To the Graduate Council:

I am submitting herewith a thesis written by Gary L. Fowler entitled "Effects of the Lysine Analog S-2-Aminoethyl-L-Cysteine on Growth and Metabolism of Germinating Barley, Hordeum vulgare L." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

Randolph R. Henke, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

X

EFFECTS OF THE LYSINE ANALOG S-2-AMINOETHYL-L-CYSTEINE ON GROWTH AND METABOLISM OF GERMINATING BARLEY, *HORDEUM VULGARE* L.

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Gary L. Fowler

August 1980

This thesis is dedicated to my family for
their encouragement and support.

ACKNOWLEDGMENTS

I am very grateful to Dr. Randolph R. Henke for his guidance throughout my initiation to research and for his advice during the writing of this thesis. I wish to express my appreciation to Dr. Beth C. Mullin for her interest in this thesis problem and for her careful review of the manuscript. I would also like to thank Dr. Raymond W. Holton for his comments and critique of this thesis.

This research was carried out at the Comparative Animal Research Laboratory operated for the U. S. Department of Energy under Contract No. DE-AC05-76OR00242 by The University of Tennessee. This research was supported in part by The University of Tennessee Agricultural Experiment Station.

I am appreciative to The University of Tennessee Agricultural Experiment Station from which the Graduate Research Assistantship was given.

ABSTRACT

The effects of the lysine structural analog, S-2-aminoethyl-L-cysteine (AEC), on germination and seedling growth of barley, Hordeum vulgare L., were studied. The AEC was inhibitory to germination and subsequent seedling growth. Lysine relieved growth inhibition caused by AEC. The appearance of α-amylase (E.C. 3.2.1.1.) activity in liquid incubation media of embryo-less barley half-seeds was inhibited by AEC. The reduced level of medium α-amylase activity did not appear to be attributable to an impaired aleurone secretory process because a parallel accumulation of enzyme activity was not found in this tissue. It may be, however, that AEC is inhibitory to secretion, to some extent, because a lower level of protein was detected in the media of half-seeds incubated in the presence of AEC. The level of reducing sugars released from endosperm starch, an indirect measure of α-amylase activity, was also decreased if half-seeds were exposed to AEC. When equimolar amounts of AEC and lysine were supplied to half-seed incubation media, the levels of α-amylase, reducing sugars and protein were restored to control values. The AEC appeared to be competing with lysine for incorporation into newly synthesized aleurone protein. These results are consistent with the hypothesis that AEC is functioning as a lysine antagonist in germinating barley seeds.

TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Much of the world's population depends on cereals as a principle protein source. The nutritional value of cereal protein is limited, however, by a deficiency in one or more of three amino acids, lysine, threonine, and tryptophan, each essential in the human diet (Hamilton, 1948; Swaminathan et al., 1969). Improving cereal protein quality, therefore, has been a primary aim of plant breeders for several decades.

A large effort has focused on altering ratios of the various storage proteins so as to render an improvement in the complement of essential amino acids. In recent years, extensive studies have provided new information about the chemical nature of the various storage proteins and on the mechanisms operative in their synthesis and deposition (Bogorad and Weil, 1977; Welsh, 1979).

The origin of amino acids for subsequent incorporation into cereal endosperm reserve protein has received relatively little attention. Delivery of preformed amino acids via the translocation stream and de novo synthesis are two likely possibilities (Sodek and DaSilva, 1977). Recent discoveries of several enzymes present in developing cereal endosperm and involved in the synthesis of lysine, methionine, and threonine have advanced the latter possibility (Gengenbach et al., 1978; Henke and Wahnbaeck, 1977, 1979; Oaks et al., 1979; Sodek, 1976, 1978; Sodek and DaSilva, 1977).

Should it be learned that de novo amino acid biosynthesis is operating at a substantial level in cereal endosperm and that it appears to

be under one or more regulatory constraints, the possibility then exists for selecting "overproducers" of essential amino acids. Such an overproducer, for example, could be a regulatory mutant which accumulates an amino acid to higher levels than normal either by failing to recognize feedback regulation or by failing to catabolize the over-accumulating species. Detecting such a mutant would require an effective selection screen.

Mutation-selection screens have been successfully used for the isolation of bacterial and fungal mutants with resistance to amino acid analogs. The lysine analog, S-2-aminoethyl-L-cysteine (AEC), is among the analogs used to select for analog-resistant strains of microorganisms (Sano and Shiio, 1970; Haidaris and Bhattacharjee, 1977, 1978; Takenouchi et al., 1977, 1979). S-2-aminoethyl-L-cysteine has also been used in screens with tobacco and carrot cells in suspension culture (Carlson, 1973; Widholm, 1972a, 1972b, 1976; Palmer and Widholm, 1975) as well as with barley seedlings (Bright et al., 1979a, 1979b). In many cases, the AEC-resistant plant-cell lines accumulate lysine to 10 times the level of the normal line.

A possible selection screen has been developed in this laboratory for the isolation of high-lysine mutants in barley seeds (Henke et al., 1979). Detection of such a mutant exploits the response involving the induction of α-amylase synthesis by gibberellic acid (GA_3) in a relatively simple assay. The assumption has been made that de novo α-amylase synthesis can be partially or wholly reliant on an available free pool of lysine in barley endosperm.

The assay proposed for this screen is the starch-agar/iodine assay (Ross, 1974). For this assay, a soluble potato starch (SPS) substrate

within an agar matrix constitutes the incubation medium. Barley seeds are cut in half transversely, the embryo-less half being placed on the medium, endosperm side face down. (The half containing the embryo can be saved for further analysis if needed.) After a period of incubation, an iodine reagent (I_2-KI) is applied to completely cover the surface of the agar. A deep blue background coloration of iodine-complexed potato starch is observed, and, if GA_3 was included in the medium, a clear region is observed around the periphery of each viable half-seed. Each clear region represents a zone where newly synthesized α-amylase, originating from the half-seed and secreted to the medium, has hydrolyzed the starch present within the agar matrix. Without GA_3 in the medium, α-amylase is not synthesized and, therefore, no zone of hydrolysis is evident upon application of the I_2-KI.

In preliminary experiments performed in this laboratory, the GA_3-induced α-amylase response was inhibited when AEC was included in the medium. Lysine supplied in combination with AEC, however, restored the response so that a clear zone of hydrolysis was observed. These observations suggested that AEC may be functioning as a lysine antagonist and may be inhibiting the development of α-amylase (i.e., the synthesis and/or subsequent secretion of a functional α-amylase enzyme) in barley half-seeds.

If conditions for the α-amylase assay on starch-agar could be manipulated so that embryo-less half-seeds respond uniformly positive (i.e., all seeds give a clear zone of hydrolysis) in the $+GA_3$ controls and uniformly negative (i.e., no zone of hydrolysis) in the presence of AEC, then the assay may be useful in a selection screen for half-seeds that render a positive response in the presence of AEC. Such half-seeds

may be resistant to the inhibitory effect of AEC on the α-amylase response because of a mutation which allows "overproduction" and accumulation of lysine to levels that are capable of outcompeting the deleterious effect of AEC.

The primary objectives of this thesis were to characterize the effect of the lysine analog AEC on the synthesis of a functional α-amylase in barley half-seeds and on the secretion of this enzyme to a liquid incubation medium. An additional question of primary importance was to determine whether the effects caused by AEC could be relieved by lysine. Information was also obtained regarding an AEC-effect on the release of half-seed protein and on the incorporation of radioactive amino acids.

CHAPTER II

LITERATURE REVIEW

Barley seeds offer a convenient system for investigating hormone-mediated biochemical responses involving several cereal seed tissues, namely the embryo, scutellum, aleurone, and starchy endosperm. Extensive studies with germinating seedlings and embryo-less half-seeds have revealed the following sequence of events which together represent a major facet of the early phase of germination: (1) imbibition facilitates diffusion of the plant hormone gibberellin (GA) from the embryo (primary site of synthesis) to the target tissue, the aleurone (Figure 1); (2) activation of some hydrolases, de novo synthesis of others, and secretion of hydrolases to the starchy endosperm are responses evoked by GA; (3) breakdown and mobilization of endosperm material occur as a result of the new hydrolase activity; (4) the hydrolyzed endosperm materials are supplied to the growing embryo through the scutellum.

Details of the GA-mediated effects on nucleic acid metabolism, enzyme synthesis and activation, membrane metabolism, and enzyme secretion and release will be described in the following sections and are illustrated in Figure 2.

The α-Amylase Response

Little understanding of the enzymatic hydrolysis of endosperm starch and protein existed prior to 1960, although the hydrolase-secreting property of cereal aleurone tissue had been suspected as early as 1890 (as referred to by Yomo and Varner, 1971; Briggs, 1973). Yomo,

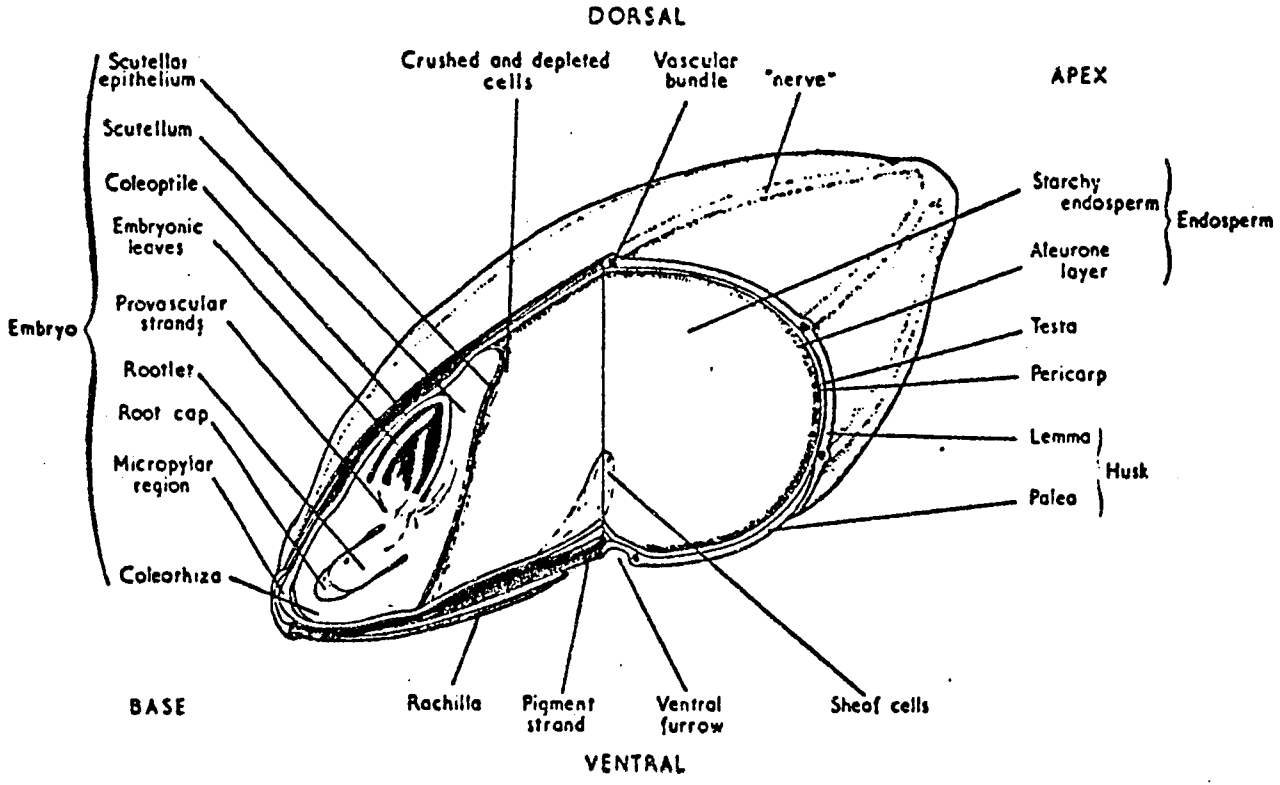


Figure 1. Anatomy of the barley grain.

Aleurone tissue lies between the testa, toward the outside of the grain, and the starchy endosperm. Aleurone is a layer of non-dividing cells (approximately three cells deep) the only living cels of the endosperm. Aleurone layers can be mechanically separated from the starch endosperm (Chrispeels and Varner, 1967a; Phillips and Paleg, 1972) and can respond to the presence of gibberellic acid with the production of hydrolases in the same manner as intact endosperm (Yomo and Iinuma, 1964; Varner, 1964).

Source: Briggs, 1973

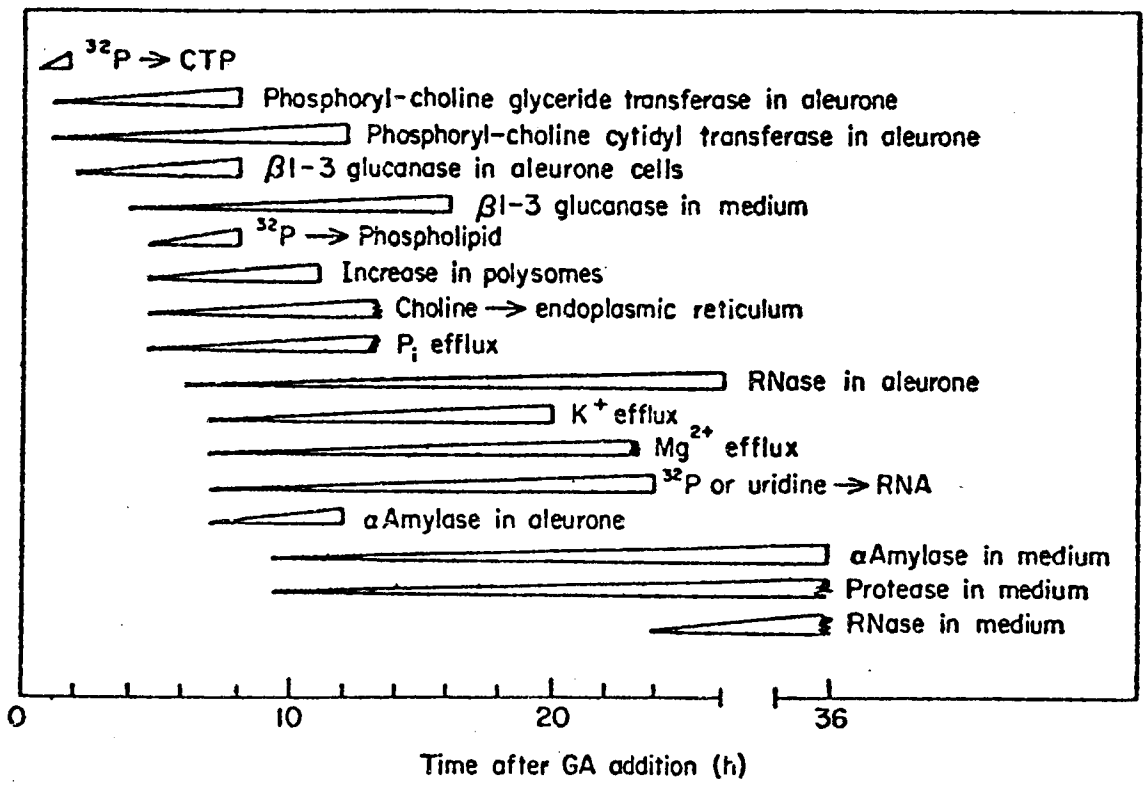


Figure 2. Metabolic changes that occur in barley aleurone cells after treatment with gibberellic acid.

Each triangle represents the time the metabolic change begins (left point) and the time the change is at its maximum (base). Triangles with serrated edges indicate that a maximum has not been determined.

Source: Trewavas, 1976.

in 1958 (according to Yomo and Varner, 1971), and Paleg (1960a) were among the first to ascertain that the factor evoking the degradation of the endosperm was gibberellin-like. It was proposed (Paleg, 1960a, 1960b) that GA, originating in the embryo, is secreted or is by some means transported to aleurone tissue to trigger a starch-hydrolyzing response, but proof for this mechanism was not available at the time of this suggestion.

An early suggestion for the mode of action of GA dealt with the hormone-mediated activation of the enzyme from an inactive form (Paleg, 1960a, 1960b). Briggs (1963), however, reported the α-amylase response to be a result of apparent GA-induced de novo synthesis. Evidence for this suggestion came from studies with ρ-fluorophenylalanine (PFP) and other amino acid analogs which reduced the incorporation of radioactive amino acids into protein (Briggs, 1963). Varner (1964) reported the incorporation of ^{14}C-phenylalanine into an α-amylase fraction, thus providing additional evidence for the de novo synthesis hypothesis.

Convincing evidence for GA-induced de novo synthesis of α-amylase came from the work of Filner and Varner (1967) with density-labeling of α-amylase molecules with "heavy water" ($H_2{}^{18}O$). Newly synthesized α-amylase labeled with ^{18}O migrated, upon centrifugation, to a position corresponding to a greater density in cesium chloride density gradient (Filner and Varner, 1967). The same technique was used to determine the mode of enhancement of protease activity (Jacobsen and Varner, 1967) and of ribonuclease and β-1,3-glucanase activities (Bennett and Chrispeels, 1972) in aleurone tissue of germinating barley. These enzymes were also demonstrated by this method to be synthesized de novo.

