# HYDROLYSIS OF ENDOSPERM PROTEINS IN GERMINATING MAIZE

# HYDROLYSIS OF ENDOSPERM PROTEINS IN GERMINATING MAIZE

by

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SCOPE AND CONTENTS:

Endosperm protein hydrolysis in germinating maize caryopses was investigated, with emphasis on:

1. The disappearance of the various classes of endosperm proteins.
2. The enzymes responsible for hydrolysis of the reserve proteins.
3. Hormonal regulation of enzyme formation and protein hydrolysis in the endosperm.

Degradation of the principal storage proteins, zein and glutelin, was found to begin within the first two days of germination. After 3 days hydrolysis proceeded rapidly until the endosperm reserves were depleted, at about 8 days. The rate of degradation

of zein and glutelin depended on the composition of the endosperm; the more abundant protein was hydrolysed most rapidly.

The disappearance of zein and glutelin during germination was correlated with proteolytic activity in the endosperm. The principal protease extracted from germinated maize endosperm had a pH optimum of 3.8, temperature optimum of 46C, and required free sulphydryl groups for its activity. The enzyme preparation which had endopeptidase activity degraded a wide range of substrate proteins. Denatured protein, such as hemoglobin, was degraded more rapidly than native proteins such as bovine serum albumin, and gliadin was found to be more readily degraded after partial acid hydrolysis. An agar gel assay was developed to permit use of the insoluble maize storage proteins as substrates. In this assay zein and glutelin were degraded at a rate comparable to hemoglobin. The ability of the preparation to degrade zein and glutelin with equal efficiency can account for the non-specific pattern of protein degradation during germination. This protease and α-amylase are absent from quiescent caryopses. These enzymes appear 2-3 days after imbibition of intact caryopses, and increase in activity throughout the course of protein and starch breakdown.

In contrast to other cereals neither the embryo nor exogenous factors were necessary for initiation or continuation

of α-amylase and protease formation in de-embryonated endosperms. Both enzyme activities appeared earlier in excised endosperms than in intact caryopses germinated at the same temperature. Zein, glutelin, and starch were also degraded more rapidly in excised endosperms. Protease and α-amylase production were found to require protein and RNA synthesis in the endosperm, hence it is tentatively concluded that these hydrolytic enzymes are synthesised de novo in the endosperm.

Enzyme production was not markedly stimulated by treating de-embryonated endosperms with phytohormones. However hydrolase production and starch and protein breakdown were strongly inhibited by abscisic acid, a hormone antagonist. This inhibition was reversed by inclusion of gibberellic acid in the incubation medium. Neither kinetin nor indoleacetic acid could overcome abscisic acid inhibition, indicating that gibberellins may have a special role in regulation of hydrolase production.

The response to gibberellic acid of a dwarf-mutant maize, thought to be deficient in endogenous gibberellins, was tested. De-embryonated endosperms of this mutant had low enzyme activities, and degraded little starch or protein when incubated in buffer. Inclusion of gibberellic acid in the incubation medium stimulated enzyme production five-fold, and accelerated starch and protein hydrolysis. Thus, as in other cereals, the gibberellins appear to influence the induction of hydrolytic enzymes in maize endosperm.

## PREFACE

The experiments described in this thesis were carried out in the Department of Biology, McMaster University, from September 1968 to July 1973. Except where others are specifically mentioned, this thesis consists entirely of my own original work. No similar thesis has been submitted to any other university.

I am indebted to my supervisor, Dr. B. A. Oaks, for her guidance and encouragement throughout this investigation. I would like to thank the National Research Council of Canada for financial support during this project. I would also like to thank Mrs. Mary Haight for her excellent typing.

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## ABBREVIATIONS

| | |
|---|---|
| A | absorbance |
| ABA | abscisic acid |
| Act. D | Actinomycin D |
| AMO 1618 | 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl Piperidine carboxylate |
| BAPA | α-benzoyl-L-arginine-p-nitroanilide |
| BSA | bovine serum albumin |
| cAMP | adenosine 3',5'-cyclic monophosphate |
| CCC | (2-chloroethyl)trimethylammonium chloride |
| CHX | cycloheximide |
| CPM | counts per minute |
| DPM | disintegrations per minute |
| EDTA | ethylenediaminetetraacetic acid |
| GA | gibberellic acid |
| IAA | indoleacetic acid |
| K | kinetin, 6-furfurylaminopurine |
| 6-MP | 6-methylpurine |
| Phosphon D | tributyl-2,4-dichlorobenzylphosphonium chloride |
| m-RNA | messenger-ribonucleic acid |
| r-RNA | ribosomal-ribonucleic acid |
| t-RNA | transfer-ribonucleic acid |
| TCA | trichloroacetic acid |

## INTRODUCTION

### Germination of Seeds

Proteins, carbohydrates and lipids which accumulate in seed endosperms or cotyledons during development are hydrolysed during germination. The hydrolysis products are transported, via the scutellum in cereals (Figure 1), to the embryo to support its initial growth. The amino acids released by storage protein hydrolysis are efficiently incorporated into embryo protein (5,47,64,129,169). In maize the endosperm proteins appear to be the preferred nitrogen source for embryo protein synthesis during early germination. For example, Srivastava (171) found that supplying nitrate did not increase protein accumulation in the germinating embryo until after depletion of the endosperm nitrogen reserves. In both cereals and dicotyledonous plants seedling vigour and crop yield have been positively correlated with mature seed protein (79,93, 94,151,158). The vigour of seedling growth appears to be determined by the protein content of the endosperm (95).

Embryos can be cultured in the absence of the seed storage tissue, but in the case of maize must be supplied with sugars (36). Oaks and Beevers (126) found that in maize embryo

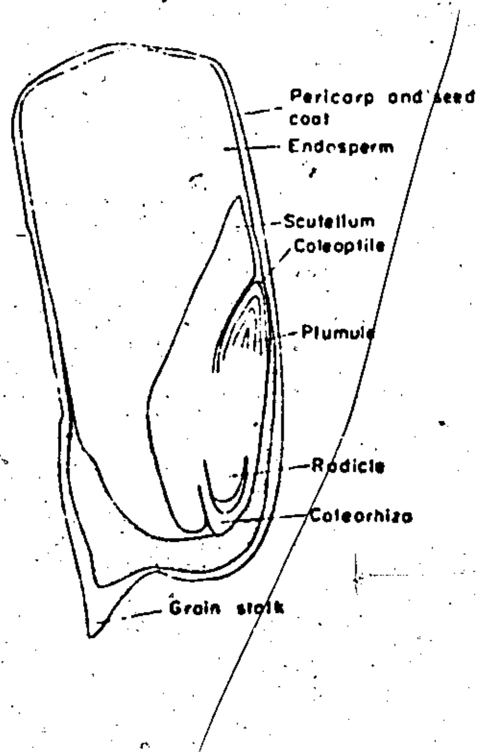



FIGURE 1

Structure of the Maize Caryopsis

(Mayer, A. M. and A. Poljakoff Mayber, 1963. The Germination of Seeds. Macmillan Co., New York.)

protein accumulation depended on an exogenous amino acid supply for the first 2-3 days after excision. An endosperm protein hydrolysate or synthetic mixture of amino acids of equivalent composition supported embryo protein synthesis most effectively. This emphasises the importance of reserve protein hydrolysis for embryo growth during normal germination. However Oaks (125) showed that the embryo has the capacity to synthesise amino acids. Excised embryos cultured without an exogenous supply of amino acids began, after a three day lag, to accumulate both amino acids and protein. It appears that during germination amino acid synthesis in the embryo may be inhibited by amino acids supplied from the endosperm.

Conversely in many instances the hydrolysis of seed reserves has been found to depend on embryo metabolism. If the embryo is killed or removed (18,98,130,131,191) the storage tissues do not degrade their reserves when exposed to conditions which normally permit starch and protein hydrolysis. Protein degradation in pea cotyledons (53,196), protein hydrolysis in squash cotyledons (138), and triglyceride metabolism in wheat (90,175) are also known to be dependent on the presence of the embryo axis. In some cases the embryo axis may simply act as a "sink" for utilization of hydrolysis products (196), but frequently an embryo extract or exogenous hormone can replace the growing axis in stimulation of reserve hydrolysis (50,130,138,165,192). Thus there are clear

interactions between the embryo and storage tissues during germination. These interactions appear to modulate both catabolic processes in the storage tissues, and embryo development.

In barley interactions between the embryo and endosperm have been extensively studied, with particular emphasis on regulation of starch hydrolysis in the endosperm. The predominant enzymes of starch hydrolysis are the amylases. α-Amylase cleaves internal linkages of the starch molecule, and β-amylase removes terminal maltose groups. Accumulation of the product maltose, appears to inhibit breakdown of raw starch by the amylases (159,165). The presence of maltase may therefore also be required for liquefaction of starch reserves in the germinated caryopsis. The ungerminated endosperms of many cereals have been found to contain β-amylase, mostly in a bound form (87,154), but little α-amylase (36,87). During germination amylase activity increases greatly. Some of this is caused by release of bound β-amylase, but it is mainly due to large increases in α-amylase (35), which has the more important role in starch hydrolysis (116).

Production of α-amylase in the endosperm of barley is regulated both by metabolites and by hormones. Sugars and other osmotica, for example polyethylene glycol, have been found to inhibit α-amylase production by isolated aleurone layers (75). Jones (75) suggested that high osmotic pressure

would reduce water availability for reserve protein hydrolysis. Since the amino acids required for enzyme synthesis in the aleurone are generated by hydrolysis of the reserve proteins (45), inhibition of protein degradation would also inhibit α-amylase synthesis. De novo synthesis of all proteins should then be affected, and this has been demonstrated by Chrispeels (27). Effects on reserve protein hydrolysis have not been investigated. Alternatively Chrispeels (27) proposed that the protein synthetic capacity of the cells might be affected directly. For example, disaggregation of ribosomes could be caused by the water stress (62).

Other researchers have also observed inhibition of α-amylase production by exogenous sugars. Yomo and Iinuma (193) observed that 0.1 M sucrose caused 50% inhibition of amylase production by endosperm fragments, whereas the same concentration had little effect on enzyme production by whole de-embryonated endosperms of barley. Different effects were also found at different sugar concentrations. Briggs and Clutterbuck (21) observed that high concentrations of sugar (>0.1 M) interfered with the ability of the aleurone to synthesise α-amylase, but 20 to 100 mM sucrose also inhibited α-amylase production. Since this inhibition could be overcome by addition of exogenous gibberellic acid they proposed that these concentrations of sugars prevented endogenous gibberellic acid from reaching the site of α-amylase synthesis. Still

lower concentrations (5 mM) have been demonstrated to suppress gibberellin biosynthesis in the scutellum (148).

Considerable evidence has accumulated to show that an embryo factor, which is almost certainly gibberellic acid, is required for initiation and continuation of hydrolase synthesis, and starch and protein breakdown in barley endosperm. These processes occur in excised endosperms only in the presence of excised embryos, embryo extracts, or exogenous gibberellic acid (191). The barley embryo and scutellum can synthesise GA during germination (148,194), and this appears to move into the endosperm. On reaching the aleurone cells GA stimulates de novo synthesis of α-amylase and protease (45,68), and also the release of these enzymes into the starchy endosperm or medium surrounding excised aleurone layers. Release of other enzymes, such as ribonuclease and β-1,3-glucanase (12) is also controlled by the hormone. Thus GA emanating from the embryo affects many aspects of endosperm metabolism.

Recently investigators have sought the primary site of action of GA in the endosperm. Early information showed that incorporation of precursors into RNA and protein, and activity of specific enzymes such as α-amylase and protease was stimulated 8-10 hours after GA treatment of isolated aleurone layers. Study of events during this 8-10 hour "lag" indicates that an entire change in metabolism is evoked by the hormone. Table 1 summarises the principal hormone effects recorded to

TABLE 1

Early Effects of Gibberellic Acid on Imbibed Barley Aleurone Tissue

| Time (hr) | Observation | References |
|---|---|---|
| 0 | GA added to isolated imbibed aleurones | |
| 1 | Change in pattern of proteins labelled by radioactive amino acids | 46 |
| 2 | Increased activity of lecithin synthesising enzymes | 72 |
| 4 | Increased $^{14}C$-choline incorporation into endoplasmic reticulum | 43 |
| | Increased polysome formation | 42 |
| | Increased $^{32}P$ incorporation into phospholipid | 88 |
| | Decreased pentosan (cell wall precursor) synthesis | 71 |
| 8 | Protease and α-amylase synthesis | 45,69 |
| | Selective incorporation of $^{3}H$-uridine into a polydisperse RNA fraction | 198 |
| 10-12 | Increase in rough endoplasmic reticulum | 74,182 |

date. The apparent diversity of the processes affected by the hormone has led Johnson and Chrispeels (71) to propose that "GA causes a structural and functional reorganization of the endomembrane system of the aleurone cell. Thus GA action may involve enhanced formation of a specific membrane complex geared to hydrolase synthesis and release." This is vague because the "target," or primary site of action of the hormone has not been discovered.

There is considerable evidence that GA also affects embryo metabolism. Intact caryopses can be stimulated to germinate with GA, and effects on embryo metabolism are apparent long before initiation of hydrolysis of the endosperm reserves (26). In barley embryos GA can stimulate α-amylase synthesis, and in dormant oat embryos it overcomes a block in sugar and amino acid utilization (119,164).

GA may not be the only hormone involved in regulation of embryo and endosperm metabolism in cereals. Hormone interactions have been demonstrated in both monocotyledons and dicotyledons, for example Eastwood _et al_. (38) found that aleurones prepared from quiescent wheat grains produced much more α-amylase when treated with GA and K than when treated with either alone. Khan (84) has suggested that these two hormones have distinctive roles in germination. He proposed that kinins and inhibitors, such as abscisic acid, interact and their balance determines the ability of a seed to germinate.

Even when this balance is favourable gibberellins are required to induce hydrolase production in the germinating seed. Experiments with barley showed that when the balance is unfavourable hydrolases cannot be induced by gibberellins (83,85).

Hence the response of a seed to exogenous hormones depends on its endogenous hormone status. Eastwood et al. (38) also observed that the kinetin requirement for α-amylase production by wheat differed when aleurones were prepared from soaked and from quiescent grains. The authors postulated that the starchy endosperm contains a cytokinin-like factor which moves into the aleurone during soaking, eliminating the requirement for exogenous kinin. An alternative explanation is consistent with Khan's hypothesis (84). Inhibitors may be present in aleurones of quiescent grains, thus cytokinins are required to annul their effects and permit GA stimulation of α-amylase synthesis. If the inhibitors leach out during soaking the requirement for cytokinin is lost. Since endogenous hormones have not been determined there is no evidence to support either of these hypotheses. This emphasises the importance of evaluating the roles of both endogenous and exogenous factors in regulation of metabolism during germination.

It is evident that many hormones and metabolites can influence embryo and endosperm metabolism during germination. To fully understand interaction of the embryo and endosperm

during hydrolysis of the endosperm reserves, it is necessary to characterise;

1. the nature of the endosperm reserves, their pattern of hydrolysis, and factors affecting this pattern.
2. the enzymes with major roles in hydrolysis of storage products.
3. the origin and involvement of hormones in regulation of production of these enzymes, and thus in regulation of hydrolysis of the endosperm reserve materials.

For this thesis a study has been made of the degradation of endosperm proteins of Zea mays during germination and the enzymes involved in that degradation. The regulation of enzyme production and protein hydrolysis has also been investigated.

Endosperm Protein Reserves

The protein reserves of a mature seed function principally as a nitrogen supply for the embryo during germination. Since these proteins have no apparent enzymatic activity, they can only be identified by their physico-chemical properties, such as electrophoretic mobility, solubility, or immunological reactivity. In 1897 Osborne (129) developed a simple classification of seed proteins, based on differential solubility. Proteins soluble in pure water

were designated albumins, while those soluble in salt solutions were called globulins. These proteins are the characteristic nitrogen reserves of dicotyledonous seeds.

In cereals the bulk of the endosperm protein is not solubilized by such mild treatments. Osborne found that another protein fraction could be extracted in aqueous alcohol. This was designated prolamine. After extraction of albumin, globulin and prolamine, most of the remaining protein was soluble in dilute alkali. This fraction was called glutelin. A small fraction (about 5%) of the total endosperm protein cannot be extracted with these solvents, and is termed scleroprotein. Nucleic acids are not considered in this fractionation of endosperm proteins since they make up less than 1% of the total endosperm nitrogen (65).

In cereals albumins and globulins are of relatively minor importance as nitrogen storage proteins, but appear to contain most of the enzymatic proteins of germinating grain (123). Ayrapaa and Nihler (4) found that α-amylase is a globulin-type protein, while β-amylase from resting or germinated barley is water-soluble and thus an albumin. Enari and Mikola (40) determined that all the proteases extractable from green malt at pH 4.9 were albumins. Other workers found that most of this protease activity was indeed associated with the albumin fraction, but a significant amount of activity was also extracted with the globulin proteins (61).

In mature caryopses, enzymes may be bound to, or associated with storage proteins. For example β-amylase is bound to wheat glutelin (154,155), and wheat proteases are extracted with both alcohol and alkali soluble storage proteins (77,184). Ory and Hennigsen (128) have reported the association of acid phosphatases and acid proteinases with protein bodies (prolamines) of ungerminated barley. It has not been suggested that any of these enzymes are in fact prolamines or glutelins.

In maize zein and glutelin are the principal storage proteins, and together comprise 60-80% of total endosperm protein (189). In all cereals the two classes of protein differ extensively in amino acid composition (2). The prolamines, for example zein, are generally rich in glutamic acid, proline and leucine, but deficient in tryptophan and lysine.

Table 2 compares the amino acid composition of maize proteins and ovalbumin, which was chosen as a "standard" protein. Zein is clearly deficient in lysine, and rather low in histidine, arginine and glycine. The amino acid compositions of globulin and glutelin are similar to that of ovalbumin.

Zein from maize can be fractionated into two components, α- and β-zein. The α-zein represents about 80% of the total, and is soluble in 95% alcohol. The minor component, β-zein, dissolves in 60% but not 95% alcohol. Both components are extracted by intermediate concentrations of alcohol (104).

TABLE 2

Amino Acid Composition of Ovalbumin and of Maize Endosperm Proteins*

| Amino Acid | mMoles Amino Acid/g Protein Nitrogen | | | Ovalbumin |
|---|---|---|---|---|
| | Globulin | Zein | Glutelin | |
| Lysine | 2.35 | 0.05 | 1.08 | 2.74 |
| Histidine | 1.56 | 0.58 | 1.84 | 0.69 |
| Arginine | 4.58 | 0.71 | 1.84 | 2.09 |
| Aspartic acid | 3.76 | 2.94 | 2.58 | 4.44 |
| Threonine | 2.04 | 1.66 | 2.22 | 2.15 |
| Serine | 3.52 | 3.24 | 3.34 | 4.92 |
| Glutamic acid | 6.12 | 11.90 | 9.37 | 7.12 |
| Proline | 2.84 | 6.77 | 7.56 | 1.99 |
| Glycine | 5.04 | 1.22 | 3.80 | 2.58 |
| Alanine | 4.39 | 8.06 | 5.40 | 4.79 |
| ½ Cystine | 1.41 | 0.27 | 1.02 | 0.98 |
| Valine | 3.04 | 2.40 | 2.90 | 3.83 |
| Methionine | 0.58 | 0.18 | 1.00 | 2.21 |
| Isoleucine | 1.67 | 2.60 | 1.58 | 3.39 |
| Leucine | 2.79 | 11.14 | 6.18 | 4.45 |
| Tyrosine | 1.24 | 2.08 | 1.96 | 1.29 |
| Phenylalanine | 1.84 | 3.59 | 1.94 | 2.94 |

*Data from Boundy, Wochik, Dimler and Wall (16).

Turner et al. (178) found that zein has a molecular weight of 44,000, and contains disulphide linked aggregrates. On electrophoresis of native zein they observed several mobile components, together the equivalent of α-zein, and an immobile fraction. Reduction of disulphide bonds rendered the latter fraction mobile, and it was identified by its solubility as β-zein. Electrophoresis of dilute solutions has shown that zein has at least six components (157). The maize prolamine clearly is not a single protein, but a group of polypeptides associated in an undefined way. Gliadin and hordein, the wheat and barley prolamines, also have many component polypeptides (14,15,108).

Glutelin from maize is also heterogeneous. In the endosperm glutelin appears to be extensively cross-linked by disulphide bridges, and cannot be extracted without disulphide bond cleavage (122). Paulis and Wall (137) detected six components of different electrophoretic mobility in alkylated-reduced glutelin. Their mobilities ranged between those of zeins and of globulins, and the amino acid composition of total glutelin is also intermediate between globulin and zein (16,91).

Modern knowledge of the heterogeneity of the Osborne protein groups makes classification of seed proteins by their solubility appear arbitrary. However in maize the distinction between zein and glutelin has physiological significance. Zein

is "compartmented" in membrane-bound protein bodies (37,188). These and starch grains are embedded in a cytoplasmic matrix of extensively cross-linked glutelin (29). Thus intracellular locations of the two classes of proteins differ, and so do their patterns of synthesis in developing grain. Glutelin accumulation begins 10 days after pollination in maize, continues at a steady rate until 42 days, and then ceases. In contrast, zein synthesis is slight until about 22 days and then becomes very rapid until 42 days. Between 22 and 42 days the rate of zein accumulation is about three-fold greater than that of glutelin (31). This pattern of prolamine and glutelin increase is also found in developing barley (67).

Further evidence for the validity of dividing endosperm proteins into the classes of zein and glutelin is that synthesis of the two groups of protein appears to be under independent genetic control. Genetic regulation of the protein composition of the grain became evident with the discovery of single gene mutants in which lysine and tryptophan content were doubled (107,121). This doubling was apparently not due to an alteration in amino acid composition of individual proteins (136), but is caused by a change in the zein to glutelin ratio of the endosperm. The mutants, called opaque-2 and floury-2, contain reduced amounts of zein and increased levels of glutelin, albumin and globulin (30,70). Zein containing protein bodies are smaller and much less

abundant in the high-lysine mutants (188). The accumulation of zein in the endosperm is therefore genetically controlled.

Environmental factors can also influence not only the total amount of protein synthesised during seed development, but also the zein to glutelin ratios of the mature endosperms. The effects of nitrogen fertilization have been investigated (55,80,145,187) and the results show that endosperm glutelin content remains fairly constant. On the other hand, the zein content increases when ample nitrogen is present, and is greatly reduced when nitrogen is limiting. Thus the zein to glutelin ratios of mature caryopses can vary enormously.

During germination both proteins are broken down, but the sequence and rates of degradation of zein and glutelin have not been studied. In the first section of this thesis development of extraction techniques for endosperm protein analysis are described, and the patterns of hydrolysis of maize endosperm proteins determined.

## Endosperm Proteases

For many years it has been recognized that proteases and peptidases occur in resting seeds and in germinating grain, and that their activities increase during germination (97,114,139). Peptidases here refer to enzymes which hydrolyse

only small peptides, and proteases to enzymes which degrade protein. Both endo- and exopeptidases fall into the latter class. Exopeptidases remove α-amino or carboxyterminal amino acids from their protein substrate, whereas endopeptidases cleave internal peptide linkages of the molecule.

During germination the insoluble reserve proteins are probably first solubilized by endopeptidases. Subsequently peptidases can complete the hydrolysis to amino acids and small peptides suitable for transport to the embryo (110). Many specific peptidases have been identified in germinating barley (89,110,143,183), and their distribution and activity during germination investigated. However endopeptidases, which would appear to have the more important role in protein breakdown in the earlier stages of germination, have been less thoroughly described.

The prominent modern reports on proteases in resting and germinated cereal grains are listed in Table 3. In many cases it is not clear whether the enzymes have endo- or exopeptidase activity, since the assays measure products characteristic of either class of enzyme. For example, both endo- and exopeptidases can release tyrosine, which may be determined by the Lowry method (96), or α-amino nitrogen measured with ninhydrin, or total nitrogen determined after Kjeldahl digestion of the hydrolysis products. Endopeptidase

TABLE 3

Characteristics of Proteases from Quiescent and Germinated Cereal Endosperms

| Grain | Activity | pH Optimum | Assay | Reference |
|---|---|---|---|---|
| Ungerminated wheat | Protease | n.d. | Change in nitrosine staining of hemoglobin | 77 |
| Ungerminated wheat | Protease | n.d. | Tyrosine release from hemoglobin | 184 |
| Ungerminated wheat | Protease | 5-6 | Tyrosine release from hemoglobin | 166 |
| Ungerminated wheat | Protease | 3.8 | Tyrosine release from hemoglobin | 101 |
| Ungerminated wheat | Endopeptidase | 3.8 | Peptide release in flour suspensions | 52 |
| | Exopeptidase | n.d. | Amino acid release in flour suspensions | |
| Ungerminated barley protein bodies | Endopeptidase | n.d. | Gelatin viscosity change<br>BAPA hydrolysis | 128 |
| Ungerminated and germinated barley | Endopeptidases | n.d. | Gelatin viscosity change | 40 |
| Ungerminated and germinated barley | Endopeptidases (5) | n.d. | BAPA hydrolysis | 24 |

.....continued.....

TABLE 3 (Continued)

| Grain | Activity | pH Optimum | Assay | Reference |
|---|---|---|---|---|
| Germinated barley | Protease | acid | α-Amino nitrogen release from gliadin | 69 |
| Germinated barley | Proteases (5) | n.d. | Total nitrogen release from gliadin<br>Tyrosine release from casein<br>α-Amino nitrogen from gliadin | 23 |
| Germinated barley | Endopeptidase | 3.8, 5-6.5 and 7.0 | Gelatin viscosity change | 173 |
| Germinating barley | Endopeptidase | Acidic | Liquifaction of gelatin<br>α-Amino acid release from N,N-dimethylhemoglobin | 13 |
| Germinating wheat | Endopeptidase<br>Protease | n.d.<br>n.d. | BAPA hydrolysis<br>Release from hemoglobin of material absorbing at 280 nm | 174 |
| Germinating oats | Protease | n.d. | Release from hemoglobin of material absorbing at 276 nm | 49 |
| Germinating sorghum | Endopeptidase | 3.6 | Tryosine release from bovine serum albumin | 22 |

*n.d. indicates not determined.

activity can be specifically measured by change in viscosity of a gelatin substrate, or hydrolysis of the synthetic peptide BAPA. Both exo- and endopeptidase activities are present in ungerminated caryopses (52,110).

Few studies have been made of protease activity during seed germination. An endopeptidase with optimal activity at pH 7.5 is present in the milky endosperm of developing maize, but has very low activity in the mature caryopsis. During germination it is present in the scutellum, but is not detectable in the endosperm (106). In kidney bean cotyledons both hemoglobin degradation and autolysis in crude extracts were measured (147). Both activities fluctuated, apparently unrelated to reserve protein hydrolysis in the cotyledons. In pea cotyledons (11,53,196), chickpea (5), rice (135), and barley (139), protease activity is known to increase during germination. In oats proteases reach their maximum activity when degradation of the storage proteins is almost completed (174).

Since activities of several of these proteases do not appear to correlate with the observed rates of protein breakdown, they may not have a crucial role in hydrolysis of the endosperm protein reserves. Furthermore several seed proteases have been found to degrade only denatured proteins, such as hemoglobin (54,167) or the synthetic endopeptidase substrate BAPA (99). Such enzymes could not attack undenatured storage proteins during germination. Criteria should therefore

be established to facilitate evaluation of the physiological function of a protease during germination. I propose the following requirements:

To fulfil an essential function in initiation and continuation of reserve protein hydrolysis a protease should be:

1. an endopeptidase,
2. capable of degrading the storage proteins in their native states,
3. sufficiently active to account for protein degradation during germination.

Maize endosperm proteolytic activity has been investigated, with reference to these criteria. Endopeptidase activity of the protease preparation has been established, and its ability to degrade the maize storage proteins investigated. Development of activity during germination is traced, and correlated with disappearance of zein and glutelin from the endosperm. The activity measured *in vitro* is calculated to be sufficient to degrade a major fraction of total endosperm protein during germination.

Hormones, Proteases and Protein Hydrolysis

Starch and protein are the principal endosperm reserves of cereals. In barley, starch and protein breakdown in the endosperm depend on gibberellic acid supplied exogenously or by the embryo. α-Amylase and protease synthesis and release by the aleurone are controlled by the hormone. Ungerminated cereal grains contain little or no α-amylase (36,117,131), which is essential for starch hydrolysis (116). Thus hormonal regulation of α-amylase synthesis can provide an excellent control of starch hydrolysis. However, the picture is less clear in the case of protein breakdown. A new protease is induced in barley aleurone by gibberellic acid (69), but the endosperms of ungerminated caryopses contain both endopeptidases and exopeptidases (52,110). Furthermore considerable protein hydrolysis occurs in isolated barley endosperms, in the absence of GA (105). As yet there is no evidence that the endosperm protease induced by gibberellic acid in barley has an important function in protein breakdown during germination. This must be established before it can be validly claimed that GA controls production of a key enzyme for protein hydrolysis, as is the case for starch hydrolysis.

Maize endosperms have been observed to rapidly degrade their reserve proteins in the absence of the embryo or exogenous

hormone (124,177). These may indicate that endosperm protein catabolism is relatively independent of the embryo or hormonal control. In contrast to this report Ingle and Hageman (65) have reported that in excised endosperms protein breakdown is stimulated three-fold by the hormone, while sugar release is completely dependent on the presence of exogenous GA.

Thus there is conflicting evidence concerning the role of hormones in regulation of protein hydrolysis in maize endosperms. This is investigated by examining the pattern of reserve protein hydrolysis and protease activities in germinating grain, in de-embryonated grain, and in excised endosperms supplied with exogenous hormones. For comparison embryo and hormonal control of amylase activity and starch hydrolysis are also determined. This study permits evaluation of the roles of the embryo and hormones in control of protease production and zein and glutelin degradation in germinating maize.

## MATERIALS AND METHODS

### Plant Material

The maize hybrid Wf9 x 38-11 was used throughout this study. Caryopses were purchased in batches from the Agricultural Alumni Association, Lafayette, Indiana, or were grown in Hamilton. Dwarf maize mutant d-5 was a gift from Professor H. Kende of Michigan State University, and later was grown in McMaster Greenhouse facilities. W64A, normal and opaque-2, were supplied by Professor Nelson of the University of Wisconsin, and later grown by the Warwick Seed Company of Blenheim, Ontario.

### Preparation of Caryopses

Commercial maize caryopses were received coated with fungicide dust. This was removed by a 30 second treatment with Javex, a domestic bleach containing 6% sodium hypochlorite. The caryopses were then thoroughly rinsed in tap water, or autoclaved distilled water for experiments requiring sterile conditions. This treatment also served to surface sterilize the caryopses.

Caryopses of maize grown in Hamilton, and some of those supplied by the Warwick Seed Company, had not been treated with fungicides. To reduce fungal contamination these were infiltrated with a commercial fungicide (Captan, Plant Products Co. Ltd.) as described by Meyer and Meyer (109) 48 hours before use. This treatment did not affect germination or hydrolysis of the endosperm reserves.

## Germination of Maize Caryopses

Caryopses were treated with Javex, rinsed thoroughly in tap water, and imbibed for 4 hours before planting in moist vermiculite. The caryopses were germinated at 28C and grown with a daily cycle of 16 hours illumination. Samples of 50 seedlings were harvested as required. As all stages of seedling growth the endosperm + scutellum could be easily excised and quick-frozen in liquid nitrogen. The de-embryonated caryopses were stored at -20C until required, with no loss of enzyme activity. Before each extraction the scutella were rapidly dissected out of the frozen endosperms, thus preventing loss of the liquid endosperm which forms in the later stages of germination.

Incubation of Excised Endosperms

Caryopses were surface sterilized in Javex, then rinsed thoroughly with sterile distilled water. After imbibition for two hours in sterile distilled water the embryos and scutella were dissected out in sterile conditions. The endosperms were transferred to sterile 50 ml Erlenmeyers, 10 endosperms per flask. Each flask contained 10 ml of acetate buffer 0.1 mM, pH 5.0, with $10^{-3}$ M calcium chloride. Penicillin (10 μg/ml), chloramphenicol (10 μg/ml) and streptomycin (250 μg/ml) were included to minimize bacterial contamination. These antibiotic concentrations were found to have no effect on endosperm starch and protein hydrolysis during incubation. A total of 50 endosperms were used per treatment. The flasks were incubated at 29C, with shaking. After the required incubation time aliquots of incubation medium were plated on nutrient agar, to test for contamination. The endosperms were frozen in liquid nitrogen and stored at -20C. The incubation medium was immediately assayed for hydrolytic enzyme activity, total nitrogen and reducing sugars.

Products of Endosperm Starch and Protein Hydrolysis

Reducing sugars, released into the medium by starch hydrolysis, were measured by Nelson's method (120). For

estimation of nitrogen released from endosperms as amino acids, peptides, and possibly polypeptides, aliquots of medium were concentrated and then digested by the Kjeldahl method (56). The nitrogen was converted to ammonium sulphate, and measured colourimetrically by reaction with Nesslers Reagent (92). The total nitrogen released into the incubation medium per endosperm could then be calculated. This includes non-protein nitrogenous compounds, but these are an insignificant percentage of total endosperm protein-nitrogen (64).

Endosperm Protein Extraction

The endosperm proteins classified by Osborne (129) were extracted by a modification of the method of Jimenez (70). Lipids were first extracted by defatting endosperm powder in a Soxhlet apparatus for 24 hours (70), or by grinding the powder with defatting solvent using a pestle and mortar. Routinely n-butanol was ground for 10 minutes with the endosperm powder, which was then pelleted and lyophilized. The butanol extract was retained for nitrogen measurement. One gram samples of the defatted endosperm powder were subjected to four consecutive extractions, with distilled water, 5% sodium chloride, 70% ethanol and 0.2% sodium hydroxide respectively. The powder was stirred for 24 hours with 50 mls of solvent at 3C, then centrifuged down at 28,000 x g for 20 minutes. The

residue was washed with cold distilled water, and the washings combined with the extraction solvent. This was repeated with all four solvents. Total nitrogen in each extract was determined by Kjeldahl digestion followed by Nesslerization. After the final extraction with alkali the insoluble residue was lyophilized, and its nitrogen content also measured. The nitrogen per gram of endosperm powder was recorded, and from this the nitrogen per endosperm could be calculated. Total nitrogen per gram endosperm powder, or per endosperm was also measured directly by Kjeldahl digestion and Nesslerization of a sample of endosperm powder. This would be compared with the total obtained from the protein analysis, and the percentage recovery of the extraction calculated.

