poly A^+ RNA was eluted from oligo d(T) cellulose column by the salt-free buffer, accounting for about 3.5% of total RNA.

— TEMPLATE ACTIVITY OF RNA

RNA preparations were translated in a rabbit reticulocyte lysate cell-free translation system essentially as described by Pelham et al(49).Magnesium concentration was very critical for the translation efficiency of the poly A^+ RNA preparations. Polysomes and poly A^+RNA had Mg^{+2} concentration optima of 3 mM and 2.4 mM respectively in the limited range of Mg^{+2} concentration tested.

Poly A^+ RNA was faithfully translated in the cell-free system. A maximum of 32-fold stimulation was obtained when poly A^+ RNA was translated in the rabbit reticulocyte lysate translation system. However, a higher stimulation (up to 101-fold) was observed when the same RNA preparation was translated in wheat germ extract (results not shown). The incorporation of radioactivity

was linear for the first 45 min for poly A^+ RNA translation.

Poly A^- RNA and total polysomal RNA had negligible template activity compared to poly A^+ RNA. The low template activity of total polysomal RNA might be the result of the inhibition by the huge amount of ribosomal RNAs present in the preparation. These observations indicate that a part of messenger RNA activity is probably polyadenylated.

F. GENERAL DISCUSSION

Early establishment of the nitrogen-carbohydrate ratio of oat grains was detected during seed development. Oat endosperms actively synthesize storage proteins shortly after pollination and continue to do so until maturity. The major protein fraction in oat storage tissue is a salt-soluble globulin with a good balance of essential amino acids.

Most storage proteins in cereals and legumes are synthesized on polyribosomes bound to the endoplasmic reticulum and transported across the membrane into its lumen, carried into vacuoles and finally deposited in protein bodies. The specific mechanism for synthesis of oat storage proteins is not known but it is possibly similar to that described for barley and maize storage protein biosynthesis (22, 26).

Globulins which are the major storage protein in oat endosperm constitute up to 50% of total protein during seed development. Globulins are separated into two major

subunits, α and β. These two subunits exist in equimolar amounts (31). It has been proposed that globulin present in oat endosperm exists in a molecule consisting of 6α and 6β subunits with a molecular weight of 300000 (31). Recent progress (Matlashewski 1981, unpublished data) in identification of *in vitro* products of polysomes and poly A^+ RNA suggests that a 60000 MW protein is synthesized *in vivo* and then post-translationally modified to the two α and β subunits. Further investigations on the identification of *in vitro* products of oat poly A^+ RNA translation are required to elucidate the nature of the proteins synthesized *in vivo*.

An efficient method for the isolation of poly A^+ RNA from polysomes of developing oat endosperms has been described in this thesis. This RNA preparation has a messenger RNA activity and is polyadenylated but it probably contains some ribosomal RNAs.

Work is in progress to fractionate the RNA preparation and further purify the poly A^+ RNA fraction which is specific for globulin and other oat storage proteins. Sucrose gradient centrifugation of total poly A^+ RNA and translation of each fraction in a cell-free protein synthesizing system is one important approach. In addition, *in vitro* products of poly A^+ RNA translations are being identified using antibodies against globulin subunits. Further characterization of poly A^+ RNA coding for storage proteins by agarose and polyacrylamide gel electrophoresis is also in progress.

The main objective of this project was to gain basic knowledge on the protein biosynthesis in developing oat endosperms. Now that a translatable poly A^+ RNA can be readily obtained from oat endosperm, the next step in studying the mechanisms involved in storage protein gene expression will be to prepare a complementary DNA to the oat poly A^+ RNA fraction. To amplify the cDNA sequences, they can be inserted into a carrier (e.g. plasmid) and cloned in E.coli. The clones may then be screened and those which are able to hybridize to globulin mRNA could be used to isolate genomic DNA in order to study the structure and organization of the globulin gene or gene family. Such studies have been successfully conducted for zein in maize and phaseolin in french bean (69,70).

The long term goal of this research is to genetically improve the quality and quantity of storage proteins in cereals by manipulating the storage protein genes. Along this line, much research has to be done to further understand the regulation of the expression of storage protein genes.

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APPENDIX 1. Statistical Analysis of Nitrogen Accumulation Data*

A detailed analysis of the data on nitrogen percentage of oat groats is discussed here. Two types of analyses have been performed on the data. First, the interactions between nitrogen percentages of seven cultivars at various stages of development were examined by the ANOVA treatment(analysis of variance). This analysis was performed only on harvest dates 2, 3,5, and 6 because only three measurements had been done for the 4th harvest date . This analysis is summarized in Table I. Nitrogen percentage of seven cultivars were highly significantly different as shown by the F value of 18.0872. The F value for stages of dvelopment is 1.7599 which indicates that the nitrogen percentages at four harvest dates tested were not significantly different. The third F value (11.7752) which represents the interaction between cultivars and stages of development shows that nitrogen percentages of seven cultivars were significantly different throughout development.

Secondly, the change in nitrogen percentage of each oat cultivar at five harvest dates was examined by plotting nitrogen percentage against stage of development (Figure 1). Apparently, the nitrogen percentage of the oat groat changes randomly during development and the pattern of this variation did not follow a logical trend of increase or decrease. As a result, it is assumed that N% of the oat groat remains constant in the course of development. However, a few cultivars show significant changes at certain periods of maturation. For example, Hinoat shows a significant change in N% between second and third

harvest dates. Also, Elgin shows a drastic change in N% in the same period. A significant decrease in N% of Harmon groats occurs between 4th and 5th harvest dates. The biological significance of these significant changes are not yet clear. They might be result of hormone actions on the synthesis of starch and protein. More research on nitrogen and carbohydrate accumulation in oat groats may provide us with more definite answers.