Ho and Varner (1978) showed that all α-amylase arising from treatment with GA_3 was a result of de novo synthesis, and not partially attributed to the activation of an inactive precursor. This was accomplished again by density labeling of α-amylase, but this time employing ^{13}C-labeled amino acids. Density-shifts as a result of incorporation of a heavy carbon were evident whether label was added at 0 hours or 12 hours after addition of GA_3. Density-shifts were accompanied by similar shifts in the radioactive profiles.

Since an increased level of α-amylase (and some protease activity) occurred as a result of de novo synthesis, rather than activation, it was appropriate to seek the source of amino acids necessary for new hydrolase production. Ultrastructural studies of aleurone cells (Jones, 1969a, 1969b) revealed the existence of a membrane-bound proteinaceous organelle with two types of inclusions. The generic term for these entities within many types of seeds is "protein bodies" (Pernollet, 1978), but as they also occur in the specialized cereal aleurone cells, they have been referred to as "aleurone grains" (Jacobsen, Knox, and Pyliotis, 1971). With exposure to GA_3, the aleurone grain was observed to swell, mainly in the peripheral proteinaceous region (Jones, 1969b). Varner and Chandra (1964) reported an increase in the size of the aleurone free amino acid pool when the barley half-seeds were incubated in the presence of GA_3. These two observations suggested that reserve protein in aleurone grains was hydrolyzed (accounting for swelling of the aleurone grain) to free amino acids for their subsequent incorporation into new hydrolase protein (Jones, 1969b).

Both α-amylase and protease begin to be synthesized and secreted between 8 and 10 hours after addition of GA_3 to the medium (Jacobsen

and Varner, 1967). This 8- to 10-hour period is often referred to as the "lag phase" prior to appearance of hydrolase activity. More recently (Higgins, Zwar, and Jacobsen, 1976), however, experiments employing *in vitro* cell-free translating systems revealed that the onset of *de novo* synthesis may be as early as two to three hours from the time GA_3 is added.

Properties of α-Amylase and the α-Amylase Response

Isozymes and Amino Acid Composition

Agar gel electrophoresis has been used to separate amylase isozymes in germinating barley seeds (Frydenberg and Nielsen, 1965). Nine was the maximum number of amylase forms found in any barley variety, five of which were shown to be α-amylase. Momotani and Kato (1966) observed three α-amylase isozymes in embryo-less half-seeds by separating a protein extract by DEAE-cellulose column chromatography and assaying the fractions for α-amylase activity. In a later study (Momotani and Kato, 1971), the microsomal fraction from isolated aleurone layers was put into a cell-free protein synthesizing system. Three isozymes of α-amylase were separated by electrophoresis. Separation of α-amylases from isolated aleurone layers revealed four isozymes, and all of these were shown to arise by *de novo* synthesis (Jacobsen, Scandalios, and Varner, 1970).

The amino acid composition has been determined for α-amylase that has been secreted from isolated aleurone layers of *Hordeum vulgare* L. var. Himilaya (Rodaway, 1978). An enzyme extract was subjected to sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis. The protein band which represented a molecular weight of 41,000, and

demonstrated to be α-amylase, was cut from the gel. The protein was eluted and subsequently subjected to an amino acid analysis in which lysine content was demonstrated to be 22 residues per molecule.

Experimental Conditions for the α-Amylase Response

Newly harvested seeds are not normally used for studies of the GA_3-enhanced response of α-amylase production as they typically contain higher amounts of "background" α-amylase (enzyme present in the dormant seed) (Jones and Varner, 1967). Investigations have typically utilized seeds that are at least four years old.

The addition of 10 mM $KBrO_3$ to incubation medium causes inhibition of hydrolysis of reserve protein in aleurone protein bodies (Melcher and Varner, 1971). Because the primary supply of amino acids (i.e., from protein body hydrolysis) for protein synthesis in the aleurone was lacking, subsequent expression of α-amylase activity (as well as other hydrolases synthesized de novo) was prevented. When the amino acids required for aleurone protein synthesis were supplied in the form of casein hydrolysate or as a mixture of the 20 protein amino acids, the expression of hydrolase activity was restored (Melcher and Varner, 1971; Ho and Varner, 1978).

When the divalent cation calcium (Ca^{2+}) was included in incubation media of detached aleurone layers, the level of α-amylase detected after GA_3-induced enzyme synthesis was comparable to the level detected in media of half-seeds (Chrispeels and Varner, 1967b). Prior to this discovery, isolated aleurone layers had yielded considerably lower levels of enzyme activity. Higher α-amylase activities were observed in Ca^{2+}-supplemented media than those observed in media containing other

divalent cations (Frydenberg and Nielsen, 1965; Chrispeels and Varner, 1967b). Alpha-amylase is apparently protected from attack by protease when Ca^{2+} is present (Yomo and Varner, 1971).

In characterizing the α-amylase response, Varner (1964) studied the effects of several metabolic inhibitors on the response. Incubation with dinitrophenol was severely inhibitory at 10^{-3} M, indicating a requirement for oxidative phosphorylation; with chloramphenicol, cycloheximide, or PFP in the medium, α-amylase activity was inhibited, suggesting a requirement for protein synthesis. In addition, half-seeds or aleurone layers could not be incubated under anaerobic conditions for maximum enhancement of α-amylase activity to be expressed (Varner, 1964).

RNA Metabolism

Several investigations have focused on the effects of GA on RNA metabolism. Studies with actinomycin D (Act D), a transcription inhibitor, revealed that α-amylase production was sensitive only if the inhibitor was added to the half-seed incubation medium within the first seven- to eight-hour period of exposure to GA_3 (Varner and Chandra, 1964; Varner, Chandra, and Chrispeels, 1965; Chrispeels and Varner, 1967b). If Act D and GA_3 were added simultaneously, expression of α-amylase activity was completely inhibited; if Act D was added four hours after GA_3, activity was inhibited less severely; if added eight hours after GA_3, Act D did not inhibit α-amylase activity at all. Furthermore, it was observed that the addition of Act D at any time resulted in inhibition of ^{14}C-uridine incorporation (Chrispeels and Varner, 1967b; Goodwin and Carr, 1972). [The amino acid analog PFP

(not itself an RNA inhibitor), however, inhibited the expression of the enzyme at any time following treatment with GA_3 (Varner, Chandra, and Chrispeels, 1965).]

It was hypothesized from the above observations (Chrispeels and Varner, 1967b; Goodwin and Carr, 1972) that a stable RNA component was synthesized in response to GA_3 between four and eight hours after addition of the hormone. Alpha-amylase formation, they proposed, is dependent on the synthesis of this component.

Another investigation into a GA_3-effect on RNA metabolism (Zwar and Jacobsen, 1972) revealed a small fraction of RNA, GA-RNA (corresponding to the 5S and 14S area of a polyacrylamide gel), that had incorporated labeled RNA precursors in response to GA_3 treatments at a greater rate than other RNA fractions. Moreover, α-amylase production and the enhancement of the GA-RNA occurred at the same time. When Act D was supplied to the incubation medium at the same time as GA_3, both the levels of GA-RNA and enzyme activity were reduced.

In more recent studies (Jacobsen and Zwar, 1974; Ho and Varner, 1974), it was demonstrated that the poly(A)-RNA fraction (presumed to account for some of the mRNA) was increased with exposure to GA_3. No effect was observed on the synthesis of rRNA or tRNA in response to GA_3-treatments (Jacobsen and Zwar, 1974b).

Ho and Varner (1974) studied the effect of cordycepin on the appearance of α-amylase activity (cordycepin is thought to terminate RNA chains during RNA synthesis). The production of α-amylase was inhibited drastically when cordycepin was added simultaneously with GA_3. Addition of cordycepin at successively longer times following addition of GA_3 resulted in successively less α-amylase inhibition until 12 hours,

when no inhibition was evident. They suggested from these results that it might be an association of mRNA in polysomes bound to endoplasmic reticulum (ER) which accounts for this accumulation or stabilization of mRNA.

More recently, Higgins, Zwar, and Jacobsen (1976) attempted to relate GA_3-stimulated α-amylase production to the level of α-amylase mRNA. Total RNA and poly(A)-RNA, separated by oligo-dT chromatography, from aleurone tissue that had been incubating in the presence of GA_3 for 0, 2, 4, 8, 12, and 16 hours, were used to program in vitro wheat embryo cell-free translation systems. Translation products of each incubation period were either separated by SDS-polyacrylamide gel electrophoresis or isolated by immunoprecipitation followed by subjection to SDS-polyacrylamide gel electrophoresis. Gibberellic acid appeared to increase the level of translatable mRNA for α-amylase. Furthermore, there was a positive correlation between the level of translatable RNA and the rate of α-amylase synthesis in vivo in response to treatments with GA_3.

Membrane Metabolism and Assimilation

Ultrastructural Studies

Barley aleurone cells from both unimbibed and imbibed seeds have been the subject of ultrastructural studies (Jones, 1969a). Endoplasmic reticulum is observed in both states. Ribosomes associated into polysomes are observed on the ER of imbibed cells.

As mentioned above, "lag phase" refers to the 8- to 10-hour period prior to detection of hydrolase activity in medium containing aleurone layers after treatment with GA_3. During this period, there appears to

be an increase in the amount of rough endoplasmic reticulum (RER) and a rearrangement or stacking of ER (Jones, 1969b).

At the time α-amylase is detected in the incubation medium, ER is seen as a membranous network throughout the aleurone cell. Beyond 14 hours and through 19 hours after treatment with GA_3, the ER appears to fragment and vesiculate. Between 19 and 22 hours, the number of vesicles decreases (Jones, 1969c).

Vigil and Ruddat (1973) also looked at barley aleurone cell ultrastructure and gave supporting evidence to a previous observation (Jones, 1969c) that vesiculation of ER in aleurone was occurring at the time aleurone tissue is secreting maximal amounts of α-amylase to the medium. The appearance of smooth vesicles, possibly ER-derived, fusing with the plasmalemma was also noted.

Biochemical Studies

Several attempts to quantify changes in the level of ER synthesis with time of treatment with GA_3 were made in order to substantiate the ultrastructural observations:

(1) Evins and Varner (1971) compared the levels of ^{14}C-choline incorporation into subcellular fractions (choline constitutes a part of lecithin, a major component of membrane phospholipids). They concluded that incorporation of label into a microsomal fraction was increased significantly in the presence of GA_3, thus suggesting that GA_3 had elevated the level of membrane (phospholipid) biosynthesis.

(2) Polysome levels were measured against time of tissue exposure to GA_3 (Evins, 1971). Enhancement of the level of polysomes

was observed as early as 3 to 4 hours of exposure to GA_3, with maximal levels measured at 12 to 15 hours.

(3) Activities of enzymes involved in the phospholipid biosynthetic pathway were measured with time of exposure to GA_3 (Johnson and Kende, 1971). Higher enzyme activities were measured after only two hours of treatment with GA_3, suggesting at least partial involvement in the GA_3-enhancement of membrane material. These enzymes were subsequently demonstrated to be activated rather than synthesized de novo in response to GA_3 (Ben-Tal and Varner, 1974).

The above observations seem to be consistent with the ultrastructural observations of enhanced levels of membranes, or ER, in the presence of GA_3. Other studies, however, revealed no particular change in membrane-related metabolism with time of exposure of tissue to GA_3:

(1) Koehler and Varner (1973) noticed no increase in ^{14}C-acetate incorporation into either chloroform-methanol-soluble (lipids) fractions or trichloroacetic acid-insoluble (protein and membrane) fractions.

(2) The level of lipid phosphorus was demonstrated to remain constant throughout the period of incubation (Koehler and Varner, 1973).

(3) The rate of total lipid- and phospholipid-synthesis remained constant throughout the first 12 hours and declined after 24 hours (Firn and Kende, 1974).

(4) The rate of ^{14}C-glycerol incorporation into a glycerolipid fraction during lag phase was reported to be no different in response to GA_3 (Firn and Kende, 1974).

Thus, no unequivocal biochemical evidence exists that shows an increase in ER metabolism or a shift in lipid synthesis from one class to another in response to GA_3.

Enzyme Secretion in Barley Aleurone: Two Conflicting Ideas

The mode of enzyme secretion in barley aleurone tissue has presented a problem in the basic understanding of the function of these specialized cells. Presently, two distinctly different ideas are expressed in the literature. One view favors packaging the hydrolases in ER-derived vesicles and eventually releasing these enzymes at the plasmalemma. The second view favors enzyme formation on RER and subsequent release into the cytoplasm as a soluble enzyme.

Secretion in Particulate Form

Ultrastructural evidence (Jones, 1969c; Vigil and Ruddat, 1973) revealing ER fragmentation and the concomitant appearance of vesiculation led to the following hypothesis: hydrolases are synthesized on RER, packaged in RER-derived vesicles, and transported in these vesicles to the plasmalemma where they are released. Vesicle-mediated transport of a hydrolase would mean that the hydrolase is isolated from the cell's own cytoplasm preventing degradation of the cell contents (Gibson and Paleg, 1975). They are transported via these vesicles to starchy endosperm where protein and carbohydrates are hydrolyzed for subsequent mobilization to the growing embryo.

Gibson and Paleg (1972), in their work with wheat aleurone, were among the first to provide evidence for α-amylase activity being associated with a subcellular particle. The suggestion was obtained from differential centrifugation studies. Pellets from spins of 20,000 x g and 105,000 x g were α-amylase enriched. Protease activity was also found in these pellets, but ribonuclease was not.

In an attempt to substantiate the particulate nature of α-amylase activity, Firn (1975) applied crude barley aleurone homogenates (after a 200 x g spin) to a Sephadex G-200 or Sepharose 4B column. Two peaks, a particulate peak and a soluble peak, of α-amylase activity were evident. Treating a homogenate with Triton X-100 eliminated the first peak in the elution profile while enhancing activity in the second peak. These results suggested the presence of a membrane-bound form of α-amylase. Percentage of particulate activity relative to total homogenate activity was increased when a dilute acid wash was incorporated into the procedure. Varner and Mense (1972) and Gibson and Paleg (1975) had shown that an acid treatment inactivated α-amylase associated with cell wall material (previously secreted enzyme) without damaging the cellular machinery. Gibson and Paleg (1975), using acid-washed wheat aleurone tissue, demonstrated approximately 80 percent α-amylase activity was of a particulate nature.

Attempts to purify a lysosome fraction from wheat aleurone homogenates using sucrose-ficoll linear density gradients were reported by Gibson and Paleg (1976). Good separations of enzyme activity were obtained: cytochrome oxidase, the mitochondrial marker; catalase, the microbody marker; and $NADH_2$-cytochrome-c reductase, an ER marker, were detected in distinct regions of the gradient. The lysosome fraction

contained both $NADH_2$-cytochrome-c reductase activity and α-amylase activity. This fraction, however, was contaminated by two types of particulates of undetermined origin.

Locy and Kende (1978) provided supporting evidence to the particulate- or membrane-associated nature of α-amylase. Results obtained with Sepharose 4B chromatography were similar to those of Firn's study (1975), i.e., a particulate α-amylase fraction was found in the void volume, and lost if treated with Triton X-100. A dilute acid treatment enhanced particulate α-amylase activity. Experiments with (^{3}H)-α-amylase in a homogenizing medium revealed that only a very small percentage of activity was attributable to non-specific binding resulting from the homogenization process.

Secretion in Soluble Form

In contrast to results mentioned above, Jones (1972) found less than 5 percent of total extractable α-amylase enzyme activity in all the particulate fractions combined following differential centrifugation. Specific activity of supernatant increased with higher centrifugation speeds, the opposite trend expected if α-amylase is particulate. Isopycnic centrifugation experiments also revealed only 4 percent of α-amylase activity to be present in the sediment fraction, with the balance remaining in the supernatant.

Several other lines of evidence also argued against the presence of a secretory intermediate form (Chen and Jones, 1974a):

(1) No lag time was observed in the release of labeled protein to the medium, a fact they cite as uncharacteristic of secretory proteins based on other studies.

(2) No appreciable α-amylase activity is evident in a microsomal pellet which does have high specific radioactivity, presumably attributable to nascent polypeptide chains associated with ER.

(3) No accumulation of label from (^{14}C)-tryptophan [characteristically, secreted hydrolases are rich in tryptophan according to Evins (1971)] is apparent in the sedimented fraction.

In another study, autoradiography was used to (1) serve as a check for cell-fractionation procedures, and to (2) serve as a visible label in a non-destructive method (Chen and Jones, 1974b). Nothing was observed that would suggest the presence of a labeled enzyme associated with a secretory organelle. Instead, label was first observed associated with ER and then, with increasing chase-time, dispersed throughout the cell cytoplasm (Chen and Jones, 1974b).