Radioactive Labelling of Endosperm Proteins

L-Proline-UL-$^{14}$C (202 mc/μM) and acetate-2-$^{14}$C (1.46 mc/μM) were obtained from New England Nuclear Corporation. The $^{14}$C-proline was purified by passage first through Dowex-50 ion exchange resin, to remove neutral and acidic compounds, then through Dowex-1-acetate to remove acidic amino acids. After this procedure no impurities were detected by chromatography. About 25% of the original radioactivity was removed during purification. Maize, growing in the field, was fed the tracer at appropriate times during seed development using

the method described by Spenser and Gear (170). The tracer (25 μCi in 1 ml water/cob) was placed in a vial, which was taped to the maize stem just below a cob. A wick was threaded through the stem at the base of the cob, and one end placed in the vial. The radioactive proline and acetate moved up the wick and into the stem. Hence it entered the transpiration stream of the plant, and was drawn into the cob. The 1 ml of tracer was completely taken up in less than 4 hours. The vial was rinsed by addition of 1 ml water. At maturity the cobs were harvested and air-dried for about a month. The dry caryopses were then removed from the cob and stored at room temperature.

## Separation of Labelled Compounds in the Endosperm

Acetate-2-$^{14}$C was expected to be incorporated into endosperm proteins, starch, and to a lesser extent lipid, while proline-UL-$^{14}$C was anticipated to selectively label storage proteins. Endosperm proteins were extracted as described previously. Initial defatting with butanol extracted the lipids, and their radioactivity was measured. The defatted endosperm powder was sequentially extracted for albumin, globulin, zein and glutelin. However starch as well as protein was extracted in the aqueous solvents. To degrade this starch each protein fraction was hydrolysed with 1 N HCl for 1 hour at 100C. The hydrolysis products were removed by

dialysis and identified as glucose (167). Proteins were not hydrolysed by this treatment since α-amino nitrogen tests on the concentrated dialysate were negative. Starch left in the endosperm residue after extraction of all protein fractions was released by mild acid hydrolysis. Residual protein was removed by hydrolysis with 6N HCl for 12 hours at 110C in an autoclave.

To determine radioactivity in each fraction aliquots were spotted onto Whatman No. 3 paper discs, and placed in vials with 10 mls of scintillation fluid (5.5 g Packard Pre-Mix M per litre toluene). No radioactivity was lost from the discs into the fluid. Radioactivity was measured with a Nuclear Chicago Model 720. Counting efficiency was constant throughout, at about 65%. The values were not converted from CPM to DPM. The total radioactivity in starch and in protein was calculated as the sum of that found in each protein extract and in the residue. No direct measurement of total starch or of total protein radioactivity was made.

The fate of proline or acetate was determined by hydrolysing the individual protein fractions in 6N HCl at 110C for 2 hours, and separating the component amino acids as described by Barnard (9) and Oaks and Johnson (127). Radioactivity in each amino acid fraction was determined (127) and the contribution of the major labelled amino acids to total radioactivity calculated.

Proteolytic Activity in Flour Suspensions

The proteolytic activity of endosperm flour was measured. Endosperms of caryopses germinated 3 days were freeze-dried and powdered. 125 mg of the flour was incubated with 2.5 ml of 5% hemoglobin and 7.5 ml of water or 0.1 M buffer. Tris, phosphate, citrate or acetate buffer was used, depending on the pH required. The flour suspensions were incubated with shaking at room temperature. Two ml samples were removed at the beginning of incubation and after 1, 2 and 3 hours, and added to 2 ml of 5% TCA to stop the reaction. Blanks with flour alone, or heated flour and hemoglobin were also incubated and assayed. Activity was measured as release of α-amino nitrogen from protein into the TCA-soluble fraction. α-Amino nitrogen was measured by the method of Yemm and Cocking (190) using alanine as the standard. TCA interferes with the ninhydrin assay for α-amino nitrogen, so the alanine α-amino nitrogen standard was constructed in the presence of appropriate amounts of TCA. Activity was expressed as μg α-amino nitrogen released per hour per 100 mg endosperm powder.

Extraction and Assay of Protease

Frozen endosperms were homogenized in an Omnimix with 0.2 M acetate buffer, pH 3.8, which contained 5 mM β-mercaptoethanol. Two mls of buffer were used per g fresh weight of endosperm tissue, and the homogenate was centrifuged at 28,000 x g for 30 minutes at 0C. The supernatant was assayed for proteolytic activity with a 5% solution of hemoglobin or gliadin as substrate. Gliadin was found to be most easily soluble if the pH was adjusted to 3.8 with dilute acetic acid. Each assay routinely contained 50 mg substrate, 1 ml acetate buffer, 0.05 M, pH 3.8 with 2.5 mM EDTA, and 0.2 ml of enzyme in a total volume of 2.5 ml. The mixture was incubated for 10 minutes at 40C, and the reaction stopped by adding an equal volume of 5% TCA. Undigested protein was allowed to precipitate for 15 minutes at room temperature. The precipitated hemoglobin was removed by filtration, gliadin by centrifugation. Activity was routinely measured by the increase in absorbance at 280 nm of the TCA-soluble fraction. This was arbitrarily calibrated against the absorbance of tryptophan at 280 nm. Release of α-amino nitrogen into the TCA soluble fraction was also measured. Release of total nitrogen from protein into the TCA-soluble fraction was measured by Kjeldahl digestion followed by Nesslerization.

Protease activity released into the medium by excised

incubated endosperms was also measured. An aliquot of fresh medium, 0.25-0.5 ml, was incubated with 50 mg protein substrate and 1.5 ml assay buffer, in a total volume of 3 ml. The reaction was stopped by addition of 3 ml of 5% TCA, and activity determined as described for the endosperm enzyme preparation. Soluble proteins in the endosperm extracts were determined by the Lowry method (96), using bovine serum albumin as a standard. Protease activity per mg protein in the extract could then be calculated.

Assay of Protease Activity on Agar Gels

Agar gels containing the substrate protein were prepared. The substrate was dissolved in acetate buffer, 0.05 M, pH 3.8, to make a 0.05% solution. 8 M Urea was included if zein or glutelin were to be dissolved. The solution was warmed to 50C. A 3% solution of agar in water was prepared, and cooled to 50C. The agar and protein solutions were mixed in equal volumes, so that the final protein concentration was 0.025%, the buffer 0.025 M, and the agar 1.5%. 25 ml aliquots of the mixture were poured into petri plates, and allowed to set in the cold room. The urea-containing gels were washed for 24 hours with frequent changes of 0.025 M acetate buffer, pH 3.8, before use. Holes 7 mm in diameter were punched through the protein-containing gels

with a cork-borer. These gels were each placed on top of a thin layer of 4% agar in a petri-plate, so that the wells had a base of 4% agar. Enzyme was pipetted into these wells, and the gels incubated for 12 hours at 30C. The plates were wrapped in polythene to prevent drying out. During this time the enzyme diffused out of the wells and could degrade the substrate protein in the gel. At the end of incubation the gels were washed with dilute acetic acid, then water, and stained with nigrosine, 0.02% in 0.2 M phosphate buffer, pH 8.0. To minimize microbial growth, the gels were kept at 3C while staining. After 8 hours or longer, the excess stain was washed off with tap water, and clear rings were visible where the substrate protein had been degraded.

Exopeptidase Assay

Activity of an exopeptidase on gliadin was measured. Bovine pancreas carboxypeptidase A, pretreated with diisopropylfluorophosphate to eliminate contaminating seine-proteases, was obtained from Sigma. The method described by Fasold and Gundlach (44) for measurement of Carboxypeptidase A activity was used.

## α-Amylase Extraction and Assay

Frozen endosperms were extracted by grinding in a pestle and mortar with 0.005 M acetate buffer, pH 5.0, which contained $10^{-3}$ M $CaCl_2$. Two mls of buffer were used per g fresh weight of endosperm tissue. The homogenate was centrifuged at 28,000 x g for 30 minutes at 0C. α-Amylase activity of the extracts was determined by the method outlined by Jones and Varner (76). When necessary the extracts were diluted with extraction buffer before assaying. Activity was expressed as mg starch degraded per minute per endosperm.

α-Amylase activity released into the medium by excised endosperms incubated in buffer was also measured. Aliquots of medium, diluted if necessary, were substituted for endosperm extract in the routine assay.

## Materials

The reagents were obtained from the following sources: Radioactive isotopes were purchased from New England Nuclear. Protease substrates hemoglobin, edestin, bovine serum albumin, zein and gliadin were obtained from Sigma. Gliadin was also purchased from British Drug Houses when not available from Sigma. α-Amylase substrate, potato starch, was obtained from Allied Chemicals. Phytohormones gibberellic acid $A_3$,

indoleacetic acid and kinetin were supplied by Sigma; and abscisic acid was a gift from Dr. Millborrow, Shell Research Centre, Sittingbourne, Kent, England. Plant growth retardants AMO 1618 and CCC were purchased from Calbiochemical Corporation, and Phosphon D was donated by the Richmond Chemical Co., Richmond, Virginia. Inhibitors cycloheximide, puromycin, streptomycin, penicillin and 6-methylpurine were obtained from Sigma. Actinomycin D was a gift of Merck, Sharp and Dohme. Nessler reagent was obtained from Paragon Co., New York, ninhydrin from Sigma, and 1,2-napthaquinone-4-sulphonic acid from Eastman Organic. Amino acids and Carboxypeptidase A were purchased from Sigma. Inorganic chemicals and solvents were supplied by Fisher Scientific Company.

# RESULTS

## I. ENDOSPERM PROTEINS

The Osborne method (129) of endosperm protein extraction was developed to give a 100% recovery and the reliability of the extraction was tested. Using this method the endosperm protein compositions of ungerminated caryopses were determined, and the sequence of protein loss during germination investigated.

### Protein Extraction

Cereal endosperm proteins were classified according to their solubility as albumins, globulins, prolamines, glutelins and scleroprotein (129). They can be extracted by consecutive treatments with water, 5% salt, 70% alcohol, and 0.2% alkali. Complete extraction of the soluble proteins is necessary if conclusions are to be drawn about the protein composition, or changes in composition of the endosperm. The extraction procedure described by Jimenez (70) was modified to obtain complete extraction of the proteins. The principal difficulties met during protein extraction were:

1. Replicate sampling. Initially there were problems with the homogenous powdering of endosperms.
2. Lipid extraction. Proteins cannot be efficiently extracted without defatting the endosperm powder, but care must be taken to avoid nitrogen losses during the lipid extraction.
3. Extraction of the protein fractions. An exhaustive extraction of endosperm powder in a minimum time was required.
4. Protein-nitrogen measurements on the extracts. The accuracy of this measurement is essential since errors are magnified in calculation of nitrogen in the total fraction.

Techniques were developed to deal with each of these difficulties:

1. Homogenization of the endosperms. The endosperms were freeze-dried, then ground in a commercial blender. Liquid nitrogen was repeatedly poured onto the powder to facilitate breakage of membrane-bound structures, such as protein bodies. A final milling was performed by hand with pestle and mortar. The powder then appeared homogeneous. Other methods, such as sonication, were not satisfactory.
2. Lipid extraction. Hexane, chloroform-methanol (2:1 v/v), acetone, ether, propanol and butanol were tested as defatting solvents (Table 4). The very volatile solvents

TABLE 4

Lipid Extraction of Maize Endosperm

Endosperm powder was defatted in a Soxhlet Apparatus and by grinding in a pestle and mortar with the solvent. Dry weight and nitrogen determinations were made before and after extraction with each solvent.

| Solvent | Extraction Method | mg N/g endosperm | | Percentage Loss During Defatting | |
|---|---|---|---|---|---|
| | | Initial | After Defatting | Weight | Nitrogen |
| Hexane | Soxhlet | 16.18 | 11.90 | 5.2 | 26.5 |
| Chloroform - Methanol | Soxhlet | 16.18 | 13.51 | 3.7 | 16.5 |
| Ether | Soxhlet | 16.18 | 14.53 | 2.8 | 10.1 |
| Acetone | Soxhlet | 16.18 | 14.96 | 3.2 | 7.5 |
| Propanol | Soxhlet | 16.18 | 14.95 | 3.0 | 7.6 |
| Propanol | Grind | 16.18 | 15.72 | 2.3 | 1.8 |
| Butanol | Soxhlet | 16.18 | 15.9 | 2.9 | 1.7 |
| Butanol | Grind | 16.18 | 16.09 | 2.1 | 0.6 |

were used only in a Soxhlet Apparatus, butanol and propanol were also ground by hand with the endosperm powder. Nitrogen in the endosperm powder (mg N/g powder) was measured before and after defatting. The percentage nitrogen loss for each solvent, and the percentage dry weight loss during defatting was calculated. There were considerable nitrogen losses with the more volatile solvents (Table 4). Extraction in a Soxhlet also resulted in greater nitrogen losses than grinding the powder by hand with the solvent. Dry weight losses were considerable from samples extracted with hexane or chloroform-methanol, but not sufficient to account for the percentage nitrogen losses.

Since grinding by hand with butanol minimized nitrogen and dry weight losses, this method was adopted for routine lipid extraction.

3. Extraction of the protein fractions. The optimal time for extraction with each solvent was tested. Extractions of albumin, globulin, zein and glutelin were each made for 6, 12, 2 x 12, 24 and 48 hours. Table 5 shows that the proteins were not fully extracted in 6 or 12 hours. No significant difference was observed in the yield after extraction for 2 x 12, 24 or 48 hours. A 24 hour extraction without change of solvent is most convenient, and was used routinely.

TABLE 5

The Optimal Extraction Time for Proteins of Maize Endosperm

One gram samples of defatted endosperm powder were extracted. Albumin, globulin, zein and glutelin were consecutively extracted by stirring at 3C with the appropriate solvent, for the time shown. In one sample (2 x 12) the solvent was changed after 12 hours and the two extracts pooled. Total nitrogen in each fraction was measured.

| Protein | Solvent | Extraction Time (h) | Extraction Volume (ml) | Yield (mg N) |
|---|---|---|---|---|
| Albumin | Water | 6 | 50 | 0.45 |
| | | 12 | 50 | 0.98 |
| | | 2 x 12 | 100 | 1.71 |
| | | 24 | 50 | 1.68 |
| | | 48 | 50 | 1.70 |
| Zein | 70% Alcohol | 6 | 50 | 3.35 |
| | | 12 | 50 | 4.43 |
| | | 2 x 12 | 100 | 5.90 |
| | | 24 | 50 | 5.99 |
| | | 48 | 50 | 6.02 |
| Glutelin | 0.2% Alkali | 6 | 50 | 4.60 |
| | | 12 | 50 | 6.55 |
| | | 2 x 12 | 100 | 7.35 |
| | | 24 | 50 | 7.30 |
| | | 48 | 50 | 7.26 |

4. Protein-nitrogen measurements. The accuracy of nitrogen measurement by Kjeldahl digestion and Nesslerization was tested. Table 6 shows results obtained in five replicate determinations made on two different samples of endosperm powder. The variability between replicate determinations was very small. Standard errors of the means were not calculated due to the small numbers of replicates. Percent variability was calculated as:

$$\frac{\text{(highest value - lowest value)}}{\text{mean value}} \times 100$$

The variability of the determinations was only 2% for both samples. Nitrogen determination is therefore not an important source of error.

The errors at each stage have been minimized as described above, but the determination of each protein fraction involves a total of the errors at each stage. Considerable variability can therefore be expected between replicate protein extractions. Table 7 shows data from three separate experiments. Three replicates were extracted in Experiment I. The percent recovery was calculated in each case by summing the nitrogen fractions and comparing with a direct measurement of total nitrogen. This was made by digestion of endosperm powder. Only 2 to 5% of the total nitrogen remained insoluble in the final residue, and the recovery was between 97 and 101% in all five samples. The efficiency of the extraction was therefore satisfactory.

TABLE 6

Reliability of Nitrogen Determination

Endosperm powder was subjected to Kjeldahl digestion and its nitrogen measured by Nesslerization. The nitrogen content was calculated as μg N/mg endosperm, and the values averaged. Variation % was calculated as $\frac{\text{highest value-lowest value}}{\text{mean}}$ X 100.

| Sample | Sample Digested mg. | Nitrogen in Sample mg. | μg N per mg sample | Mean | Variation % |
|---|---|---|---|---|---|
| W64A-$O_2$ | 52.4 | 1.110 | 21.18 | | |
| | 53.0 | 1.129 | 21.31 | | |
| | 86.0 | 1.791 | 20.82 | | |
| | 105.0 | 2.210 | 21.05 | | |
| | 110.7 | 2.413 | 21.08 | 21.09 | 2.3 |
| W64A | 52.5 | 1.324 | 25.43 | | |
| | 53.0 | 1.320 | 24.91 | | |
| | 82.6 | 2.088 | 25.28 | | |
| | 101.5 | 2.555 | 25.17 | | |
| | 108.3 | 2.730 | 25.21 | 25.20 | 2.1 |

TABLE 7

Reliability of Endosperm Protein Extraction

Three separate experiments, one with triplicate extractions, are presented. The percentage recovery is calculated by comparison of total nitrogen obtained by summing the protein fractions, and total nitrogen measured directly on endosperm powder.

| Nitrogen Fraction | Solvent | Nitrogen mg per gram endosperm powder I | II | III | Mean |
|---|---|---|---|---|---|
| Lipid | n-butanol | 0.15, 0.18, 0.08 | 0.09 | 0.05 | 0.11 |
| Albumin + Dialysable N | water | 1.50, 1.93, 1.42 | 1.50 | 1.45 | 1.56 |
| Globulin | 5% salt | 0.55, 0.55, 0.39 | 0.70 | 0.62 | 0.56 |
| Zein | 70% ethanol | 4.95, 5.31, 6.56 | 6.31 | 5.95 | 5.82 |
| Glutelin | 0.2% alkali | 7.75, 7.40, 7.17 | 7.17 | 7.36 | 7.37 |
| Residue | | 0.85, 0.70, 0.69 | 0.51 | 0.82 | 0.71 |
| Total | | 15.75, 16.07, 16.31 | 16.28 | 16.25 | 16.13 |
| Total nitrogen (measured on endosperm powder) | | | | | 16.16 |
| Percentage Recovery | | 97 99 101 | 101 | 100 | |

Examination of the replicates for each protein-fraction shows that there was considerable variability in the measurements. The values obtained for zein ranged from 4.95 to 6.56 mg zein-nitrogen per g endosperm powder, for glutelin the range for 7.17 to 7.75 mg glutelin-nitrogen per g endosperm. Standard errors of the mean values were not calculated since the number of replicates was small. A larger number of replicates would increase the reliability of the mean value, but this was impossible on a routine basis. To further test the suitability of the method, caryopses of the W64A wild-type and W64A opaque-2 mutant were analyzed. The opaque-2 mutant is known to be deficient in zein (107). It may have increased levels of albumin, globulin and glutelin in partial compensation for the reduction in zein. The results in Table 8 show that zein is reduced by 66% and total nitrogen by 20% in the opaque-2 mutant. Albumins and globulins were doubled in the mutant, and glutelin increased by 15%. The differences between wild-type and the mutant are readily detectible using this method of protein analysis. This indicates that the extraction procedure can be used routinely to measure gross changes in the pattern of protein distribution in the endosperm of maize, for example the changes which occur during germination.

## TABLE 8

## A Test of Endosperm Protein Analysis

The endosperm proteins of W64A and W64A-$O_2$ were analysed as described in METHODS. The caryopses were supplied by Warwick Seed Company in 1971 and analysed in 1972.

| Nitrogen Fraction | Nitrogen mg/10 endosperms | | | | | |
|---|---|---|---|---|---|---|
| | W64A | | | W64A-$O_2$ | | |
| | i | ii | Mean | iii | iv | Mean |
| Lipid | 0.3 | 0.5 | 0.4 | 0.4 | 0.5 | 0.4 |
| Albumin | 2.1 | 1.7 | 1.9 | 3.5 | 4.4 | 4.0 |
| Globulin | 0.9 | 1.7 | 1.3 | 2.8 | 2.2 | 2.5 |
| Zein | 16.7 | 17.5 | 17.1 | 6.0 | 5.1 | 5.6 |
| Glutelin | 14.8 | 12.9 | 13.9 | 15.4 | 16.5 | 15.9 |
| Residue | 3.0 | 3.8 | 3.4 | 2.0 | 2.4 | 2.2 |
| TOTAL | 37.8 | 38.2 | 38.0 | 30.1 | 31.1 | 30.6 |
| Total - Measured on endosperm powder | | | 40.2 | | | 31.7 |
| Recovery (%) | | | 95 | | | 97 |

Protein Composition of Ungerminated Corn Endosperm

Endosperm proteins were extracted from several different batches of the hybrid Wf9 x 38-11. Table 9 shows the results from four different batches of caryopses. Both the total nitrogen and protein composition varied considerably from batch to batch. The lowest protein caryopses contained 29.95 mg and the highest 42.5 mg nitrogen per endosperm. The nitrogen content of each protein fraction varied, but the variations were most pronounced in the case of zein. Zein nitrogen ranged from 6.0 to 21.5 mg per 10 endosperms, whereas glutelin content was between 14.5 and 18.2 mg per 10 endosperms. This is in agreement with observations that zein is the protein fraction increased by nitrogen fertilization during development of the caryopses (80,187). Calculation of the zein to glutelin ratios of the caryopses illustrates the differences in their protein composition. The caryopses analysed (Table 9) had zein to glutelin ratios of 0.3, 0.7, 1.0 and 1.5. Both the protein content and the pattern of distribution of nitrogen between the storage proteins can vary enormously from batch to batch of the same maize hybrid.

TABLE 9

Protein Composition of Mature Ungerminated Maize Endosperm

Four different batches of the hybrid Wf9 x 38-11 were used. The proteins were analysed, and total nitrogen per endosperm also measured directly on endosperm powder. The zein:glutelin ratio was calculated for each batch. Batches B and D were supplied by the Agricultural Alumni Association, Lafayette, Indiana. Batches A and C were grown in Hamilton.

| Batch | mg protein-nitrogen/10 endosperms | | | Total Nitrogen/ endosperm | Zein / Glutelin |
|---|---|---|---|---|---|
| | Albumin+ Globulin | Zein | Glutelin | | |
| A | 3.1 | 6.0 | 18.2 | 29.9 | 0.3 |
| B | 5.8 | 11.50 | 17.2 | 39.5 | 0.7 |
| C | 4.1 | 16.6 | 15.7 | 41.50 | 1.0 |
| D | 4.2 | 21.5 | 14.5 | 42.5 | 1.5 |

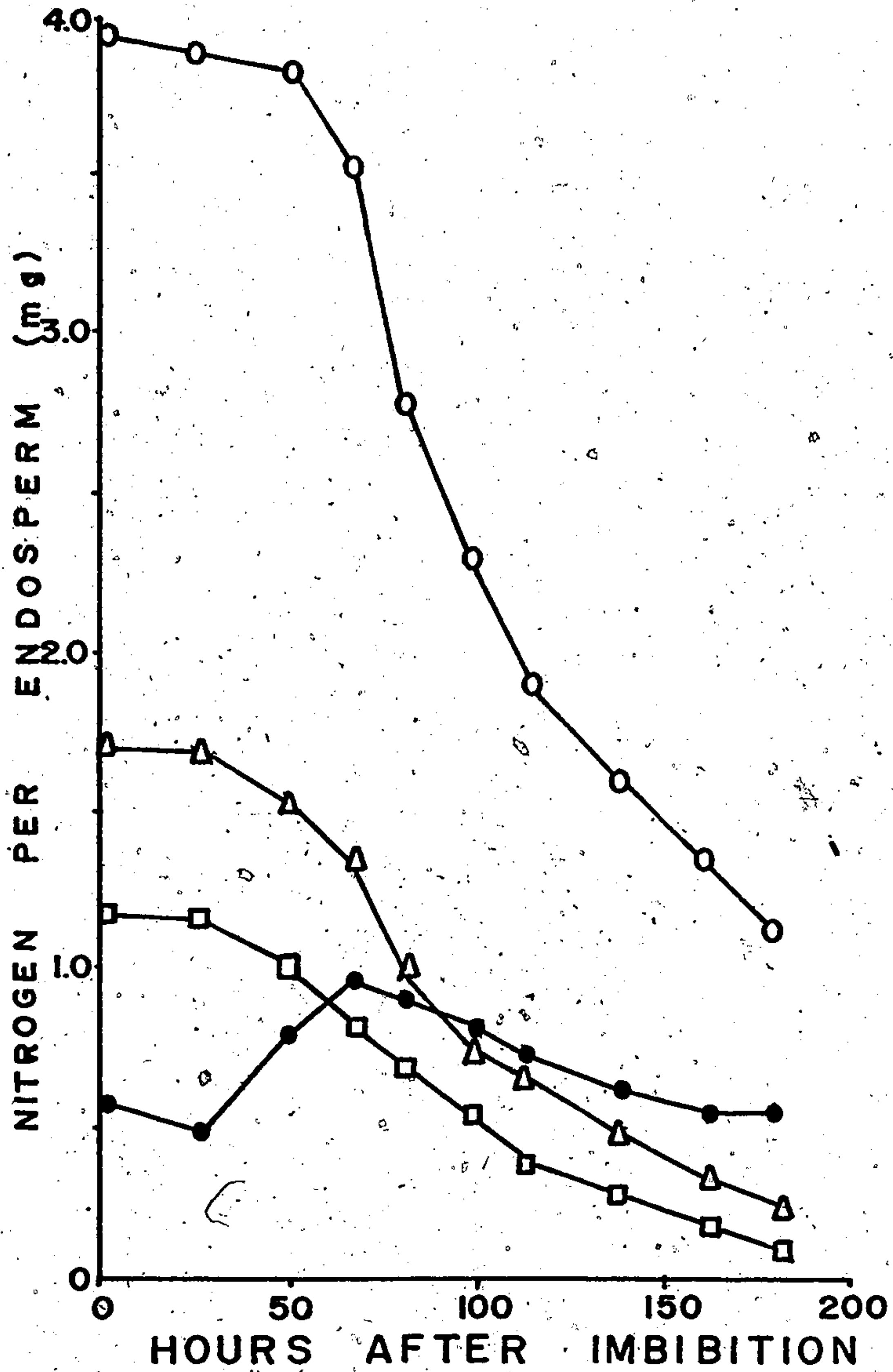
Pattern of Protein Breakdown During Germination

Caryopses were germinated and at approximately 20 hour intervals samples of 50 seedlings were taken for endosperm protein analysis. Figure 2 shows the pattern of hydrolysis of the major endosperm protein fractions. Data for albumin and globulin fractions were pooled, since they followed a similar trend. The albumin + globulin fraction includes a dialysable nitrogen component, chiefly amino acids and small peptides. Changes in total endosperm nitrogen were also measured. During the first day of germination total nitrogen and the albumin + globulin fraction declined slightly. During the second day zein and glutelin loss began. This was not initially reflected in loss of total nitrogen, due to increases in the salt and water soluble nitrogen. This increase was mainly in dialysable nitrogen, probably the products of zein and glutelin degradation. Increases in water soluble fraction during the first days of germination were also observed by Ingle, Beevers and Hageman (64). After 2 days total nitrogen, zein and glutelin declined rapidly. The water and salt soluble fraction continued to rise, doubling between 24 and 68 hours. Glutelin degradation appeared more rapid than zein hydrolysis from 30 to 80 hours, then both continued at similar rates until the protein reserves were depleted. By 7½ days 90% of the zein and glutelin had

FIGURE 2

Changes in Endosperm Proteins during Germination

Caryopses were germinated as described in MATERIALS AND METHODS. At daily intervals 50 seedlings were harvested and the endosperms freeze-dried and powdered. Total nitrogen per endosperm (O) was measured directly on endosperm powder. Endosperm proteins glutelin (Δ), zein (□), and albumin + Globulin + dialysable nitrogen (●) were extracted and measured as described in MATERIALS AND METHODS. The difference between the sum of the protein fractions and the total nitrogen was insoluble nitrogen left in the residue after extraction.


NITROGEN PER ENDOSPERM (mg)
4.0
3.0
2.0
1.0
0
0
50
100
150
200
HOURS AFTER IMBIBITION

## TABLE 10

### Changes in Endosperm Proteins During Germination

Caryopses were germinated as described in MATERIALS AND METHODS. At daily intervals 50 seedlings were harvested and the endosperms freeze-dried and powdered. Total nitrogen per endosperm was measured directly on endosperm powder. Endosperm proteins glutelin, zein, and albumin + globulin + dialysable nitrogen were extracted and measured as described in MATERIALS AND METHODS. The difference between the sum of the protein fractions and the total nitrogen was insoluble nitrogen left in the residue after extraction. Total nitrogen and proteins are expressed in mg.

| Time after imbibition hrs | Water and salt soluble Protein | Zein | Glutelin | Total Nitrogen |
|---|---|---|---|---|
| 0 | 0.58 | 1.15 | 1.72 | 3.98 |
| 26 | 0.48 | 1.00 | 1.66 | 3.96 |
| 52 | 0.80 | 1.03 | 1.49 | 3.92 |
| 68 | 0.99 | 0.81 | 1.23 | 3.53 |
| 80 | 0.90 | 0.63 | 0.99 | 2.78 |
| 100 | 0.81 | 0.54 | 0.77 | 2.35 |
| 114 | 0.74 | 0.38 | 0.65 | 1.91 |
| 142 | 0.64 | 0.28 | 0.48 | 1.61 |
| 162 | 0.59 | 0.18 | 0.34 | 1.35 |
| 185 | 0.61 | 0.13 | 0.21 | 1.11 |

been degraded, but 30% of the total endosperm nitrogen remained. This was mainly albumin, globulin and insoluble nitrogen left in the residue after extraction. This pattern of protein degradation was similar whether the caryopses were grown in the presence or absence of nitrate.

The more rapid initial loss of glutelin than zein is interesting since it could imply independent degradation of the two storage proteins. However it is consistent with either of two hypothesis:

1. The rates of hydrolysis of the two proteins are independent, and glutelin generally is the major supplier of amino acids to the embryo during early germination.
2. The more abundant protein of the endosperm is degraded most rapidly.

These possibilities were tested by examining protein loss in caryopses of different protein composition. Caryopses of the four batches of Wf9 x 38-11 analysed in Table 9 were germinated for approximately three and a half days, then harvested for protein analysis. At 3½ days the total protein losses ranged from 20 to 30%, values consistent with the losses seen in Figure 2. Table 11 shows that the high glutelin caryopses, batches A and B, again degraded glutelin more rapidly than zein. The converse was true of the high zein caryopses, batch D, where zein was more rapidly degraded than

TABLE 11

Endosperm Protein Breakdown During Germination of Caryopses with Different Zein to Glutelin Ratios

Caryopses were germinated for 3½ days, and their endosperm proteins analysed. The zein to glutelin ratios were: A, 0.3; B, 0.7; C, 1.0; and D, 1.5. Batches B and D were supplied by the Agricultural Alumni Association, Lafayette, Indiana. Batches A and C were grown in Hamilton.

| Batch | Initial Values mg N/10 endosperms | | | mg N degraded during 3½ days germination | | |
|---|---|---|---|---|---|---|
| | Total | Zein | Glutelin | Total | Zein | Glutelin |
| A | 30.0 | 6.0 | 18.0 | 9.5 | 2.2 | 7.0 |
| B | 39.5 | 11.5 | 17.0 | 13.5 | 4.8 | 7.0 |
| C | 41.5 | 16.5 | 16.2 | 8.5 | 5.5 | 5.5 |
| D | 42.5 | 21.5 | 14.5 | 13.8 | 12.25 | 6.0 |

glutelin during the germination period. Batch C initially had the same amount of zein and glutelin, and equal amounts of the proteins were degraded during the experiment. These results show that the more abundant protein was degraded most rapidly.

Radioactive Labelling of Endosperm Proteins

Caryopses containing a specifically labelled endosperm storage protein would be useful in measuring breakdown rates of the protein during germination, and in determining the mode of protein degradation. Identification of the hydrolysis products could indicate the nature and specificity of proteases in the endosperm.

In 1966 developing caryopses were fed radioactive tracers by Oaks, as described by Spenser and Gear (170). It was decided to label zein rather than glutelin since at that time zein was considered to be the principal endosperm storage protein (17). In maize starch synthesis is rapid for 30 days after pollination, and then slows to a negligible rate. Glutelin accumulates at a steady rate between 10 and 42 days after pollination, whereas zein is rapidly synthesised between 22 and 42 days. Zein increases from 30 to 45% of the total endosperm protein over this interval, and so is synthesised more rapidly than the other endosperm components in

the later stages of development (31). With late administration of radioactive tracers zein should be preferentially labelled. Zein is rich in proline, hence administration of $^{14}$C-proline should lead to selective labelling of zein. $^{14}$C-Acetate was also fed, since it might be expected to label lipid, starch and protein. In the initial experiments the tracers were administered at 19 and 26 days after pollination, and in a second experiment in 1969 $^{14}$C-proline was fed at 33 days. At maturity, 50 days after pollination, the cobs were harvested and stored at room temperature.