*This Appendix was reffered to on pages 43 and 60.

TABLE I

ANALYSIS OF VARIANCE

(ANOVA SUMMARY TABLE)

Source	Degree of Freedom	Sum of Squares	Mean Squares	F value[a]
Cultivars	6	20.3762	3.39604	18.0872***
Stages	3	0.775068	0.258356	1.7599*
Cultivars x stages	18	3.37966	0.187759	11.7752***
Error	224	3.50498	0.015945	

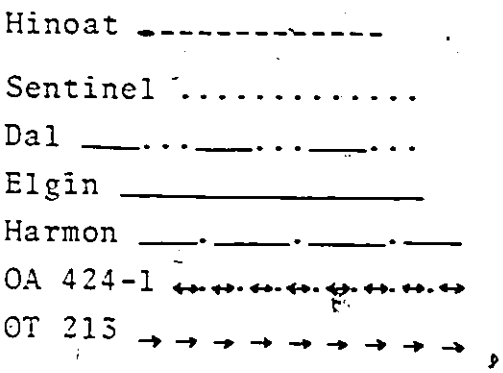
*** highly significant

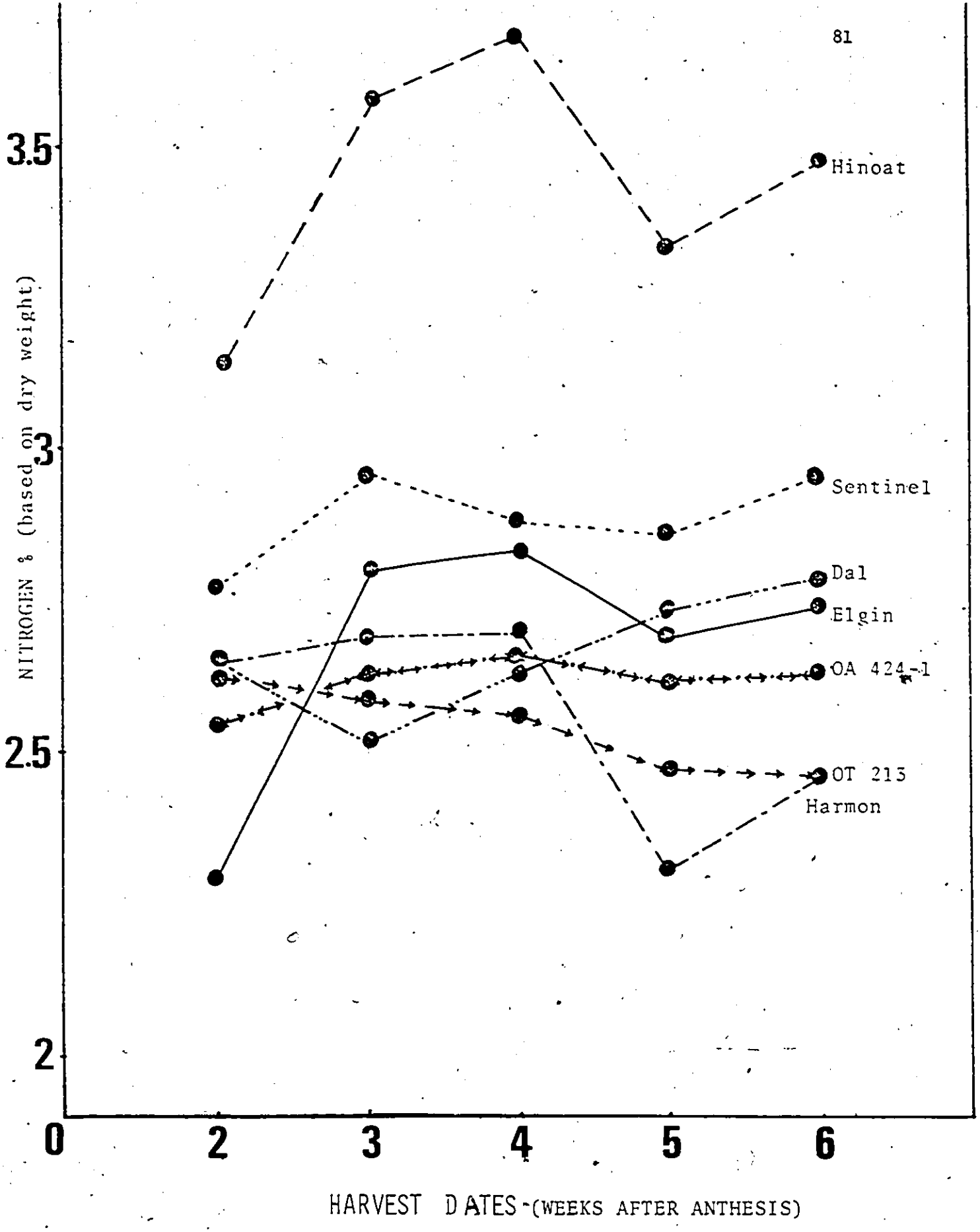
* not significant

a: F values are calculated by the following formula:

$$F = \frac{\text{Mean squares (Treatment)}}{\text{Mean squares (Interaction)}}$$

Figure 1.
Changes in Nitrogen Content of Oat Groat during Development. The N content of oat groats was measured at 5 weekly intervals by the standard Kjeldahl method(56).




3.5
3
2.5
2
0
2
3
4
5
6
NITROGEN % (based on dry weight)
HARVEST DATES - (WEEKS AFTER ANTHESIS)
Hinoat
Sentinel
Dal
Elgin
OA 424-1
OT 215
Harmon