A subsequent study by Jones and Chen (1976) employed an immunohistochemical technique combined with ultracentrifugation of aleurone tissue pieces. Autofluorescence again was evidently not associated with a particular organelle. Thus, no conclusive evidence that overwhelmingly favors one of the hypotheses of secretion over the other currently exists in the published literature.

Amino Acid Analogs; S-2-Aminoethyl-L-Cysteine (AEC)

Amino acid analogs have long been recognized as important tools in genetic and metabolic studies. Prior to discussion of their utility, however, it would be appropriate to mention some of their characteristics. To be classified as an analog, a molecular species must exhibit basic similarities in molecular structure and in charge distribution to

its naturally occurring amino acid. Analogs commonly inhibit growth when included in growth media by interrupting some biochemical function in metabolism; inhibition can often be relieved by including, at a sufficient level, the naturally occurring amino acid in combination with the inhibitor. For example, growth of bacterial strains, Lactobacillus arabinosus and Leuconostoc mesinteroides, is antagonized by AEC (see Figure 3), a structural analog of lysine (Shiota, Folk, and Tietze, 1958). Inhibition of growth is relieved when lysine or a lysinyl-peptide is supplied in ample quantity to the growth medium.

A variety of biological studies have been made with amino acid analogs as metabolic probes. Such studies include investigations into (1) the mode of enzyme regulation in amino acid biosynthesis, (2) amino acid transport, (3) mechanism of action, substrate specificity, and reactive sites of enzymes, and (4) nucleotide biosynthesis (Umbarger, 1970; Lea and Norris, 1976).

Analogs have also been important in their utilization as selective pressures in mutation screens. For example, resistance in some organisms to what are normally inhibitory concentrations of analogs has been observed following mutagenesis. Strains of Brevibacterium flavum that grow in the presence of normally inhibitory concentrations of AEC have been isolated (Sano and Shiio, 1970). Lysine levels in these AEC-resistant lines are characteristically high; in addition, these mutants are not inhibited by the exogenous addition of L-threonine. Taken together, these observations suggested a mutation at the allosteric site of one of the branchpoint enzymes of the aspartate family of amino acids, e.g., aspartokinase (see Figure 4).

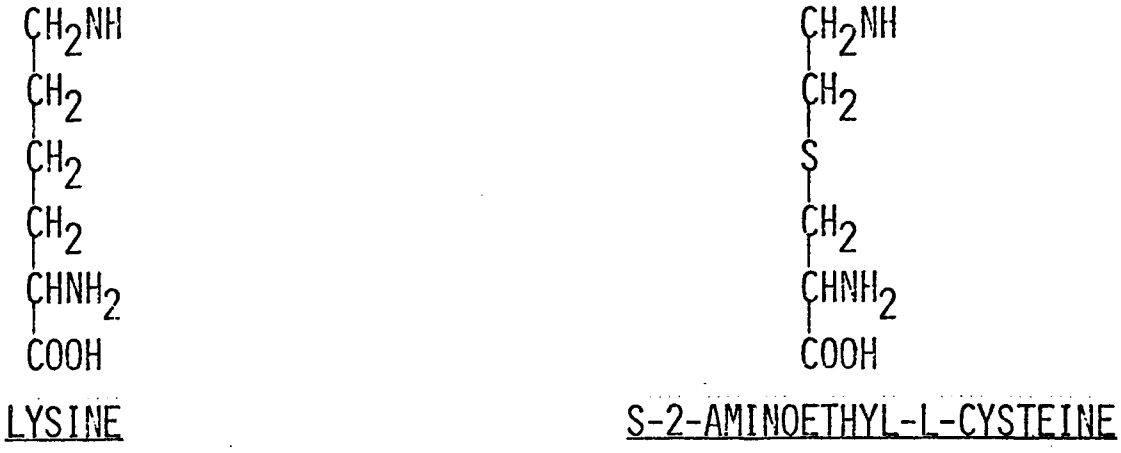
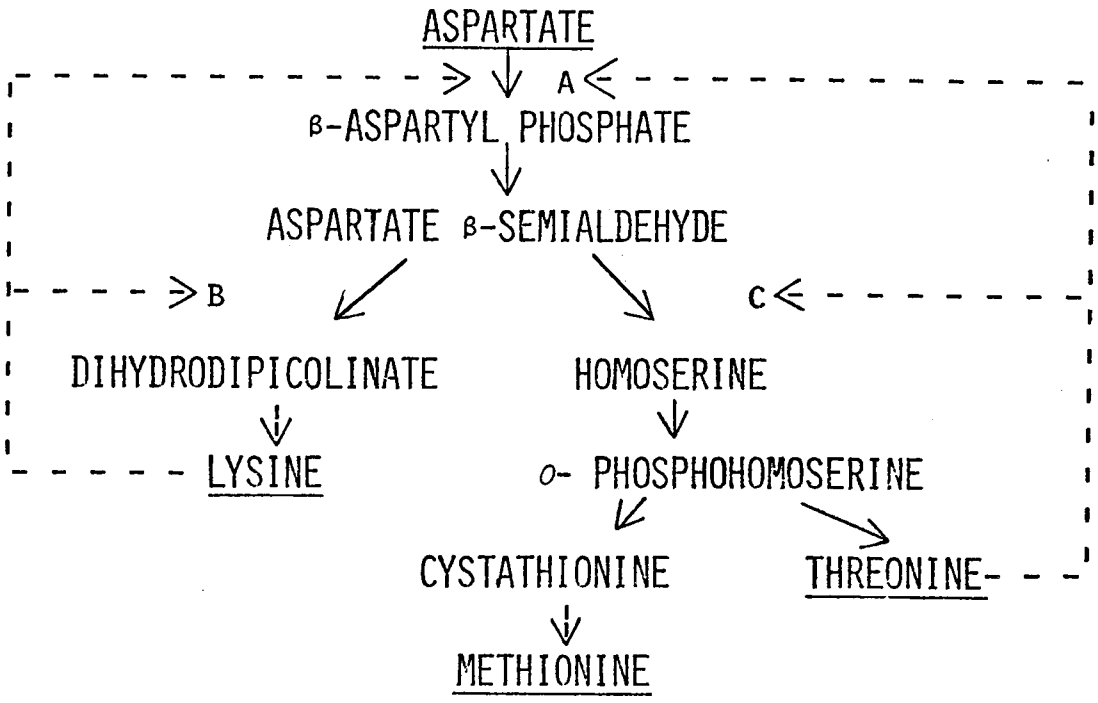


Figure 3. Chemical structures of the amino acid and one of its analogs, S-2-aminoethyl-L-cysteine (AEC).

The AEC molecule is structurally similar to lysine and functions as a lysine antagonist in barley growth and development.



A β-ASPARTATE KINASE

B DIHYDRODIPICOLINATE SYNTHASE

C HOMOSERINE DEHYDROGENASE

Figure 4. Simplified version of the biosynthetic pathway for the aspartate family of amino acids.

Dashed lines from L-threonine and L-lysine represent possible patterns of feedback regulation.

Mutant strains of a yeast, Saccharomyces cervisiae, with resistance to AEC, have been isolated. These organisms also produce high levels of lysine and excrete it to the medium (Haidaris and Bhattacharjee, 1977, 1978).

In studies with higher plants, analogs have been employed in selecting resistant lines of cells in suspension culture that accumulate higher levels of the corresponding amino acid than normal lines. For example, 5-methyltryptophan-resistant lines of Daucus carota, Nicotiana tabacum, and Solanum tuberosum have been isolated (Widholm, 1977), each of which is observed to accumulate over 20 times the amount of free tryptophan of normal lines.

Para-fluorophenylalanine-resistant lines of D. carota and N. tabacum have also been isolated (Palmer and Widholm, 1975). Accumulation of phenylalanine is observed in these resistant lines.

Lysine analogs, AEC and α-aminocaprylic acid, have been used to select resistant lines of D. carota (Miflin, 1975). More recently, lines of N. tabacum were found resistant to δ-hydroxylysine (also a lysine analog) and AEC (Widholm, 1976). In the same paper, reports were made of resistant D. carota lines to the proline analog, hydroxyproline, and the methionine analog, ethionine. Widholm (1976) reports that the level of the corresponding natural counterpart in each of these lines exceeded the level of normal by 10 times.

Amino acid analogs have been employed in studies on barley germination physiology as discussed above. In early studies, PFP was supplied to media containing barley half-seeds in order to observe the effects on α-amylase activity (Varner, 1964). Activity of the enzyme was substantially reduced when this analog was present in a concentration of

10^{-4} M. This was one of the experiments which led to investigations dealing with the nature of the appearance (i.e., de novo synthesis) of α-amylase activity in germinating barley.

CHAPTER III

MATERIALS AND METHODS

Plant Material: Preparation and Culture Conditions

Seedling growth studies were carried out with barley, Hordeum vulgare L. var. Atlas-57. All remaining studies were carried out with Himilaya barley, a huskless variety, obtained from Dr. Robert Nilan, Washington State University, Pullman, Washington. The seed material used was at least four years postharvest. More recently harvested seed material characteristically contains a higher level of background α-amylase activity (Jones and Varner, 1967), an undesirable trait for use in the α-amylase assays described below. The seed stocks were stored at 4°C. Whole seeds were used in the axenic seedling growth studies; embryo-less half-seeds were used in the remainder of the experiments. Half-seeds were prepared by cutting whole seeds transversely, approximately 4 mm from the distal end, with a scalpel.

Acid Dehusking

Atlas-57 seeds were dehusked according to the method of Briggs (1963). Up to 200 seeds were placed in a 100 mm x 80 mm pyrex storage dish containing 200 ml of 50 percent (v/v) sulfuric acid. The seeds were swirled on a magnetic stirplate for 90 minutes and then washed five times with 100-ml volumes of double-distilled water (ddH_2O). Next, the seeds were stirred for 20 minutes in a suspension of calcium carbonate (1 percent) and then rinsed with 100 ml of ddH_2O three more times.

Surface Sterilization of Seeds

Up to 200 half-seeds were surface-sterilized in 100 ml of a 1 percent NaOCl solution (commercial bleach diluted five times) and a few drops of a wetting agent (7-X detergent) for 20 minutes on a magnetic stirplate. Seeds were rinsed 5 times with sterile ddH_2O, washed for 10 minutes in 0.1 N HCl in order to remove residual NaOCl (Abdul-Baki, 1974), and rinsed 10 more times (or the number of rinses necessary to eliminate the smell of the bleach) with sterile ddH_2O.

Seedling Growth Studies

The effects of AEC and lysine, alone and in combination, on barley seedling growth were determined by measuring the height of the primary leaf after seven days of growth on water alone or on Murashige and Skoog basal nutrient medium (see Table XIII, Appendix, for medium formulation; Murashige and Skoog, 1962).

All media were prepared at twice the final concentration (AEC and/or lysine were added at this point) with the pH adjusted to 5.5. The media were filter sterilized with 0.20 micron Millipore filters, mixed gently with an equal volume of 1.6 percent agar, still hot from autoclaving, and dispensed to the bottom halves of sterile petri dishes (15 mm x 100 mm) in 100-ml volumes per petri dish. After the media solidified, 10 acid-dehusked, surface sterilized seeds were placed on the surface of the medium, evenly spaced, with the embryo side of the seed facing up (the pigment strand facing down). Sterile glass cylinders, 100 mm x 300, mm were affixed by a Parafilm wrap to the top edge of the petri dish containing the seeds. A petri dish cover was placed over the top of the cylinder and was sealed in place with a Parafilm

wrap. These sealed cylindrical incubation chambers were placed in an environment-controlled chamber for the seeds to be grown in constant light (approximately 1000 foot-candles) at 30°C for seven days.

Effects of AEC on the Expression of α-Amylase Activity

The effects that AEC and lysine, alone and in combination, have on the level of α-amylase activity present in and released from half-seeds were examined. Quantification of α-amylase activity released from the aleurone tissue after 24 hours or 48 hours incubation was carried out with either of two colorimetric assays, a reducing sugar assay, and a starch/iodine assay. Each assay is described below.

Incubation Conditions

The conditions for incubation of half-seeds were similar to those of Nicholls and Paleg (1963). Two surface-sterilized half-seeds were placed in an autoclaved specimen vial (24 mm x 50 mm) containing 1.0 ml of an incubation medium consisting of 10^{-6} M GA_3, 20 mM $CaCl_2$, and the appropriate concentrations of AEC and/or lysine. Buffer conditions and preincubation conditions are specified within the description of each assay. One of the vials per treatment condition contained incubation medium but no half-seeds; this sample was used for preparation of a blank in either assay. All vials were sealed, placed on a rotatory shaker in an environment-controlled chamber, and incubated with shaking at 100 rpm for 24 hours or 48 hours at 27°C in the dark.

Reducing Sugar Assay

The presence of free reducing groups of sugars released to incubation media was measured by the method of Coombe, Cohen, and

Paleg (1967). This measurement was regarded as an indication of α-amylase hydrolysis of its native substrate, starchy endosperm. Under strictly controlled conditions, the amount of copper (II) reduced in alkaline solution, is directly proportional to the concentration of reducing sugars in solution. Copper (II) ions react with exposed aldehydic carbons of carbohydrates to form a red copper oxide (CuO) precipitate. Upon addition of an arsenomolybdate reagent, a quantitative reduction of CuO occurs. A blue solution is formed in the reaction and the absorbance is measured at 560 nm (Clark, 1964).

Half-seeds were incubated in sterile ddH_2O instead of buffer. In some experiments, the surface-sterilized half-seeds were given a preincubation treatment which consisted of storing the half-seeds at 4°C for 24 hours in a glass petri dish containing enough sterile ddH_2O to cover the bottom of the petri dish, while not submerging the seeds completely. The release of reducing sugars was measured following half-seed incubation in the presence of AEC (from 1 mM to 30 mM), of lysine (at 5, 10, 20, or 30 mM) and of both AEC and lysine at several concentrations.

After incubation, 9 ml of ddH_2O and approximately one gram of ion exchange resin (Amberlite IR-120H) were added to each vial; all vials were then shaken for five minutes. Resin and half-seeds were separated from the diluted medium by Millipore vacuum filtration, using Whatman 540, 2.4 cm hardened ashless filter discs. One milliliter of Somogyi's alkaline copper reagent (see Figure 11, Appendix, for preparation of reducing sugar assay reagents) was added to 1 ml aliquots of diluted medium in clean test tubes. The solution was placed in a boiling water bath for exactly 10 minutes, after which it was placed in

an ice bath for approximately 5 minutes. One milliliter of the arsenomolybdate reagent was then added to the tubes. Contents of the test tubes were mixed thoroughly with a vortex mixer, diluted to 10 ml, and mixed again. The absorbance of this solution was determined at 560 nm with a Beckman Model 25 double beam spectrophotometer. Each absorbance measurement was determined against a blank which contained every assay component and treatment component in the same concentrations as were present in the treatments themselves.

Starch/Iodine Assay for α-Amylase Activity

The α-amylase activity was determined by measuring spectrophotometrically the hydrolysis of a soluble starch substrate. The starch remaining after a short incubation with the enzyme (present in half-seed incubation medium) was stained with I_2-KI and the absorbance of a starch/iodine complex was determined at 620 nm.

Preparation of the Soluble Potato Starch (SPS) Substrate

The starch substrate used in the assay for α-amylase was prepared according to the method of Ho and Varner (1978). A 0.15 percent stock substrate solution of SPS was prepared by adding 300 mg of SPS (obtained from Sigma Chemical Co.) to 200 ml of a solution which contained 25 mM KH_2PO_4 and 20 mM $CaCl_2$. This mixture was brought to a boil for 1 minute and then centrifuged at 12,000 x g for 15 minutes. The resulting clear, soluble starch supernatant was decanted to a flask and stored at 4°C. Starch solutions were prepared fresh the day the assays were performed.

Preparation of the Iodine Reagent

The I_2-KI was prepared fresh minutes before use by diluting 1 ml of I_2-KI stock solution (6 g KI and 600 mg I_2 per 100 ml ddH_2O, stored in the dark at room temperature) to 100 ml with 0.05 N HCl (Ho and Varner, 1978).

Preparation of Enzyme (α-Amylase)

After incubation, medium from each vial (1 ml/2 half-seeds/vial) was transferred quantitatively to an 8-ml test tube. Half-seeds and vials were washed with 1 ml of 50 mM KH_2PO_4 and 20 mM $CaCl_2$. The wash solution was combined with the incubation medium. If further dilution was necessary (as in the 48-hour treatments), 2 ml of 25 mM KH_2PO_4/20 mM $CaCl_2$ solution was added to bring the total volume of the enzyme preparation to 4 ml. Half-seeds that were to be homogenized for enzyme extraction were left in the incubation vials and frozen until time for extraction.