Samples of $^{14}$C-acetate (1966) and $^{14}$C-proline (1966 and 1969) labelled caryopses were analysed, to determine the specificity of incorporation. Endosperm proteins were extracted as described in MATERIALS AND METHODS. The caryopses grown in 1966 and 1969 differed considerably in total nitrogen and in zein content (Table 12). Table 13 shows the total incorporation of each tracer into starch, protein and lipid. Total incorporation was best when the tracers were fed 19 days after pollination, and greater in caryopses fed with $^{14}$C-acetate than $^{14}$C-proline. A higher percentage of the total radioactivity was incorporated into protein in caryopses fed with $^{14}$C-proline than with $^{14}$C-acetate, and those fed proline at 33 days incorporated the largest proportion of their total radioactivity into protein (77%). Table 13 also shows labelling of the major endosperm protein fractions,

## TABLE 12

## Protein Composition of Endosperms from 1966 and 1969 Caryopses

50 Endosperm samples of the 1966 and 1969 caryopses were extracted and their proteins analysed as described in MATERIALS AND METHODS. Total endosperm nitrogen was measured directly on endosperm powder, to allow calculation of % recovery.

| Protein Fraction | Caryopses mg nitrogen/g endosperm | |
|---|---|---|
| | 1966 | 1969 |
| Lipid | 0.7 | 0.3 |
| Albumin + Globulin | 1.9 | 2.4 |
| Zein | 4.4 | 9.5 |
| Glutelin | 13.4 | 10.1 |
| Residue | 1.8 | 1.8 |
| TOTAL | 22.2 | 23.1 |
| Total nitrogen measured on endosperm powder | 22.4 | 23.6 |
| % Recovery | 100 | 98 |
| Zein/Glutelin | 0.3 | 0.9 |

TABLE 13

Incorporation of $^{14}C$-Proline and $^{14}C$-Acetate into Maize Endosperm

Tracers were fed (25 µci/cob) at 19 and 26 days after pollination in August 1966, and at 33 days after pollination in August 1969. Fifty endosperm samples of each treatment were analysed for radioactivity in lipid, starch and endosperm proteins, extracted by Osborne's method. Radioactivity is expressed as CPM per gram of endosperm powder.

| Fraction | 1966 Caryopses | | | | 1969 Caryopses |
|---|---|---|---|---|---|
| | Acetate 19 days | Acetate 26 days | Proline 19 days | Proline 26 days | Proline 33 days |
| Lipid | 1,200 | 1,000 | 800 | 600 | 900 |
| Starch* | 41,300 | 33,200 | 25,000 | 18,400 | 7,100 |
| Protein | 46,500 | 47,800 | 47,000 | 34,000 | 26,000 |
| Albumin | 3,900 | 5,700 | 2,800 | 3,400 | 1,600 |
| Globulin | | | | | |
| Zein | 9,300 | 10,000 | 7,500 | 4,400 | 13,500 |
| Glutelin | 31,200 | 31,000 | 35,300 | 25,700 | 10,400 |
| Residue | 2,100 | 1,100 | 1,400 | 500 | 500 |
| TOTAL | 89,000 | 82,000 | 73,000 | 53,00 | 34,000 |

*>95% of the total $^{14}C$ in starch was identified as glucose.

albumin + globulin, zein, and glutelin. In 1966 glutelin represented the major storage protein (Table 12) and most of the radioactivity of $^{14}$C-acetate and proline was incorporated into this fraction. In 1969 there was an appreciable accumulation of zein which is reflected in the larger incorporation of $^{14}$C-proline into this fraction. The specific activities of the endosperm proteins were calculated from the data in Tables 12 and 13 and are shown in Table 14. Zein and glutelin were labelled with similar specific activity (2,100-2,400 cpm per mg protein nitrogen) in the caryopses fed with $^{14}$C-acetate. Glutelin had a slightly higher specific activity than zein in caryopses fed with $^{14}$C-proline at 19 or 26 days, but in those fed at 33 days the zein and glutelin specific activities were again very similar.

To determine whether proline was extensively converted to other amino acids the proteins were hydrolysed and the distribution of radioactivity determined. The amino acids in the hydrolysates were separated as described in MATERIALS AND METHODS, and their radioactivity measured. The data presented in Table 15 show that: (a) proline + glutamic acid + glutamine contained 70 to 80% of the total radioactivity of zein and glutelin, and (b) the distribution of $^{14}$C was the same whether the caryopses had been fed radioactive proline or acetate.

TABLE 14

Specific Activity of Endosperm Proteins

The radioactivity per fraction (Table 12) and the nitrogen per fraction (Table 11) were used to calculate the specific activities of ablumin + globulin, zein and glutelin.

| Tracer | Total Protein CPM/g endosperm | Specific Radioactivity CPM/mg protein | | |
|---|---|---|---|---|
| | | Albumin+ Globulin | Zein | Glutelin |
| Acetate-19 | 45,500 | 3,000 | 2,100 | 2,300 |
| 26 | 46,800 | 2,900 | 2,300 | 2,400 |
| Proline-19 | 47,000 | 1,600 | 1,700 | 2,600 |
| 26 | 33,000 | 1,800 | 1,000 | 2,000 |
| Proline-33 | 26,000 | 1,400 | 1,600 | 1,200 |

TABLE 15

Distribution of Radioactivity in Zein and Glutelin

The tracers (25 μC/cob) were fed at 26 days. Endosperm proteins were extracted and hydrolysed. The amino acids were separated and their radioactivity determined as described in MATERIALS AND METHODS. Most label was in glutamic + glutamine and in proline, and their contribution to the total radioactivity of zein and of glutelin was calculated. The percent of total radioactivity in proline was 15% in the $^{14}C$-acetate fed caryopses, and 11% in the $^{14}C$-proline fed caryopses.

| Tracer | Zein | | | Glutelin | | |
|---|---|---|---|---|---|---|
| | CPM | % Proline | % Glutamic Glutamine | CPM | % Proline | % Glutamic Glutamine |
| Acetate | 9,900 | 25 | 47 | 31,800 | 27 | 45 |
| Proline | 4,400 | 26 | 54 | 26,000 | 22 | 54 |

From the data for labelling of protein and starch it is apparent that both tracers were extensively metabolised before incorporation into the endosperm reserves. Incorporation into starch was initially high, at 19 days after pollination, but was much reduced in caryopses fed at later times (25,000 and 7,100 cpm/g endosperm in caryopses fed $^{14}C$-proline at 19 and 33 days respectively). Protein synthesis, measured by total incorporation of radioactivity into protein, also declined with time after pollination. This drop was less than in starch labelling, confirming that protein synthesis continues longer than starch synthesis in the developing caryopsis. The incorporation patterns appear to indicate that most radioactivity was incorporated into the fraction being synthesized most rapidly at the time of feeding the tracer. Since radioactivity was incorporated into all protein fractions, the project to specifically label zein was unsuccessful. This work has not been continued.

Recently a method of specifically labelling glutelin has been published. Soedek and Wilson (168) have studied the lysine metabolism of normal maize and of opaque-2 high lysine mutants. Their data for normal maize showed that when $^{14}C$-lysine was fed at 12 days after pollination, 78% of the total radioactivity remained in lysine. Most of this radioactivity was extracted with glutelin, and only 10% was in zein. Table 16 reproduces their data for normal maize, and

TABLE 16

Specific Labelling of Endosperm Glutelin with $^{14}C$-Lysine

Calculations from the data of Soedek and Wilson, Arch. Biochem. Biophys. 140, 29-38, 1970 (168).
Caryopses Inbred line R802 (normal maize, not opaque-2).
Treatment: 2µCi $^{14}C$-lysine injected at 12 days, caryopses harvested at 50 days after pollination.

| Protein Fraction | DPM/endosperm | mg nitrogen/endosperm | Specific activity DPM/mg protein |
|---|---|---|---|
| Total | 5,200 | 2.55 | 2,000 |
| Glutelin | 44.2% = 2,300 | 32.8% = 0.85 | 2,700 |
| Zein | 9.7% = 520 | 38.6% = 0.98 | 500 |
| Residue | 33.8% = 1,700 | 24.2% = 0.62 | 2,700 |

shows calculations of the specific activities of the labelled proteins. Glutelin had a specific activity of 2,700 dpm/mg protein nitrogen, approximately five times that of zein. However 24% of the total nitrogen and 34% of the total radioactivity remained in the residue after extraction. Residual protein is normally 5 to 10% of total endosperm nitrogen, so this fraction must have included storage proteins. The specific activity of the residue protein is the same as that of glutelin. Should this fraction prove to be glutelin, it would be evident that feeding $^{14}C$-lysine is a good method of selectively labelling a specific endosperm protein.

## II. ENDOSPERM PROTEOLYTIC ENZYMES

An assay for endosperm proteolytic activity was developed. The assay was optimised so that activity of unpurified enzyme could be reliably measured with different substrates, or in endosperms of different ages. The capacity of the protease(s) to degrade various substrates, including the maize endosperm proteins, and its mode of action were investigated. The changes in endosperm proteolytic activity during germination were followed, and correlated with the degradation of the endosperm proteins.

### Proteolytic Activity in Endosperm Powder

Proteolytic activity of endosperm powder suspensions was measured in experiments designed to indicate the pH range most favourable for endosperm protease activity and the time of highest protease activity during germination. Activity was measured by the release of α-amino nitrogen from protein into the TCA soluble fraction. The endosperms of caryopses germinated 0, 3 or 5 days were lyophilised and powdered. The flour was incubated in water with hemoglobin as substrate, and TCA soluble α-amino nitrogen measured after 0 and 4 hours incubation. Flour was incubated alone to test for autolysis. Hemoglobin blanks were also included.

A boiled flour + hemoglobin blank was not included since the flour suspension became gelatinous if heated above 65C. Table 17 shows that activity in ungerminated caryopses was low. Some α-amino nitrogen was released when the flour was incubated alone, and activity was enhanced by the presence of exogenous substrate, hemoglobin. No α-amino nitrogen was released from hemoglobin in the absence of endosperm powder. Activity was calculated on a per endosperm basis, as well as per g endosperm powder, since endosperm dry weight changes during germination. Both measurements showed that proteolytic activity increased during germination. The endosperms of caryopses germinated 5 days had most autolytic activity, and degraded hemoglobin most rapidly.

The effect of pH on proteolytic activity was tested by incubating endosperm powder at pH 4.0, 6.0 and 8.0 in 0.1 M acetate, phosphate and Tris buffers respectively. Flour from seeds germinated 5 days was used, and incubated for 2 hours, with and without hemoglobin. Table 18 shows that activity was best at pH 4.0, in 0.1 M acetate buffer. At pH 6.0 the activity was comparable to that observed in the previous experiment, where water was used instead of buffer.

TABLE 17

Proteolytic Activity in Endosperm Powder

Endosperms from caryopses germinated 0, 3 or 5 days were lyophilized and powdered. Flour was suspended in 10 ml water and incubated ± hemoglobin for 4 hours at room temperature, with shaking. The TCA soluble α-amino nitrogen of each suspension was measured at the beginning of incubation and after 4 hours. Activity was expressed as μg α-amino nitrogen released per hour per g endosperm powder, and also per endosperm. The dry weights per endosperm were 0.255, 0.232 and 0.205 respectively in ungerminated seeds and after 3 and 5 days germination.

| Caryopses Germination time (days) | Endosperm Powder (g) | Hemoglobin (g) | μg α-$NH_2$ in TCA soluble fraction | | μg α-$NH_2$ N/h | |
|---|---|---|---|---|---|---|
| | | | 0 h | 4 h | per g powder | per endosperm |
| 0 | 0.5 | 0 | 90 | 114 | 12 | |
| | 0 | 0.25 | 30 | 30 | | |
| | 0.5 | 0.25 | 122 | 180 | 29 | 7 |
| 3 | 0.5 | 0 | 112 | 144 | 16 | |
| | 0 | 0.25 | 30 | 33 | | |
| | 0.5 | 0.25 | 140 | 310 | 85 | 19 |
| 5 | 0.5 | 0 | 214 | 280 | 33 | |
| | 0 | 0.25 | 30 | 28 | | |
| | 0.5 | 0.25 | 250 | 700 | 225 | 45 |

## TABLE 18

## Effect of pH on Proteolytic Activity of Endosperm Flour Suspensions

Lyophilized endosperm powder from caryopses germinated 5 days was suspended in 10 ml of 0.05 M buffer. Acetate buffer was used at pH 4.0, phosphate at pH 6.0, and Tris at pH 8.0. The suspensions were shaken for 2 hours at room temperature. TCA soluble α-amino nitrogen was measured before and after incubation, and activity expressed as μg α-amino nitrogen released per hour per g endosperm powder.

| Flour mg | Hemoglobin mg | pH | Activity μg α-$NH_2$ N released from hemoglobin | |
|---|---|---|---|---|
| | | | per h in assay | per h per g flour |
| 125 | 0 | 4.0 | 7 | |
| 0 | 125 | | 0 | |
| 125 | 125 | | 57 | 400 |
| 125 | 0 | 6.0 | 5 | |
| 0 | 125 | | 0 | |
| 125 | 125 | | 31 | 208 |
| 125 | 0 | 8.0 | 4 | |
| 0 | 125 | | 1 | |
| 125 | 125 | | 20 | 132 |

Extraction and Assay of Endosperm Protease

The initial experiments with endosperm powder indicated that endosperm protease(s) should be most active at low pH, after 5 days germination. Accordingly, in preliminary extractions for endosperm protease(s), endosperms from 5 day germinated caryopses were used. The frozen endosperms were homogenized in 0.2 M acetate buffer, pH 4.0, and assayed in 0.05 M acetate buffer at the same pH. Initial assays were conducted at 37C. In the early experiments hemoglobin was used as substrate, since it is readily soluble in a wide range of assay conditions. Later, when conditions for the assay had been established, gliadin was also used as a standard substrate. Activity was measured as the increase in absorbance at 280 nm of the TCA soluble fraction. This measures the release from protein of aromatic amino acids, whether free or bound in TCA soluble peptides. The absorbance at 280 nm of the TCA soluble fraction was arbitrarily calibrated against the absorbance of tryptophan dissolved in the same concentration of TCA (2.5%). Activity could thus be expressed as the change in absorbance of TCA soluble fraction, or as the release of tryptophan equivalents (μg) from protein during the assay.

(a). Linearity of the Assay

Figure 3A shows that with 0.3 ml of enzyme per assay product formation, measured by the increase in absorbance at 280 nm, increased linearly for 15 minutes. At this time the change in absorbance was 0.68 units. With 0.1 ml enzyme per assay product formation continued linearly for 25 minutes, until absorbance of the TCA soluble fraction had increased by 0.36 units. The results in Figure 3B show that activity was proportional to enzyme concentration (0.1-0.3 ml/assay) between 0 and 15 minutes incubation. Hence in all subsequent assays enzyme concentration was adjusted so that the increase in absorbance would be between 0.2 and 0.65 units after 10 minutes incubation. Within these limits product formation should depend directly on the activity and concentration of enzyme extract in the assay.

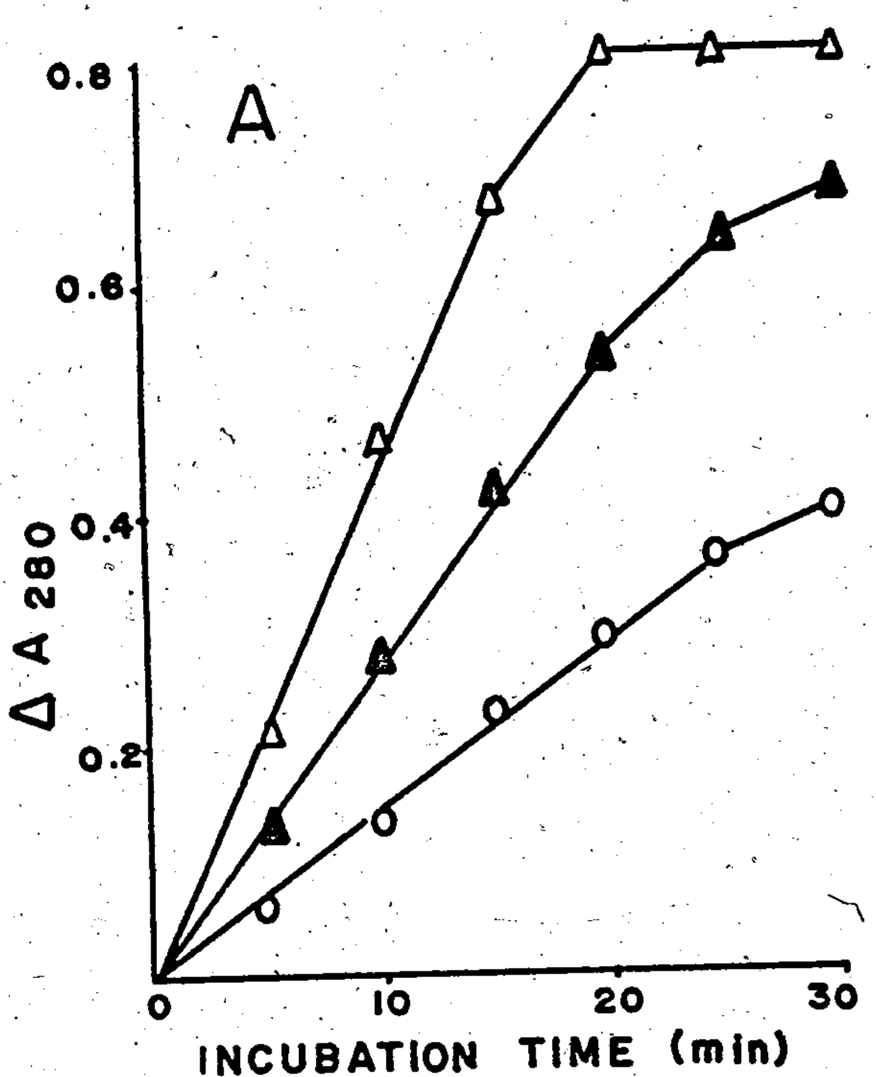
(b) pH of the Assay

The pH optimum for assay of protease(s) was determined. Citrate buffer was used from pH 3.0 to 4.0 and 5.0 to 6.0, acetate from pH 3.6 to 5.2, phosphate from pH 5.8 to 7.8, and Tris from pH 7.2 to 9.0. All assay buffers were 0.05 M. The results in Figure 4A show that activity was obtained at pH 3.8. The curve is not symmetrical, indicating that more

## FIGURE 3

### Linearity of the Protease Assay

Enzyme was extracted in 0.2 M acetate buffer, pH 4.0, from endosperm of caryopses germinated 5 days. Activity with hemoglobin substrate was assayed in 0.05 M acetate buffer at the same pH, at 37C. Activity was measured as the increase in absorbance at 280 nm of the TCA soluble fraction, between 0 and 30 minutes incubation. Activity of 0.1 (O), 0.2 (▲) and 0.3 (Δ) ml of enzyme per assay was measured. A shows the relationship between incubation time and product formation for the three enzyme concentrations. B shows the relationship between enzyme concentration and product formation after 5, 10, 15, 20 and 25 minutes incubation.


A
0.8
0.6
0.4
0.2
Δ A 280
0
10
20
30
INCUBATION TIME (min)

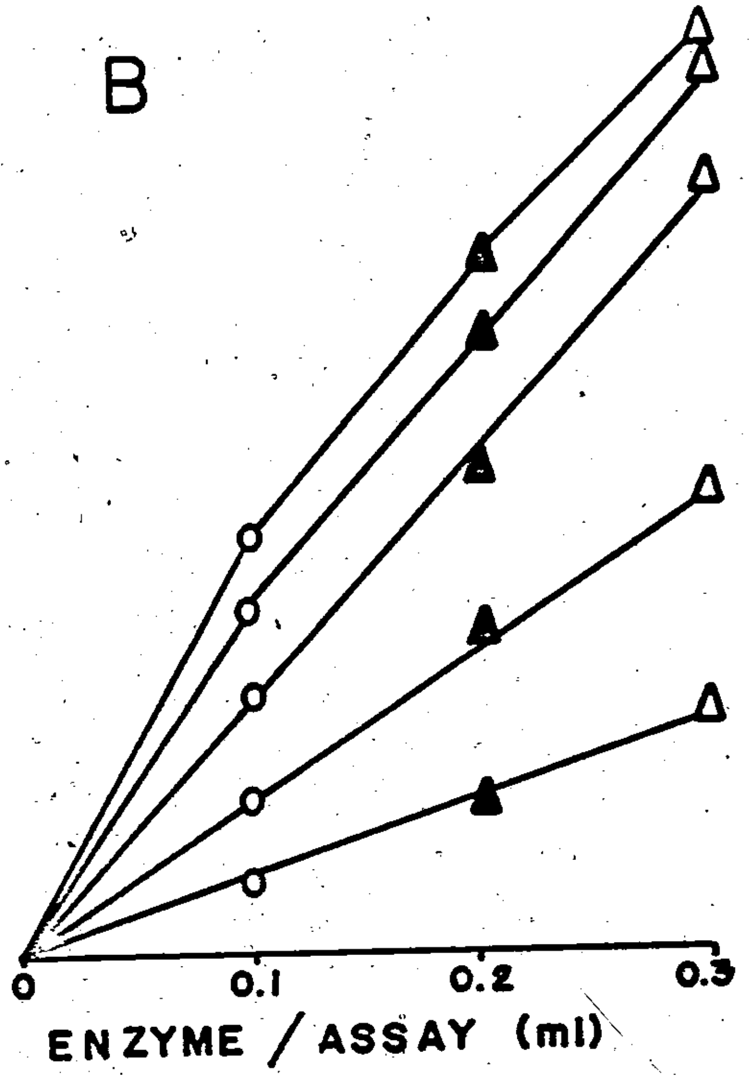

B
0
0.1
0.2
0.3
ENZYME / ASSAY (ml)

FIGURE 4

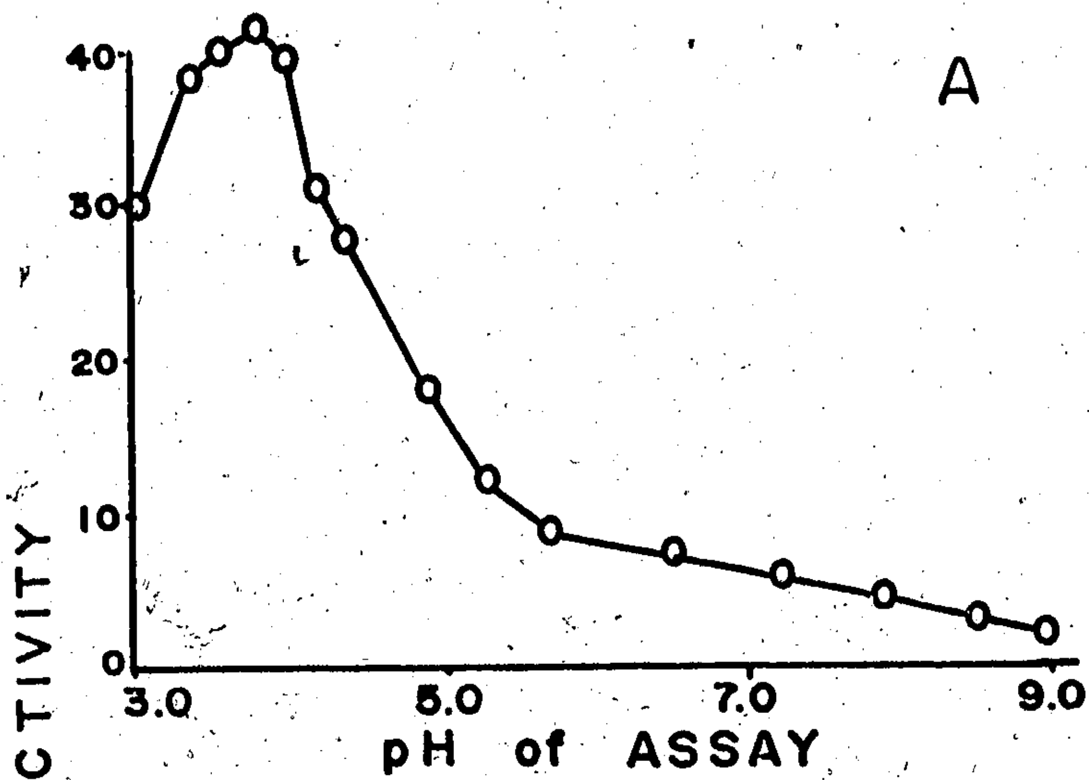
Effects of Buffer Molarity and pH on Protease Activity

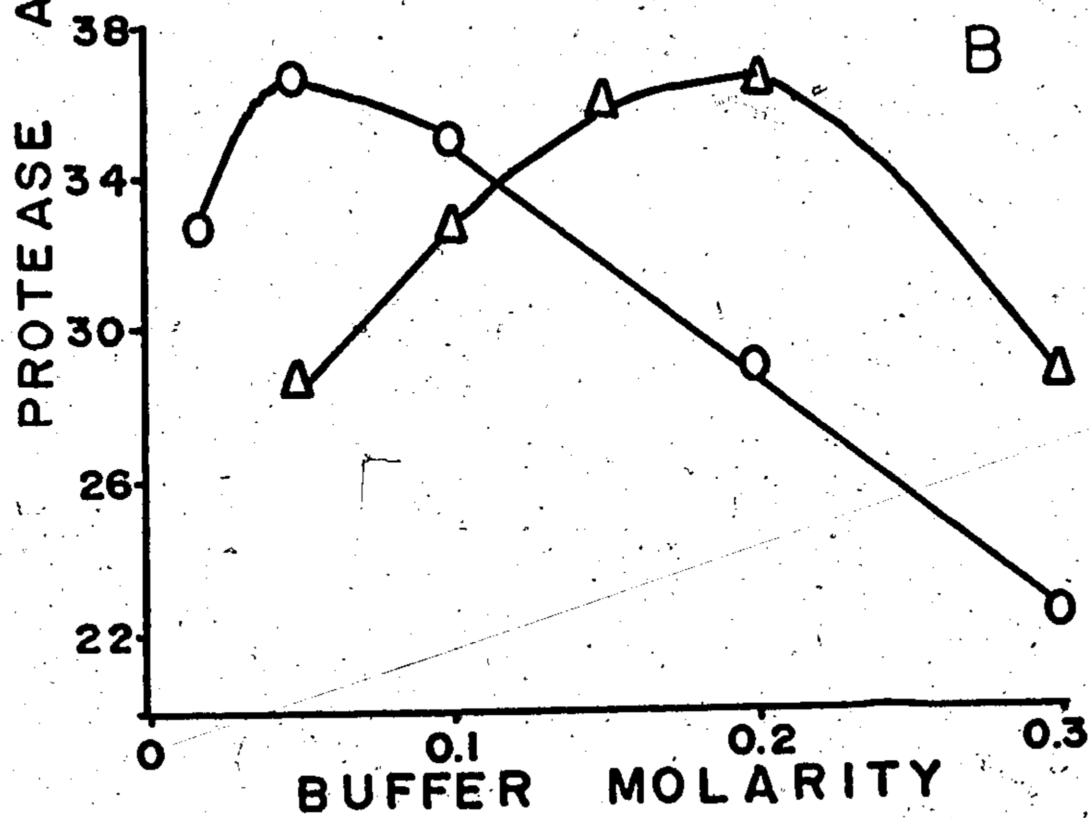
A. Endosperms were extracted in 0.2 M acetate buffer, containing 5 mM β-mercaptoethanol. Activity was assayed at 40C using 0.05 M buffers in the following pH ranges; pH 3.0-4.0, citrate; pH 3.6-5.2, acetate; pH 5.0-6.0, citrate; pH 5.8-7.8, phosphate; and pH 7.2-9.0, Tris.

Endosperms of caryopses germinated for 5 days were used. Hemoglobin was used as substrate in all assays and activities are expressed as μg tryptophan equivalents released per minute per mg protein in the endosperm extract.

B. Endosperms were extracted in 0.2 M acetate buffer, pH 3.8, containing 5 mM β-mercaptoethanol, and activity assayed with 0.02 to 0.30 M acetate buffer, pH 3.8. The experiments where assay buffer molarity was varied are shown by (O).

Extraction buffer molarity was also varied between 0.05 and 0.30 M, and activity was assayed with 0.05 M acetate buffer, pH 3.8. The experiments where extraction buffer molarity was varied are represented by (Δ).


A
40
30
20
10
0
3.0
5.0
7.0
9.0
pH of ASSAY
PROTEASE ACTIVITY


B
38
34
30
26
22
0
0.1
0.2
0.3
BUFFER MOLARITY

than one proteolytic enzyme in the crude extract may be active at low pH. Subsequent extractions and assays were routinely conducted at pH 3.8.

(c) Extraction and Assay Buffers

Enzyme was extracted with 0.2 M acetate buffer, pH 3.8, and assayed with 0.02 to 0.30 M acetate buffer at the same pH. Figure 4B shows that maximum activity was obtained with 0.05 M assay buffer. Extracts were than made with various concentrations of extraction buffer, 0.05 to 0.30 M, and activity assayed with 0.05 M buffer. Most active enzyme was prepared with 0.2 M extraction buffer. Extracts were therefore routinely made in 0.2 M acetate buffer and activity assayed in 0.05 M buffer.

(d) Optimum Assay Temperature and Heat Stability

Activity was assayed at temperatures between 25 and 52.5C. The results in Figure 5B show that activity was highest at 46C. The enzyme appeared to be inactivated at higher temperatures. Assays were routinely conducted at 40C.

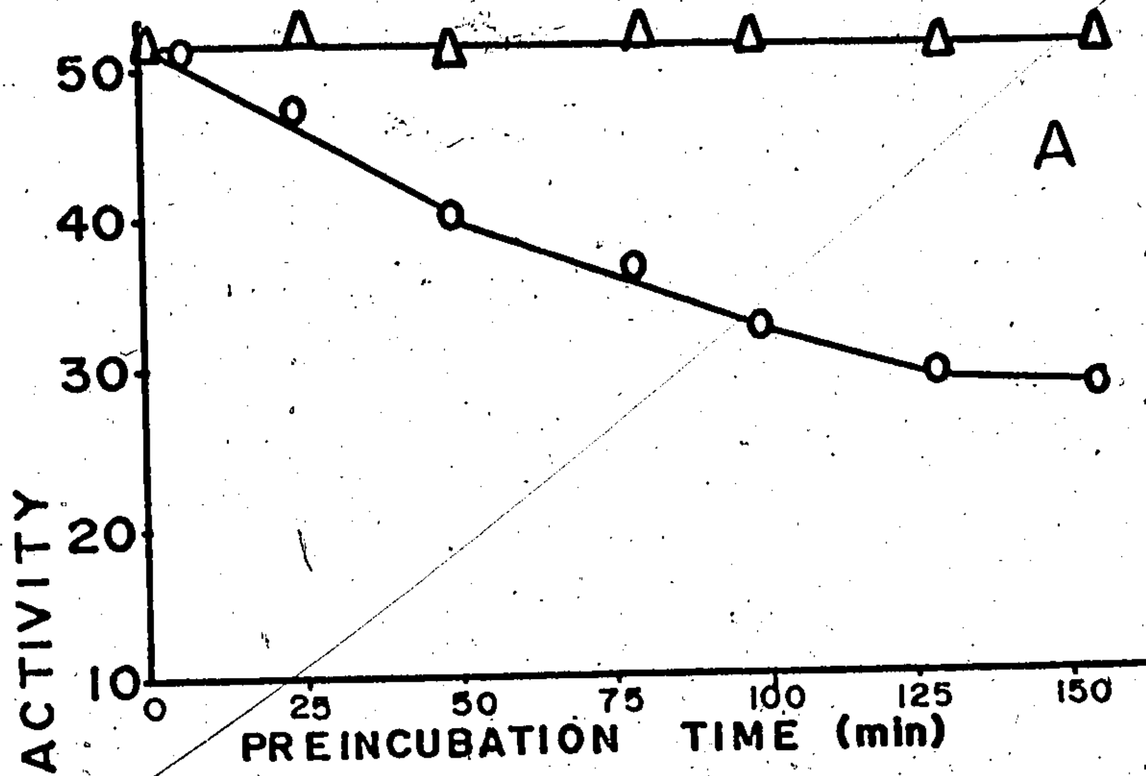
Stability of the protease was tested at the assay temperature. Enzyme incubated at 40C in the absence of substrate lost activity rapidly (Figure 5A). Forty percent

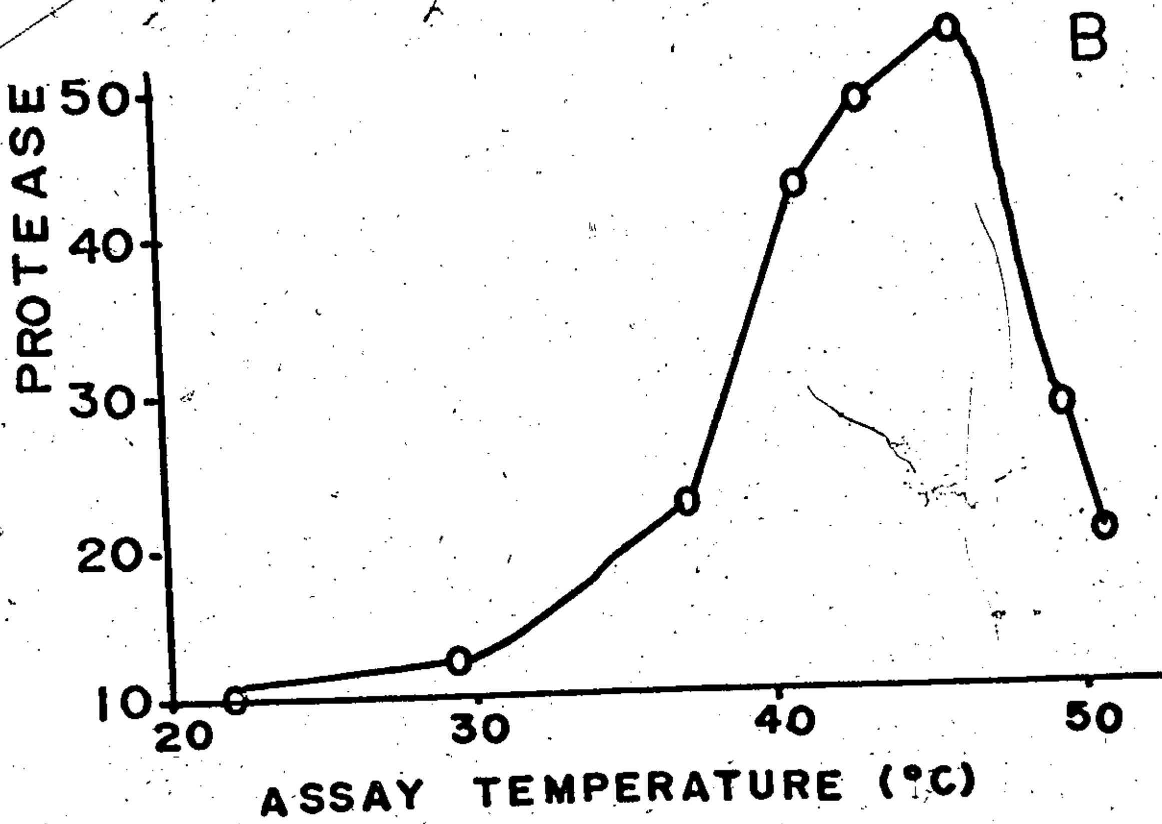
## FIGURE 5

## Temperature Optimum for Assay for Endosperm Protease, and Lability of the Enzyme

A. The enzyme was allowed to stand for 0-155 minutes at 40C (O) or on ice (Δ) before assaying its activity at 40C.

B. The assay temperature was varied between 22 and 52C. Endosperms of 5 day germinated caryopses were extracted with 0.2 M acetate buffer, pH 3.8, containing 5 mM β-mercaptoethanol. Activity was assayed with 0.05 M acetate buffer, pH 3.8, with hemoglobin as substrate. Activity is expressed as μg tryptophan equivalents released per minute per mg protein in the endosperm extract.


A
ACTIVITY
50
40
30
20
10
0
25
50
75
100
125
150
PREINCUBATION TIME (min)


B
PROTEASE
50
40
30
20
10
20
30
40
50
ASSAY TEMPERATURE (°C)

of the total activity was lost in 2 hours, and then activity continued to decline very slowly. The biphasic curve seen in Figure 5A could be due to the presence of two proteases, one heat-labile and one heat stable, in the crude extract. No activity was lost if the enzyme was allowed to stand on ice for three hours before the assay.

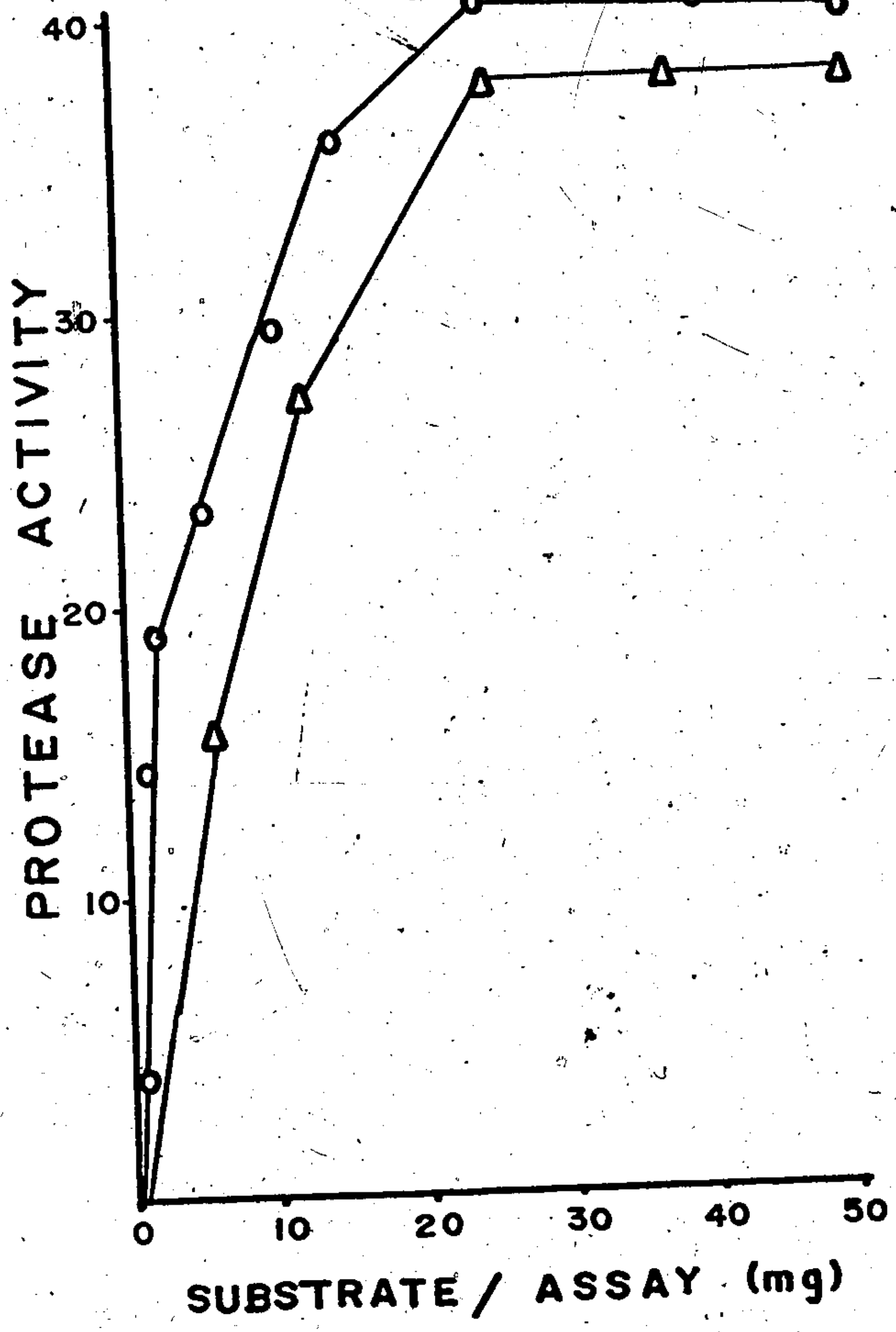
(e) Optimal Substrate Concentration

Optimal substrate concentration per assay was determined. Figure 6 shows that with 0.2 ml enzyme extracted from seeds germinated for 5 days, 20 mg hemoglobin per assay was necessary to achieve the maximum rate of hemoglobin hydrolysis. In routine assays 50 mg hemoglobin (1 ml of 5% solution) was used to provide excess substrate. No substrate inhibition was observed at high substrate concentrations. Data for gliadin concentration are also included. These indicate that a similar gliadin concentration is required for maximum velocity, although this was a separate experiment with a different enzyme preparation. Again no inhibition was observed at high substrate concentrations. Fifty mg gliadin per assay was therefore used routinely.

## FIGURE 6

### Optimal Substrate Concentration for Assay of Protease Activity

Endosperms of caryopses germinated 5 days were extracted in 0.2 M acetate buffer, pH 3.8, containing 5 mM β-mercaptoethanol. The assays were conducted at 40C in 0.05 M acetate buffer pH 3.8, which contained 2.5 mM EDTA. The substrate concentration (mg protein/assay) was as shown in the Figure. Activity was expressed as μg tryptophan equivalents released per minute per mg protein in the endosperm extract for hemoglobin (O) and for gliadin (Δ).


PROTEASE ACTIVITY
40
30
20
10
0
10
20
30
40
50
SUBSTRATE / ASSAY (mg)

(f) Storage of Enzyme Preparations

The enzyme extract remained stable if stored on ice for 12 hours (Figure 7), then gradually lost activity. Figure 7 also shows that freezing the enzyme for storage caused an initial loss of activity of 5%, then activity continued to decline at a rate of about 10% per day. The enzyme was therefore always freshly extracted, and kept on ice for no longer than 12 hours before assaying its proteolytic activity.

(g) Sulphydryl Reagents

Inclusion of β-mercaptoethanol in the extraction buffer enhanced protease activity. Table 19 shows that 5 mM β-mercaptoethanol in the extraction buffer was most effective. Activity was not increased by its inclusion in the assay buffer. The effectiveness of various sulphydryl reagents in stimulating activity are compared in Table 20. Cysteine and dithiothreitol were used. Enzyme was extracted without β-mercaptoethanol and preincubated for 3 hours with the concentration of reagent shown in the Table, before assay of activity. The assay buffer contained no sulphydryl reagent, therefore the concentration during the assay was about ten-fold lower than during preincubation of the enzyme. Table 20 shows

FIGURE 7

Storage of the Endosperm Protease

Protease was extracted from endosperms of caryopses germinated for 5 days, with 0.2 M acetate buffer, pH 3.8, containing 5 mM β-mercaptoethanol. The extracts were allowed to stand on ice (Δ), or frozen in liquid nitrogen and stored at -20C (O) for 0 to 5 days. Activity was assayed with 0.05 M acetate buffer, pH 3.8, and hemoglobin substrate. Activity is expressed as μg tryptophan equivalents released per minute per mg protein in the endosperm extract.

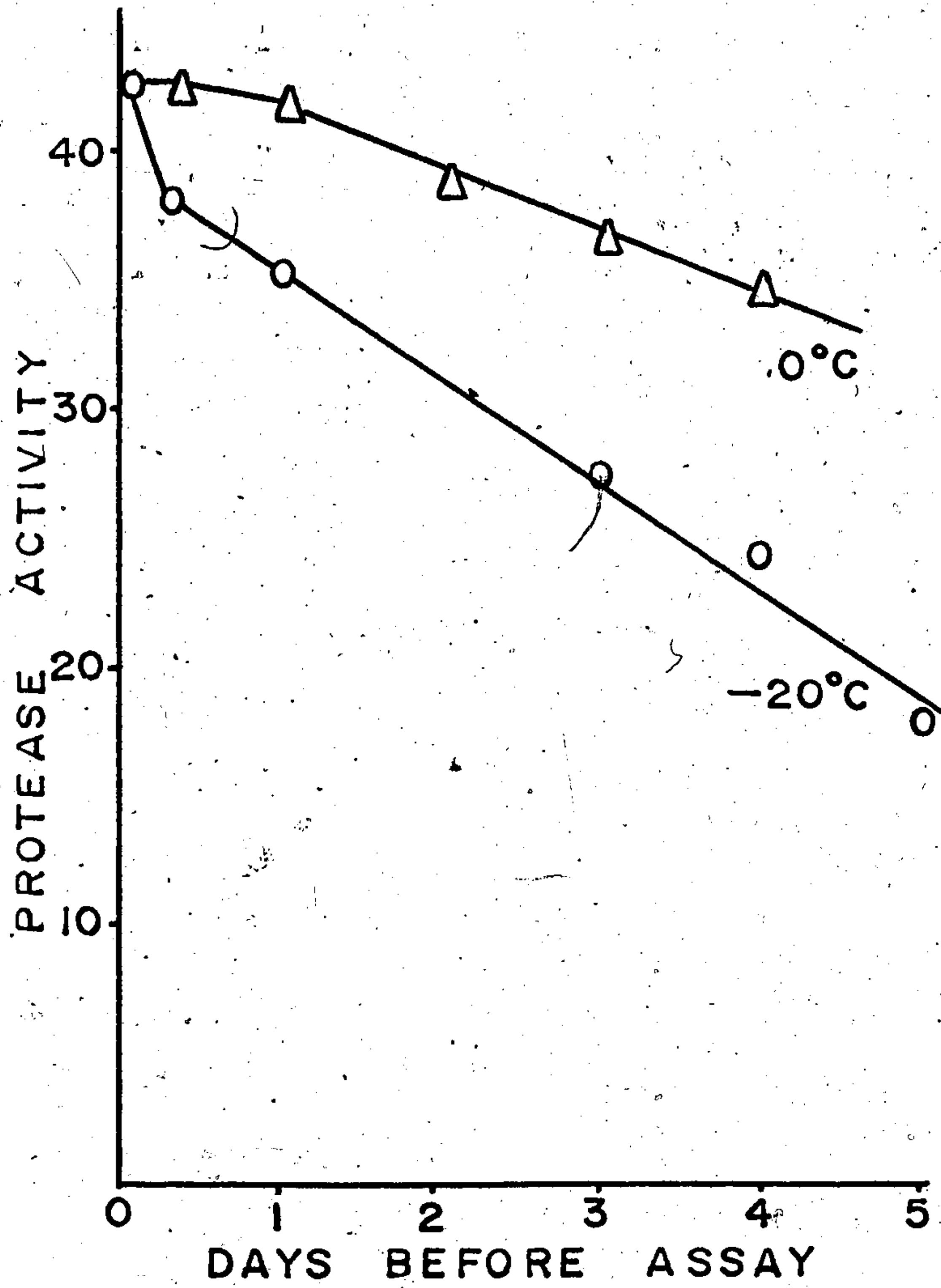

PROTEASE ACTIVITY
40
30
20
10
0°C
−20°C
0
1
2
3
4
5
DAYS BEFORE ASSAY

TABLE 19

Effects of β-Mercaptoethanol on Extraction and Assay of Endosperm Protease

Enzyme from caryopses germinated 5 days was extracted in 0.2 M acetate buffer, pH 3.8, containing 0 to 10 mM β-mercaptoethanol. The extracts were assayed in 0.05 M acetate buffer, pH 3.8, containing 0 or 5 mM β-mercaptoethanol. The substrate was hemoglobin, and the assay mixture was incubated 10 minutes at 40C before stopping the reaction with an equal volume of 5% TCA. Activity was measured as μg tryptophan equivalents released per minute per mg protein in the endosperm extract.

| β-Mercaptoethanol mM | | Protease Activity |
|---|---|---|
| Extraction buffer | Assay buffer | |
| 0 | 0 | 40.3 |
| 2.5 | 0 | 42.6 |
| 5.0 | 0 | 49.2 |
| 10.0 | 0 | 48.5 |
| 5 | 5 | 48.5 |
| 0 | 5 | 39.9 |

TABLE 20

Effect of Various Sulphydryl Reagents on Protease Activity

Enzyme was extracted in 0.2 M acetate buffer, pH 3.8, and preincubated 3 hours with reagents at the concentrations shown. Activity was assayed with hemoglobin substrate, in 0.05 M acetate buffer, pH 3.8, with 2.5 mM EDTA. Activity of the untreated control was set at 100, and relative activities of the treated enzymes calculated.

Experiment I: 100 = 30.5 µg tryptophan equivalents released per minute per mg protein.

Experiment II: 100 = 27.0 µg tryptophan equivalents released per minute per mg protein.

| Experiment | Reagent | Concentration | Activity |
|---|---|---|---|
| I | - | - | 100 |
| | β-mercaptoethanol | 5 | 120 |
| | Cysteine | 1 | 101 |
| | Cysteine | 5 | 110 |
| | Cysteine | 10 | 100 |
| II | - | - | 100 |
| | Dithiothreitol | 1 | 110 |
| | Dithiothreitol | 5 | 126 |
| | Dithiothreitol | 10 | 116 |

that the largest stimulation of activity (26%) was obtained with 5 mM dithiothreitol. β-Mercaptoethanol at the same concentration stimulated activity by 20%, and cysteine by 10%. These data indicate that the enzyme contains sulphydryl groups necessary for its activity.

The effects of sulphydryl blocking agents were tested, to investigate the sulphydryl nature of the enzyme. Enzyme extracted without β-mercaptoethanol was preincubated with the inhibitors for 3 hours, as previously described. Activity was again measured without addition of sulphydryl reagents to the assay buffer.

The results in Table 21 show that 1 mM para-chloromercuribenzoate (PCMB), N-ethylmaleimide and iodoacetate inhibited activity by 30 to 50%. Inhibition was stronger at higher concentrations of the reagents. 5 mM PCMB completely inhibited activity of the protease. Hydrogen peroxide, which oxidises sulphydryl groups, also completely inhibited enzyme activity. These data provide further evidence that the enzyme requires free sulphydryl groups for its activity. Phenylmethylsulphonylfluoride, which inhibits serine proteases, had no effect on the maize protease preparation (Table 21).

TABLE 21

Effects of Sulphydryl Blocking Agents and a Serine Protease Inhibitor on Protease Activity

Enzyme was extracted in 0.2 M acetate buffer, pH 3.8, and preincubated 3 hours on ice with reagents at the concentrations shown. Activity was assayed with gliadin substrate in 0.05 M acetate buffer, pH 3.8, with 2.5 mM EDTA. Activity of the untreated control was set at 100, and relative activities of the treated enzymes calculated.
100 = 41 μg tryptophan equivalents released per minute per mg protein in the endosperm extract.

| Treatment | Reagent Concentration mM | Activity |
|---|---|---|
| - | - | 100 |
| Iodoacetic acid | 1 | 72 |
| | 5 | 26 |
| N-ethylmaleimide | 2 | 61 |
| | 5 | 54 |
| Para-chloromercuribenzoate | 1 | 54 |
| | 5 | 0 |
| Hydrogen peroxide | 1 | 0 |
| Phenylmethylsulphonylfluoride | 0.5 | 100 |
| | 5 | 94 |

(h) Requirement for EDTA and Inhibition by Metal Ions

The enzyme appears to have no requirement for metal ions since inclusion of EDTA in the assay buffer did not inhibit proteolytic activity. Table 22 shows that inclusion of 1 to 5 mM EDTA in the assay buffer had no significant effect on activity when hemoglobin was the substrate, but 2.5 mM EDTA doubled the activity when gliadin was used as substrate. Dialysis of gliadin before the assay also increased activity but was less effective than inclusion of EDTA in the assay buffer. The assay buffer routinely contained 2.5 mM EDTA whichever substrate was used.

Heavy metal ions were found to inhibit activity of the enzyme. Metal ions (5 mM) were included in the assay buffer and activity measured with hemoglobin as substrate. Table 23 shows that mercury, lead, copper and zinc inhibited activity between 37 and 53%. Magnesium, manganese and calcium were only slightly inhibitory.

(i) Ammonium Sulphate Fractionation

A preliminary purification of the enzyme with ammonium sulphate was tried. Crude enzyme preparations were brought to 20, 40 and 60% saturation with ammonium sulphate. Between each addition the preparation was stirred gently on

TABLE 22

Effect of EDTA on Protease Activity with Hemoglobin and Gliadin Substrates

Enzyme was extracted in 0.2 M acetate buffer, pH 3.8, containing 5 mM β-mercaptoethanol. Activity was assayed with hemoglobin or gliadin as substrate in 0.05 M acetate buffer, pH 3.8, containing 0 to 5 mM EDTA. Gliadin previously dialysed for 24 hours at 3C was also used as substrate. Activity was measured as μg tryptophan equivalents released per minute per mg protein in the endosperm extract.

| | EDTA mM | Protease Activity |
|---|---|---|
| Hemoglobin | 0 | 43.2 |
| | 1 | 44.7 |
| | 2.5 | 45.3 |
| | 5.0 | 44.9 |
| Gliadin | 0 | 21.3 |
| | 1 | 29.7 |
| | 2.5 | 46.8 |
| | 5.0 | 43.8 |
| Dialysed Gliadin | 0 | 37.5 |
| | 2.5 | 46.5 |

## TABLE 23

### Inhibition of Protease Activity by Metal Ions

Enzyme was extracted in 0.2 M acetate buffer, pH 3.8, containing 5 mM β-mercaptoethanol. Activity was measured with hemoglobin as substrate, using 0.05 M acetate buffer, pH 3.8, and no EDTA in the assay. Metal ions were included in the assay buffer. Activity of the control was set at 100 and relative activities of the other treatments calculated.
100 = 42 μg tryptophan equivalents released per minute per mg protein in the endosperm extract.

| Metal | Concentration mM | Activity |
|---|---|---|
| - | - | 100 |
| $Hg^{++}$ | 5 | 51 |
| $Pb^{++}$ | 5 | 47 |
| $Cu^{++}$ | 5 | 63 |
| $Zn^{++}$ | 5 | 64 |
| $Mn^{++}$ | 5 | 82 |
| $Mn^{++}$ | 10 | 82 |
| $Ca^{++}$ | 5 | 95 |
| $Ca^{++}$ | 10 | 90 |
| $Mg^{++}$ | 5 | 100 |
| $Mg^{++}$ | 10 | 91 |

TABLE 24

Ammonium Sulphate Fractionation of Endosperm Protease

Protease was extracted from endosperms of 6 day germinated caryopses. The crude extract was subjected to ammonium sulphate fractionation, and the activity of each fraction measured with hemoglobin as substrate.

| $(NH_4)_2SO_4$ Fraction | Total Volume | ΔA 280/ 10 min/0.2 ml | Total Activity ΔA 280/min | Percentage Activity |
|---|---|---|---|---|
| 0 | 43 ml | 0.495 | 10.6 | 100 |
| 0-20% Pellet | 10 ml | 0.116 | 0.58 | 5 |
| 20-40% Pellet | 20 ml | 0.859 | 8.59 | 81 |
| 40-60% Pellet | 10 ml | 0.110 | 0.55 | 5 |
| 60% Supernatant | 43 ml | 0.030 | 0.65 | 6 |
| TOTAL | | | 10.37 | 98 |

ice for 15 minutes, and then centrifuged for 20 minutes at 10,000 x g. The pellets were resuspended in extraction buffer. Eighty percent of the total activity precipitated between 20 and 40% ammonium sulphate, and only very small amounts of activity were associated with the other fractions (Table 24).

Activity against hemoglobin was not altered by dialysis of the fractions. Activity against gliadin could only be tested with dialysed preparations, since this substrate was precipitated by very small amounts of ammonium sulphate. Over 80% of the gliadin degrading activity was also precipitated between 20 and 40% ammonium sulphate. Activity was additive when any pair of fractions were mixed and assayed, indicating that the crude extract contains no inhibitory or stimulatory factors.

(j) Reproducibility

Reproducibility of the protease extraction and assay method were investigated. Endosperms were harvested from grain germinated 4 and 6 days. Four extracts were prepared from each batch of endosperms. Soluble protein per ml of extract was measured, and each extract assayed in triplicate. The average specific activity of each extract could thus be calculated. The data in Table 25 show that the

TABLE 25

Reproducibility of the Protease Extraction and Assay

Endosperms from caryopses germinated 4 and 6 days were extracted with 0.2 M acetate buffer, pH 3.8, containing 5 mM β-mercaptoethanol. Soluble protein per ml extract was measured. Activity of 0.2 ml aliquots of enzyme was assayed with 0.05 M acetate buffer, pH 3.8, containing 2.5 mM EDTA. Hemoglobin was used as substrate, and the assay mixture incubated at 40C for 10 minutes, before stopping the reaction with an equal volume of 5% TCA. The increase in absorbance of the TCA soluble fraction was measured and activity expressed as μg tryptophan equivalents released per minute per mg protein in the endosperm extract.

| Caryopses | Extract # | mg protein per ml extract | $\Delta A_{280}$ in assay | Protease Activity |
|---|---|---|---|---|
| 4 days germinated | 1 | 0.94 | 0.34, 0.33, 0.36 | 32.8 |
| | 2 | 0.91 | 0.33, 0.34, 0.35 | 33.8 |
| | 3 | 0.96 | 0.37, 0.36, 0.33 | 35.2 |
| | 4 | 0.99 | 0.35, 0.39, 0.37 | 35.4 |
| 6 days germinated | 1 | 0.80 | 0.52, 0.50, 0.49 | 56.2 |
| | 2 | 0.84 | 0.50, 0.49, 0.54 | 55.5 |
| | 3 | 0.89 | 0.51, 0.53, 0.51 | 53.2 |
| | 4 | 0.89 | 0.50, 0.55, 0.53 | 55.4 |

reproducibility of extraction, judged by soluble protein per ml extract, was good. Variability of the assay was also low, judged by the increases in absorbance of the TCA soluble fractions in the three replicate assays for each enzyme extract. The average specific activity of protease from the 4 day germinated caryopses was 33.8 g tryptophan/min/mg protein. The individual values for the four extracts ranged from 32.8 to 35.4 or ±4% around the mean. The average protease specific activity for the 6 day germinated caryopses was 55.1 μg tryptophan equivalents released/min/mg protein, with values ranging between 53.2 and 56.1, or ±3%. In subsequent experiments where comparisons of activity are made, extracts were prepared in duplicate, pooled, assayed in triplicate and the values averaged. In physiological experiments activity has been expressed on a "per endosperm" basis because soluble protein per endosperm changes during germination. Standard errors have not been indicated on graphs or in tables since too few independent replicates were performed for determination of confidence limits of the means.

These experiments, contained in paragraphs (a) to (j), describe the development of the extraction and assay method which is outlined in MATERIALS AND METHODS, and has been used throughout the remainder of this research.

Endopeptidase Activity of the Protease

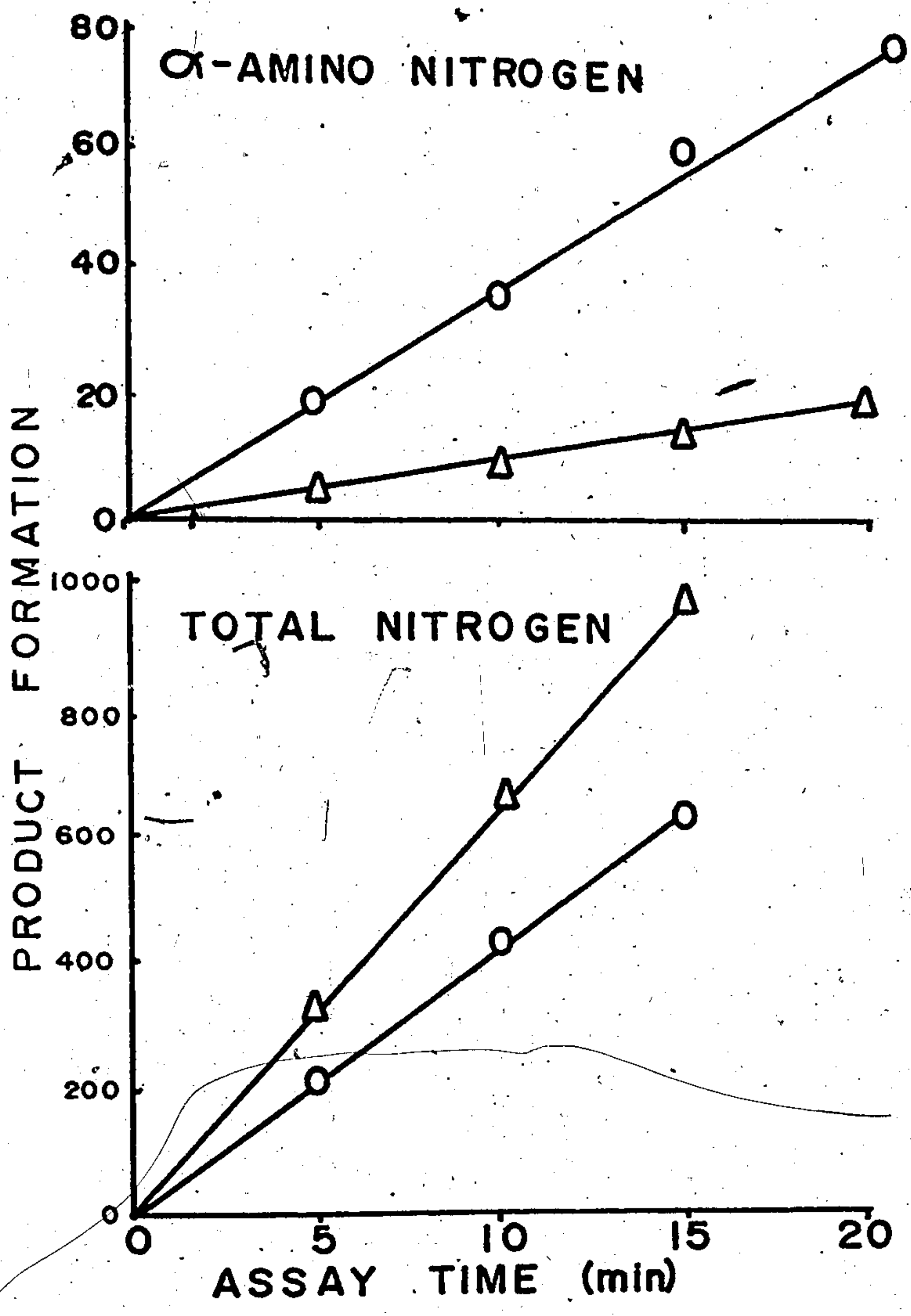
A protease with exopeptidase activity should release free amino acids and amides from protein, whereas an endopeptidass should release peptides. If only amino acids were released the ratio of total nitrogen to α-amino nitrogen of the TCA soluble hydrolysis products should be between one and two. For example for casamino acids this ratio was found to be 1.3 and from the amino acid composition was calculated to be 1.37. If peptides were the major hydrolysis products the ratio would be well in excess of two. The actual ratio would depend on the size of the peptides and the nitrogen content of their predominant amino acids. The results in Figure 8 show that total nitrogen and α-amino nitrogen release from protein increased linearly during a 15 minute assay, with either hemoglobin or gliadin as substrate. With each substrate much more total nitrogen than α-amino nitrogen was released. After 10 minutes incubation 650 μg total nitrogen and 15 μg α-amino nitrogen had been released from gliadin, resulting in a ratio of 43. With hemoglobin as substrate 430 μg total nitrogen and 37 μg α-amino nitrogen were released per assay in 10 minutes. This gives a total nitrogen to α-amino nitrogen ratio of 12. Similar values were also found in the experiments summarized in Table 26. The ratios obtained are independent of substrate concentration,

FIGURE 8

Hydrolysis of Substrate Proteins:

Release of Total Nitrogen and α-Amino Nitrogen

Enzyme was extracted from 5 day germinated caryopses and assayed as described in MATERIALS AND METHODS, with hemoglobin (O) and gliadin substrates (Δ). Activity was measured as μg total nitrogen released per assay into the TCA soluble fraction, and as μg α-amino nitrogen released per assay into the TCA soluble fraction.


α-AMINO NITROGEN
TOTAL NITROGEN
PRODUCT FORMATION
ASSAY TIME (min)
80
60
40
20
0
1000
800
600
400
200
0
0
5
10
15
20

even with substrate concentrations which limit the reaction rate. This was observed with both hemoglobin and gliadin. To check the validity of these ratios the hydrolysis products of exopeptidase activity on gliadin was analysed. Carboxypeptidase A was used, and the total nitrogen to α-amino nitrogen ratio of the hydrolysis products was found to be 1.4 (Table 26). It is therefore clear that the maize protease had endopeptidase activity with either hemoglobin or gliadin as substrate. The ability of the protease to degrade other substrate proteins was also investigated (Table 26). When measuring total nitrogen release, greatest activity was obtained with gliadin as substrate and least activity was found with bovine serum albumin. The ratios of the hydrolysis products were high in each case, again suggesting an endopeptidase activity.

The ability of the protease to degrade its natural substrates, zein and glutelin, was also tested. These proteins could not be dissolved in conditions suitable for the assay of protease activity, and were too hydrophobic to form substrate suspensions. However, they could be rendered sparingly soluble by partial hydrolysis. Zein was hydrolysed in 1 N HCl, and glutelin in 1 N NaOH, for 4 hours at 50C. Free amino acids and small peptides were dialysed out of the protein hydrolysates, which were then lyophilized and redissolved in water at pH 3.8 before use as substrates. Gliadin was also

## TABLE 26

## Effectiveness of Maize Protease with Various Protein Substrates

Zein was partially hydrolysed in 1 N HCl and glutelin in 1 N NaOH by heating for 4 hours at 50C. Gliadin was hydrolysed with 1 N HCl for 1 hour at 50C. The hydrolysates were dialysed, lyophilized and redissolved in a minimum of water at pH 3.8 before use as substrates. The amount of substrate nitrogen per assay was determined for unhydrolysed and partially hydrolysed protein substrates. Enzyme was prepared from endosperms of caryopses germinated for 6 days. Activity was measured by μg total nitrogen and μg α-amino nitrogen released per assay from the substrate into the TCA soluble fraction. The total nitrogen to α-amino nitrogen ratio of the hydrolysis products was calculated for each substrate and substrate concentration.

TABLE 26

Effectiveness of Maize Protease with Various Protein Substrates

| Substrate | | Activity | | Product |
|---|---|---|---|---|
| Protein | mg N/assay | mg N released per assay | mg $\alpha$-$NH_2$ N released per assay | $\frac{\text{Total N}}{\alpha\text{-}NH_2\text{ N}}$ |
| Hemoglobin | 9.7 | 600 | 51 | 12 |
| | 4.4 | 620 | 48 | 13 |
| | 1.2 | 370 | 37 | 10 |
| Gliadin | 9.7 | 900 | 22 | 41 |
| | 4.4 | 900 | 20 | 45 |
| | 1.2 | 660 | 18 | 37 |
| Edestin | 7.3 | 600 | 24 | 26 |
| Bovine Serum Albumin | 4.1 | 240 | 10 | 24 |
| Partially Hydrolysed Proteins | | | | |
| Gliadin | 4.4 | 1,900 | 175 | 11 |
| Zein | 1.4 | 500 | 62 | 8 |
| Glutelin | 1.1 | 400 | 27 | 15 |
| Carboxypeptidase Hydrolysed Protein | | | | |
| Gliadin | 13.6 | 96 | 70 | 1.4 |

partially hydrolysed with 1 N HCl so that its properties as a substrate could be compared before and after the treatment. Partially hydrolysed gliadin was degraded at about twice the rate of native gliadin, judging by the release of total soluble nitrogen. The ratio of total nitrogen to α-amino nitrogen of products released from partially hydrolysed gliadin was lower, and in fact comparable to that obtained with hemoglobin. This observation suggests that denatured and partially hydrolysed proteins are more susceptible to enzyme attack. Changes in tertiary structure of the substrate during acid or alkaline treatment may increase accessibility to endopeptidases, or cleavage of the protein during the partial hydrolysis may release more amino- and carboxy-terminals for exopeptidase attack. Either or both of these effects could reduce the total nitrogen to α-amino nitrogen ratio of the enzymatic hydrolysis products.

Partially hydrolysed zein and glutelin are also degraded by the protease, but since the substrate concentrations were low the rates of degradation are probably not very meaningful (Table 26). The ratios of total nitrogen to α-amino nitrogen, a value apparently not affected by substrate concentration, were 8 and 15 respectively. These results show that denatured endosperm storage proteins are degraded by the protease, and that there is endopeptidase activity.