Alpha-Amylase Assay

The assay for α-amylase was performed according to the method of Ho and Varner (1978). One-half milliliter of the 0.15 percent SPS substrate solution was added to 1 ml of enzyme preparation with mixing to initiate the enzyme reaction. The 1.5 ml reaction mixture contained 0.05 percent SPS, 20 mM $CaCl_2$, and 25 mM KH_2PO_4. The reaction mixture was incubated for one to five minutes at room temperature. The reaction was stopped with the addition of 1 ml of I_2-KI. Four milliliters of ddH_2O was added to the final reaction mixture. Blanks were prepared by taking the same size aliquot from the treatment vials without seeds as those from the enzyme preparations and diluting them to

1.5 ml with a 25 mM KH_2PO_4/20 mM $CaCl_2$ solution. One milliliter of the I_2-KI and 4 ml of ddH_2O were added to this blank reaction mixture. Absorbance of the enzyme reaction mixture was determined at 620 nm with a Beckman Model 25 double beam spectrophotometer. The amount of enzyme and the length of incubation were adjusted so that the final absorbance reading was between 0.550 and 0.800. The arbitrary unit of α-amylase activity is expressed as ΔA_{620} $ml^{-1}min^{-1}$.

Extraction of α-Amylase from Half-seed Tissue

The effect of AEC and/or lysine on the level of α-amylase activity present in half-seed tissue was determined using the starch/iodine assay described above. Half-seeds were homogenized in 1 ml of a solution containing 25 mM KH_2PO_4 and 20 mM $CaCl_2$ with a hand-held glass homogenizer. The homogenate was transferred to a 15 ml clinical centrifuge tube. One milliliter of fresh homogenizing solution was used to wash the homogenizer and this wash was combined with the original extract. The extract was centrifuged at 500 x g for 10 minutes to remove cell debris. The supernatant was carefully removed from the pellet with a Pasteur pipet to a test tube. This extract was used as the source of α-amylase.

Radioisotope Incorporation Studies

The incorporation ^3H-(G)-L-leucine (sp. act. 55 μCi/μmol) and ^{14}C-(U)-L-lysine (sp. act. 270 μCi/μmol) into two half-seed fractions was determined, an ethanol soluble fraction and an ethanol-insoluble fraction (half-seed residue), after 24 hours of incubation. The effect

that several concentrations of AEC had on the incorporation of ^{3}H-leucine and ^{14}C-lysine into these fractions was determined.

Incubation Conditions

Three surface-sterilized half-seeds were placed in a vial containing filter sterilized media that was 10^{-6} M GA_3, 20 mM $CaCl_2$, 1 mM acetate buffer (pH 4.8), ^{3}H-G-leucine (0.5 µCi/ml) at 10^{-5} M, ^{14}C-U-lysine (0.1 µCi/ml) at 10^{-5} M, and various concentrations of AEC. Each vial was sealed and shaken on a rotatory shaker at 100 rpm in an environment-controlled chamber for 24 hours at 27°C in the dark.

Ethanol-Soluble and Residue Fractions

After incubation, the medium was drawn away from the half-seeds with a Pasteur pipet and placed in a test tube. Each vial containing half-seeds was washed with 2 ml of incubation buffer (1 mM acetate, 20 mM $CaCl_2$) that included 0.2 percent bovine serum albumin (BSA). The washes were combined with the respective incubation media in designated test tubes.

The three half-seeds per treatment were removed from the vial and placed in a screw cap test tube. One milliliter of 20 percent ethanol, which included lysine and leucine at 1 mM each, was added to each tube containing three half-seeds. Test tubes were capped tightly, placed in a boiling water bath for five minutes, removed from the bath and allowed to cool. The ethanol extract was removed with a Pasteur pipet and placed in another tube. This ethanol extraction procedure was repeated twice more. Pooled ethanol extracts were mixed and, if necessary, centrifuged to eliminate the cloudiness.

The remaining ethanol-insoluble residues were placed in cocktail vials containing 1.5 ml NCS tissue solubilizer (Amersham Corp.). These vials were placed in a drying oven at 50°C overnight prior to counting.

Liquid Scintillation Counting Method

All fractions described above were prepared for liquid scintillation counting on a Searle Mark III (Searle Analytic Inc.). Ten milliliters of Aquasol-2 were delivered to the vials containing the residual fractions. The samples were given from 0.5 to 1 ml of ddH_2O and were stored in the dark for 24 hours at 4°C so that chemiluminescence would be avoided. One milliliter of the pooled ehtanol extracts was placed in a cocktail vial and 10 ml Aquasol-2 was added to this fraction.

Determination of Protein Released from Half-Seeds

The level of protein released from half-seeds to the incubation media was measured by a modified microbiuret (Itzhaki and Gil, 1964) using BSA as a standard. Half-seeds were incubated for 24 hours at 27°C (as described above) in 1 ml of media containing 10^{-6} GA_3, 20 mM $CaCl_2$, 1 mM acetate buffer (pH 4.8), and appropriate amounts of AEC and lysine. After incubation, the media were pipetted away from the half-seeds. The half-seeds in the incubation vial were washed with 1 ml of fresh medium. The wash was combined with the medium. Protein was precipitated by mixing an equal volume of trichloroacetic acid (final concentration of 10 percent) with an aliquot of the diluted medium. The mixture was allowed to stand for 30 minutes in ice before centrifuging in a clinical centrifuge. The resulting pellet was resuspended in 1 ml of 0.1 N NaOH for subsequent protein determination.

CHAPTER IV

RESULTS

Growth Studies

The following experiments were performed in order to (1) observe the effect that lysine and one of its analogs, AEC, had on the germination and growth of barley seedlings and to (2) compare seedling growth in the presence of AEC on media with and without a nutrient supplement.

Measurements of primary leaf heights after seven days were used as an indication of barley seedling growth. The effect AEC had on seedling growth in H_2O or in Murashige and Skoog (MS) nutrient medium (Murashige and Skoog, 1962) is shown in Table I. Growth of seedlings on either medium was severely inhibited by millimolar levels of AEC. It appeared that seedlings grown on nutrient-supplemented media were slightly more resistant to the higher concentrations of AEC.

The ability of lysine to prevent the inhibition of growth by AEC was tested. Results of this experiment (Table II) indicate that lysine can partially prevent inhibition imposed by AEC. Complete prevention of growth inhibition was never obtained. This may be explained by the fact that lysine itself is partially inhibitory to growth at concentrations above 3 mM (Table III).

The Starch/iodine Assay for α-Amylase

Assays for α-amylase were performed with the media of various incubation treatments of embryo-less barley half-seeds. After 24 hours or 48 hours, each incubation medium was assayed for α-amylase activity; i.e., for a functional α-amylase that had been synthesized in the seeds

TABLE I

EFFECT OF S-2-AMINOEHTYL-L-CYSTEINE (AEC) ON GROWTH OF BARLEY SEEDLINGS[a]

Treatment (mM)		Shoot length (mm) after seven days of growth ± S. D.			
		H_2O	Percent of Control	MS	Percent of Control
AEC	0 (Control)	115 ± 43	100	157 ± 50	100
	0.02	136 ± 27	118	158 ± 13	101
	0.04	90 ± 21	78	134 ± 29	85
	0.06	86 ± 17	75	127 ± 16	81
	0.10	66 ± 30	57	83 ± 26	53
	0.20	56 ± 24	49	61 ± 18	39
	0.40	48 ± 23	42	47 ± 14	30
	0.60	45 ± 20	39	46 ± 13	29
	0.80	40 ± 20	35	43 ± 13	27
	1.00	37 ± 22	32	40 ± 14	25
	1.20	28 ± 21	24	35 ± 19	22
	1.40	23 ± 21	20	35 ± 14	22
	1.60	15 ± 16	13	37 ± 15	24
	1.80	13 ± 15	11	33 ± 15	21
	2.00	11 ± 16	10	32 ± 12	20

[a]Seedlings were grown axenically on 0.8% agar with or without a Murashige and Skoog nutrient medium, designated MS or H_2O, respectively. Each value represents the mean of 50-60 seeds of Atlas-57 from at least two experiments performed on separate occasions.

TABLE II

EFFECT OF S-2-AMINOETHYL-L-CYSTEINE (AEC) AND LYSINE (LYS) ON GROWTH OF BARLEY SEEDLINGS[a]

Treatment (mM)			Shoot length after seven days of growth ± S. D.	
			mm	Percent of control
Control (- amino acids)			161 ± 46	100
AEC	1.0		40 ± 14	25
	2.0		32 ± 12	20
AEC	1.0 + LYS	1.0	58 ± 24	36
		2.0	92 ± 39	57
		3.0	92 ± 39	57
		4.0	72 ± 45	45
		5.0	82 ± 47	51
AEC	2.0 + LYS	1.0	22 ± 19	14
		2.0	29 ± 17	18
		3.0	29 ± 24	18
		4.0	61 ± 28	38
		5.0	73 ± 31	45

[a]Seedlings were grown axenically on 0.8% agar containing a Murashige and Skoog nutrient supplement. Each value represents the mean of at least 20 seedlings grown in two petri dishes.

TABLE III

EFFECT OF LYSINE (LYS) ON GROWTH
OF BARLEY SEEDLINGS[a]

Treatment (mM)		Shoot length after seven days of growth ± S. D.	
		mm	Percent of control
LYS	0 (Control)	180 ± 28	100
	0.1	177 ± 23	98
	0.5	165 ± 31	92
	1.0	143 ± 38	79
	2.0	151 ± 30	84
	3.0	150 ± 17	83
	4.0	130 ± 27	72
	5.0	114 ± 20	63
	6.0	87 ± 28	48
	7.0	93 ± 13	52
	8.0	84 ± 16	47
	9.0	69 ± 25	38

[a]Seedlings were grown axencially on 0.8% agar containing a Murashige and Skoog nutrient supplement. Each value represents the mean of at least 30 seedlings from two experiments performed on separate occasions.

and secreted to the medium. Soluble potato starch was used as a substrate for the medium-derived α-amylase. After a period of incubation (one to five minutes) of α-amylase with SPS, the remaining unhydrolyzed potato starch was stained blue with I_2-KI. The change in absorbance was a linear function of enzyme concentration (see Figure 9, Appendix) and of time (see Figure 10, Appendix) if the reaction was terminated when the final absorbance reading was in the range between 0.550 and 0.800 (Varner and Mense, 1972; Ho and Varner, 1978). Arbitrary units of α-amylase activity are defined as the change in absorbance at 620 nm per milliliter of enzyme per minute ($\Delta A_{620} ml^{-1} min^{-1}$).

Background α-Amylase

A measurable level of α-amylase activity has been detected in imbibed half-seeds that had never been exposed to GA_3; such enzyme activity has been referred to by Jones and Varner (1967) as "background" α-amylase (perhaps a small percentage of the background activity can also be attributed to β-amylase).

For each experiment in this study, a measurement was made of apparent α-amylase activity that was not attributable to GA_3-induced *de novo* synthesis. Measurements of background α-amylase activity were made from incubation media consisting of 1 mM acetate buffer (pH 4.8) and 20 mM $CaCl_2$ without GA_3. The value obtained for background α-amylase activity in a given experiment was subtracted from each value determined for the experimental treatments incubated with GA_3 in the same experiment. Background α-amylase activity was usually less than 15 percent of the total α-amylase activity measured in the control treatment.

Effect of AEC on α-Amylase Activity (Starch/iodine Assay)

Experiments dealing with the effect of AEC on α-amylase expression (described in the following sections) reflect the level of enzyme activity present in the medium following synthesis and secretion by half-seeds incubated in the presence of AEC for a given period of time. In this experiment, however, AEC was introduced into the assay mixture of a -AEC control sample for the purpose of determining whether AEC affected α-amylase function. The highest concentrations ever provided in the incubation media (e.g., 10 mM and 20 mM) were tested in the assay mixture, albeit the AEC in the experimental treatments was always diluted at least 100-fold in the normal procedure. It was determined that 10 mM or 20 mM AEC had no effect on enzyme activity.

Effects of AEC and/or Lysine on Expression of α-Amylase

The α-amylase response to GA_3 in half-seeds was tested with various treatments of AEC (0, 1, 5, 10, 20, and 30 mM). The levels of enzyme activities after 24 hours and 48 hours of incubation appear in Figure 5, and Tables IV and V. A higher level of enzyme activity in the control and in every AEC treatment was evident at 48 hours in comparison to the levels determined in the 24-hour experiment.

When the data are plotted in terms of percent of the control levels of α-amylase activity, half-seeds incubated for 24 hours were more sensitive to inhibition by AEC than those incubated for 48 hours (Figure 6). The inhibitory effect of AEC on the ability of the half-seed to synthesize and release a functional α-amylase could be completely prevented when lysine was included in combination with AEC in the incubation medium (Figure 7, Tables IV and V).

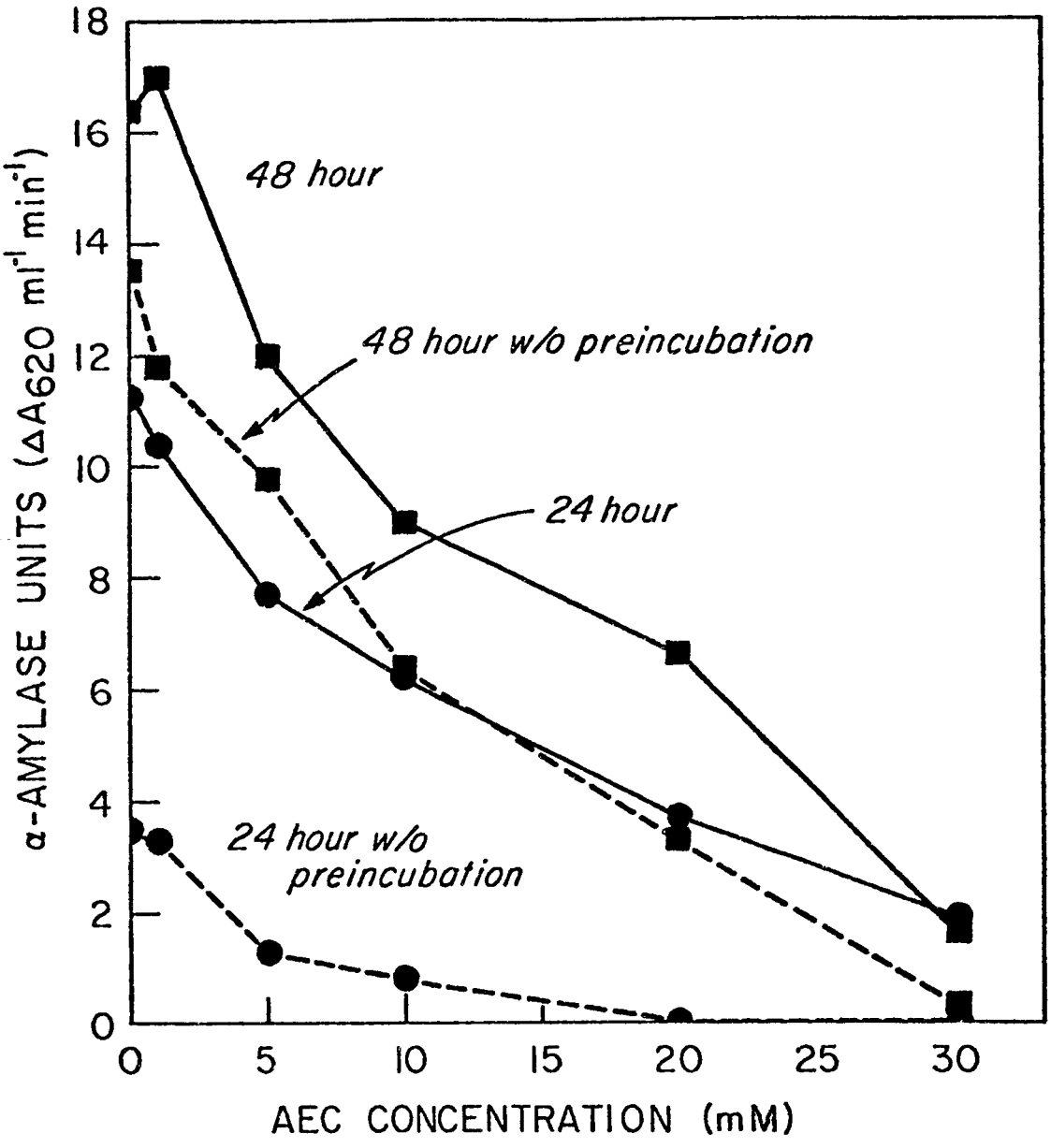


Figure 5. Effect of S-2-aminoethyl-L-cysteine (AEC) on the level of α-amylase detected in the incubation media of barley half-seeds.

Two surface-sterilized half-seeds were incubated in specimen vials containing 1 ml of 10^{-6} GA_3, 20 mM $CaCl_2$, 1 mM acetate buffer (pH 4.8), and AEC at the concentrations indicated. Vials were placed on a rotatory shaker set at 100 rpm and kept in darkness at 27°C for the duration of the incubation periods. Aliquots of the media were assayed for α-amylase according to the method of Ho and Varner (1978). Activities from half-seeds that received a preincubation treatment (partial submersion in sterile ddH_2O at 4°C for three days) are represented on the solid line. Each point on the solid line represents the mean value of six vials from two experiments performed on separate occasions. Each point on the dotted line represents the mean value of three vials.

TABLE IV

LYSINE RELIEF OF S-2-AMINOETHYL-L-CYSTEINE- (AEC) INHIBITION OF α-AMYLASE FORMED BY BARLEY HALF-SEEDS AFTER 24 HOURS[a]

Treatment (mM)	With preincubation[b]		Without preincubation[c]	
	Units of α-amylase ± S. D. ($\Delta A_{620} ml^{-1} min^{-1}$)	Percent of control	Units of α-amylase ± S. D. ($\Delta A_{620} ml^{-1} min^{-1}$)	Percent of control
Control	12.6 ± 1.4	100	3.7 ± 2.7	100
AEC 10.0	6.4 ± 2.3	51	0.8 ± 0.3	22
20.0	3.9 ± 1.8	31	0	0
LYS 10.0	13.6 ± 0.8	108	-	-
20.0	12.3 ± 0.8	98	4.4 ± 0.9	119
AEC 10.0 + LYS 5.0	9.0 ± 1.0	71	2.1 ± 0.06	57
10.0	12.1 ± 1.3	96	2.7 ± 0.08	73
20.0	12.4 ± 1.7	98	2.7 ± 1.4	73

[a]Two surface-sterilized embryo-less barley half-seeds were incubated in specimen vials containing 1 ml of 10^{-6} GA_3, 20 mM $CaCl_2$, 1 mM acetate buffer (pH 4.8), and AEC and/or lysine at the concentrations indicated. Vials were placed on a rotatory shaker set for 100 rpm and kept in darkness at 27°C. Aliquots of the media were assayed for α-amylase according to the method of Ho and Varner (1978).

[b]Half-seeds were preincubated (partially submersed in sterile ddH_2O) for three days at 4°C. Each value is the mean of at least six measurements froim six treatment replicates (two experiments of three vials per treatment).

[c]Each value is the mean of three measurements from three treatment replicates.

TABLE V

LYSINE RELIEF OF S-2-AMINOETHYL-L-CYSTEINE- (AEC) INHIBITION OF α-AMYLASE FORMED BY BARLEY HALF-SEEDS AFTER 48 HOURS[a]

Treatment (mM)	With preincubation[b]		Without preincubation[c]	
	Units of α-amylase ± S. D. ($\Delta A_{620} ml^{-1} min^{-1}$)	Percent of control	Units of α-amylase ± S. D. ($\Delta A_{620} ml^{-1} min^{-1}$)	Percent of control
Control	19.8 ± 1.5	100	13.5 ± 1.5	100
AEC 10.0	10.5 ± 2.3	53	6.2 ± 1.6	46
20.0	8.7 ± 2.9	44	3.7 ± 0.6	27
LYS 10.0	20.3 ± 0.5	103	-	-
20.0	19.6 ± 1.1	99	12.0 ± 0.4	89
AEC 10.0 + LYS 5.0	16.3 ± 2.9	82	10.5 ± 1.9	78
10.0	18.4 ± 3.4	93	11.7 ± 1.6	87
20.0	19.8 ± 3.9	100	13.4 ± 1.9	99

[a]Two surface-sterilized embryo-less barley half-seeds were incubated in specimen vials containine 1 ml of 10^{-6} GA_3, 20 mM $CaCl_2$, 1 mM acetate buffer (pH 4.8), and AEC and/or lysine at the concentrations indicated. Vials were placed on a rotatory shaker set for 100 rpm and kept in darkness at 27°C. Aliquots of the media were assayed for α-amylase according to the method of Ho and Varner (1978).

[b]Half-seeds were preincubated (partially submersed in sterile ddH_2O) for three days at 4°C. Each value is the mean of at least six measurements froim six treatment replicates (two experiments of three vials per treatment).

[c]Each value is the mean of three measurements from three treatment replicates.

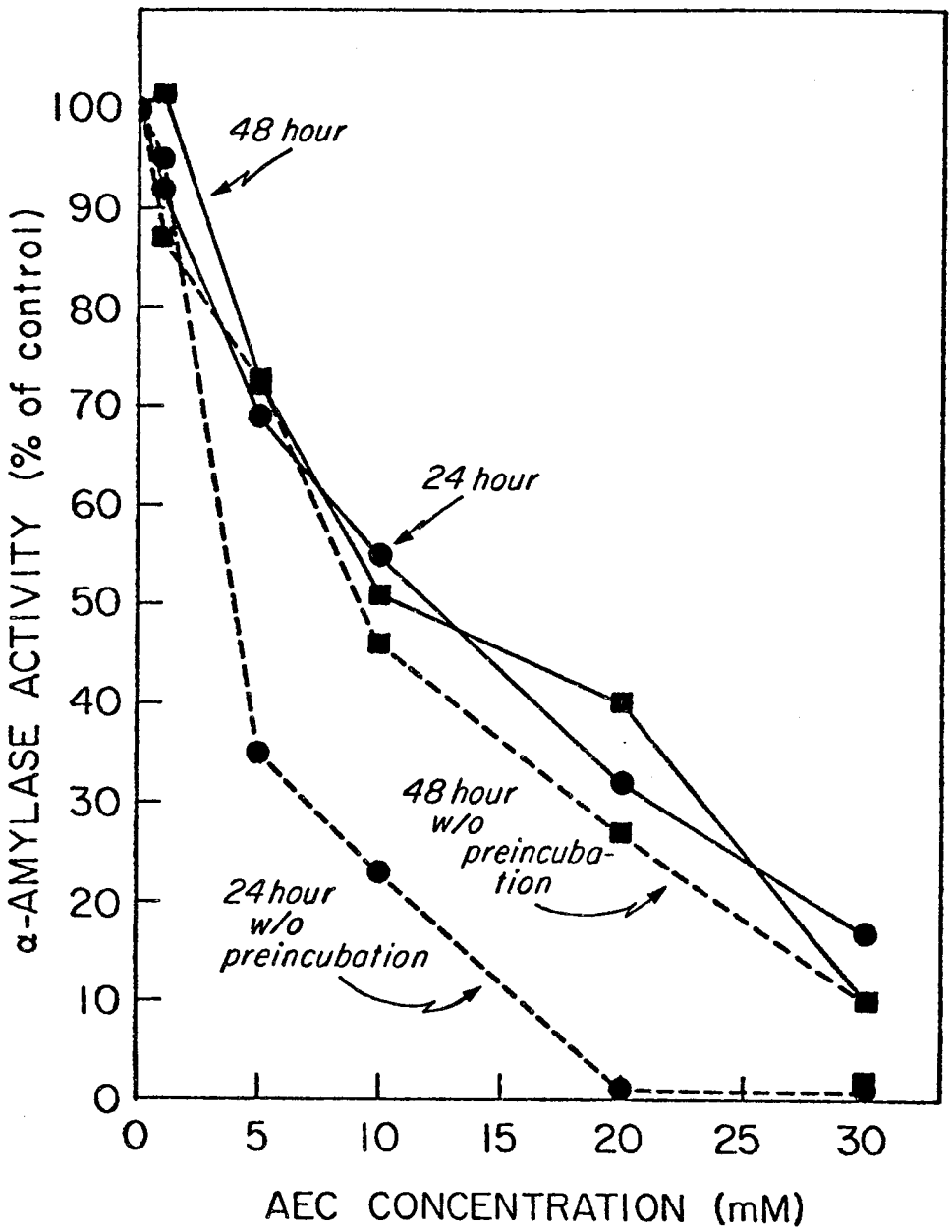


Figure 6. Relative effects of S-2-aminoethyl-L-cysteine (AEC) on the level of α-amylase detected in the incubation media of barley half-seeds.

Measurements from half-seeds that received a preincubation treatment (partial submersion for three days in ddH_2 at 4°C) are represented on the solid line. The data in this figure is another representation of the data appearing in Figure 5.

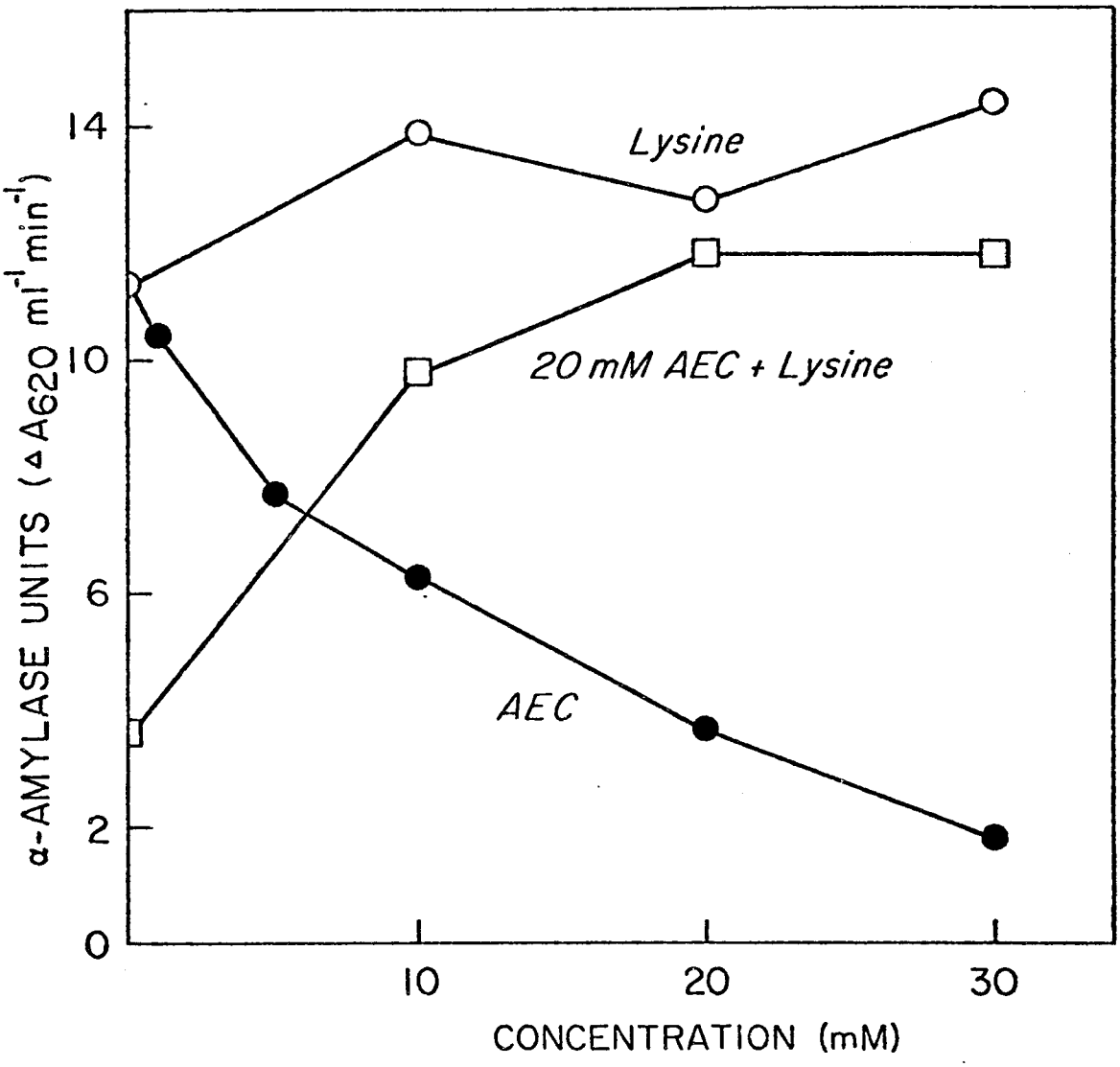


Figure 7. Lysine relief of S-2-aminoethyl-L-cysteine- (AEC) inhibition after 24 hours.

Half-seeds were preincubated three days in sterile ddH_2O at 4°C.

Effect of Preincubation

A higher level of enzyme activity was found in the incubation medium when seeds were given a three-day preincubation treatment in sterile ddH_2O at 4°C (Figure 5). [Structural changes occur in the cells of preincubated aleurone tissue in the absence of GA_3 (Jones, 1969a). Specifically, there appears to be a proliferation of ER and an organization of ribosomes into polysomes. There also appear to be changes in the ultrastructure of the proteinaceous aleurone grains. These observations suggest a preparation by the cells during imbibition for protein synthesis. Imbibed seeds, therefore, undoubtedly have a "head start," with respect to the onset of GA_3-induced protein synthesis, on unimbibed seeds.]

The experiments comparing α-amylase activities from treatments with and without preincubation are represented in terms of percent control activities in Tables IV and V. These data indicate that half-seeds preincubated at 4°C prior to 24 hours or 48 hours of incubation at 27°C in AEC were less sensitive to AEC than half-seeds that were not preincubated. The preincubation treatment enhanced the control level of α-amylase activity detected in the medium more than 300 percent (3.5 units of activity to 11.4 units) in the 24-hour experiment versus only 25 percent (13.5 units of activity to 16.7 units) in the 48-hour experiment. This suggests that preincubation may be affecting the lag phase prior to the α-amylase response rather than the rate of α-amylase synthesis.

Extraction of α-Amylase from Half-Seeds

Alpha-amylase activity was extracted from half-seeds (preincubated in sterile ddH_2O for 72 hours at 4°C) that had been incubated in the presence of AEC and/or lysine for 24 hours or 48 hours. The levels of extractable α-amylase from half-seed tissue of the various treatments, as well as the corresponding levels of activity in the media, are shown in Table VI and in Table VII. A decline in α-amylase activity obtained from half-seed tissue incubated in AEC was observed, though less dramatic than the decline in medium levels. A slightly higher level of α-amylase activity remained in the tissue of 10 mM and 20 mM AEC than that remaining in the tissue of the control treatment in the 48-hour experiment. More α-amylase activity remained in the tissue of the control treatments after 24 hours than after 48 hours despite the fact that total activity (tissue plus medium) was higher after 48 hours.

Effect of ρ-fluorophenylalanine (PFP)

The phenylalanine analog, PFP, has been used as an inhibitor of the de novo synthesis of a functional α-amylase (Varner, 1964; Varner and Chandra, 1964). Because the effect of PFP on α-amylase has been well documented, it was chosen for use in this study to provide a basis for comparison with the AEC-effect on α-amylase in half-seeds.

An α-amylase activity (24 hours incubation) dose response curve to PFP is shown in Figure 8. Inhibition of α-amylase activity by 10 mM PFP was relieved completely by 20 mM phenylalanine (Figure 8). The level of inhibition imposed by both the lysine analog (AEC) and the phenylalanine analog (PFP) on α-amylase formation and expression is similar. It is also evident that the inhibition of enzyme activity by each analog is

TABLE VI

EFFECT OF S-2-AMINOEHTYL-L-CYSTEINE (AEC) ON α-AMYLASE ACTIVITY (1) PRESENT IN THE MEDIUM AND (2) REMAINING IN TISSUE AFTER 24 HOURS INCUBATION[a]

Treatment (mM)	Units α-amylase ($\Delta A_{620} ml^{-1} min^{-1}$)			
	Medium	Tissue	Total	Percent of Control
Control	9.6	3.3	12.9	100
AEC 10.0	4.8	2.5	7.3	57
20.0	2.8	2.5	5.3	41
LYS 20.0	9.4	3.2	12.6	98
AEC 10.0 + LYS 20.0	9.1	3.1	12.2	95

[a]Half-seeds were preincubated three days in sterile ddH_2O at 4°C.

TABLE VII

EFFECT OF S-2-AMINOEHTYL-L-CYSTEINE (AEC) ON α-AMYLASE ACTIVITY (1) PRESENT IN THE MEDIUM AND (2) REMAINING IN TISSUE AFTER 48 HOURS INCUBATION[a]

Treatment (mM)	Units α-amylase ($\Delta A_{620} ml^{-1} min^{-1}$)			
	Medium	Tissue	Total	Percent of Control
Control	15.8	1.1	16.9	100
AEC 10.0	12.1	1.8	13.9	82
20.0	10.3	1.5	11.8	70
LYS 20.0	15.1	1.0	16.1	95
AEC 10.0 + LYS 20.0	13.8	1.1	14.9	88

[a]Half-seeds were preincubated three days in sterile ddH_2O at 4°C.