Natural Substrates

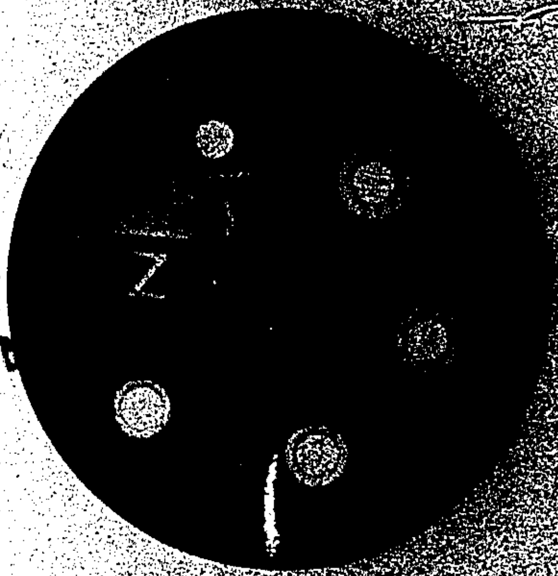
Since native maize endosperm protein could not be used in the standard assay, an agar gel method was developed to demonstrate activity of the protease against unhydrolysed zein and glutelin. These proteins were extracted in bulk from endosperms of ungerminated caryopses then dialysed and lyophilised. All proteins used as substrates in the standard assay were also tested in the gel assay. The proteins were dissolved or suspended in agar gels as described in MATERIALS AND METHODS. Enzyme introduced into wells in the gel diffused out and degraded the substrate proteins. Staining the gels for protein then revealed clear rings around wells which had contained active protease. Figure 9 shows a protease dilution series tested on zein containing gels. Activity was observed at dilutions X1, X2, X4, X8, and X16, but not at higher dilutions. Table 27 records activity of the protease against a range of substrates. With gliadin as substrate clear rings were formed with dilutions of up to 32 times of the protease preparation. Sixteen-fold was the maximum enzyme dilution clearly showing activity with hemoglobin, zein, glutelin and edestin. No degradation of bovine serum albumin was detectable. Gliadin was therefore the best substrate in this assay. Zein and glutelin were degraded with the same efficiency, judging by the maximum enzyme dilution capable of degrading

FIGURE 9

The Agar Gel Method of Assaying Endosperm Protease Activity

Enzyme extracted from caryopses germinated 6 days was diluted in a geometric series. Aliquots (0.15 ml) of each dilution was pipetted into wells in a zein (0.025%) containing gel. The wells of Gel A, moving clockwise from the arrow, contained boiled enzyme, undiluted enzyme, enzyme diluted X2, X4, and X8. The wells of Gel B, again moving clockwise from the arrow, contained enzyme diluted X8, X16, X32, X64 and X128. The gels were incubated 12 hours at 30C, washed, and stained with 0.02% nigrosine. Clear rings around the wells indicate activity of the protease dilution against the substrate.

A



B

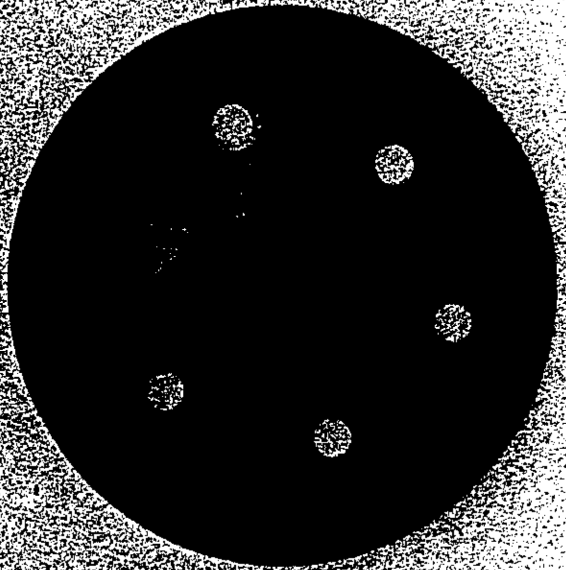


TABLE 27

Activity of the Protease with Various Substrate Proteins,

Measured by the Agar Gel Assay

Enzyme extracts were prepared from caryopses germinated 6 days, and activity assayed as described in the legend for Figure 9. The gels contained 0.025% protein solutions or suspensions. If a clear ring was formed around the enzyme-containing well, its diameter was measured in mm and recorded.

| Substrate | Enzyme Dilution | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Boiled | X1 | X2 | X4 | X8 | X16 | X32 | X64 | X128 |
| Hemoglobin | - | 15 | 13 | 11 | 10 | 9 | ± | - | - |
| Edestin | - | 15 | 13 | 12 | 11 | 9 | - | - | - |
| Bovine Serum Albumin | - | - | - | - | - | - | - | - | - |
| Gliadin | - | 17 | 15 | 13 | 12 | 11 | 9 | - | - |
| Zein | - | 14 | 13 | 11 | 10 | 9 | ± | - | - |
| Glutelin | - | 13 | 12 | 11 | 10 | 9 | ± | - | - |
| None | - | - | - | - | - | - | - | - | - |

the substrate. The size of the clear ring does not seem to be a good criterion for estimation of the activity of the protease against a particular substrate. For example the rings formed by undiluted protease acting on hemoglobin were 2 mm larger than those formed with glutelin, although in both cases the maximum enzyme dilution showing activity was sixteen-fold.

The data from this assay correlate well with the standard assay. In both assays gliadin was the best unhydrolysed protein substrate, and bovine serum albumin the poorest. It can be concluded that the maize endosperm storage proteins are degraded by the endosperm protease.

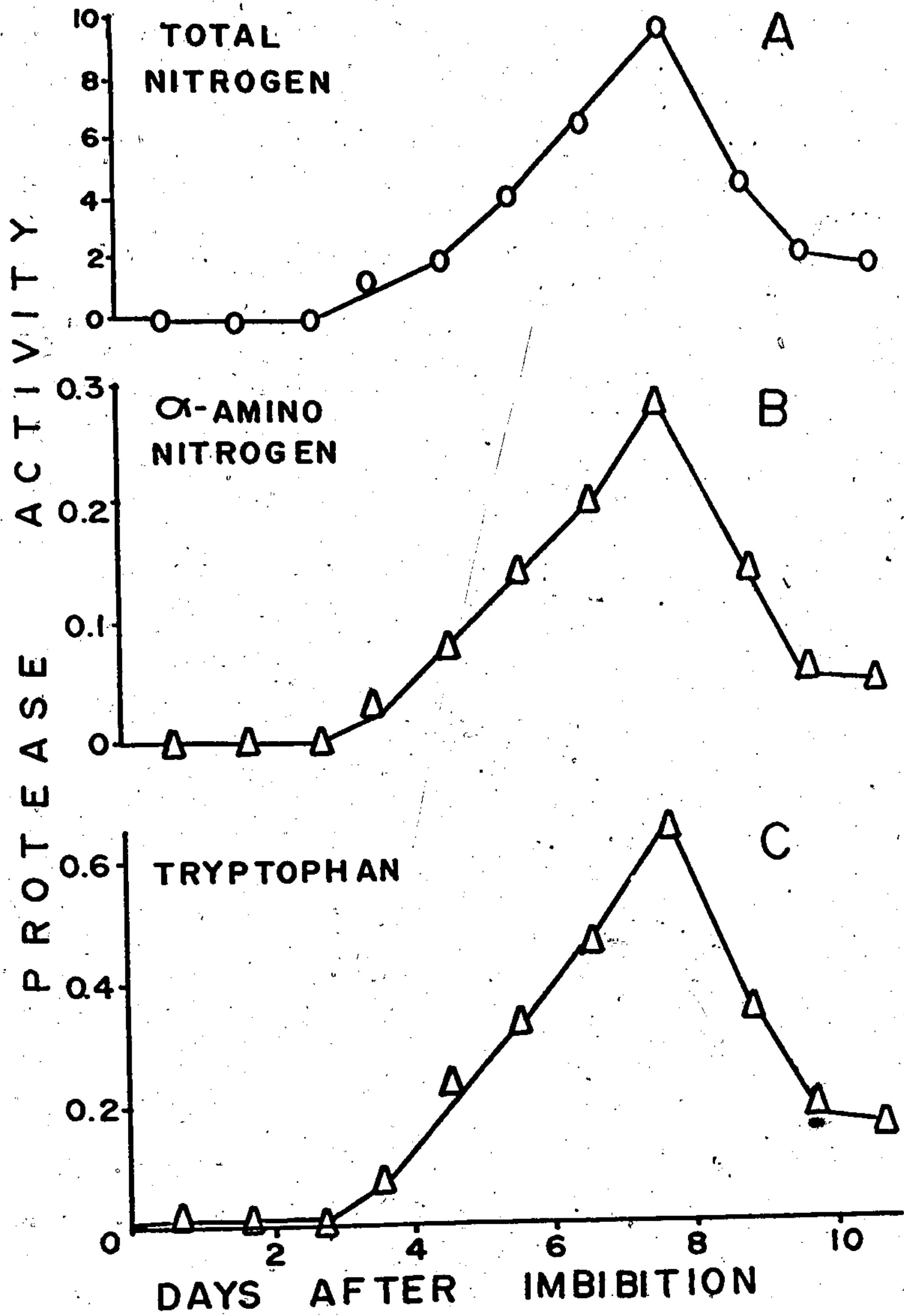
Protease Activity during Germination

Changes in activity of the protease during germination were investigated. Batches of 50 endosperms were harvested daily for 11 days after imbibition of caryopses. The endosperms were frozen in liquid nitrogen and stored at -20C prior to extraction and assay. Protease activity was measured by the standard assay with gliadin as substrate, and by the agar gel assay with zein and gliadin substrates. In the standard assay activity was measured by release of tryptophan (Figure 10C) and α-amino nitrogen (Figure 10B), and total nitrogen (Figure 10A) from gliadin substrate. With each of these assays

FIGURE 10

Changes in Endosperm Protease Activity During Germination

Endosperms were harvested at intervals for 11 days after imbibition of caryopses. Protease was extracted and assayed as described in MATERIALS AND METHODS. Gliadin was the substrate. Activity is expressed as mg tryptophan equivalents, α-amino nitrogen and total nitrogen released per hour per endosperm.



TOTAL NITROGEN
A
α-AMINO NITROGEN
B
TRYPTOPHAN
C
PROTEASE ACTIVITY
DAYS AFTER IMBIBITION
0
2
4
6
8
10
0.1
0.2
0.3
0.4
0.6

no activity was detected until 3 days after imbibition of the grain. A maximum was reached at 8 days, after which the protease activity declined. The total nitrogen to α-amino nitrogen ratio of the products was calculated from Figures 10A and B. The lowest ratio was 36, at 7½ days germination, and the highest 48 after 4½ days germination. Endopeptidase activity was therefore present throughout this growth period.

The change in protease activity measured by the agar gel method is shown in Figure 11. The maximum enzyme dilution active against zein and gliadin substrates at each time was plotted on semi-log scale. No activity was detected before day 3, and a maximum was reached after 8 days germination. This pattern is very similar to that observed with the standard assay, indicating that activity of the same enzyme(s) is measured by both methods.

Another endosperm hydrolytic enzyme, α-amylase, was also measured for comparison of its pattern of activity during germination. Figure 12 shows that it was detectible slightly earlier than the protease, at about two days after imbibition of the seeds. It reached its maximum activity at 10 days, 48 hours later than the protease. The curves for appearance, increase and then decline in activity show the same trend for both hydrolytic enzymes.

Loss of endosperm dry weight and nitrogen during

## FIGURE 11

### Changes in Endosperm Protease Activity During Germination, Measured by the Agar Gel Assay

Endosperms were harvested after 0, 3, 5½, 8 and 10 days germination. Extracts were prepared, and if necessary diluted so that 0.15 ml extract was equivalent to ½ endosperm. These extracts were diluted in geometric series and the activity of 0.15 ml of each dilution tested with zein (Δ) and gliadin (O) substrates as described in the legend for Plate 1. The maximal dilution of enzyme showing activity at each sampling time is recorded on semi-log scale. The enzyme extracted from ungerminated seeds showed no activity.

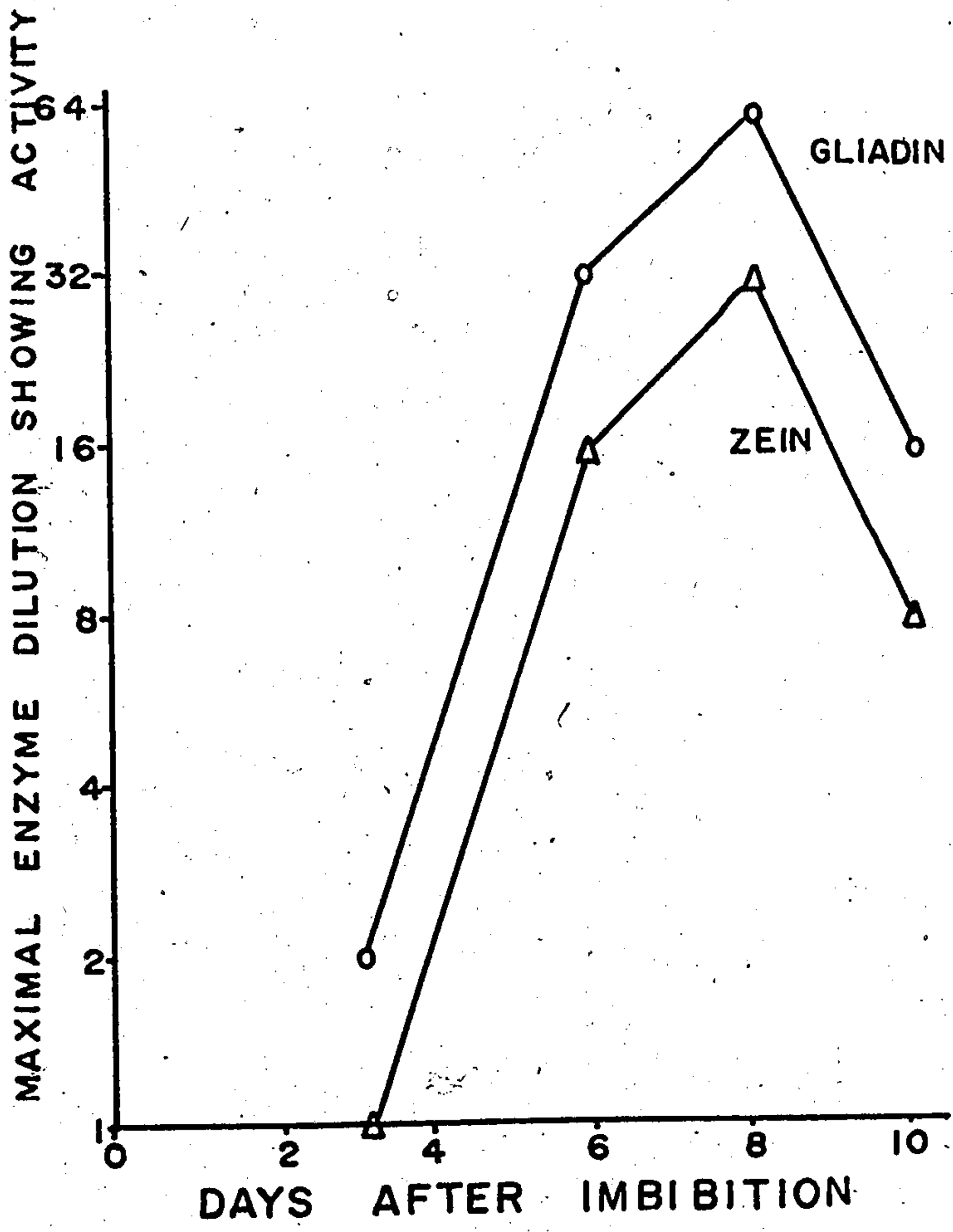
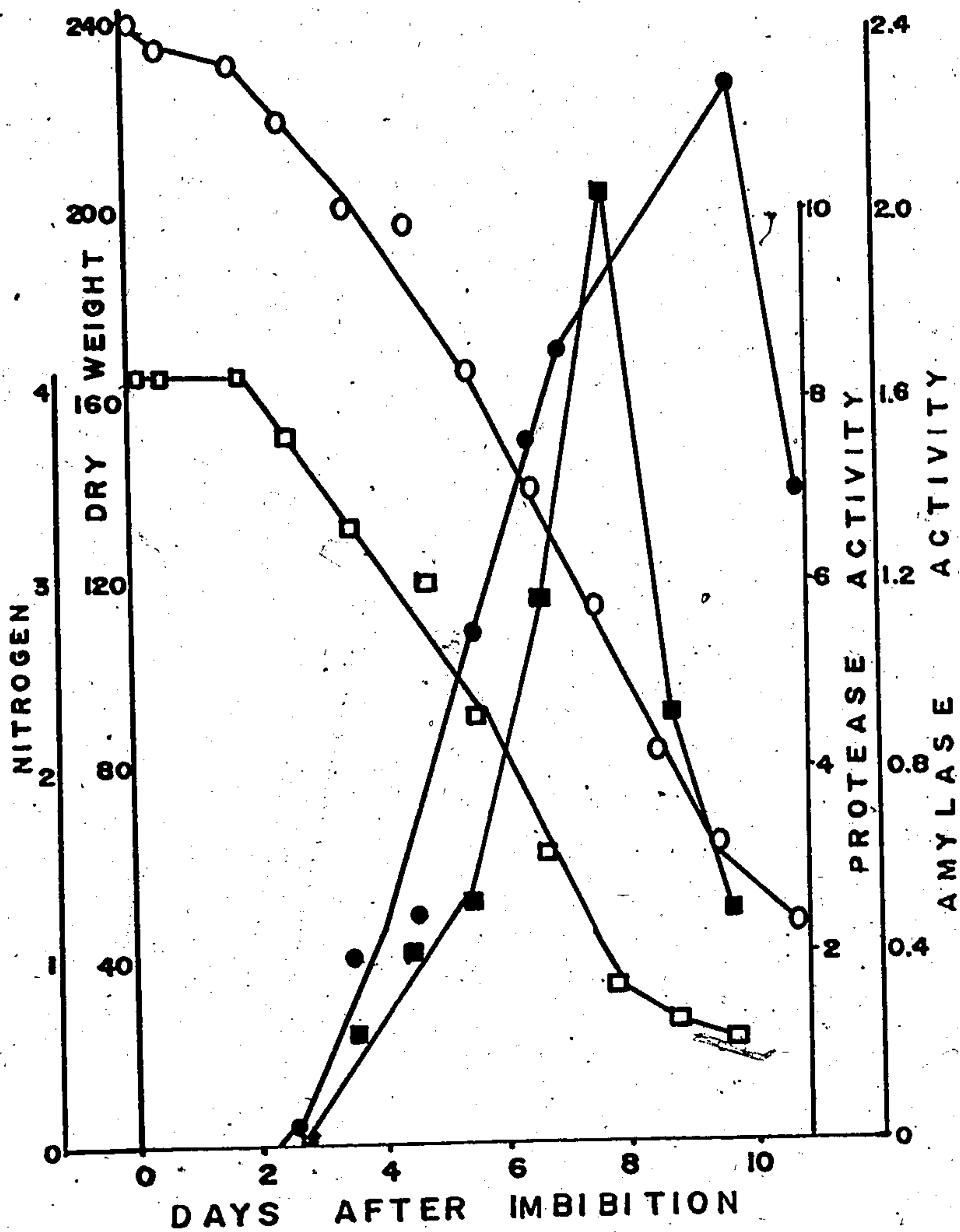

MAXIMAL ENZYME DILUTION SHOWING ACTIVITY
64
32
16
8
4
2
1
0
2
4
6
8
10
DAYS AFTER IMBIBITION
GLIADIN
ZEIN

FIGURE 12

Changes in Endosperm Hydrolytic Enzyme Activity and Total Nitrogen and Dry Weight during Germination

Endosperms were harvested between 0 and 11 days after imbibition. α-Amylase and protease were extracted and assayed as described in MATERIALS AND METHODS. Gliadin was used as substrate in the protease assay. Protease activity (■) is expressed as mg nitrogen released per hour per endosperm and α-amylase activity (●) as mg starch degraded per minute per endosperm. Dry weight per endosperm is indicated as (O), and endosperm total nitrogen as (□).


NITROGEN
DRY WEIGHT
PROTEASE ACTIVITY
AMYLASE ACTIVITY
DAYS AFTER IMBIBITION
0
1
2
3
4
40
80
120
160
200
240
0
2
4
6
8
10
0
0.4
0.8
1.2
1.6
2.0
2.4

germination is also shown in Figure 12. Dry weight loss is taken to represent starch breakdown, and nitrogen loss represents protein breakdown. A small dry weight loss occurred during the first 48 hours of germination. No nitrogen loss was evident in this interval, but protein analysis has shown changes in the distribution of nitrogen between the protein fractions at this time (Figure 2). Some protein hydrolysis therefore does occur between 0 and 48 hours after the beginning of germination. After 48 hours starch and protein loss become rapid. Protein breakdown continued at a steady rate from about 2 to 8 days, then levelled off. This reduction in rate of hydrolysis occurred when nitrogen reserves were almost depleted, and protease had reached its maximum activity.

The linear rate of starch breakdown continued until 10 days, when α-amylase reached its maximum activity. The decline in both protease and α-amylase activity began when endosperm reserves were almost depleted. The rapid phase of starch and protein breakdown coincided with the period of increasing protease and α-amylase activity.

## Protease Production by Excised Endosperms

During incubation of excised endosperms hydrolytic enzymes and hydrolysis products are released into the medium.

TABLE 28

Comparison of Protease in Extracts of Intact Germinated Caryopses and Excised Endosperms with Protease Released into the Medium during Incubation of Excised Endosperms

Extracts were prepared from endosperms of caryopses germinated for 6 days and from endosperms incubated for 4 days. Activities of these extracts and of the 4 day incubation medium were measured with gliadin as substrate. Protease activity is expressed as mg tryptophan equivalents released per hour per endosperm. The total nitrogen to α-amino nitrogen ratio of the hydrolysis products were measured, and pH and temperature optima determined as previously described.

| | Intact Caryopses | Excised Endosperms | |
|---|---|---|---|
| | Endosperm Extract | Endosperm Extract | Incubation Medium |
| Protease Activity | 0.66 | 0.34 | 0.37 |
| Total Nitrogen / α-Amino Nitrogen | 42 | 43 | 40 |
| pH Optimum | 3.8 | 3.8 | 3.8 |
| Temperature Optimum | 46 | 46 | 46 |

TABLE 29

Enzyme Production, Starch Hydrolysis and Protein Hydrolysis in Endosperms Incubated in Different Volumes of Buffer

Endosperms were incubated with 0.5, 1.0, 2.0 and 3.0 ml buffer/endosperm. After 3½ days incubation protease and α-amylase activities were assayed in the incubation medium and endosperm extracts. Total protease activity was expressed as mg tryptophan equivalents released per hour per endosperm, and total α-amylase activity as mg starch degraded per minute per endosperm. Total nitrogen and reducing sugar in the incubation medium were assayed, and the results expressed as mg nitrogen and mg reducing sugar released per endosperm.

| Buffer Volume ml/endosperm | Total Protease | Total α-Amylase | Nitrogen Release | Reducing Sugar Release |
|---|---|---|---|---|
| 0.5 | 0.58 | 1.34 | 2.26 | 20.0 |
| 1.0 | 0.64 | 1.20 | 2.13 | 18.5 |
| 2.0 | 0.55 | 1.27 | 2.09 | 21.1 |
| 3.0 | 0.69 | 1.36 | 2.34 | 19.3 |

Total enzyme production by excised endosperms is obtained by measuring activities in both the medium and endosperm extracts. In Table 28 the properties of the enzyme obtained from excised endosperms and their incubation medium are compared with those of the enzyme extracted from endosperms of intact germinated caryopses. Protease from each source had the same temperature and pH optima. In addition the total nitrogen to α-amino nitrogen ratios of the hydrolysis products released from gliadin were about 40. The same or very similar enzymes are released into the incubation medium and retained in the endosperms. Also incubation of excised endosperms in 0.5, 1.0, 2.0 and 3.0 ml buffer per endosperm has shown that enzyme production and protein and starch hydrolysis are independent of the volume of incubation medium (Table 29). Hence it is valid to compare total enzyme production by excised endosperms with enzyme activities in endosperms of germinated caryopses.

## Protease Stability

The *in vitro* stabilities of proteases in the incubation medium and in excised endosperms were compared. Excised endosperms were incubated for 70, 90 or 110 hours. Enzyme preparations from the medium and endosperms were preincubated at 40C for 0 to 90 minutes before assaying their

proteolytic activities. The results in Figure 13 show that the endosperm enzyme is less stable than the medium enzyme, and surprisingly that enzyme stability in both the medium and endosperm extracts increases with increasing age.

When endosperm extracts and medium were mixed, their combined activity was less labile than predicted (Figure 14). It appears that the medium contains a factor which can, *in vitro*, partially stabilize the endosperm protease.

To examine this stabilizing factor further, medium from a 110 hour incubation was passed through Sephadex G-25, to remove small molecules. The *in vitro* stability of the medium protease, and its ability to stabilize the endosperm protease were then tested. The medium protease lost some activity in passage through Sephadex G-25, and also became unstable to preincubation at 40C (Figure 15). When mixed with endosperm extract it still caused some increase in endosperm protease stability, but was much less effective than the untreated medium. These results indicate that the "stabilizing factor" is a small molecule.

In the absence of the "stabilizing factor" the medium and endosperm proteases showed similar stability profiles. Hence the same or very similar proteases are present in the endosperms and in the incubation medium. Incubation with buffer alone had little effect on the stability of the endosperm protease, hence the "factor" is released from the

FIGURE 13

In Vitro Stability of Endosperm and Medium Protease

Excised endosperms of Wf9 x 38-11 (1969) were incubated for 70, 90, and 110 hours. Stability of the extracted and media proteases was tested by preincubation of enzyme at 40C prior to the assay. 0.5 ml aliquots of medium and 0.25 ml aliquots of extract were used. After preincubation of the enzyme, assay buffer (1 ml of 0.05 M acetate, pH 3.8, mM EDTA) and substrate (1 ml of 5% gliadin) were added and activity assayed in the normal way. Activity of each protease sample, without preincubation, was set at 100. Relative activities of the samples preincubated 30, 60 and 90 minutes were calculated. The activities of enzymes which were not preincubated were:

| | | | |
|---|---|---|---|
| Medium | (▲) | 70 hr | 100 = 0.19 mg tryptophan equivalents |
| | | 90 hr | 100 = 0.34 released per hour per |
| | | 100 hr | 100 = 0.37 endosperm. |
| Endosperm | (●) | 70 hr | 100 = 0.28 mg tryptophan equivalents |
| | | 90 hr | 100 = 0.34 released per hour per |
| | | 100 hr | 100 = 0.36 endosperm. |

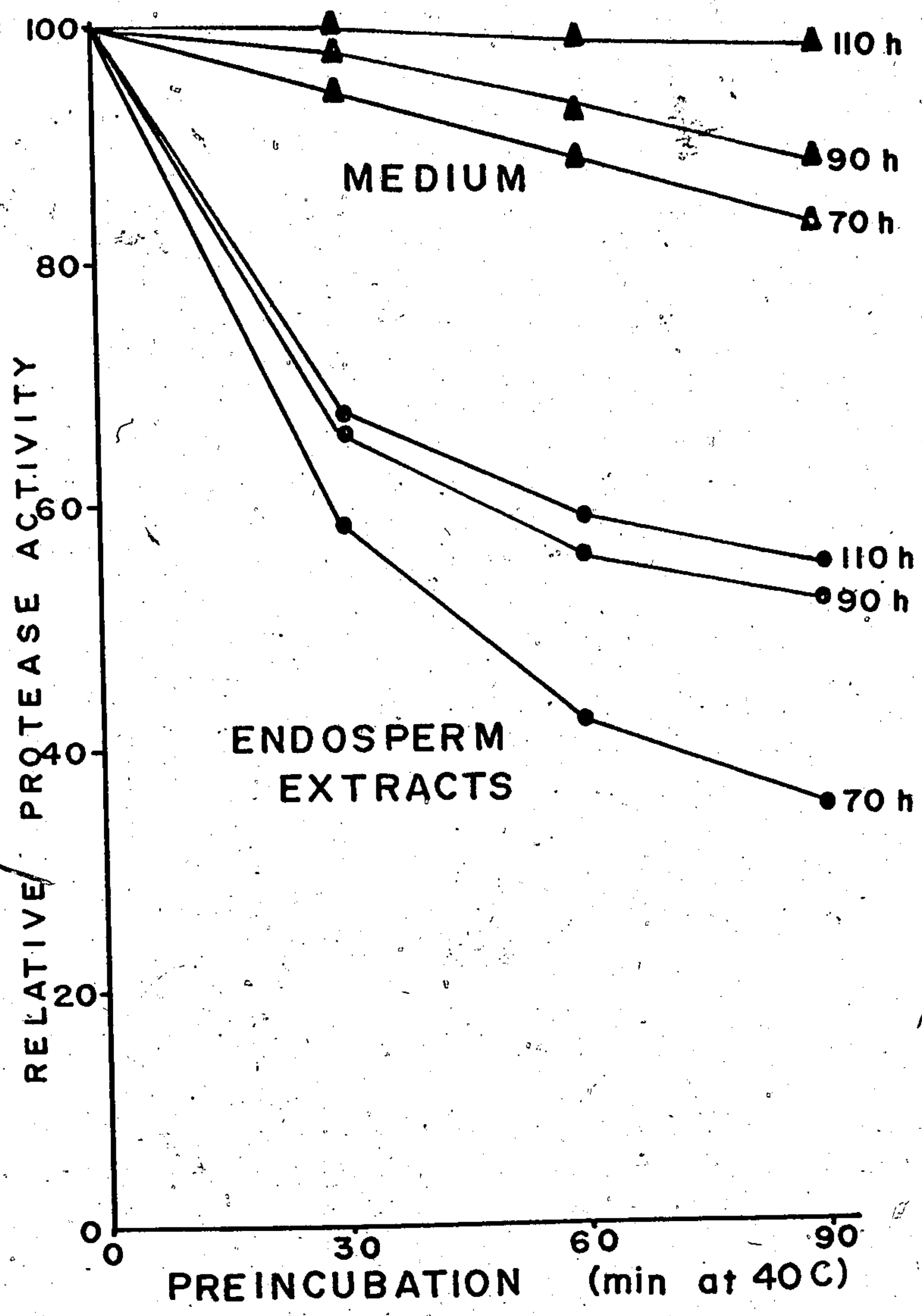
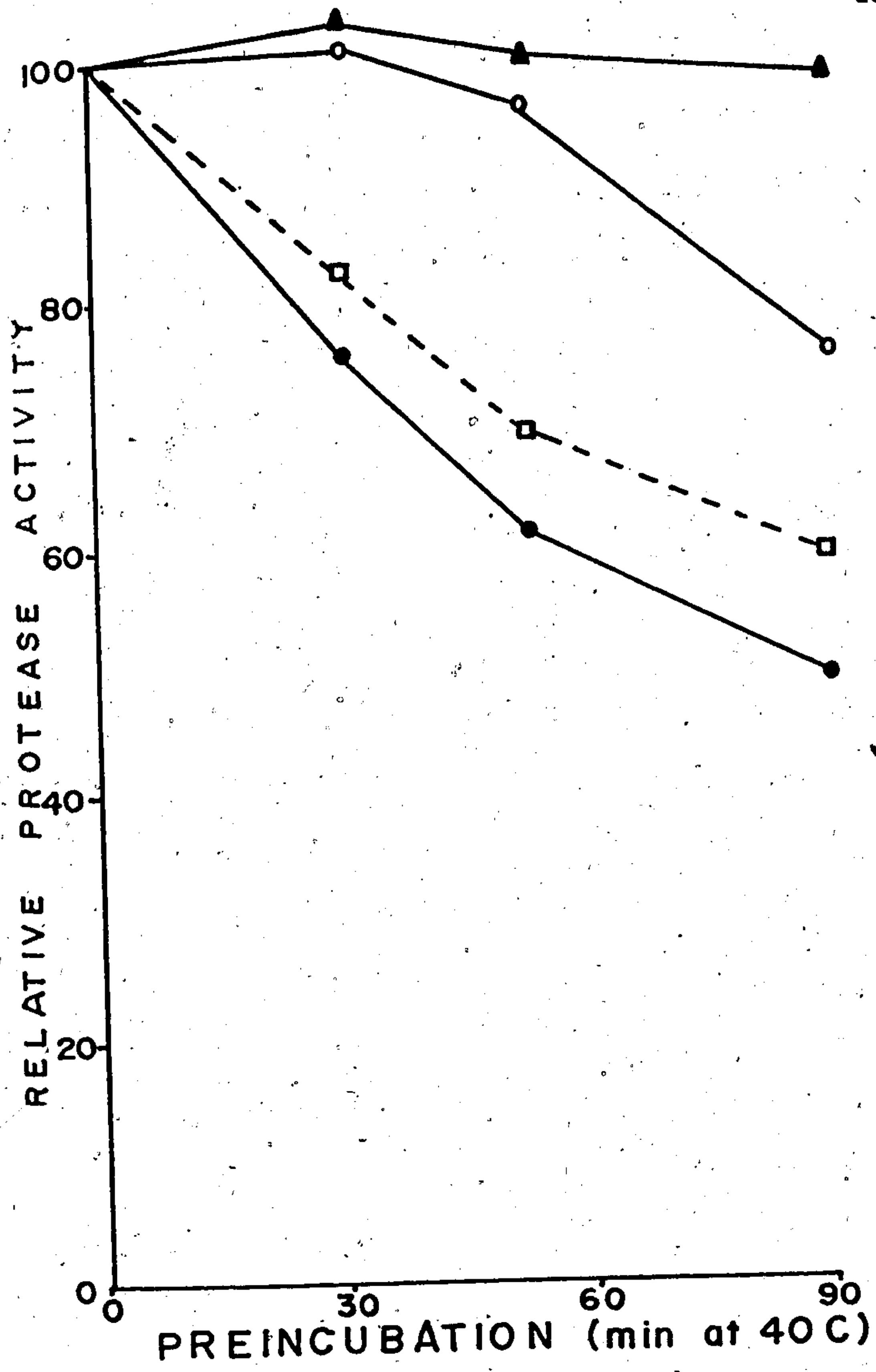

RELATIVE PROTEASE ACTIVITY
100
80
60
40
20
0
MEDIUM
110 h
90 h
70 h
ENDOSPERM
EXTRACTS
110 h
90 h
70 h
0
30
60
90
PREINCUBATION (min at 40 C)