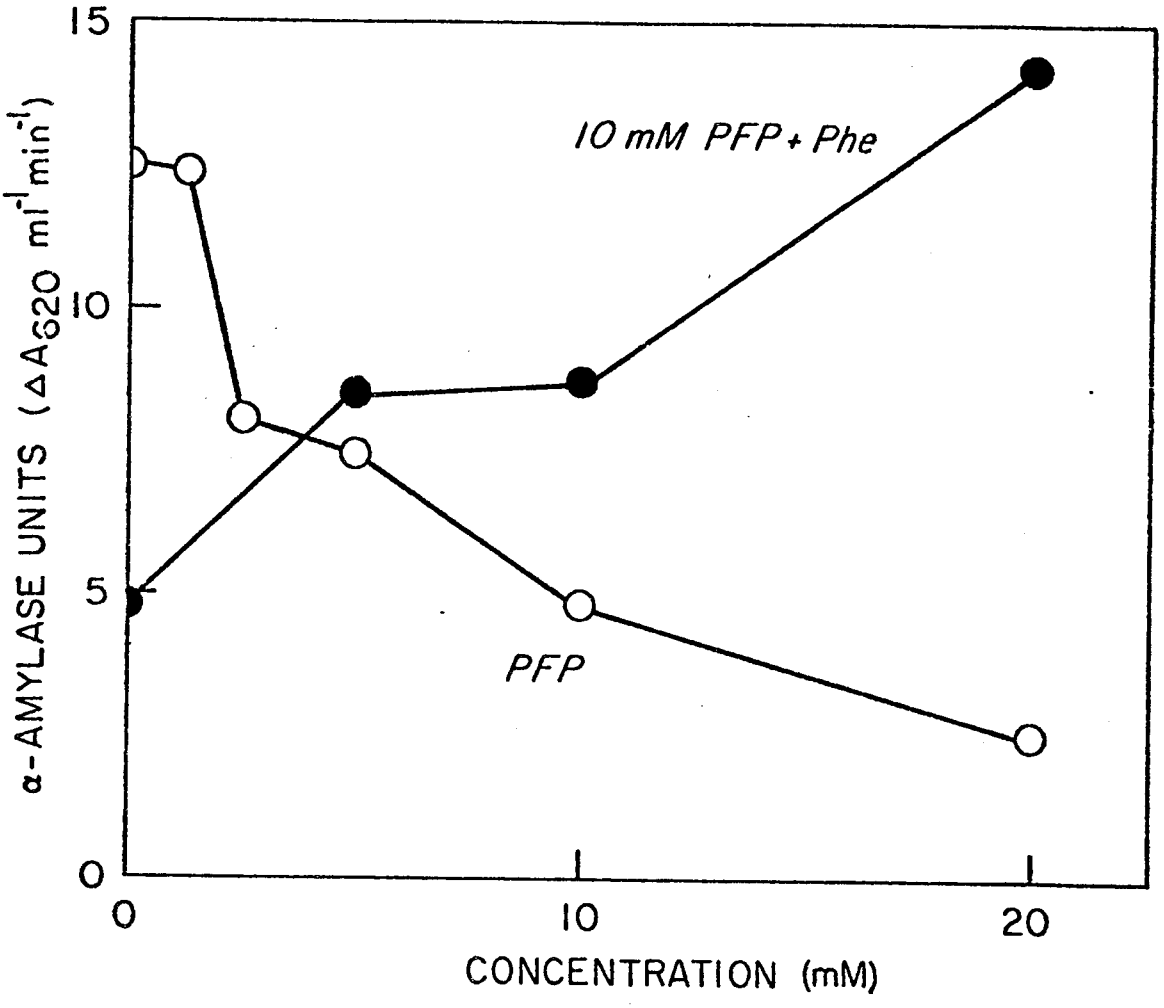


Figure 8. Effect of ρ-fluorophenylalanine (PFP) on the expression of α-amylase and relief of the PFP-effect by phenylalanine (Phe).

Half-seeds were preincubated three days in sterile ddH_2O at 4°C.

capable of being relieved by their respective naturally occurring amino acids, suggesting that each one acts in the manner of a competitive inhibitor.

Effect of Bromate, an Inhibitor of Proteolysis

Bromate is known to inhibit proteases present in endosperm and is used in the malting process of the brewing industry (Macey and Stowell, 1961). Melcher and Varner (1971) demonstrated the inhibitory effect of bromate on the development of α-amylase activity from aleurone tissue. They explained this inhibitory effect in terms of a reduced availability of free pool amino acids for synthesis of α-amylase because of a reduced level of hydrolytic activity of proteases on reserve protein.

The effect of bromate on α-amylase activity was tested in this study with Himalaya barley. The results are shown in Table VIII. Half-seeds preincubated in 10 mM $KBrO_3$ rather than H_2O for three days yielded less α-amylase activity in the medium after 24 hours incubation in 10 mM bromate (Table VIII). These results substantiate those collected by Melcher and Varner (1971).

Effect of Cycloheximide

Cycloheximide was used in the early studies in Varner's laboratory (Varner, Chandra, and Chrispeels, 1965) to determine if the α-amylase response to GA_3 was dependent on protein synthesis. They found that 5 μg/ml was approximately 90 percent inhibitory to α-amylase formation. In the experiments performed for this thesis, 5 μg/ml completely inhibited the formation of this enzyme, while 1 μg/ml was over 60 percent inhibitory.

TABLE VIII

EFECT OF BROMATE ON THE LEVEL OF α-AMYLASE ACTIVITY DETECTED IN THE MEDIA OF BARLEY HALF-SEEDS[a]

Treatment (mM)	α-Amylase $\Delta A_{620} ml^{-1} min^{-1}$			
	24 hours		48 hours	
	Units	Percent of control	Units	Percent of control
Bromate 0	12.3	100	19.5	100
1.0	8.4	68	12.6	65
2.5	5.8	47	7.7	39
5.0	5.0	41	6.0	31
7.5	2.6	21	2.6	13
10.0	1.0	8	1.0	5
10.0[b]	0	0	-	-

[a]Half-seeds were preincubated three days in sterile ddH_2O at 4°C.

[b]Half-seeds were preincubated three days at 4°C 10 mM $KBr)_3$ rather than in ddH_2O.

The Reducing Sugar Assay

Barley half-seed incubation media were assayed for reducing sugars after the half-seeds had incubated for 24 hours or 48 hours in various concentrations of AEC. Reducing sugars represent the products of enzymatic conversion of endosperm starch to mono- and disaccharide units; therefore, this assay served as an alternative, though indirect, measure of α-amylase activity on its "natural" substrate.

The reducing sugar assay was reliable when the final absorbance readings were between 0.5 and 1.5 (see Figure 11, Appendix). Table IX shows the levels of reducing sugars released by half-seeds in response to various treatments with AEC during 24 hours or 48 hours of incubation. The treatments within the 24-hour experiment evoke a slightly more sensitive response, i.e., a greater level of inhibition, than the same treatments of the 48-hour experiment if the results are considered in terms of percent of control value.

Paleg (1960) subjected half-seeds to a preincubation step wherein half-seeds were soaked in incubation media for 18 to 20 hours prior to exposure to GA_3. In the experiments reported in this thesis, all preincubation (24 hours at 4°C) and incubation treatments were made in sterile ddH_2O.

Prevention of inhibition by AEC was achieved in the reducing sugar response when lysine was included in the incubation medium in sufficient concentration (Table X). Lysine at 20 mM completely restored the level of reducing sugar released in the presence of 10 mM AEC to the level of the control. Lysine at 30 mM did not completely relieve inhibition by 20 mM AEC, but it did restore the "activity" to 75 percent of the control value from approximately 10 percent.

TABLE IX

EFFECT OF S-2-AMINOEHTYL-L-CYSTEINE (AEC) ON THE RELEASE OF REDUCING SUGARS FROM BARLEY HALF-SEEDS[a]

Treatment (mM)	Incubation time: 24 hours, µg sugar ± S. D.	24 hours, Percent of control	48 hours, mg sugar ± S. D.	48 hours, Percent of control
Control	285 ± 28	100	1.61 ± 0.20	100
AEC 1.0	238 ± 47	84	1.33 ± 0.20	83
5.0	90 ± 14	32	0.95 ± 0.11	59
10.0	85 ± 21	30	0.72 ± 0.27	45
20.0	54 ± 9	19	0.29 ± 0.10	18

[a]Two surface-sterilized embryo-less barley half-seeds were incubated in specimen vials containing 1 ml of 10^{-6} M GA_3, 20 mM $CaCl_2$, and the components indicated. Vials were placed on a rotatory shaker set at 100 rpm and kept in darkness at 27°C for the duration of the incubation periods. Following incubation, aliquots of the media were assayed for reducing sugars by the method of Coombe, Cohen, and Paleg (1967). Measurements were made from each of three vials per treatment per experiment. An average absorbance was calculated and the reducing sugar value was read for this average from the standard curve. The values in the table are mean values of at least four experiments performed on separate occasions.

TABLE X

LYSINE RELIEF OF S-2-AMINOETHYL-L-CYSTEINE- (AEC) INHIBITION OF THE RELEASE OF REDUCING SUGARS FROM BARLEY HALF-SEEDS[a]

Treatment (mM)			µg sugar released in 24 hours	Percent of control
Control			253	100
AEC	10.0		54	21
	20.0		23	9
LYS	10.0		229	91
AEC	10.0 + LYS	5.0	113	45
		10.0	227	90
		20.0	269	106
AEC	20.0 + LYS	10.0	124	49
		20.0	133	53
		30.0	191	75

[a]Two surface-sterilized embryo-less barley half-seeds were incubated in specimen vials containing 1 ml of 10^{-6} GA_3, 20 mM $CaCl_2$, and the components indicated. Vials were placed on a rotatory shaker set at 100 rpm and kept in darkness at 27°C for the duration of the incubation period. Aliquots of the media were assayed for reducing sugars by the method of Coombe, Cohen, and Paleg (1967).

Radioactive Amino Acid Incorporation Studies

The incorporation of two radioisotopes, ^{3}H-leucine and ^{14}C-lysine, into embryo-less half-seeds was observed with seeds that had been incubating in buffer containing various concentrations of AEC (0, 2, 5, 10, and 20 mM). In this experiment, incorporation of the isotopes into an ethanol-soluble fraction (free pool amino acids from the tissue), and an ethanol-insoluble residue fraction (presumed to represent protein) was observed.

Incorporation of label into the ethanol-soluble and ethanol-insoluble fractions is shown in Table XI. An approximate 80 percent increase in the level of ^{3}H-leucine incorporated into the insoluble fraction was observed with increasing AEC concentration. A less dramatic increase (approximately 25 percent) increase in the incorporation level of ^{14}C-lysine was observed in this same fraction. Corresponding decreases in the level of each isotope were observed in the ethanol-soluble fractions.

Effect of AEC on the Release of Protein

The protein present in half-seed incubation media was measured after half-seeds were incubated with AEC and/or lysine for 24 hours. It was apparent that AEC reduced the level of protein detected in the medium (Table XII). When lysine was provided in treatments containing AEC, the level of medium protein was restored to the level observed for control treatments.

TABLE XI

EFFECT OF S-2-AMINOETHYL-L-CYSTEINE (AEC) ON THE INCORPORATION OF ^{14}C-LYSINE AND ^{3}H-LEUCINE INTO TWO FRACTIONS IN BARLEY HALF-SEEDS[a]

		EtOH-Soluble		EtOH-Insoluble	
AEC (mM)	Isotope	dpm	Percent of control	dpm	Percent of control
0	^{14}C	3027	100	4356	100
	^{3}H	31268	100	34011	100
2	^{14}C	2861	95	4373	100
	^{3}H	30059	96	36859	108
5	^{14}C	2660	88	5032	116
	^{3}H	28263	90	46668	137
10	^{14}C	2409	80	5087	117
	^{3}H	25685	82	54560	160
20	^{14}C	2385	79	5473	126
	^{3}H	25076	80	61595	181

[a]Half-seeds were preincubated in sterile ddH_2O for three days at 4°c. Three half-seeds were incubated in darkness for 24 hours at 27°C in vials containing 1 ml of 1 mM acetate buffer (pH 4.8), 20 mM $CaCl_2$, AEC at the concentrations indicated in the table, cold lysine and leucine at 1 mM each, ^{14}C-lysine (0.1 µCi/ml; sp. act. 270 µCi/µml) at 10^{-5} M. Vials were placed on a rotatory shaker set at 100 rpm.

TABLE XII

EFFECT OF S-2-AMINOEHTYL-L-CYSTEINE (AEC) AND/OR LYSINE (LYS) ON THE RELEASE OF PROTEIN BY HALF-SEEDS TO INCUBATION MEDIA[a]

Treatment (mM)			µg protein released	Percent of control
AEC	0 (Control)		850	100
	2		810	95
	5		760	89
	10		560	66
	20		425	50
LYS	20		665	78
	30		675	79
AEC	20 + LYS	10	815	96
		20	830	98
		30	865	102

[a]Surface-sterilized half-seeds were preincubated in sterile ddH_2O for three days at 4°C.

[b]Three half-seeds were incubated in specimen vials containing 1 ml of 10^{-6} GA_3, 1 mM acetate buffer (pH 4.8), and 20 mM $CaCl_2$, plus the components indicated in the table. Vials were placed on a rotatory shaker set for 100 rpm and were kept in darkness for 24 hours at 27°C. Following incubation, the media were assayed for protein by a modified microbiuret assay (Itzhaki and Gil, 1964).

CHAPTER V

DISCUSSION

Effects of the lysine analog, AEC, on the germination and growth of barley seedlings from excised embryos and unimbibed seeds in axenic culture has been studied in this laboratory (Henke, unpublished results). It was demonstrated that AEC inhibits seedling growth in embryo-derived plants at considerably lower concentrations (approximately 10-fold lower) than those which impose the same levels of inhibition in plants grown from entire seeds. It was also observed that lysine, when supplemented in growth media containing AEC, could prevent this inhibition of growth. From these observations, it appeared that the presence of endosperm increased the seedlings' resistance to AEC. It was postulated that lysine, present in the endosperm tissue, may be in sufficient quantity to compete with AEC and consequently reduce the sensitivity of the seedlings to AEC-imposed growth inhibition.

The barley seedling growth experiments reported in this thesis were carried out in order to substantiate some of the work performed previously in this laboratory. If at least part of the growth inhibition by AEC in H_2O-grown seedlings (Table I, p. 36) can be explained by insufficient nutrient availability for the germinating embryo, it may be that seedling growth on MS nutrient media is less sensitive to AEC. Although the experiment represented in Table I represents an indirect approach to the question of how growth is inhibited, the data suggests that seedlings grown on MS media are less susceptible to inhibition by AEC at concentrations from 1.2 to 3 mM. Another experiment performd previously in this laboratory demonstrated that the growth of seedlings from

excised embryos on MS media was very similar to the growth from entire seeds, while no growth occurred when embryos were incubated on H_2O alone.

It is recognized that the different sensitivities to the high AEC concentrations reported here (Table I, p. 36) may be explained by other phenomena. The MS components, for example, may be causing a significant reduction in the uptake of AEC into the growing seedling.

The experiments described above, together with the observation that lysine could at least partially relieve AEC-inhibition of growth (Table II, p. 37), allow the suggestion that AEC is behaving as a lysine antagonist that is inhibiting one or more points of metabolism in the early phase of germination and/or growth. S-2-aminoethyl-L-cysteine has been shown previously to function as a lysine antagonist in microorganisms (Sano and Shiio, 1970), in plant cells in suspension culture (Widholm, 1976, 1977), as well as in barley seedlings (Bright et al., 1979a, 1979b).

Besides inhibiting barley growth, AEC retards the liquification process, or hydrolysis, of the starchy endosperm, as detected by visual inspection. This suggested that the function of the specialized aleurone tissue, i.e., the synthesis and/or activation and subsequent secretion of hydrolase protein may have been impaired. If hydrolases are less abundant or if their ability to function normally has been affected, then the nutrients required by the embryo, and normally provided via endosperm hydrolysis in the early phase of germination, may not be available.

Previous studies in this laboratory revealed that AEC inhibited the GA_3-induced α-amylase response, as expressed in the qualitative

starch-agar/iodine assay (see Introduction, p. 2; Henke et al., 1979), from barley half-seeds. Inhibition was relieved (the positive α-amylase response was restored) if lysine was supplied to the incubation medium in combination with the normally inhibitory concentration of AEC.

The inhibitory effect of AEC on the α-amylase response in half-seeds was more closely investigated (in this thesis work) by assaying for α-amylase activity in liquid incubation media. The assay used for this purpose was the starch/iodine assay (Varner and Mense, 1972; Ho and Varner, 1978). This assay is similar to the qualitative starch-agar assay in several respects: (1) the reaction substrate employed in each is SPS; (2) I_2-KI, in each assay, is used to stain the SPS remaining at the termination of an appropriate period of substrate hydrolysis by the enzyme; and (3) for enzyme to be detected in either assay, it is required that α-amylase be synthesized in response to GA_3 and secreted from the half-seed to the incubation medium.

The α-amylase response in AEC treatments of half-seeds was greater (less inhibition by AEC) if the seeds received a preincubation treatment (Figure 5, p. 41). Perhaps the preincubation treatment allowed earlier protiolytic attack of reserve protein, thus providing an increased availability of free lysine to compete with AEC. Higher levels of α-amylase activity were detected in media of 48-hour treatments than in media of 24-hour treatments (Figure 5; Table IV, p. 42; Table V, p. 43). These observations should be considered in determining potential conditions for the visual starch-agar/iodine assay (Introduction, p. 2). Length of the incubation period and preincubation conditions are two of the parameters (including SPS concentration, substrate volume, GA_3

concentration, and temperature) that will be tested in order to find a set of parameters which yield an optimal and uniform α-amylase response.

The α-amylase activity detected in the medium was restored to the levels observed for control treatments when lysine was supplied in combination with AEC in incubation media (Figure 7, p. 45; Table IV, p. 42 and Table V, p. 43). Whether the response to 10 mM or 20 mM AEC was being tested, the minimum level of lysine required to relieve AEC-inhibition was an equimolar concentration.

There are several potential explanations which may account for a reduced level of detectable α-amylase activity measured in the incubation medium. One possibility is a reduced capacity of the aleurone tissue, in the presence of AEC, to secrete the enzyme; the synthesis of normal α-amylase enzyme, in this case, may be unaffected. An impaired function of secretion, an active process requiring protein synthesis (Varner and Mense, 1972), may prevent the escape of enzyme from the aleurone tissue, resulting in an accumulation of α-amylase activity within the tissue. Both incubation media and tissue (aleurone plus endosperm) extracts were assayed for α-amylase activity (Table VI, p. 48 and Table VII, p. 49). No accumulation in the tissue of a functional α-amylase was evident.

A decrease in the level of the medium protein was evident if half-seeds had incubated in the presence of AEC (discussed further below). This fact alone would suggest an AEC-effect on secretion of protein. Because α-amylase activity did not accumulate in half-seed tissue, however, it was concluded that a loss of functional α-amylase activity occurred, possibly via insertion of AEC residues into protein in place of functionally or structurally critical lysine residues.

An alternative assay for α-amylase, the reducing sugar assay, was utilized in order to substantiate the results obtained from the starch/iodine assay for the AEC-effect on α-amylase activity. This assay is an indication of endosperm hydrolysis insofar as it measures the level of reducing sugars (present in the medium after a period of half-seed incubation) that have been cleaved from the enzyme's native substrate, endosperm starch (Paleg, 1960). Although several hydrolases, including α-amylase, are involved in hydrolyzing starch to eventually yield the reducing sugar product, this assay is a valid measurement of α-amylase activity in barley seeds for the following reason: the reduction of starch to reducing sugar units is dependent upon the GA_3-induced synthesis of α-amylase, whereas the synthesis and activation of the other hydrolases involved in starch breakdown are not GA_3-dependent.

Measurements with the reducing sugar assay were made in treatments similar to those of the starch/iodine assay experiments, i.e., treatment solutions contained concentrations of AEC alone (Table IX, p. 54) or of lysine and AEC supplied together (Table X, p. 55). Results from experiments with each assay (reducing sugar and starch/iodine) appeared to substantiate the other in terms of (1) relative levels of inhibition by AEC and (2) the concentrations of lysine required to restore each response to the levels of the controls.

Protein measurements in incubation media after 24 hours indicated that there was less protein from treatments that included AEC (Table XII, p. 58). From these results alone, several possible explanations may be offered: (1) secretion of protein by the half-seeds to

incubation media was impaired; (2) protein synthesis was inhibited; or (3) an increase in proteolytic activity occurred.

Lysine at 30 mM was able to restore the level of protein present in the medium from 50 percent of the control value, in the presence of 20 mM AEC, to slightly above 100 percent. The experiment showed that this particular response to AEC is reversible and the competitive behavior of lysine and its analog was demonstrated once again.

Experiments involving the use of radioactive amino acids provided some grounds for speculation with respect to incorporation into the residue fractions (Table XI, p. 57). Incorporation of ^{3}H-leucine increased by more than 70 percent in the presence of 20 mM AEC. Approximately 20 percent more ^{14}C-lysine was incorporated in the same treatment. Two of the possible explanations for these results are the following:

(1) Secretion of protein by aleurone is hindered, while protein synthesis may or may not be affected. If secretion of protein is being affected by AEC, the build-up of protein within the tissue might be reflected by an increased level of radioactivity. Although a functional α-amylase was not demonstrated to be accumulating in tissue, it is possible that a non-functional species of the molecule exists with some AEC residues replacing lysine residues in functionally or structurally critical positions [22 lysine residues have been reported present in barley α-amylase (Rodaway, 1978)].

(2) Secretion is not affected, but the quantity and/or quality of protein is altered. Increased radioactivity in the residue fraction may reflect a change from the complement of proteins

normally produced by the aleurone. S-2-Aminoethyl-L-cysteine may be interrupting the process so as to induce the synthesis of polypeptide fragments, or incomplete proteins, rather than complete molecules (Prouty, Karnovsky, and Goldberg, 1975). This, in part, would explain the lack of accumulation of a functional α-amylase in the tissue and also, the reduction in the level of protein recovered from the incubation medium.

Incorporation of label into the protein fraction that is present in the medium following incubation would presumably be considerable, because a significant portion of the medium protein is de novo synthesized hydrolase protein. The attempt to isolate this protein fraction and to subsequently measure the incorporation of label yielded inconclusive results. Protein was precipitated with phosphotungstic acid (PTA), supplied in a one-to-nine ratio. Nearly half of the 0.1 μCi ^{14}C-lysine provided in the 20 mM AEC incubation treatment appeared in the PTA-insoluble fraction following 24 hours of incubation. One-tenth as much radioactivity was incorporated into the insoluble fraction of the control treatments (minus AEC).

To determine whether or not the apparent incorporation was biological, labeled lysine (0.1 μCi) was added to the control medium after half-seed incubation, protein was precipitated, the insoluble fraction was washed with cold lysine, and the fraction was hydrolyzed and counted. Again, half the label initially provided was associated with this fraction. It was concluded that the apparent incorporation was not a biological phenomenon.

It was later determined that PTA precipitates amino acids, present in millimolar concentrations, from solution. This occurrence would

appear to account for much of the radioactivity in the PTA-insoluble fraction. Further attempts to monitor incorporation into the medium protein fraction could be attempted using a different precipitating agent, such as trichloroacetic acid.

To investigate more closely the possibility of AEC-replacement of lysine residues in de novo synthesized protein, at least two methods of approach are available. One approach involves the use of ^{14}C- or ^{35}S-labeled AEC molecule. Following a suitable period of half-seed incubation in the presence of labeled AEC, protein can be extracted, subsequently separated by polyacrylamide gel electrophoresis (under denaturing conditions) and prepared for autoradiography. Labeled protein bands can be detected on the autoradiogram. If a labeled polypeptide would migrate to a position corresponding to an α-amylase standard, it may indicate that the AEC molecule had been incorporated into the α-amylase enzyme.

A second approach involved the isolation of barley protein that has been secreted to the medium by half-seeds incubating in the presence of cold AEC. This protein is isolated and analyzed by gas-liquid chromatography or an amino acid analyzer to determine if AEC had been incorporated into de novo synthesized protein.

CHAPTER VI

CONCLUSIONS

From the data presented in this work, it was evident that the lysine analog, AEC, (1) inhibits barley seedling growth during the first seven days of germination, (2) induces the loss of functional α-amylase activity in barley half-seeds, (3) inhibits the release of reducing sugars from endosperm starch to the incubation medium, and (4) possibly obstructs the process of secretion of total protein from aleurone tissue. When lysine is supplied to incubation media with AEC, the rate of seedling growth is partially restored, and functional α-amylase activity, the level of reducing sugars, and protein, all detected in the media, are all restored to the levels observed for the controls.

It was concluded from the above observations that AEC was behaving in the manner of a lysine antagonist, since the inhibitory effects imposed by the analog were capable of being competitively prevented when lysine was provided in sufficient concentration. However, still remaining to be unequivocally determined is the point of metabolism at which AEC confers its inhibitory effect during the early phase of germination. It has been speculated that AEC molecules may be incorporated into protein (including α-amylase) instead of the structurally similar lysine residues. Insertion of AEC might alter the structure and/or function of the α-amylase enzyme in such a way as to be rendered inactive or less active.

LIST OF REFERENCES

Abdul-Baki, A. A. 1974. Hypochlorite and tissue sterilization. Planta 115, 373-376.

Bennett, P. A. and M. J. Chrispeels. 1972. De novo synthesis of ribonuclease and 1,3-glucanase by aleurone cells of barley. Plant Physiol. 49, 445-447.

Ben-Tal, Y. and J. E. Varner. 1974. An early response to gibberellic acid not requiring protein synthesis. Plant Physiol. 54, 813-816.

Bogorad, L. and J. H. Weil, eds. 1977. Nucleic Acids and Protein Synthesis in Plants. Plenum Press, New York, 417 pp.

Briggs, D. E. 1963. Biochemistry of barley germination: Action of gibberellic acid on barley endosperm. J. Inst. Brew. 69, 13-19.

Briggs, D. E. 1973. Germinating cereal grains. In: Biosynthesis and Its Control in Plants, B. V. Milborrow, ed. Academic Press, London, pp. 219-277.

Bright, S. W. J., M. M. Leggatt and B. J. Miflin. 1979a. Amino acid levels in carrot cell suspension culture. Plant Physiol. 63, 586-588.

Bright, S. W. J., P. B. Norbury and B. J. Miflin. 1979b. Isolation of a recessive barley mutant resistant to S-(2-aminoethyl)-L-cysteine. Theor. Appl. Genet. 55, 1-4.

Carlson, P. S. 1973. Methionine sulfoximine-resistant mutants of tobacco. Science 180, 1366-1368.

Chen, R. and R. L. Jones. 1974a. Studies on the release of barley aleurone cell proteins: Kinetics of labelling. Planta 119, 193-206.

Chen, R. and R. L. Jones. 1974b. Studies on the release of barley aleurone cell proteins: Autoradiography. Planta 119, 207-220.

Chrispeels, M. J. and J. E. Varner. 1967a. Gibberellic acid-enhanced synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol. 42, 398-406.

Chrispeels, M. J. and J. E. Varner. 1967b. Hormonal control of enzyme synthesis: On the mode of action of gibberellic acid and abscisin in aleurone layers of barley. Plant Physiol. 42, 1008-1016.

Clark, J. M. Jr., ed. 1964. Experimental Biochemistry. W. H. Freeman and Company, San Francisco, pp. 12-14.

Coombe, B. G., D. Cohen and L. G. Paleg. 1967. Barley endosperm bioassay for gibberellins. I. Parameters of the response system. Plant Physiol. 42, 105-112.

Evins, W. 1971. Enhancement of polyribosome formation and induction of tryptophan-rich proteins by gibberellic acid. Biochemistry 10, 4295-4303.

Evins, W. H. and J. E. Varner. 1971. Hormone-controlled synthesis of endoplasmic reticulum in barley aleurone cells. Proc. Natl. Acad. Sci. U. S. A. 68, 1631-1633.

Filner, P. and J. E. Varner. 1967. A test for de novo synthesis of enzymes: Density labeling with H_2O^{18} of barley α-amylase induced by gibberellic acid. Proc. Natl. Acad. Sci. U. S. A. 58, 1520-1526.

Firn, R. D. 1975. On the secretion of α-amylase by barley aleurone layers after incubation in gibberellic acid. Planta 125, 227-233.

Firn, R. D. and H. Kende. 1974. Some effects of applied gibberellic acid on the synthesis and degradation of lipids in isolated barley aleurone layers. Plant Physiol. 54, 911-915.

Frydenberg, O. and G. Nielsen. 1965. Amylase isozymes in germinating barley seeds. Hereditas 54, 123-139.

Gengenbach, B. G., T. J. Walter, C. E. Green and K. A. Hibberd. 1978. Feedback regulation of lysine, threonine, and methionine biosynthetic enzymes in corn. Crop Sci. 17, 472-476.

Gibson, R. A. and L. G. Paleg. 1972. Lysosomal nature of hormonally induced enzymes in wheat aleurone cells. Biochem. J. 128, 367-375.

Gibson, R. A. and L. G. Paleg. 1975. Further experiments on the α-amylase-containing lysosomes of wheat aleurone cells. Aust. J. Plant Physiol. 2, 41-49.

Gibson, R. A. and L. G. Paleg. 1976. Purification of gibberellic acid-induced lysosomes from wheat aleurone cells. J. Cell Sci. 22, 413-425.

Goodwin, P. G. and D. J. Carr. 1972. Actinomycin D and the hormonal induction of amylase synthesis in barley aleurone layers. Planta 106, 1-12.

Haidaris, C. G. and J. K. Bhattacharjee. 1977. High lysine excreting mutants of Saccharomyces cerevisiae. J. Ferment. Technol. 55, 189-192.

Haidaris, C. G. and J. K. Bhattacharjee. 1978. Lysine production by thia-lysine-resistant mutants of Saccharomyces cerevisiae. J. Ferment. Technol. 56, 189-192.

Hamilton, T. S. 1948. Nutritive value of corn protein. In: Protein in Corn. ATSA, Chicago, p. 96.

Henke, R. R. and R. C. Wahnbaeck. 1977. β-aspartokinase from developing endosperm of maize. Biochem. Biophys. Res. Comm. 79, 38-45.

Henke, R. R. and R. Wahnbaeck-Spencer. 1979. Variations in β-aspartate kinase activity during the development of maize endosperm. FEBS Letters 99, 113-116.

Henke, R. R., G. Fowler, R. Wahnbaeck-Spencer and M. J. Constantin. 1979. A possible screen for detecting increases in the level of soluble lysine in barley endosperm. Plant Physiol. (suppl.) 63, 68.

Higgins, T. J. V., J. A. Zwar and J. V. Jacobsen. 1976. Gibberellic acid enhances the level of translatable mRNA for α-amylase in barley aleurone layers. Nature 260, 166-169.

High-Quality Protein Maize. 1975. CIMMYT-Purdue University. International Symposium on Protein Quality in Maize, Bauman, L. F., E. T. Mertz, A. Carballo and E. W. Sprague, eds. Dowden, Hutchinson and Ross, Inc., Stroudsburg, PA, p. 524.

Ho, D. T. and J. E. Varner. 1974. Hormonal control of messenger ribonucleic acid metabolism in barley aleurone layers. Proc. Natl. Acad. Sci. U. S. A. 71, 4783-4786.

Ho, D. T. and J. E. Varner. 1978. Density labeling of proteins with ^{13}C-labeled amino acids (no accumulation of an inactive α-amylase precursor in barley aleurone cells). Arch. Biochem. Biophys. 187, 441-446.

Itzhaki, R. F. and D. M. Gill. 1964. A micro-biuret method for estimating proteins. Analytical Biochem. 9, 401-410.

Jacobsen, J. V. and J. E. Varner. 1967. Gibberellic acid-induced synthesis of protease by isolated aleurone layers of barley. Plant Physiol. 42, 1596-1600.

Jacobsen, J. V. and J. A. Zwar. 1974a. Gibberellic acid causes increased synthesis of RNA which contains poly(A) in barley aleurone tissue. Proc. Natl. Acad. Sci. U. S. A. 71, 3290-3293.

Jacobsen, J. V. and J. A. Zwar. 1974b. Gibberellic acid and RNA synthesis in barley aleurone layers: Metabolism of rRNA and tRNA and of RNA containing polyadenylic acid sequences. Aust. J. Plant Physiol. 1, 343-356.

Jacobsen, J. V., J. G. Scandalios and J. E. Varner. 1970. Multiple forms of amylase induced by gibberellic acid in isolated barley aleurone layers. Plant Physiol. 45, 367-371.

Jacobsen, J. V., R. B. Knox and N. A. Pyliotis. 1971. The structure and composition of aleurone grains in the barley aleurone layer. Planta 101, 189-209.

Johnson, K. D. and H. Kende. 1971. Hormonal control of lecithin synthesis in barley aleurone cells: Regulation of the CDP-choline pathway by gibberellin. Proc. Natl. Acad. Sci. U. S. A. 68, 2674-2677.

Jones, R. L. 1969a. The fine structure of barley aleurone cells. Planta 85, 359-375.

Jones, R. L. 1969b. Gibberellic acid and the fine structure of barley aleurone cells. I. Changes during the lag-phase of α-amylase synthesis. Planta 87, 119-133.

Jones, R. L. 1969c. Gibberellic acid and the fine structure of barley aleurone cells. II. Changes during the synthesis and secretion of α-amylase. Planta 88, 73-86.

Jones, R. L. 1972. Fractionation of the enzymes of the barley aleurone layer: Evidence for a soluble mode of enzyme release. Planta 103, 95-109.

Jones, R. L. and R. Chen. 1976. Immunohistochemical localization of α-amylase in barley aleurone cells. J. Cell Sci. 20, 183-198.

Jones, R. L. and J. V. Jacobsen. 1978. Membrane and RNA metabolism in the response of aleurone cells to GA. Bot. Mag. Tokyo Special Issue 1, 83-99.