FIGURE 14

In Vitro Stability of Mixed Endosperm and Medium Proteases

Excised endosperms (Wf9 x 38-11, 1969) were incubated for 110 hours in buffer. In vitro stability of endosperm extracts, incubation medium, and a mixture of endosperm extract and incubation medium was tested as described for Figure 13. Assays contained 0.25 ml endosperm extract, 0.5 ml incubation medium, or 0.25 ml extract + 0.25 ml medium in a total volume. Activity after 0, 30, 60 or 90 minutes preincubation was measured with gliadin as substrate. The theoretical activity loss of the mixture was calculated, assuming that each component of the mixture would lose activity at its normal independent rate. Activity of each protease preparation, without preincubation, was set at 100, and relative activities of each treatment were calculated. Activities prior to preincubation were:

| | | |
|---|---|---|
| Medium | (▲) | 100 = 22 μg tryptophan equivalents released per assay |
| Endosperm Extract | (●) | 100 = 43 μg |
| Medium + Extract | (○) | 100 = 51 μg |
| Theoretical Medium + Extract | (□) | 100 = 54 μg |

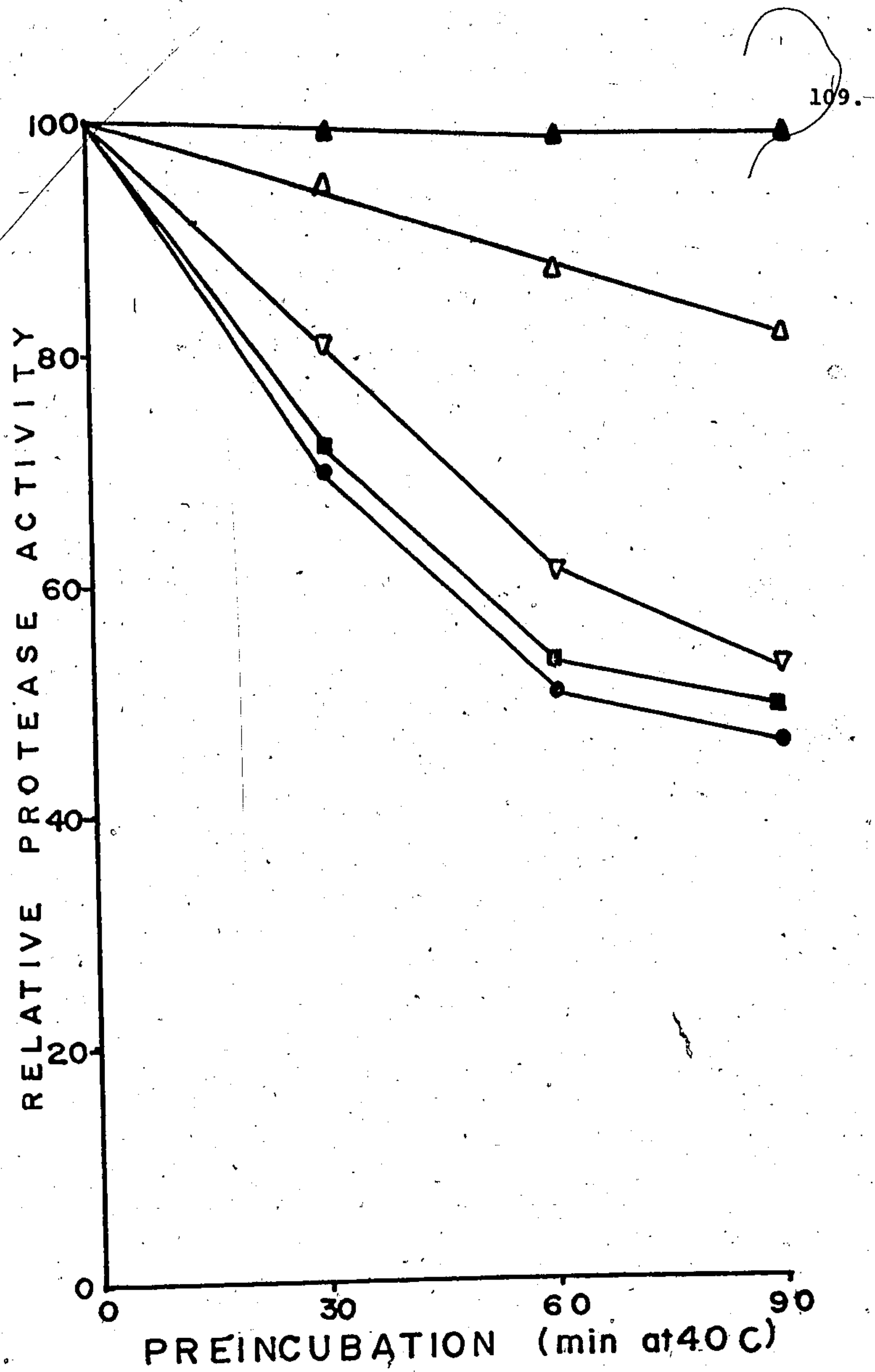

RELATIVE PROTEASE ACTIVITY
100
80
60
40
20
0
0
30
60
90
PREINCUBATION (min at 40 C)

## FIGURE 15

### *In Vitro* Stability of Medium Protease after Passage Through Sephadex G-25

Endosperms (Wf9 x 38-11, 1969) were incubated for 110 hours in buffer. Half the incubation medium was passed through Sephadex G-25 at 3C. *In vitro* stability was measured with endosperm extract, incubation medium, Sephadex treated medium, endosperm extract mixed with medium, and endosperm extract mixed with treated incubation medium. Samples of each were preincubated for 0, 30, 60, or 90 minutes before assaying proteolytic activity. Each assay contained 0.25 ml of extract, 0.5 ml medium, or 0.25 ml extract + 0.25 ml medium, in a final volume of 3.0 ml. Gliadin was used as substrate in the assays. Activity of each enzyme preparation prior to preincubation was set at 100, and relative activities of the preincubated enzymes were calculated. Activities prior to preincubation were:

| | | |
|---|---|---|
| Endosperm Extract | (●) | 100 = 40 μg tryptophan equivalents |
| Incubation Medium | (▲) | 100 = 19 μg released per assay |
| Medium - Sephadex G-25 | (■) | 100 = 10 μg |
| Medium + Extract | (Δ) | 100 = 48 μg |
| Sephadex Medium + Extract | (∇) | 100 = 43 μg |


100
80
60
40
20
0
RELATIVE PROTEASE ACTIVITY
0
30
60
90
PREINCUBATION (min at 40 C)

endosperms during germination and accumulates in the incubation medium. The rate of inactivation or degradation of protease *in vivo* may be modified by accumulation of this factor.

# III. REGULATION OF RESERVE PROTEIN HYDROLYSIS

Excised endosperms were used to investigate the regulation of protease production and reserve protein hydrolysis in maize. Since induction of α-amylase is well understood in barley (45) its production and starch hydrolysis were also measured as a control system. Neither the presence of the embryo nor exogenous hormone was necessary for enzyme production or for the hydrolysis of storage reserves in maize. However in some caryopses gibberellic acid (GA) was found to stimulate these processes. Experiments were performed with abscisic acid, gibberellin synthesis inhibitors, and seeds of dwarf maize mutants. The results indicate that GA is important in control of production of hydrolytic enzymes, and that endosperms probably have an endogenous supply of the hormone. Protein synthesis was found to be necessary for protease and α-amylase production throughout incubation. 6-Methylpurine, an RNA synthesis inhibitor, also effectively blocked enzyme production.

## Influence of the Embryo on Endosperm Hydrolytic Processes

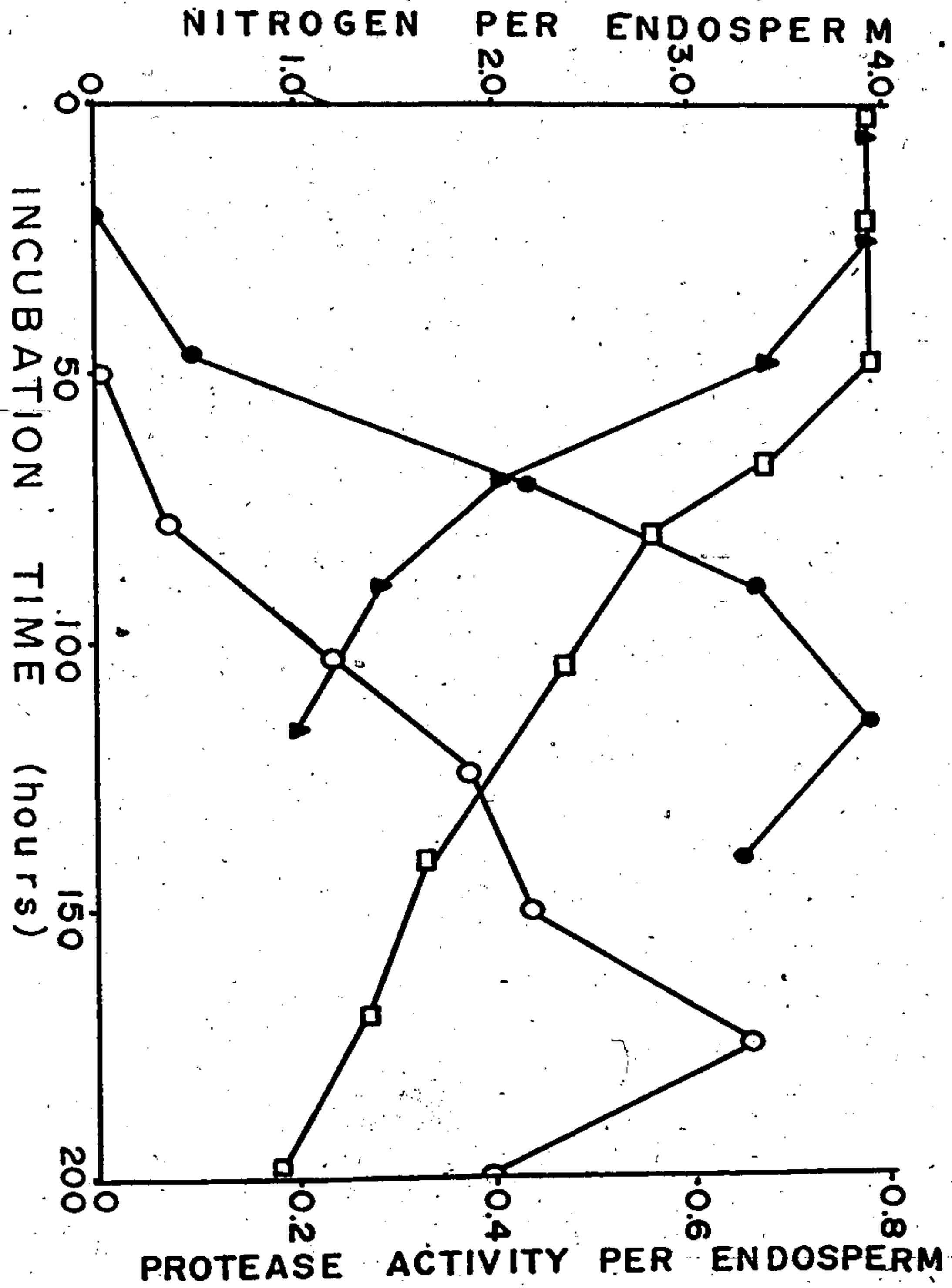
The results in Figures 16, 17 and 18 compare enzyme production and endosperm degradation in intact germinating caryopses with that in excised endosperms incubated in buffer.

Caryopses were surface sterilized and soaked in sterile water for 2 hours. Endosperms were excised under aseptic conditions, and incubated in sterile buffer at 28C. Samples were taken at about 20 hours intervals, and the endosperms frozen. Protease and α-amylase activities in the incubation media were assayed immediately, following the procedure described in MATERIALS AND METHODS. Total nitrogen in the incubation medium was measured and nitrogen loss per endosperm calculated. Endosperm protease and α-amylase were extracted and assayed. The total enzyme production during incubation was obtained by summing the activity extracted per endosperm and the activity released per endosperm into the incubation medium. Figure 16 shows the increase in total protease activity in endosperms incubated in buffer, compared with protease production in endosperms of intact caryopses germinated in vermiculite at the same temperature. Protease activity appeared earlier in the excised endosperms, and increased rapidly. Protease activity increased more slowly in the intact caryopses, but finally reached a level only 20% lower than the maximum activity reached in excised endosperms. Endosperm protein breakdown also began later and progressed more slowly in the intact caryopses (Figure 16), thus the rate of protein breakdown correlated with the measured protease activity. In both excised endosperms and intact caryopses the maximum proteolytic activity occurred when about 75%

## FIGURE 16

**Protease Activity and Protein Loss from Incubated Endosperms and Germinated Caryopses**

Caryopses (Wf9 x 38-11, 1969) were germinated at 28C (open symbols) and excised endosperms were incubated at 28C (solid symbols). Samples were harvested at intervals and total nitrogen per endosperm (□ , ▲) determined. Protease in the endosperms was extracted and assayed, and protease released into the medium by incubated endosperms was also measured. Gliadin was used as substrate in the protease assays. Total protease per endosperm was calculated and activity expressed as mg tryptophan equivalents released per hour per endosperm (O,●).


NITROGEN PER ENDOSPERM
0
1.0
2.0
3.0
4.0
INCUBATION TIME (hours)
0
50
100
150
200
PROTEASE ACTIVITY PER ENDOSPERM
0
0.2
0.4
0.6
0.8

of the endosperm protein had been degraded.

The patterns of zein and glutelin degradation were also compared in excised endosperms and germinated caryopses (Figure 17). As with the total protein, degradation of zein and glutelin began later in germinating intact caryopses, and progressed more slowly than in excised endosperms. There was no qualitative difference in the sequence of zein and glutelin breakdown in the presence and absence of the embryo.

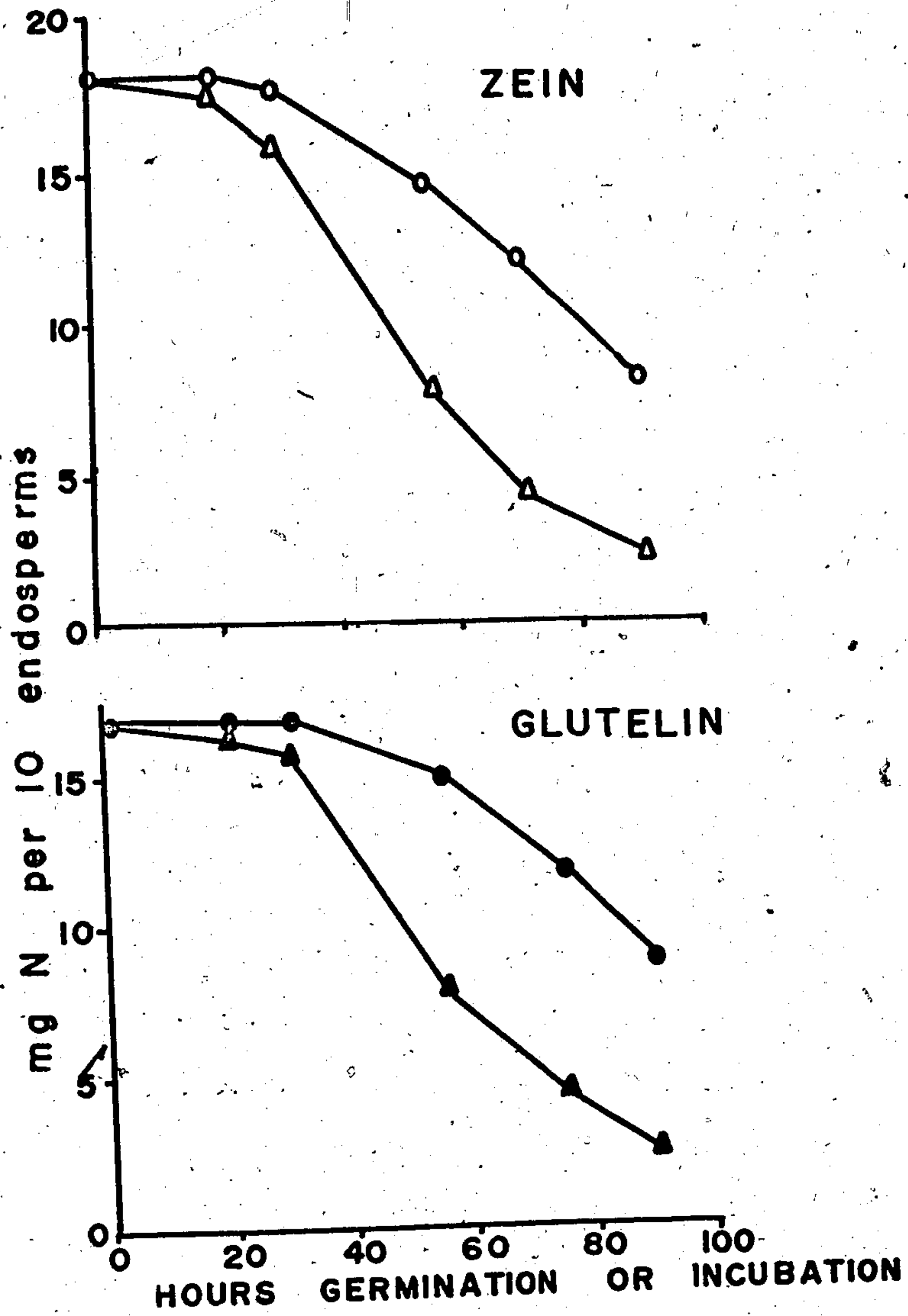
The results in Figure 18 show that α-amylase also appeared earlier in the excised endosperms, but in this case the linear rates of enzyme accumulation appeared to be similar in excised endosperms and germinating caryopses. This is in contradiction of Dures observations (36) that in maize α-amylase is produced by the scutellum, not the endosperm. Total enzyme production cannot be compared, since α-amylase activities were still increasing at the end of the experiment.

The differences in timing of enzyme production and reserve hydrolysis in intact caryopses and excised endosperms are not an artifact of comparing endosperms immersed in buffer with those germinating in moist vermiculite. Whole caryopses, excised endosperms, and excised endosperms + excised embryos were incubated in buffer and the losses in nitrogen and dry weight were measured (Table 30). After 2½ days the losses from excised endosperms were considerable, while losses from endosperms of intact caryopses were negligible. In excised

## FIGURE 17

## Comparison of Zein and Glutelin Loss from Germinated Caryopses and from Excised Endosperms

Caryopses (Wf9 x 38-11, 1969) were germinated at 28C and excised endosperms were incubated at 28C. Fifty endosperm samples were taken at 0, 20, 30, 55, 75, and 90 hours. Endosperm proteins were analysed as described in MATERIALS AND METHODS. Proteins in the endosperms of germinated caryopses are represented as (O,●), and (Δ,▲) represents proteins present in excised endosperms.



ZEIN
GLUTELIN
20
15
10
5
0
mg N per 10 endosperms
0
20
40
60
80
100
HOURS GERMINATION OR INCUBATION

## FIGURE 18

## α-Amylase Production by Incubated Endosperms and by Germinated Caryopses

Caryopses (Wf9 x 38-11, 1969) were germinated at 28C, and excised endosperms were incubated at 28C. Samples were harvested at intervals and α-amylase extracted and assayed. α-Amylase activity in the endosperms of germinated caryopses (O) and the total activity of excised endosperms (Δ) are shown. Activity is expressed as mg starch degraded per minute per endosperm.

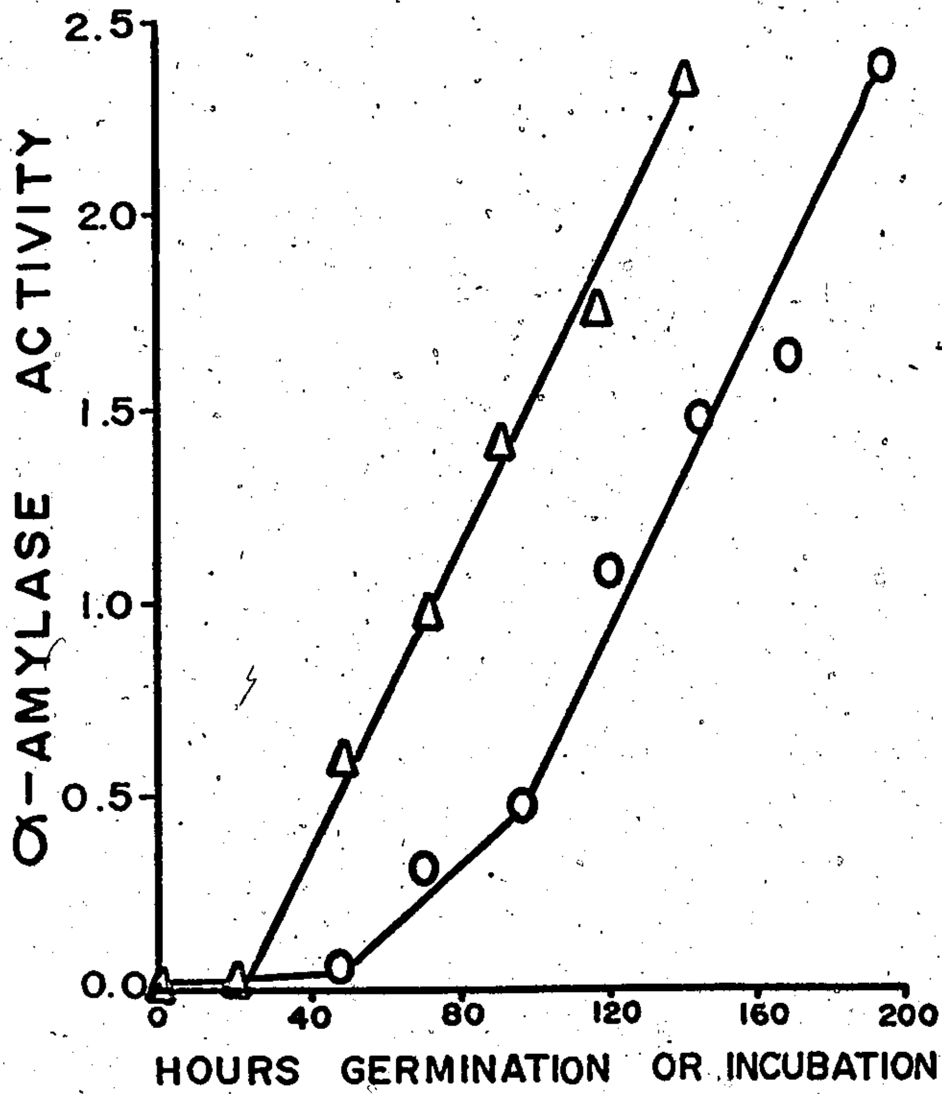

α-AMYLASE ACTIVITY
2.5
2.0
1.5
1.0
0.5
0.0
0
40
80
120
160
200
HOURS GERMINATION OR INCUBATION

TABLE 30

Incubation of Intact Caryopses, Excised Endosperms, and Excised Endosperms + Excised Embryos in Buffer

Excised endosperms, excised endosperms + excised embryos, and intact caryopses of Wf9 x 38-11, 1969, were incubated for 2 days in buffer. Endosperm dry weight was measured to indicate its starch content, and endosperm nitrogen to measure protein content. Each endosperm initially has a dry weight of 242 mg and contains 3.95 mg nitrogen. Results are expressed as mg lost per endosperm during incubation.

| | Intact Caryopses | Excised Endosperms + Excised Embryos | Excised Endosperms |
|---|---|---|---|
| Nitrogen loss mg | 0.07 | 0.56 | 0.86 |
| Dry weight loss mg | 20 | 78 | 97 |

endosperms incubated with excised embryos the losses were less than in excised endosperms incubated alone, but much more than intact caryopses. The embryo thus apparently delays endosperm starch and protein hydrolysis in the intact caryopses. The effect is less apparent when excised embryos are incubated with excised endosperms.

This comparison of hydrolytic enzyme production and endosperm protein breakdown in intact and embryoless caryopses demonstrates that the presence of the embryo or an embryo factor is not necessary for initiation or continuation of hydrolytic processes in maize endosperm.

Influence of Hormones on Endosperm Hydrolysis

The effects of plant hormones on hydrolytic processes in the endosperm were investigated using excised endosperms. Hormones, at the desired concentrations, were included in the incubation medium. Nitrogen and α-amylase release into the medium were assayed as convenient indicators of hormone effects on endosperm hydrolytic processes. In later experiments total production of protease and α-amylase was assayed to give a more precise measurement of hormone or inhibitor effects.

(a) Gibberellic Acid

Excised endosperms were incubated with a range of gibberellic acid concentrations, from 0 to 100 μM. Six separate experiments were performed in 1970 with various hybrids. The amounts of nitrogen and α-amylase released during the 3 day incubation period (Table 31) were variable from hybrid to hybrid, and between batches of the same hybrid. Replication in independent experiments, using the same batches of caryopses, was good (Table 31). The response to gibberellic acid appeared to depend on the batch of caryopses tested. No GA stimulation of α-amylase or nitrogen release was observed with Wf9 x 38-11 (1968) or with 1097 x 1126 (1961). However with Wf9 x 38-11 (1967) gibberellic acid (5 μM) almost doubled α-amylase release, and 0.15 μM gave a minor stimulation of protein breakdown. With Pride-5 (1967) all concentrations of GA up to 15 μM increased α-amylase release, but again only the lowest concentration (0.15 μM) stimulated protein breakdown. High concentrations of GA (100 μM) were generally inhibitory, even to endosperms of caryopses which did not respond at all to lower concentrations of the hormone. It appears that exogenous GA has a variable effect on endosperm hydrolytic processes, and that the effect observed depends on the batch of caryopses tested. In barley endosperms gibberellic acid causes a 10-fold increase in

TABLE 31

The Effect of Gibberellic Acid on the Release of Nitrogen and α-Amylase from Incubated Endosperms

In separate experiments excised endosperms from various maize hybrids were incubated for 3 days in buffer. Independent replicates are represented by a and b. Nitrogen (mg) and α-amylase (mg starch degraded per minute) in the incubation medium were assayed, and release per endosperm calculated. Experiments were performed in 1970.

| Gibberellic Acid (μM) | α-Amylase Release per Endosperm into the Incubation Medium | | | | | |
|---|---|---|---|---|---|---|
| | Wf9 x 38-11 1968 | | Wf9 x 38-11 1967 | | 1097 x 1126 1961 | Pride-5 1967 |
| | a | b | a | b | | |
| 0 | 0.44 | 0.50 | 0.68 | 0.62 | 0.80 | 0.60 |
| 0.15 | 0.39 | 0.42 | 0.68 | 0.69 | 0.56 | 0.85 |
| 5.0 | 0.30 | 0.37 | 1.27 | 1.01 | 0.64 | 0.86 |
| 15.0 | 0.31 | 0.37 | 0.91 | 0.83 | 0.60 | 0.88 |
| 100.0 | - | - | 0.48 | 0.50 | 0.54 | 0.89 |
| | **Nitrogen Release per Endosperm into the Incubation Medium** | | | | | |
| 0 | 1.25 | 1.22 | 1.12 | 1.13 | 1.50 | 1.36 |
| 0.15 | 1.29 | 1.20 | 2.00 | 1.91 | 1.45 | 1.72 |
| 5.0 | 1.12 | 1.25 | 1.44 | 1.50 | 1.00 | 0.80 |
| 15.0 | 0.84 | 0.92 | 1.12 | 1.24 | 1.35 | 0.44 |
| 100.0 | - | - | - | - | 1.00 | 0.76 |

α-amylase release (131), but "background" values for release in the absence of hormone are very low. In maize "background" α-amylase release is high, so that hormone effects may be difficult to detect.

(b) Indoleacetic Acid and Kinetin

Hormones other than gibberellic acid have also been implicated in regulation of endosperm hydrolytic processes (36,38,63). The effects of kinetin (K) and indoleacetic acid (IAA), alone and in the presence of GA, were tested in maize. Table 32 shows that endosperms did not respond to low concentrations of either hormone, and at higher concentrations both were inhibitory. Low concentrations of K and IAA applied with GA did not influence the normal response of the endosperms to gibberellic acid.

(c) Abscisic Acid

Abscisic acid (ABA) has been classified as a gibberellin antagonist (176), however it also inhibits responses to other plant hormones (185). The data in Table 33 show that inclusion of ABA in the incubation medium of excised endosperms inhibited protease and α-amylase production. A low ABA concentration (2 μM) inhibited protease production by 76% and

## TABLE 32

## Effects of Kinetin and Indoleacetic Acid on Release of α-Amylase and Nitrogen by Excised Endosperms

Endosperms of Wf9 x 38-11 (1969) were incubated in buffer with the kinetin (K), indoleacetic acid (IAA), and gibberellic acid (GA) at the concentrations indicated. After 3½ days α-amylase and nitrogen release into the medium were measured.
For α-Amylase: 100 = 0.86 mg starch degraded per minute per endosperm.
For Nitrogen: 100 = 1.68 mg nitrogen released per endosperm.

| Treatment | Release per Endosperm into Incubation Medium | |
|---|---|---|
| | α-Amylase | Nitrogen |
| Control | 100 | 100 |
| K 0.5 μM | 101 | 100 |
| 1.0 μM | 100 | 98 |
| 10.0 μM | 75 | 70 |
| IAA 0.5 μM | 100 | 100 |
| 1.0 μM | 99 | 100 |
| 10.0 μM | 78 | 88 |
| GA 15.0 μM | 114 | 117 |
| K 1 μM + GA 15 μM | 112 | 116 |
| IAA 1 μM + GA 15 μM | 114 | 114 |

## TABLE 33

### Inhibition of Protease and α-Amylase Production by Abscisic Acid, and Reversal of Inhibition by Gibberellic Acid

Endosperms of Wf9 x 38-11 (1971) were incubated for 3 days with the hormone concentrations indicated. Protease and α-amylase activities were assayed in endosperm extracts and the incubation media. Gliadin substrate was used for the protease assays.
For Protease 100 = 0.15 μg tryptophan equivalents released per hour per endosperm,
and α-Amylase 100 = 0.73 mg starch degraded per minute per endosperm.

| Hormone | Concentration μM | Protease | α-Amylase |
|---|---|---|---|
| - | - | 100 | 100 |
| ABA | 2 | 24 | 35 |
| | 10 | 5 | 5 |
| GA | 3 | 114 | 124 |
| | 30 | 132 | 146 |
| ABA<br>GA | 2<br>3 | 63 | 93 |
| ABA<br>GA | 2<br>30 | 109 | 110 |
| ABA<br>GA | 10<br>3 | 33 | 51 |
| ABA<br>GA | 10<br>30 | 45 | 54 |

α-amylase by 65%. Higher concentrations inhibited enzyme production almost completely.

Gibberellic acid alone (3 and 30 μM) gave a small but measurable stimulation of protease and α-amylase production. When both hormones were included in the incubation medium, the inhibitory effect of abscisic acid was reduced. GA (30 μM) overcame the effect of 2 μM ABA, and reduced inhibition due to 10 μM ABA from 95% to 50%.

Kinetin and indoleacetic acid were also tested (Table 34). These hormones did not overcome the inhibition caused by ABA. Indoleacetic acid reduced the inhibition by 2 μM ABA slightly, but did not alleviate inhibition due to 10 μM ABA. These results show that the inhibition of endosperm hydrolases by abscisic acid is reversed specifically by gibberellic acid.

Maize Endosperm Gibberellic Acid

Interaction of ABA and GA suggest that gibberellic acid has a function in the regulation of maize endosperm hydrolytic processes. However it is not normally effective when applied exogenously. Gibberellic acid could be present in adequate amounts in mature endosperms of maize, or it should be synthesised in the endosperm during incubation or germination. The following experiments were designed to test

TABLE 34

Effects of Kinetin and Indoleacetic Acid on Abscisic Acid Inhibition of Hydrolytic Enzyme Production

Excised endosperms of Wf9 x 38-11 were incubated for 4 days with the hormone concentrations indicated. Suitable controls were incubated for the kinetin and indoleacetic acid experiments, to permit correction for effects of solvents used to dissolve these hormones. Enzyme activities were measured in endosperm extracts and in the incubation media. Total α-amylase activity is expressed as mg starch degraded per minute per endosperm, and total protease activity as mg tryptophan released per hour per endosperm. Gliadin was used as the protease substrate.

| Experiment | Hormone | Concentration μM | α-Amylase | Protease |
|---|---|---|---|---|
| 1 | Control | - | 1.21 = 100 | 0.26 = 100 |
| | ABA | 2 | 35 | 28 |
| | ABA | 10 | 6 | 4 |
| 2 | Control | - | 0.80 = 100 | 0.21 = 100 |
| | K | 10 | 91 | 93 |
| | K + ABA | 10 + 2 | 32 | 24 |
| | K + ABA | 10 + 10 | 6 | 3 |
| 3 | Control | - | 1.6 = 100 | 0.29 = 100 |
| | IAA | 10 | 108 | 106 |
| | IAA + ABA | 10 + 2 | 51 | 37 |
| | IAA + ABA | 10 + 10 | 8 | 4 |

these possibilities:

(a) Dwarf Mutants

Certain single-gene mutants in maize can be converted to normal phenotype by repeated treatment with gibberellic acid (140). The mutants are deficient in the ability to synthesise gibberellic acid. Endosperms of these mutants could be deficient in GA, and hence more responsive to exogenously applied hormone than endosperms of normal maize. Excised endosperms of dwarf mutant d-5 were incubated with 0, 1 and 10 μM GA. Since isogenic lines were not available excised endosperms of Wf9 x 38-11 were similarly treated for comparison. Total protease and α-amylase production was measured in each treatment. Nitrogen and reducing sugar release per endosperm were also measured as indicators of protein and starch hydrolysis.

Endosperms of d-5 incubated without GA had low protease and α-amylase activities, and degraded little protein or starch (Table 35). Inclusion of 10 μM GA in the incubation medium stimulated protease production by over 500%, and α-amylase production by over 300%. Starch and protein hydrolysis were also stimulated, to a lesser extent. In Wf9 x 38-11 10 μM GA produced minor stimulations of α-amylase (47%) and protease (29%) activities.

TABLE 35

Effect of Gibberellic Acid on Hydrolase Production and Starch and Protein Hydrolysis in Dwarf and Normal Maize

Excised endosperms of d-5 and normal Wf9 x 38-11 (1971) were incubated in the presence of 0, 1, and 10 μM GA for 3½ days. Enzyme activities in the incubation medium and in endosperm extracts were measured, and added to give total enzyme production. Protease activity was assayed with gliadin (BDH) as substrate, and activity is expressed as mg tryptophan equivalents released per hour per endosperm. α-Amylase activity is expressed as mg starch degraded per minute per endosperm. Nitrogen (mg) and reducing sugar (mg) release per endosperm into the medium was also measured. Figures in brackets represent the absolute values.

| GA Concentration (μM) | Wf9 x 38-11 | | | Dwarf-5 | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 10 | 0 | 1 | 10 |
| α-Amylase | 100 (1.14) | 111[a] (1.26) | 147 (1.68) | 100 (0.32) | 284 (0.91) | 322 (1.03) |
| Protease | 100 (0.34) | 120 (0.38) | 129 (0.44) | 100 (0.06) | 333 (0.20) | 533 (0.32) |
| Nitrogen Release | 100 (1.90) | 112 (2.13) | 111 (2.11) | 100 (0.51) | 214 (1.09) | 223 (1.13) |
| Reducing Sugar | 100 (20.2) | 106 (21.3) | 103 (20.8) | 100 (9.1) | 160 (14.5) | 166 (15.1) |

These results indicate that GA is required for hydrolytic enzyme production and hydrolysis of endosperm storage products. A reasonable interpretation is that exogenous hormone replaces a deficiency in d-5 mutants, whereas sufficient gibberellic acid may already be present in normal varieties.

(b) Effect of Gibberellic Acid Synthesis Inhibitors on Endosperms of Normal Maize

Endogenous GA could be present in the mature endosperms, or GA could be synthesised in the imbibed endosperms. Specific inhibitors of GA biosynthesis can be used to test the latter possibility. CCC, AMO 1618, and Phosphon D inhibit GA synthesis (33) but do not interfere with the response of barley endosperms to exogenous GA (133). They were incubated with excised endosperms, and total protease and α-amylase production measured. The data in Table 36 show that AMO 1618 was the most potent inhibitor. A concentration of 200 μM inhibited enzyme production by about 60%. Phosphon D (300 μM) was slightly inhibitory, and CCC had little effect even at very high concentrations.

If these compounds inhibit by blocking GA synthesis in the endosperm, then their effects should be overcome by addition of exogenous gibberellin to the incubation medium.

## TABLE 36

**Effect of Inhibitors of Gibberellic Acid Synthesis on Protease and α-Amylase Production by Excised Endosperms**

Excised endosperms of Wf9 x 38-11 (1971) were incubated for 4 days with the concentrations of inhibitors shown. Protease and α-amylase activities were assayed in the incubation media and in endosperm extracts. Gliadin (BDH) was used as the protease substrate. Activity in the control was set at 100, and relative activity in each treatment calculated.
For Protease 100 = 0.42 mg tryptophan released per hour per endosperm.
and α-Amylase 100 = 1.24 mg starch degraded per minute per endosperm.

| Inhibitor | Concentration μM | Protease | α-Amylase |
|---|---|---|---|
| Control | - | 100 | 100 |
| CCC | 100 | 100 | 108 |
| | 600 | 95 | 100 |
| | 6,000 | 98 | 70 |
| Phosphon D | 30 | 95 | 113 |
| | 300 | 69 | 88 |
| AMO 1618 | 20 | 88 | 98 |
| | 200 | 34 | 41 |

TABLE 37

Effect of Interacting GA and AMO 1618 on Enzyme Production

Excised endosperms of Wf9 x 38-11 (1971) were incubated for 3 days with the concentrations of AMO 1618 and GA shown. Protease and α-amylase activities were assayed in incubation media and endosperm extracts. Gliadin (BDH) was used as protease substrate. Enzyme activities in the control were set at 100, and relative activities of each treatment calculated.

For Protease: 100 = 0.34 mg tryptophan equivalents released per hour per endosperm.

and α-Amylase: 100 = 1.17 mg starch degraded per minute per endosperm.

| Treatment | Concentration µM | Protease | α-Amylase |
|---|---|---|---|
| Control | - | 100 | 100 |
| GA | 3 | 103 | 99 |
| GA | 30 | 117 | 105 |
| AMO | 125 | 44 | 47 |
| AMO | 250 | 24 | 27 |
| AMO<br>GA | 125<br>3 | 56 | 65 |
| AMO<br>GA | 125<br>30 | 56 | 73 |
| AMO<br>GA | 250<br>30 | 34 | 41 |

The results in Table 37 show that exogenous GA does not overcome the effect of concentrations of AMO1618 which only partially inhibit protease and α-amylase production. AMO 1618 appears not to inhibit enzyme production by blocking gibberellin biosynthesis in the endosperms. The results suggest that maize endosperms have sufficient endogenous GA to induce hydrolytic processes after imbibition.

Protein Synthesis Requirement for Hydrolytic Enzyme Production

Hydrolytic enzymes may be activated or may be synthesised de novo in the endosperm. The involvement of protein synthesis in hydrolytic enzyme production was tested by including cycloheximide, a protein synthesis inhibitor, in the incubation medium of excised endosperms. The results in Figure 19 show that cycloheximide (5 μg/ml) stopped protease and α-amylase production, if added at any time during incubation.

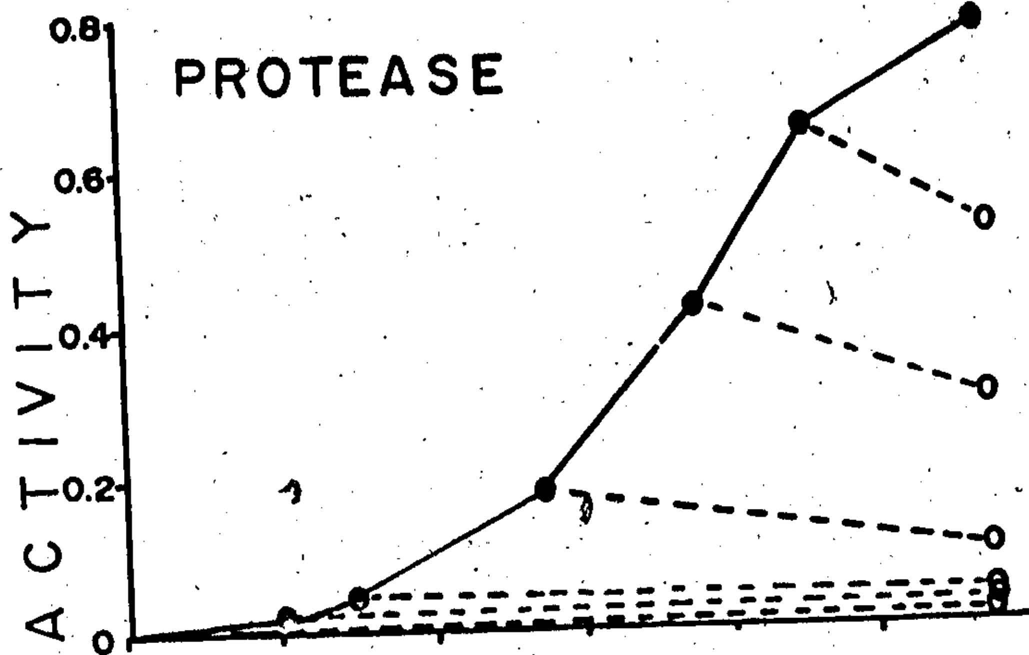
Endosperm hydrolysis was measured by release of total nitrogen from protein, and reducing sugars from starch into the incubation medium. Addition of cycloheximide at the beginning of incubation (0 or 20 hours) completely prevented endosperm starch and protein hydrolysis (Figure 20). If the inhibitor was added at 30 or 55 hours starch and protein hydrolysis continued, but at slower rates than in the minus cycloheximide control. Addition of cycloheximide after

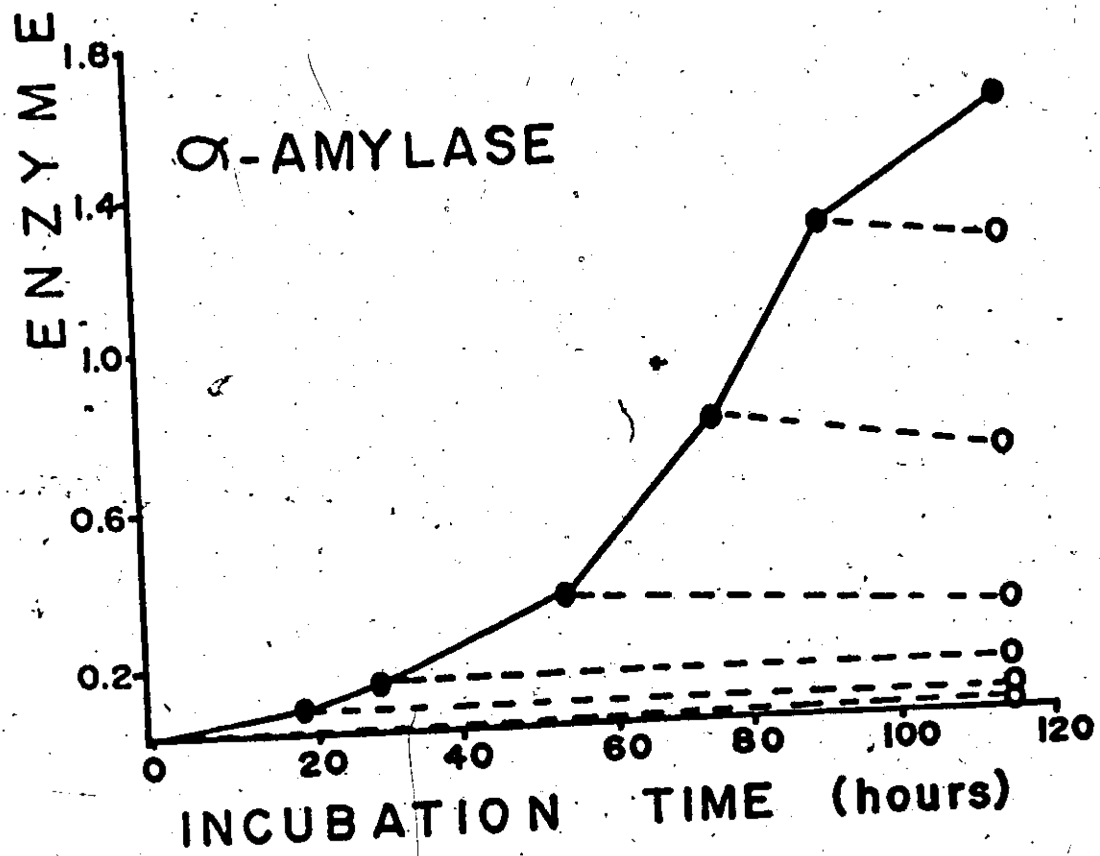
## FIGURES 19 AND 20

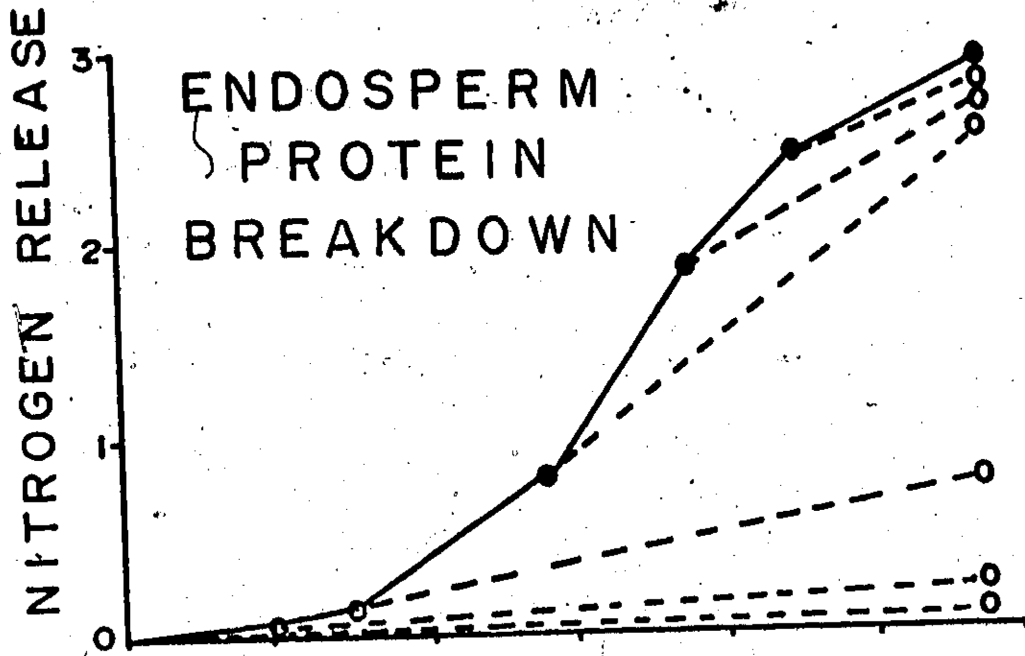
### Effects of Cycloheximide on Hydrolytic Enzyme Production and Endosperm Hydrolysis in Excised Endosperms

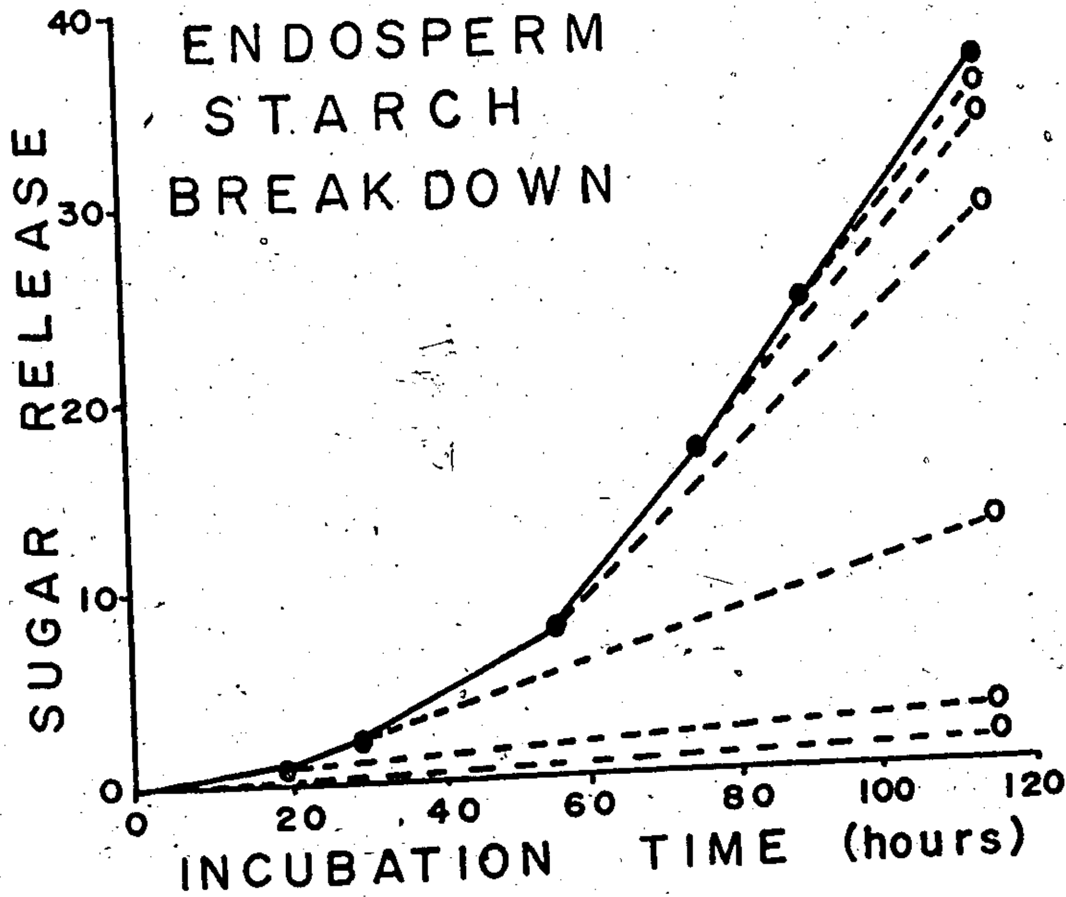
Excised endosperms of Wf9 x 38-11 (1969) were incubated in buffer. Cycloheximide (5 µg/ml) was added at the times indicated by arrows. Enzyme activaties were assayed in the incubation media and in endosperm extracts. Protease activity was measured with gliadin as substrate, and activity expressed as µg tryptophan equivalents released per hour per endosperm. α-Amylase activity was measured as mg starch degraded per minute per endosperm. Protein hydrolysis was measured by total nitrogen (mg) release into the medium per endosperm, and starch breakdown as reducing sugar (mg) release per endosperm into the medium.

Solid lines and symbols represent the controls incubated without cycloheximide, broken lines and open symbols represent the cycloheximide treatments.


PROTEASE
0.8
0.6
0.4
0.2
0
ACTIVITY


ENZYME
1.8
1.4
1.0
0.6
0.2
α-AMYLASE
0
20
40
60
80
100
120
INCUBATION TIME (hours)


NITROGEN RELEASE
ENDOSPERM
PROTEIN
BREAKDOWN
3
2
1
0


SUGAR RELEASE
ENDOSPERM
STARCH
BREAKDOWN
40
30
20
10
0
0
20
40
60
80
100
120
INCUBATION TIME (hours)

70 hours incubation had little effect on the rate of endosperm starch and protein hydrolysis, although enzyme production was inhibited. Apparently enough enzyme is already present by 70 hours to continue degradation of endosperm starch and protein at the control rate.

The effect of cycloheximide on zein and glutelin degradation was investigated, to determine whether the hydrolysis of each protein was inhibited to the same extent. Treatment times were chosen so that endosperm protein hydrolysis would be partially inhibited. Cycloheximide was therefore added after 35 and 55 hours incubation, and endosperms from the ± cycloheximide treatments were used for protein analysis. The results in Figure 21 show that zein and glutelin loss were affected similarly.

The constant requirements for protein synthesis in protease and α-amylase production indicates that the enzymes are synthesised de novo in the endosperms.

## RNA Synthesis

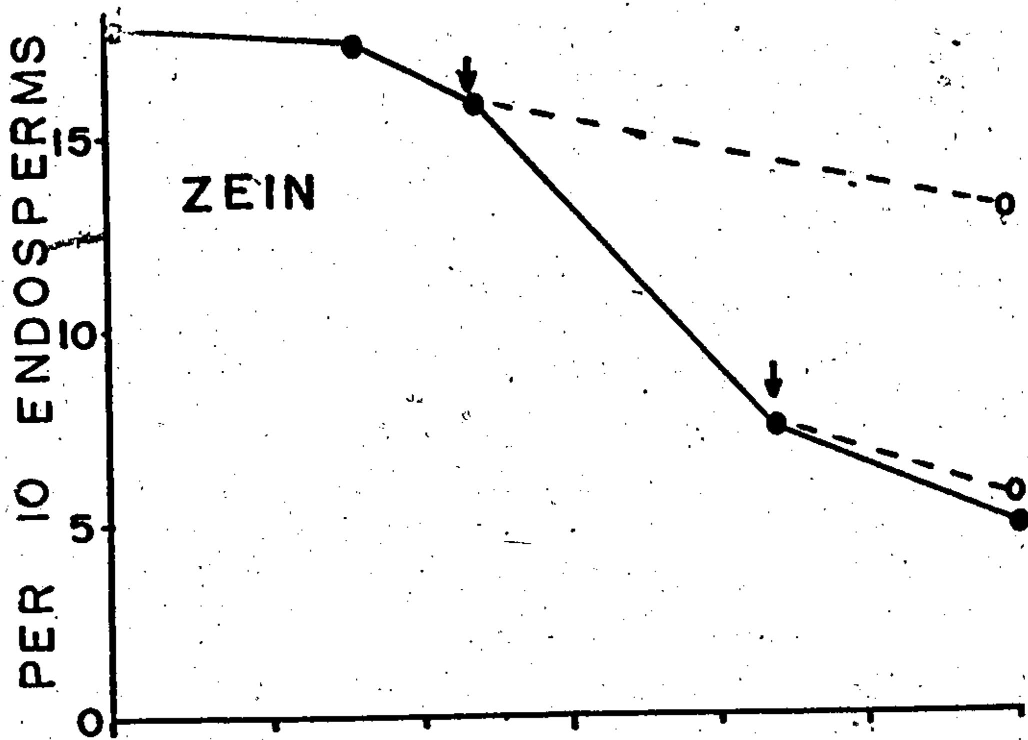
The RNA synthesis requirement for hydrolytic enzyme production was also tested. Two RNA synthesis inhibitors, Actinomycin D and 6-methylpurine, were included in the incubation media of excised endosperms (Table 38). Enzyme production and hydrolysis of starch and protein were strongly inhibited by

FIGURE 21

Effects of Cycloheximide on Zein and Glutelin Loss from Excised Endosperms

Excised endosperms (Wf9 x 38-11, 1969) were incubated in buffer. Cycloheximide (5 μg/ml) was added as indicated by the arrows. Fifty endosperm samples were removed for protein analysis after 0, 20, 50 and 75 hours incubation of the minus cycloheximide controls, and at 75 hours samples were taken from the two cycloheximide treatments.

The solid lines and symbols represent zein and glutelin in the controls, and the broken lines and open symbols show zein and glutelin content of the cycloheximide treated endosperms.


ZEIN
PER 10 ENDOSPERMS
15
10
5
0

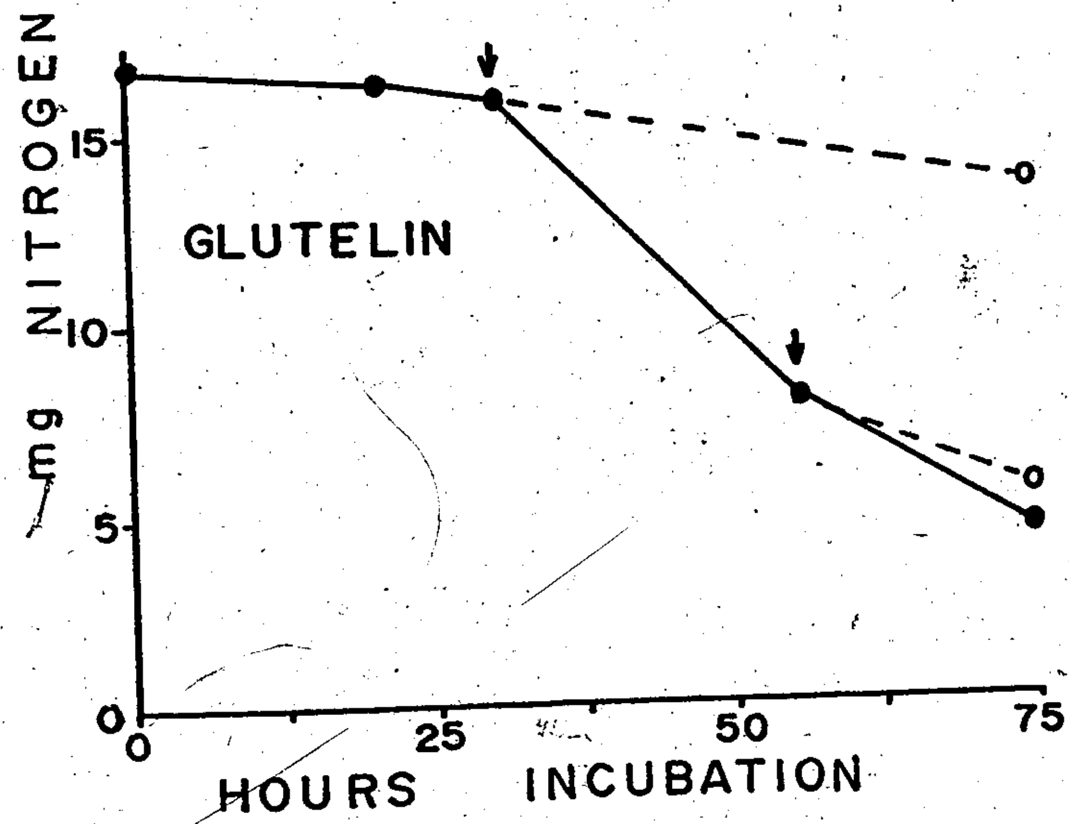

GLUTELIN
mg NITROGEN
15
10
5
0
0
25
50
75
HOURS INCUBATION

TABLE 38

Effect of RNA Synthesis Inhibitors on Hydrolytic Enzyme Production and Starch and Protein Hydrolysis

Excised endosperms of Wf9 x 38-11, 1971, were incubated for 3½ days. Enzyme activities were assayed in the incubation medium and endosperm extracts. Hemoglobin substrate was used in the protease assays. Nitrogen and reducing sugars in the incubation medium were also measured.

For Protease: 100 = 0.30 mg tryptophan equivalents per hour per endosperm.
α-Amylase: 100 = 1.19 mg starch degraded per minute per endosperm.
Nitrogen: 100 = 1.92 mg per endosperm.
Reducing Sugar: 100 = 2.2 mg per endosperm.

| Inhibitor | Concentration μg/ml | Protease | α-Amylase | Nitrogen | Sugar |
|---|---|---|---|---|---|
| Control | - | 100 | 100 | 100 | 100 |
| 6-Methylpurine | 5 | 12 | 0 | 12 | 3 |
| | 50 | 10 | 0 | 8 | 2 |
| Actinomycin D | 5 | 109 | 91 | 100 | 101 |
| | 50 | 62 | 63 | 77 | 83 |

6-methylpurine, and partially inhibited by high concentrations of Actinomycin D. This resembles the results of Jacobsen and Varner (69). In barley aleurone layers they observed total inhibition of enzyme production with 5 μg/ml 6-methylpurine, and approximately 50% inhibition with 50 μg/ml Actinomycin D. 6-Methylpurine has no effect on respiration in barley aleurone tissue, and appears to inhibit RNA synthesis directly (57). Thus RNA synthesis appears necessary for normal enzyme synthesis and hydrolysis of starch and storage protein in maize endosperms.

## DISCUSSION

### Endosperm Protease Activity

Proteolytic activity has been studied in ungerminated wheat (54,77,101) because of its relevance to flour and bread making, and in resting and germinated barley (13,40,173) for the malting and brewing industries. Flour suspensions and crude extracts of germinating grain have been found to show maximum proteolytic activity at low pH (52,101,166), and high incubation temperatures, 40 to 50C (101,184). The major proteolytic enzymes in cereal endosperms thus appear to be acid proteases with high temperature optima. Such enzymes have been partially purified from germinated sorghum (49), and from wheat endosperm (166), however most investigations have been performed with crude extracts, or with proteases distinguished by their specificity to synthetic substrates.

Early investigators considered that the endosperm proteases were all of the papain-type, requiring free sulphydryl groups for their activity (7,41). More recently sulphydryl enzymes have been shown to account for only part of the total proteolytic activity of flour (101). Sundblom and Mikola (173) have shown that barley endosperms produce several

sulphydryl-activated enzymes, pH optima 3.9 and 5.0 to 6.5, but also enzymes which are insensitive to sulphydryl-blocking agents. These enzymes are metal-activated, and active in a higher pH range, than the sulphydryl enzymes. These enzymes were shown to be endopeptidases, and the sulphydryl enzyme with a pH optimum of 3.9, was most active. The pH of the barley endosperm has been measured to decline from 4.5 in the imbibed caryopsis to 4.0, after 6 days growth (20). Hence this enzyme may account for most protein breakdown in the endosperm of the germinating caryopsis.

The maize endosperm protease activity is optimal at pH 3.8, is strongly inhibited by sulphydryl-blocking agents, and has a high temperature optimum (46C). It is therefore comparable to proteases of other cereals (Table 3), and to the sulphydryl enzyme which Sundblom and Mikola consider to be the principal barley endosperm protease. The maize endosperm protease preparation has endopeptidase activity. It may contain one or several endopeptidases, and exopeptidases may also be present.

No attempt has been made to purify the proteases present in the maize extracts. Properties of the enzyme or enzymes were investigated to permit development of reliable extraction and assay procedures. Principal emphasis was placed on the physiological function of the proteases, hence activity with the natural substrates zein and glutelin were investigated.

Few seed proteases have been demonstrated to degrade their seed reserve proteins. Shinano and Furishimo (163) have purified a lotus seed protease which increased in activity during germination, but which only degraded denatured proteins. Native proteins, including lotus seed globulin, were not hydrolysed. Hanford (54) showed that hemoglobin degrading enzymes extracted from wheat flour did not attack wheat gluten. In contrast to these reports, Mountfield (114) found that extracted wheat storage protein were degraded more slowly than native glutelin. He proposed that denaturation during extraction made the proteins more resistant to hydrolysis by wheat protease. Hence demonstration that the maize acid protease can degrade partially hydrolysed zein and glutelin should not be interpreted as evidence that it can degrade native zein and glutelin in the endosperm.

An alternative assay method was developed to permit use of insoluble proteins as substrates. The method was based on the starch gel assay for α-amylase described by Simpson and Naylor (165) and Kaminsky and Bushuk's (77) electrophoretic method of detecting and separating proteases. Substrates were incorporated into agar gels, and the activity of successive protease dilutions tested. The maize protease degraded both zein and glutelin, with efficiency similar to hemoglobin and edestin (Table 27). The same enzyme(s) may be active in both this and the standard

assay, since the time courses of increases in activity during germination were comparable (Figures 10 and 11). The agar-gel experiments do not show that native storage proteins are degraded by the protease, but do show that extracted and thus partially denatured zein and glutelin can be degraded. The protease displayed no specificity for either protein, since both were degraded with similar efficiency.

Protease Production by Excised Endosperms

The investigation of regulation of endosperm zein and glutelin hydrolysis is simplified by experimenting with de-embryonated endosperms incubated in buffer. Hence proteases obtained from incubated endosperms and from the incubation medium of excised endosperms were compared with protease extracted from germinated intact caryopses. Enzymes from the three sources had the same pH and temperature optima, and the hydrolysis products released from gliadin substrate had similar total nitrogen to α-amino nitrogen ratios(Table 28). This indicates that the same enzyme or enzymes are released into the incubation medium as are retained in the endosperm during incubation. Sundblom and Mikola (173) have recently made a similar observation with barley.

In contrast to these observations, proteases from the

incubation medium and from extracts of excised endosperms displayed strikingly different stabilities to preincubation at 40C. The enzyme in the incubation medium was stable, losing little activity during a 90 minute preincubation at 40C, but the protease extracted from excised endosperms lost about 50% of its activity during the same period. Stability of the enzyme preparations also depended on the age of the material. Both endosperm and medium proteases were more stable when obtained from 5 day than from 3 day incubations of excised endosperms (Figure 13).

Mixing of endosperm and medium proteases resulted in stabilization of the endosperm protease. This was due to a factor released from the endosperm during incubation, since incubation buffer alone had no stabilizing effect. Passage of the medium protease through Sephadex G-25 reduced the stability of the enzyme, and reduced its ability to stabilize the endosperm protease (Figure 15). This treatment removed only small molecules from the medium, hence a small molecule released from the endosperm during incubation stabilises the maize protease. Investigations of the "stabilising factor" were not carried further.

Peptide "activators" of Carboxypeptidase B have been documented (197), but no records of seed protease "stabilizing factors" have been found in the literature. The possible physiological functions of this "stabilising factor" require

further investigation. For instance accumulation and disappearance of such a factor could contribute to regulation of rates of protease turn-over in the endosperm during germination.

Evidence for the occurrence of protease turn-over, both synthesis and inactivation or degradation, is provided by experiments with cycloheximide (Figures 19 and 20). Addition of this inhibitor to the incubation medium of excised endosperms at any time inhibited all further increases in activity of protease and of α-amylase. This indicates that the enzymes are synthesised *de novo* in the endosperm. When new enzyme production was blocked by cycloheximide, protease activity remained approximately constant in the incubation medium, but declined in the endosperms. This decline represents the degradation or inactivation phase of enzyme turn-over. Amylase activity declined less markedly than protease, indicating that the protease may be turning-over more rapidly *in vivo*.

## Regulation of Endosperm Hydrolysis

Analysis of the endosperm proteins showed that zein and glutelin degradation began during the first 48 hours germination in all caryopses, but the rate of loss of either protein appeared to depend on the protein composition of the

caryopsis. Those rich in glutelin initially degraded glutelin more rapidly than zein (Figure 2), and the converse was true of high-zein caryopses (Table 11). Thus the most abundant storage protein was degraded most rapidly in the maize endosperm. This is indicative of non-specific proteolysis, and is in agreement with observations that the endosperm protease preparation degrades zein and glutelin with equal efficiency (Table 27).

Measurement of endosperm proteolytic activity at daily intervals after imbibition of caryopses showed that the protease detected by my assay appeared at 3 days, and rapidly increased in activity until about 8 days germination. Comparison of protease activity with the course of zein and glutelin breakdown shows that the protease did not appear until after the initiation of protein hydrolysis, and reached its maximum activity when degradation of zein and glutelin was almost complete.

Similarly development of α-amylase activity in the endosperm was compared with loss of dry weight, an indication of starch breakdown in the endosperm (Figure 12). Appearance of α-amylase activity approximately coincided with the beginning of starch loss, but again maximum hydrolase activity was reached only when degradation of the reserve material was almost complete.

Calculations of total proteolytic activity and amylolytic

activity indicate these hydrolases were produced in excess of requirements to degrade the endosperm starch and protein reserves. Protease extracted from endosperms of caryopses germinated for 3½ days could release 1.3 mg nitrogen per hour, or 0.03 mg α-amino nitrogen per hour from gliadin substrate, in the standard assay conditions. Each endosperm initially contains about 4 mg protein-nitrogen, and this is degraded over a 6 day period (Figure 12). Even if activity measured in vitro is arbitrarily reduced 20-fold to compensate for less favourable conditions in vivo, it is still adequate to degrade all zein and glutelin in less than 6 days.

A similar calculation can be made for α-amylase and starch breakdown. Each endosperm initially has about 150 mg starch, and this is degraded between 2 and 9 days germination (Figure 12). After 3½ days germination the amylase extracted from one endosperm was sufficient to degrade 0.4 mg starch per minute. If this activity is arbitrarily reduced 20-fold to compensate for less favourable conditions in vivo, it would also be enough to degrade all endosperm starch in less than the normal 7 days.

Theoretically it might be predicted that hydrolase activity would be highest during the rapid phase of substrate breakdown, and that a constant level of activity would be maintained while the rate of substrate hydrolysis is linear.

This possibility is represented in Figure 22A. Proteases which remain approximately constant in activity throughout the phase of reserve protein degradation have been found in cotyledons of chickpea (5), in lettuce seed (161), and pea cotyledons (32). Pusztai and Duncan (147) observed that trypsin-like activity and leucine aminopeptidase activity remained constant throughout kidney bean germination, and in barley endosperm several peptidases did not change in activity during protein breakdown (110,144). In these instances there is a clear correlation between rate of protein breakdown and enzyme activity.

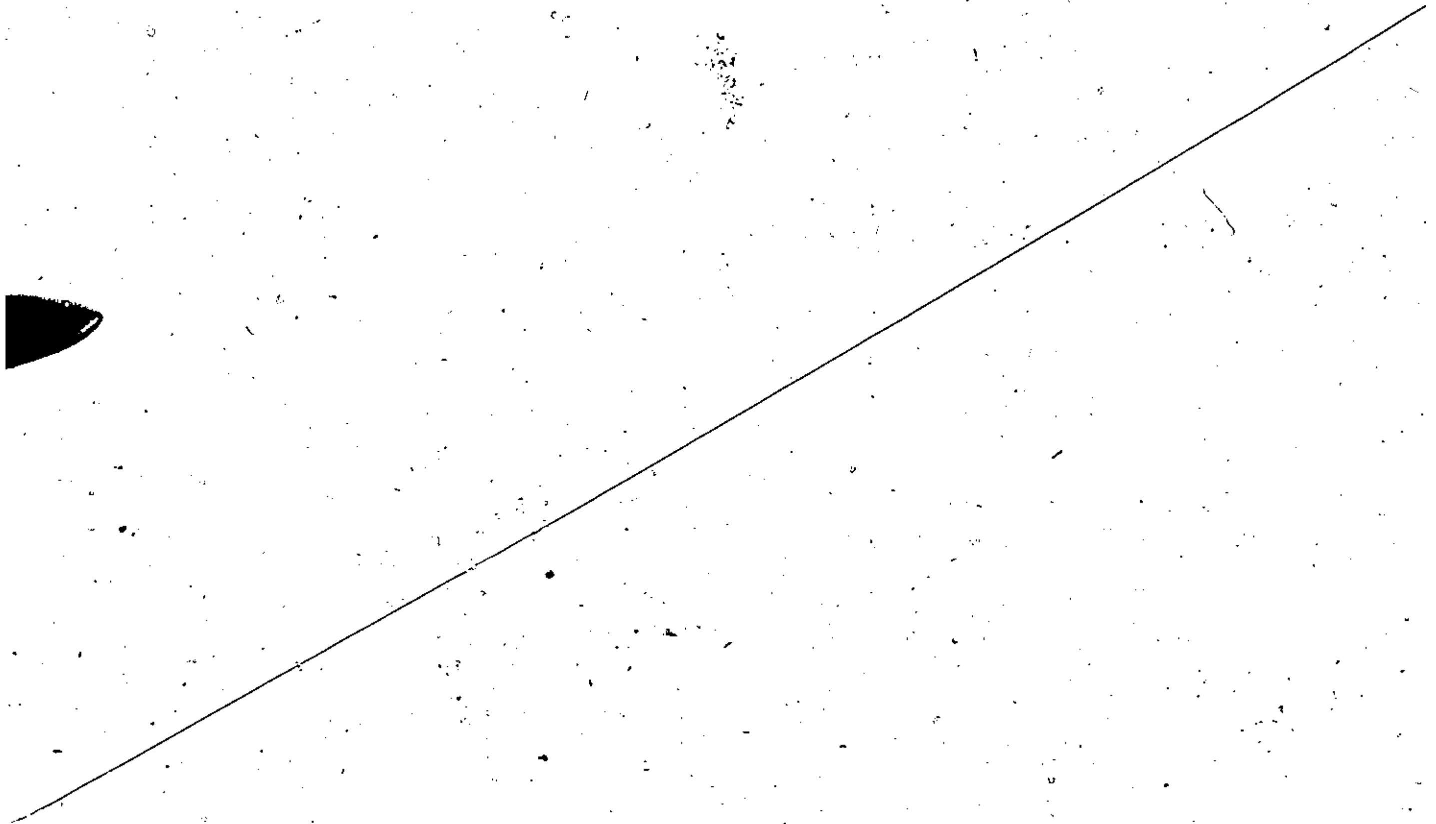
In maize (Figure 12) massive increases in protease activity occur between 3 and 8 days germination, and in α-amylase between 2 and 9 days. This pattern of development is represented in Figure 22B. Maximum protease activity after depletion of storage protein reserves has also been observed in germinating pea (10,53,196), rice (115), kidney bean (147), and oats (174).

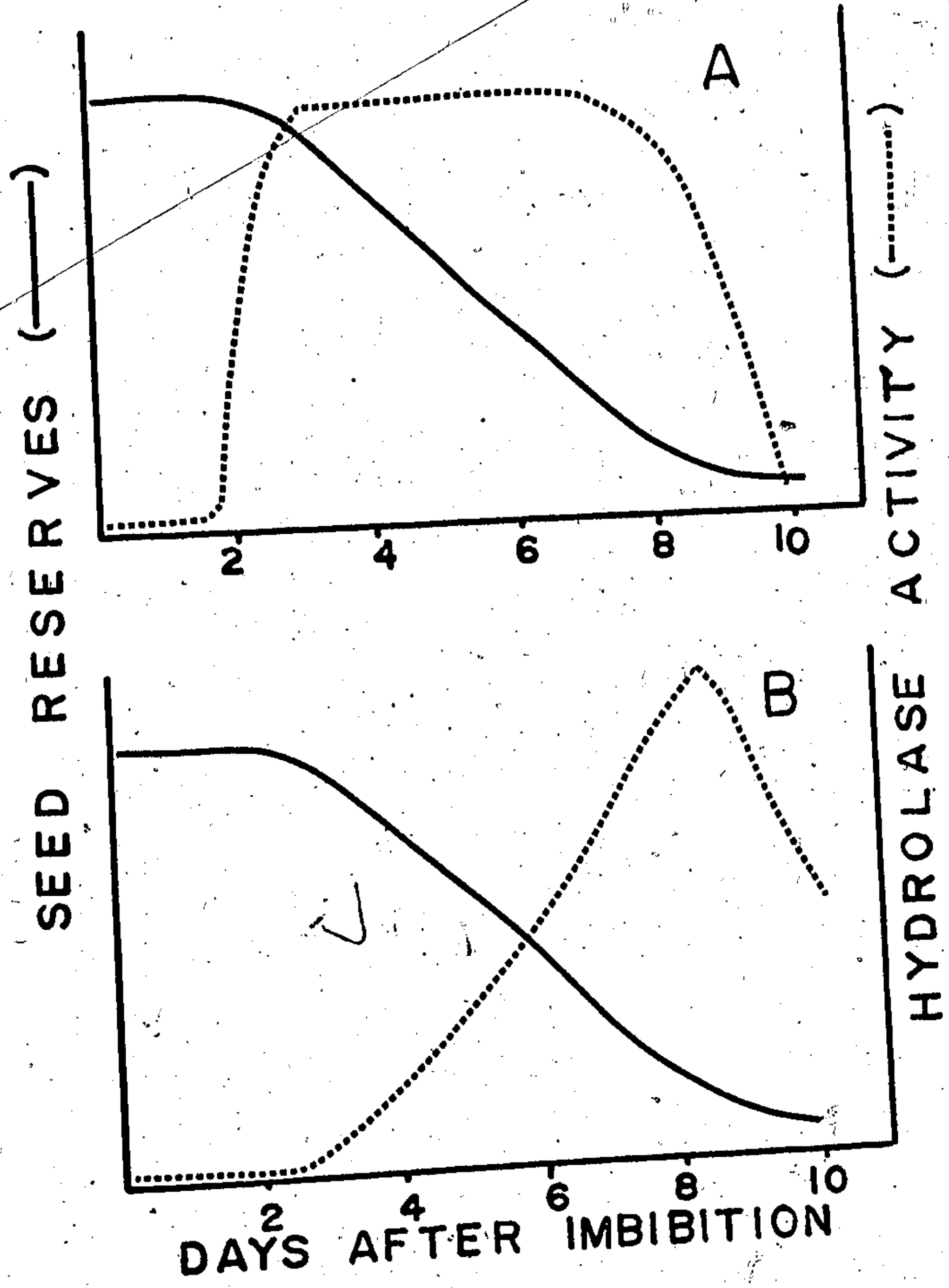
Sutcliffe and Baset (174) have put forward two hypotheses to explain this phenomenon. They observed that in oats endosperm hydrolysis proceeds as a front moving from the dorsal to the ventral side of the caryopsis and so assume that activity must be confined to a specific zone at any given time. They propose that either:

1. As substrate is depleted in a zone the enzyme molecules

## FIGURE 22

## Models for Enzyme Activity and Substrate Breakdown During Germination




A
B
SEED RESERVES (——)
HYDROLASE ACTIVITY (·······)
2
4
6
8
10
DAYS AFTER IMBIBITION

become inactivated, although still able to contribute to total activity measured *in vitro*. Thus new enzyme would be produced to degrade substrate in adjacent zones.

or:

2. As the zone of hydrolysis spreads the enzyme present is diluted, and so more must be produced to maintain the active concentration necessary for substrate hydrolysis.

Thus in both cases the high enzyme activities produced during germination are required for endosperm hydrolysis. Neither model was experimentally tested.

In maize experiments with cycloheximide have shown that continuous enzyme production is not necessary to maintain the normal rate of protein and starch hydrolysis (Figures 19 and 20). This indicates simply that excess enzyme is produced by germinated or incubated endosperms. Very large accumulations of hydrolytic enzymes have been observed in leaves during their senescence (103,113), apparently in excess of requirements for mobilization of nutrients. Loss of control of hydrolase production may be a phenomenon common to senescing tissues and organs.

The problem remains that enzyme activity measured *in vitro* increases steadily during germination, but the rate of substrate hydrolysis in the endosperm remains approximately constant (Figures 12 and 22B). Thus activity of the protease does appear to be progressively inhibited *in vivo*. Evidence in the literature indicates two possible mechanisms; accumulation

of endogenous protease inhibitors, or amino acid (end product) inhibition of activity. The former appears improbable since I have found no indication of maize protease inhibitors in the germinated caryopsis. If inhibitors do accumulate during germination, homogenates prepared from older endosperms might be expected to inhibit activity of protease extracted from younger endosperms. This did not occur. Similarly stepwise ammonium sulphate precipitation of proteins in crude extracts of germinated maize did not yield any fraction which inhibited protease or α-amylase activity. Endosperms of caryopses at different stages of germination were tested.

Protease inhibitors which have been isolated from ungerminated maize (59) and other caryopsis storage tissues are generally ineffective against the endogenous proteases (86,111). Metabolism of trypsin-inhibitory proteins has been carefully studied in pea (58) and kidney bean (146). In both cases the authors concluded that the inhibitors were not involved in regulation of protease activity during germination of the seeds.

End-product inhibition of protein breakdown in germinating caryopses is a more likely possibility, since it has already been observed in maize (124) and pea (196). To see a difference between *in vivo* and *in vitro* activities, enzyme activity in the endosperm rather than enzyme production

should be inhibited by accumulations of hydrolysis products. Oaks found that if maize caryopses were germinated with an amino acid mixture resembling endosperm protein (about 0.08 mg nitrogen/ml), protein breakdown in the endosperm was inhibited. Protease activities were not assayed, so either protease activity or production may have been inhibited. The effect of exogenous amino acids on protein breakdown or protease production in excised maize endosperms has not been tested. However excised endosperms incubated in small volumes of buffer accumulated very high concentrations of soluble nitrogen in the medium (over 2 mg/ml), and no inhibition of protease production or protein breakdown was observed (Table 29).

In peas Guardiola and Sutcliffe (53) observed no correlation between soluble nitrogen accumulation in the cotyledons and the rate of protein hydrolysis or protease production, with excised organs or intact caryopses. However Yomo and Varner (196) found that addition of 1% casamino acids (about 0.2 mg nitrogen/ml) to the incubation medium at the beginning of incubation inhibited protein breakdown in cotyledons or germinating peas. Extractable protease activity was also reduced, indicating that enzyme production rather than enzyme activity was inhibited by amino acids. It appears that addition of amino acids before the beginning of endosperm hydrolysis inhibits either protease production or activity (124,196), but accumulation of similar or greater concentrations

of amino acids during endosperm hydrolysis is not inhibitory (53 and Table 29). Furthermore there appears to be little accumulation of protein hydrolysis products in endosperms at the later stages of germination (64 and Figure 2). Thus end-product inhibition by accumulated amino acids does not readily explain the progressive discrepancy between _in vivo_ and _in vitro_ activity of proteases in the germinating seed. This requires further careful investigation, with study of the effects of exogenous amino acids on protease production by endosperms, at various stages of germination, or incubation of de-embryonated caryopses.

## Initiation of Storage Protein Degradation

Degradation of zein and glutelin in intact caryopses begins between 30 and 50 hours after imbibition (Figures 2 and 17), but acid protease activity does not appear until 3 days. Low activities of the acid protease could be present between 0 and 3 days, but not detected by the assay methods used. However this seems improbable since similar techniques were used to demonstrate traces of proteolytic activity in flour suspensions prepared from endosperms of ungerminated caryopses (Table 61). Thus the acid protease probably is not responsible for initiation of storage protein degradation. Both endo- and exo-peptidases are known to be present in ungerminated

caryopses (110,184) and proteolytic activity was measured in flour prepared from quiescent maize endosperm (Table 17). On imbibition of the seed these enzymes may initiate zein and glutelin hydrolysis.

Proteases have been demonstrated in protein bodies of ungerminated barley (128), hempseed (172), and squash (1). Abdel-Gawad and Ashton (1) have shown that protein bodies extracted from ungerminated squash seeds undergo autolysis when endogenous proteases are activated with cysteine. Paleg (132) found that barley aleurone protein bodies begin to disintegrate after 18 hours gibberellin treatment, and Yomo and Varner (195) observed vacuolation of aleurone cells due to disintegration of protein and lipid bodies during the first 48 hours of germination of barley. In maize the protein bodies may also have their complement of enzymes, and may begin to disintegrate before the acid protease activity appears at 3 days. However such enzymes only appear capable of limited proteolysis. When appearance of new protease activity was prevented by inhibitors such as cycloheximide and 6-methylpurine, only a small amount of total endosperm protein was degraded (Figure 20 and Table 38). This limited proteolysis may be adequate to denature the reserve proteins, since scission of only a small number of peptide bonds can cause extensive denaturation of storage protein (149). During early germination the enzymes present in the mature seed may: (i) break down

internal structure to make the zein contained in protein bodies and the cytoplasmic glutelin equally accessible, and (ii) denature the proteins to facilitate further degradation by the protease(s) which appear later. Careful study of the locations of proteases in the mature maize grain, and their activity during the initial hours of germination would clarify this problem.

Starch hydrolysis is not apparent until α-amylase activity appears in the endosperm of germinating caryopses or excised endosperms (Figures 12 and 20). This would indicate that newly synthesised α-amylase initiates breakdown of the starch reserves. However the methods used to determine endosperm starch breakdown were only approximate, dry weight loss and sugar release were measured. Internal cleavages of starch polymers could occur without loss in dry weight, or release of sugars. Hence starch breakdown could begin, undetected, before the appearance of α-amylase activity in the endosperm. A highly specific enzyme might be required to initiate degradation of the starch storage grains (6), and the activity of such an enzyme might not be detected by the α-amylase assay used. The experiments performed therefore do not conclusively show that newly synthesised α-amylase is responsible for initiation of endosperm starch hydrolysis. In view of this no clear comparison can be made of the initiation of starch and protein hydrolysis in maize endosperm.

Protein hydrolysis in suspensions of flour of ungerminated maize occurs in the absence of activating agents (Table 17). This contrasts with the requirements for sulphydryl agents in activation of squash protease (1), and for sulphydryl agents or protease in activation of wheat β-amylase (155). Thus initial protein breakdown in maize endosperm requires only imbibition, but subsequent protein hydrolysis depends on endopeptidase production. In many cereals this second stage of storage protein hydrolysis is regulated by interaction between the embryo or exogenous hormones, and the endosperm.

## Role of the Embryo and Hormones

Isolated maize endosperms produce hydrolytic enzymes and degrade their starch and protein reserves in the absence of the embryo or exogenous hormones (Table 31). Protease and α-amylase essential for protein and starch hydrolysis appear to be synthesised *de novo* in the endosperms (Figures 19 and 20). Enzyme activities appear earlier in the de-embryonated grains and correspondingly starch and protein hydrolysis proceed more rapidly than in intact grains germinating at the same temperature (Figures 16 and 18). MacLeod *et al.* (98) have also observed earlier production of hydrolytic enzymes in endosperm slices than in germinating grain. Hence the presence of the

embryo appears to retard hydrolytic processes in the endosperm. This negative regulation of hydrolase production is not described in the literature on cereal germination, and has not been further investigated in maize.

Autonomy of maize endosperm hydrolysis is noted in the literature (177), but conflicting reports also appear. Dure (36) observed that endosperm hydrolysis depended on the presence of the embryo, and α-amylase was principally produced by the scutellum, not the endosperm. Ingle and Hageman (66) found that some protein but little or no starch breakdown occurred in de-embryonated endosperms. Gibberellic acid was required for starch breakdown, and stimulated α-amino nitrogen release from protein three-fold. The exogenous hormone was found to replace a stimulus which moved from the embryo to the endosperm after 36 hours imbibition. On the other hand, all batches of maize which I have tested displayed little or no response to gibberellic acid, indoleacetic acid, kinetin, or combinations of these hormones. Since my experimental conditions were similar to those used by Ingle and Hageman (66), it can only be assumed that different batches of seed can respond differently to treatment with exogenous hormone.

Endosperms of non-dormant strains of oats can also produce α-amylase in the absence of exogenous GA (118). Since GA requirements in oats can be partially overcome by supplying amino acids (117), Naylor has suggested that amino

acid availability may limit enzyme synthesis in the aleurone. GA may regulate production or activity of a protease which releases the amino acids required for *de novo* synthesis of enzyme proteins. In GA-independent strains of oats the protease should be produced autonomously. Thus protein breakdown should preceed α-amylase synthesis and starch hydrolysis. This is not true of maize, since starch and protein hydrolysis begin simultaneously in both the GA-dependent maize (66) and in the GA-independent strains (Figure 12).

Variability in response to exogenous GA is also observed among batches of barley (68). Isolated endosperms or aleurone layers of some batches of grain produce no α-amylase in the absence of GA, others produce considerable amounts of the enzyme. These are called "high background" grain, and respond less markedly to GA than the "low background" varieties. This may be attributed to loss of gibberellin regulation, or to induction of α-amylase production by endogenous hormone.

Free and bound hormones have been found in many caryopses (8,100,150,160). Auxin esters and glycosides have been isolated from mature maize caryopses (39,179) and bound cytokinins and gibberellins from immature maize kernels (73, 112,142). Ross and Bradbeer (153) have suggested that bound hormones may be liberated during germination to stimulate growth processes. Hence a tissue or organ could be at least partially independent of an exogenous source of hormone.

To investigate the role of hormones in hydrolase synthesis in maize endosperm, abscisic acid was used. The mechanism of action of this growth regulator is disputed, but in many instances it appears to act as a hormone antagonist (176, 185). For example GA-induction of α-amylase in barley aleurone is inhibited by abscisic acid, and the ABA inhibition can be annulled by increasing the GA concentration (28). Similarly in maize addition of ABA to the incubation medium of excised endosperms strongly inhibited hydrolase production, as well as protein and starch breakdown. Addition of 3 μM GA reduced inhibition due to 2 μM ABA, and 30 μM GA completely overcomes this inhibition. Inhibitions due to higher concentrations of ABA were also reduced, but not abolished by 30 μM GA. Neither indoleacetic acid nor kinetin reversed the effects of abscisic acid (Tables 33 and 34). The specific interaction of ABA and GA indicates that gibberellic acid has a special role in regulation of hydrolase production in maize.

These observations (Tables 33 and 34) are contrary to Khan's evidence that kinetin overcomes ABA inhibition more effectively than GA (84). He proposed that a balance between cytokinins and inhibitors such as ABA controls germination, while gibberellins directly induce hydrolase production when this balance is favourable. The observations on GA-ABA interaction in control of hydrolase production in maize

endosperm cannot be reconciled with this hypothesis. Instead ABA appears to inhibit the actions of gibberellic acid directly, indicating that gibberellins are involved in the normal regulation of hydrolase production in maize endosperm.

In many seeds production of hydrolytic enzymes is not induced by GA, for example α- and β-amylases in pea cotyledon (196) and carboxypeptidase and isocitratase in cottonseed (63). In these cases enzyme production is sensitive to abscisic acid inhibition, but this cannot be reversed with gibberellins, or other hormones. Hence maize may contain a supply of gibberellins which stimulate the synthesis of protease and α-amylase when the grain takes up water. This process is inhibited by ABA and the inhibition can be overcome by supplementing the endogenous gibberellins with an exogenous supply.

To further investigate the role of gibberellic acid in regulation of enzyme production in maize, caryopses expected to be deficient in endogenous GA were used. Some dwarf-mutants of maize can be restored to normal growth habit by treatments with gibberellic acid (140). These single-gene mutations involve enzymes in the GA biosynthetic pathway (78,156), and both shoot and root tissues of the plants are deficient in gibberellins (141). Endosperms of mutant d-5 were found to produce low levels of α-amylase and protease when incubated in buffer, and addition of gibberellic acid

stimulated enzyme production five-fold (Table 35). This provides strong evidence that GA is involved in stimulation of protease and α-amylase production, and that normal maize endosperms have an adequate endogenous supply of the hormone.

Gibberellic acid could be present in the mature endosperm of maize, or could be synthesised in the imbibed endosperm. Kessler and Kaplan (81) found that isolated barley endosperms or aleurone layers have the capacity to produce GA, when treated with cAMP. In the absence of exogenous GA, cAMP causes an increase in endogenous GA, and α-amylase production. Both processes are prevented by inclusion of AMO-1618, CCC or Phosphon D (50-100 μM) in the incubation medium. These compounds block specific steps in the GA biosynthetic pathway (3,33,34,152,186), hence it appears that gibberellic acid is synthesised in cAMP-treated barley endosperms. Indirect evidence to the contrary is provided by Galsky and Lippencott (48). They found that these GA synthesis inhibitors (15-1500 μM) did not affect cAMP stimulation of α-amylase production by barley aleurone layers. This discrepancy has not been explained.

To determine whether GA biosynthesis occurs in maize endosperm, the effects of these inhibitors on α-amylase and protease production were tested. Concentrations which completely inhibited GA and α-amylase synthesis in barley (81) had little effect on enzyme production by maize endosperms

(Table 36). High concentrations partially inhibited protease and α-amylase production, but this appeared to be non-specific since the inhibition was not overcome by addition of gibberellic acid (Table 37). It appears, therefore, that enzyme production and endosperm hydrolysis in maize do not depend on synthesis of GA in the imbibed endosperm.

By elimination it appears that mature maize endosperms must contain bound or free GA, or GA precursors which may be converted to active forms of the hormone when the caryopses take up water. Measurement of endogenous hormones in resting and imbibed endosperms of dwarf d-5 and normal maize is necessary before definite conclusions can be made. However this research does indicate that endogenous gibberellins play a key role in stimulation of protease and α-amylase synthesis in maize endosperm.

## Site of Action of Gibberellins

The mechanism of hormonal stimulation of protease and α-amylase synthesis has not been determined. In cereals in the presence of GA α-amylase and protease are synthesised *de novo* (45,69, and Figure 19), and RNA synthesis is necessary for this process (18,117,180 and Table 38). The hormone may act at the level of transcription, promoting synthesis of m-RNA encoding the enzyme proteins (198).

Alternatively its action may be post-transcriptional (25), stimulating translation of existing m-RNA into protein. The m-RNA may be continuously synthesised, but not translated in the absence of the hormone, or a stable m-RNA may be present.

If a stable m-RNA is present, RNA synthesis inhibitors should not prevent enzyme synthesis. For example in cottonseed m-RNA for carboxypeptidase is produced at a defined stage of embryogenesis, but is not translated into enzyme protein until the caryopsis has matured and germinated. During this time RNA synthesis inhibitors do not block enzyme production (63).

RNA synthesis inhibitors do prevent enzyme production in cereal endosperms, but this is not necessarily proof that m-RNA synthesis is required for enzyme induction. The inhibitors might block production of other species of RNA essential for enzyme synthesis. Goodwin and Carr (51) have shown that synthesis of more than one type of RNA is necessary for GA induction of α-amylase in barley aleurone. They found that actinomycin D inhibits enzyme synthesis only if present during a short interval late in "lag" phase, between administration of GA and appearance of α-amylase. At later times the inhibitor penetrates the tissue, but does not affect enzyme synthesis. Other inhibitors, such as 6-methylpurine and 8-azauracil are inhibitory if added at any time during GA

treatment (28). A stable RNA may be synthesised late during "lag" phase, while other species of RNA whose syntheses are insensitive to actinomycin D may be made continuously.

The functions of these two types of RNA in GA-induction of α-amylase are not evident. Ribosomes do increase in number after GA treatment of aleurone tissue (42), and this involves new synthesis of RNA (60). However 5-fluorouracil, an inhibitor of ribosomal and transfer RNA synthesis in plants (82), does not inhibit α-amylase production in the endosperm (198). Gibberellic acid induction of α-amylase thus appears to require synthesis of RNA species other than r-RNA and t-RNA.

Zwar and Jacobsen (198) have observed stimulation of synthesis of polydisperse RNA in GA treated aleurone layers. This is first detectable at 4 hours after GA treatment, and increases steadily. It and α-amylase production are inhibited by actinomycin D and by abscisic acid, while neither are inhibited by 5-fluorouracil. Thus a correlation is established between GA-induced RNA and α-amylase synthesis. The RNA fraction synthesised is not r-RNA or t-RNA, and could be m-RNA for the various enzymes induced by the hormone.

Induction of synthesis of this polydisperse RNA fraction is unlikely to be the primary effect of GA in the aleurone, since the pattern of proteins being synthesised in the tissue changes within one hour of exposure to the hormone (46).

Effects on synthesis of lecithin also occur in the first hours of GA treatment (72). Thus we do not know the primary site of action of the hormone, nor all the specific processes stimulated by GA, nor whether it acts at the level of m-RNA synthesis or at a post-transcriptional level in regulation of hydrolase synthesis in barley aleurone. Observation of the effects of RNA and protein synthesis inhibitors on maize endosperms do not permit any speculation on the mechanism of action of gibberellins in this tissue.

## Model for Protease Activity and Storage Protein Degradation in Maize Endosperm during Germination

To summarise the information on storage protein degradation, protease activity, and hormonal control of these processes in maize, a simple model has been constructed (Figure 23). This model proposes the following sequence of events:

Day 0: The endosperm of the quiescent grain contains undenatured zein and glutelin, free or bound gibberellin, and inactive protease.

Days 1 and 2: Imbibition of the caryopsis activates latent protease, which denatures the zein and glutelin. No loss of total nitrogen occurs since small losses in storage proteins are compensated by increases in the soluble fraction (Figure 2). Imbibition may also permit release of bound gibberellin.

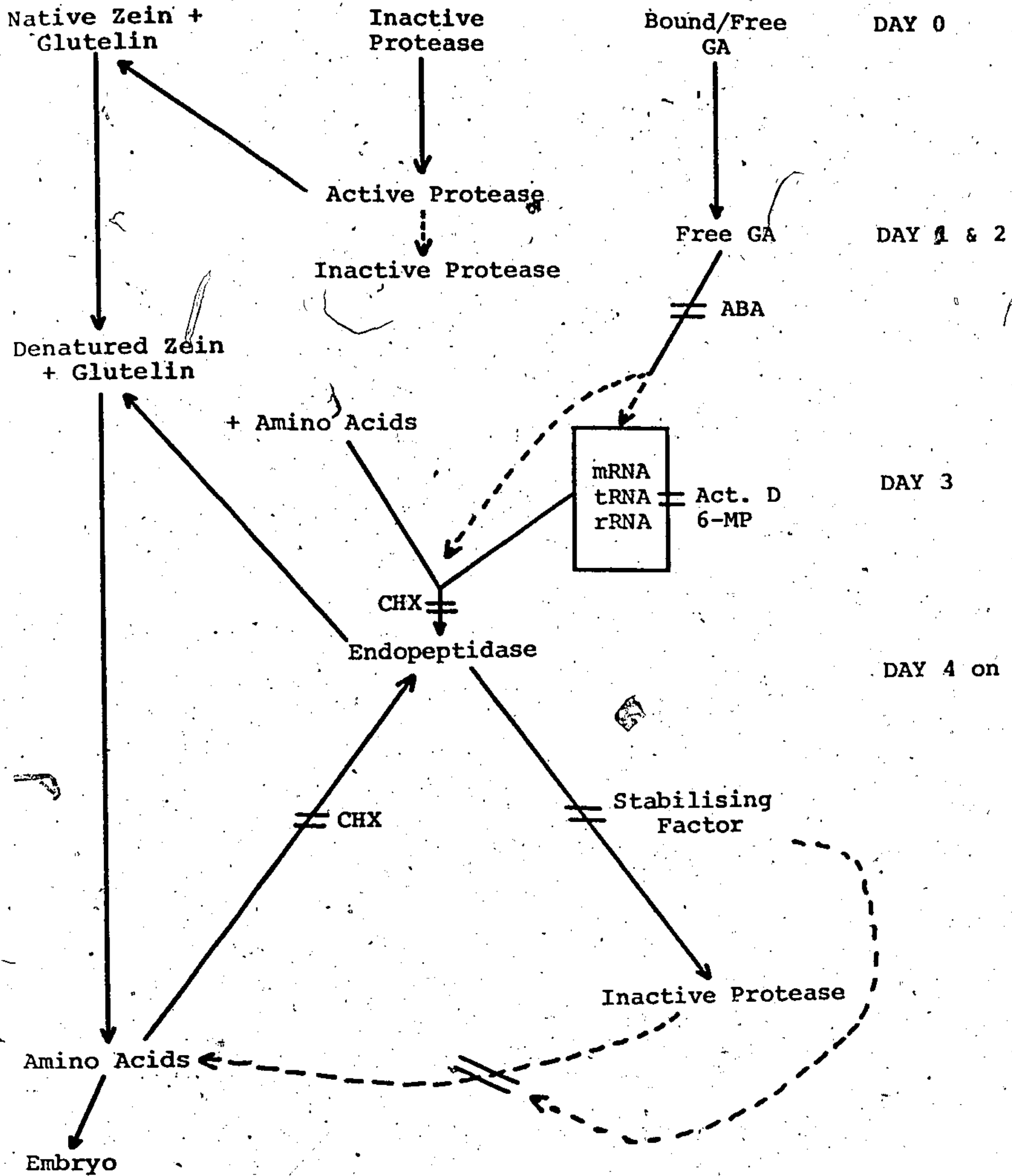
Day 3: Synthesis of acid protease(s) begins (Figure 10). This is stimulated by endogenous GA, but the site of action of the hormone is not known.

Days 4 to 9: Zein and glutelin are degraded with equal efficiency by the new protease(s) (Table 27) and consequently the more abundant protein disappears most rapidly. Amino acids released from zein and glutelin are transported to the embryo, or are incorporated into new endosperm proteins, for example protease and α-amylase. Protease activity increases steadily, synthesis exceeding inactivation. A small molecule "stabilising factor" may accumulate as endosperm hydrolysis progresses, and could modify the rate of inactivation of the protease(s).

Day 8: Zein and glutelin reserves are depleted, and proteolytic activity begins to decline.

FIGURE 23

Model for Development of Protease Activities and Protein Degradation during Germination of Maize


Native Zein + Glutelin
Inactive Protease
Bound/Free GA
DAY 0
Active Protease
Inactive Protease
Free GA
DAY 1 & 2
ABA
Denatured Zein + Glutelin
+ Amino Acids
mRNA
tRNA
rRNA
Act. D
6-MP
DAY 3
CHX
Endopeptidase
DAY 4 on
CHX
Stabilising Factor
Inactive Protease
Amino Acids
Embryo

Although this model is incomplete, it provides new insight into protein catabolism in germinating grain. The sequence of events in protein breakdown and activities of proteases can be compared. This permits realistic evaluation of the roles of hormones and hormonally stimulated enzymes in reserve protein hydrolysis in the germinating grain.

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APPENDIX

## TABLE 39

### Reproducibility of Protease Extraction

Two batches of endosperms were used. Batch A were from 5 day old seedlings, and Batch B from 7 day old seedlings. Four extracts were prepared from each batch. Twenty-five endosperms were used for each extract. Gliadin was employed as substrate, and protease activity was determined in triplicate for each extract. These values were averaged and expressed as μg tryptophan equivalents released per minute. Soluble protein per extract was determined as described in MATERIALS AND METHODS.

| Endosperms | | Protein in extract mg | Protease Activity | | |
|---|---|---|---|---|---|
| | | | Total extract | Per mg protein | Per endosperm |
| A | 1 | 3.14 | 85.0 | 27.0 | 3.40 |
| | 2 | 3.13 | 87.0 | 27.6 | 3.48 |
| | 3 | 2.90 | 83.4 | 28.6 | 3.34 |
| | 4 | 2.96 | 84.0 | 28.4 | 3.34 |
| B | 1 | 2.74 | 126.0 | 45.8 | 5.04 |
| | 2 | 2.85 | 137.5 | 48.2 | 5.50 |
| | 3 | 2.83 | 135.5 | 47.8 | 5.42 |
| | 4 | 2.80 | 132.0 | 47.0 | 5.28 |

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