Jones, R. L. and J. E. Varner. 1967. The bioassay of gibberellins. Planta 72, 155-161.

Koehler, D. E. and J. E. Varner. 1973. Hormonal control of ortho-phosphate incorporation into phospholipids of barley aleurone layers. Plant Physiol. 52, 208-214.

Lea, P. J. and R. D. Norris. 1976. Review: The use of amino acid analogues in studies on plant metabolism. Phytochemistry 15, 585-595.

Locy, R. and H. Kende. 1978. The mode of secretion of α-amylase in barley aleurone layers. Planta 143, 89-99.

Macey, A. and K. C. Stowell. 1961. Use of gibberellic acid in malting and brewing. I. Development of gibberellic acid treatment in malting. J. Inst. Brew. 67, 396-404.

Melcher, U. and J. E. Varner. 1971. Protein release by barley aleurone layers. J. Inst. Brew. 77, 456-461.

Miflin, B. J. 1975. Potential for improvement of quantity and quality of plant proteins through scientific research. In: Fertilizer Use and Protein Production: Proceedings of the 11th Colloquium of the International Potash Institute, pp. 53-74.

Momotani, Y. and J. Kato. 1966. Isozymes of α-amylase induced by gibberellic acid in embryo-less grains of barley. Plant Physiol. 41, 1395-1396.

Momotani, Y. and J. Kato. 1971. α-amylase synthesis by a cell free system and plant growth substances. Plant Cell Physiol. 12, 405-410.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

Nelson, N. 1944. A photometric adaption of the Somogyi method for the determination of glucose. J. Biol. Chem. 153, 375-380.

Nicholls, P. B. and L. G. Paleg. 1963. A barley endosperm bioassay for gibberellins. Nature 199, 823-824.

Oaks, A., K. E. Jones, D. W. Ross, I. Boesel, D. Lenz and S. Misra. 1979. Enzymes of nitrogen assimilation in developing seeds. In: Seed Protein Improvement in Cereals and Grain Legumes: Proceeding of a Symposium. IAEA, Vienna, pp. 179-186.

Paleg, L. G. 1960a. Physiological effects of gibberellic acid. I. On carbohydrate metabolism and amylase activity of barley endosperm. Plant Physiol. 35, 293-299.

Paleg, L. G. 1960b. Physiological effects of gibberellic acid. II. On starch hydrolyzing enzymes of barley endosperm. Plant Physiol. 35, 902-906.

Paleg, L. G. 1961. Physiological effects of gibberellic acid. III. Observations on its mode of action on barley endosperm. Plant Physiol. 36, 829-837.

Palmer, J. E. and J. Widholm. 1975. Characterization of carrot and tobacco cell cultures resistant to -fluorophenylalanine. Plant Physiol. 56, 233-238.

Pernollet, J. 1978. Protein bodies of seeds: Ultrastructure, biochemistry, biosynthesis and degradation. Phytochemistry 17, 1473-1480.

Phillips, M. and L. G. Paleg. 1972. The isolated aleurone layer. In: Plant Growth Substances, D. J. Carr, ed. Springer-Verlag, New York, pp. 396-406.

Prouty, W. F., M. J. Karnovsky and A. L. Goldberg. 1975. Degradation of abnormal proteins in Escherichia coli: formation of protein inclusions in cells exposed to amino acid analogs. J. Biol. Chem. 250, 1112-1122.

Rodaway, S. J. 1978. Composition of α-amylase secreted by aleurone layers of grains of Himalaya barley. Phytochemistry 17, 385-389.

Rodaway, S. J. and H. Kende. 1978. No effect of 5-fluorouracil on the properties of purified α-amylase from barley half-seeds. Plant Physiol. 61, 1-6.

Ross, C. W., ed. 1974. Plant Physiology Laboratory Manual. Wadsworth Publishing Company, Belmont, CA, pp. 131-133.

Sano, K. and I. Shiio. 1970. Microbial production of L-lysine. III. Production by mutants resistant to S-(2-aminoehtyl)-L-cysteine. J. Gen. Appl. Microbiol. 16, 373-391.

Shiota, T., J. E. Folk and F. Tietze. 1958. Inhibition of lysine utilization in bacteria by S-(β-aminoehtyl)cysteine and its reversal by lysine peptides. Arch. Biochem. Biophys. 77, 372-377.

Sodek, L. 1976. Biosynthesis of lysine and other amino acids in developing maize endosperm. Phytochemistry 15, 1903-1906.

Sodek, L. 1978. Partial purification and properties of diaminopimelate decarboxylase from maize endosperm. Revta. Brasil. Bot. 1, 65-69.

Sodek, L. and W. J. daSilva. 1977. Glutamate synthase: a possible role in nitrogen metabolism of developing maize endosperm. Plant Physiol. 60, 602-605.

Swaminathan, M. S., A. Austin, A. K. Kau and M. S. Naik. 1969. Genetic and agronomic enrichment of quantity and quality of proteins in cereals and pulses. In: New Approaches to Breeding for Improved Plant Protein: Proceedings of a Symposium. IAEA, Vienna, pp. 71-96.

Takenouchi, E., D. K. Nikolova, K. Awano, K. Soda and H. Tanaka. 1977. Excretion of lysine by lysine sulfur analogue resistant-mutants of Candida pelliculosa. Agric. Biol. Chem. 41, 615-616.

Tekenouchi, E., T. Yamamoto, D. K. Nikolova, H. Tanaka and K. Soda. 1979. Lysine production by S-(2-aminoehtyl)-L-cysteine resistant mutanst of Candida pelliculosa. Agric. Biol. Chem. 43, 727-734.

Trewavas, A. J. 1976. Plant growth substances: V. Gibberellic acid and enzyme secretion. In: Molecular Aspects of Gene Expression in Plants, J. Bryant, ed. Academic Press, New York, pp. 269-276.

Umbarger, H. E. 1971. Metabolite analogs as genetic and biochemical probes. Adv. Genet. 16, 119-140.

Varner, J. E. 1964. Gibberellic acid controlled synthesis of α-amylase in barley endosperm. Plant Phyisol. 39, 413-415.

Varner, J. E. and G. R. Chandra. 1964. Hormonal control of enzyme synthesis in barley endosperm. Proc. Natl. Acad. Sci. U. S. A. 52, 100-106.

Varner, J. E. and R. M. Mense. 1972. Characteristics of the process of enzyme release from secretory plant cells. Plant Physiol. 49, 187-189.

Varner, J. E., G. R. Chandra and M. J. Chrispeels. 1965. Gibberellic acid-controlled synthesis of α-amylase in barley endosperm. J. Cell. Comp. Physiol. 66, 55-68.

Varner, J. E., D. Flint and R. Mitra. 1976. Characterization of protein metabolism in cereal grain. In: Genetic Improvement of Seed Proteins. National Academy of Science/National Research Council, Washington, pp. 309-328.

Virgil, E. L. and M. Ruddat. 1973. Effect of gibberellic acid and actinomycin D on the formation and distribution of rough endoplasmic reticulum in barley aleurone cells. Plant Physiol. 51, 549-558.

Welsh, C. N., ed. 1979. Seed Protein Improvement in Cereals and Grain Legumes: Proceedings of a Symposium. IAEA, Vienna, 2 Vols.

Widholm, J. M. 1972a. Cultured Nicotiana tabacum cells with an altered anthranilate synthetase which is less sensitive to feedback inhibition. Biochem. Biophys. Acta 261, 52-58.

Widholm, J. M. 1972b. Anthranilate synthetase from 5-methyltryptophan-susceptible and resistant cultured Daucus carota cells. Biochim. Biophys. Acta 279, 48-57.

Widholm, J. M. 1976. Selection and characterization of cultured carrot and tobacco cells resistant to lysine, methionine, and proline analogs. Can. J. Bot. 54, 1523-1529.

Widholm, J. M. 1977. Selection and characterization of biochemical mutants. In: Proceedings in Life Sciences: Plant Tissue and Cutlure and Its Biotechnological Application, W. Barz, E. Reinhard and M. H. Zenk, eds. Springer-Verlag, Berlin, pp. 112-122.

Yomo, H. and H. Iinuma. 1964. The enzymes of the aleurone layer of barley endosperm. Proc. Amer. Soc. Brew. Chem., pp. 97-102.

Yomo, H. and J. E. Varner. 1971. Hormonal control of a secretory tissue. In: Current Topics in Developmental Biology, Vol. 6, A. A. Moscona and A. Monroy, eds. Academic Press, New York, pp. 111-144.

Zwar, J. A. and J. V. Jacobsen. 1972. A correlation between a ribonucleic acid fraction selectively labeled in the presence of gibberellic acid and amylase synthesis in barley aleurone layers. Plant Physiol. 49, 1000-1006.

APPENDIX

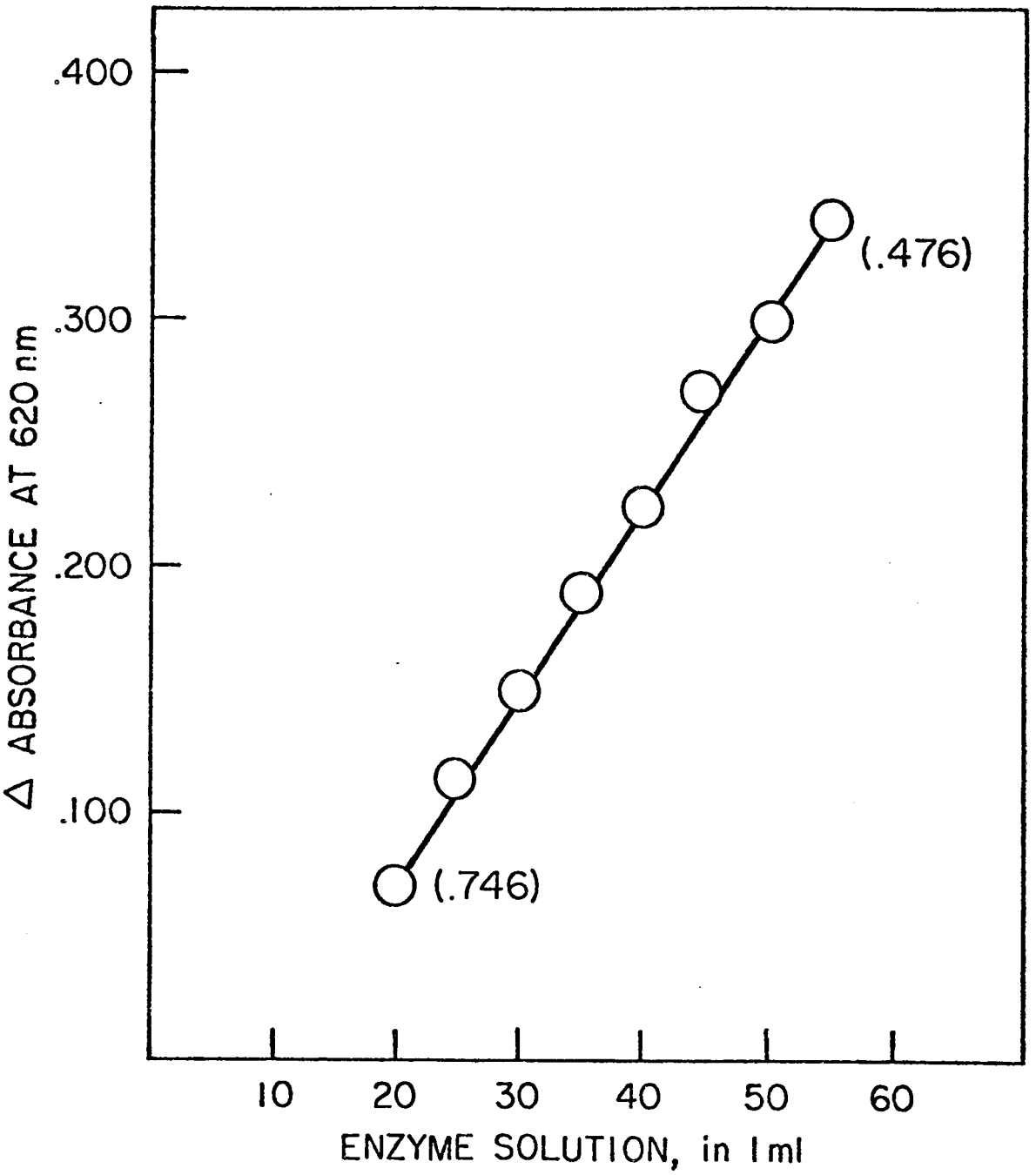


Figure 9. Change in absorbance (ΔA_{620}) versus amount of enzyme solution.

Aliquots from the diluted incubation medium were incubated with soluble potato starch for 2.5 minutes. According to Ho and Varner (1978), the assay is linear with amount of enzyme is the final absorbance reading lies between 0.750 and 0.550. The numbers in parentheses are A_{620} values ready directly from the spectrophotometer.

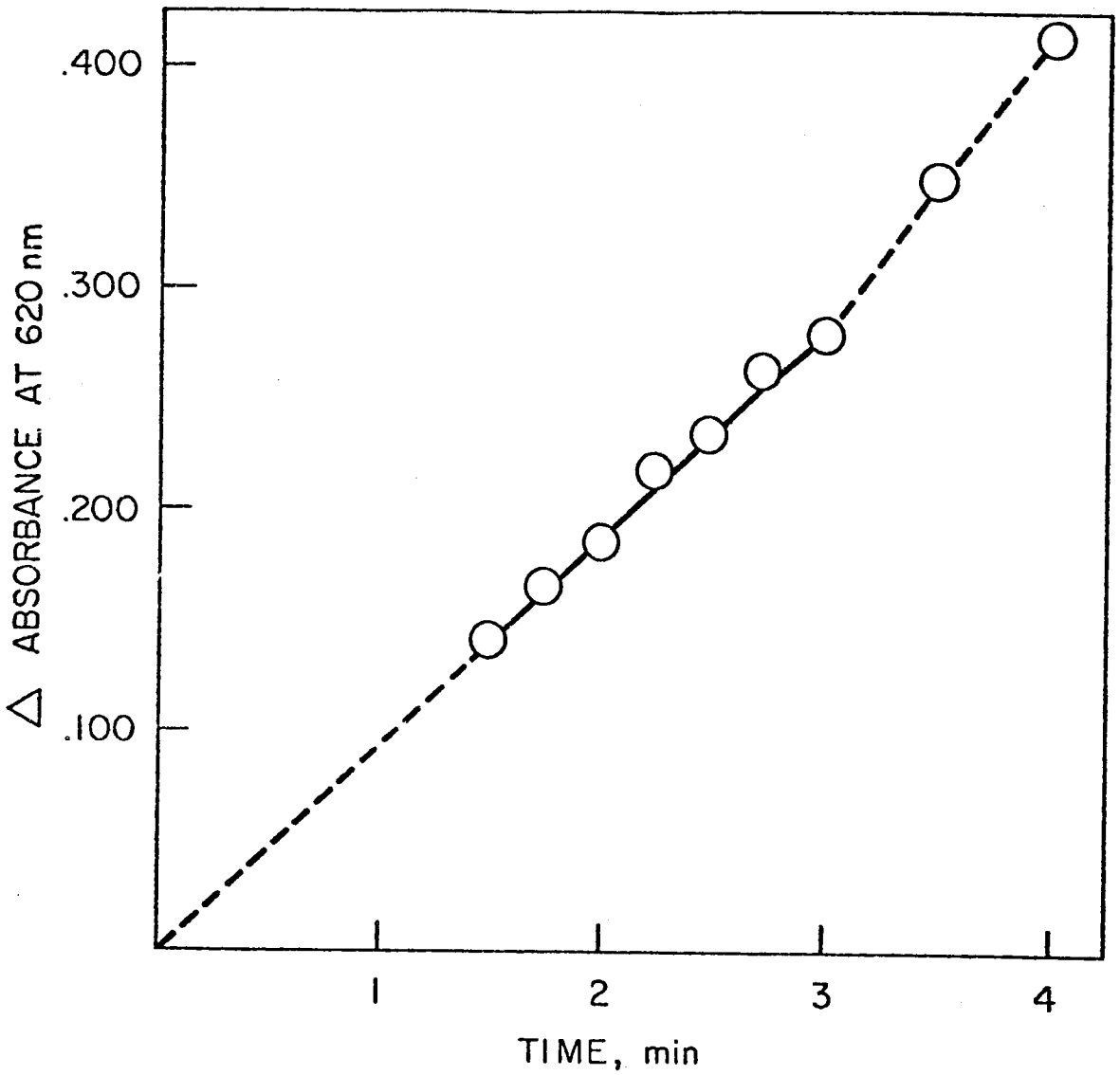


Figure 10. Change in absorbance (ΔA_{620}) versus assay reaction time.

An aliquot from the diluted incubation medium was incubated with soluble potato starch from 1.5 to 4 minutes. According to Ho and Varner (1978), the assay is linear with time when final absorbance reading lies between 0.750 and 0.500.

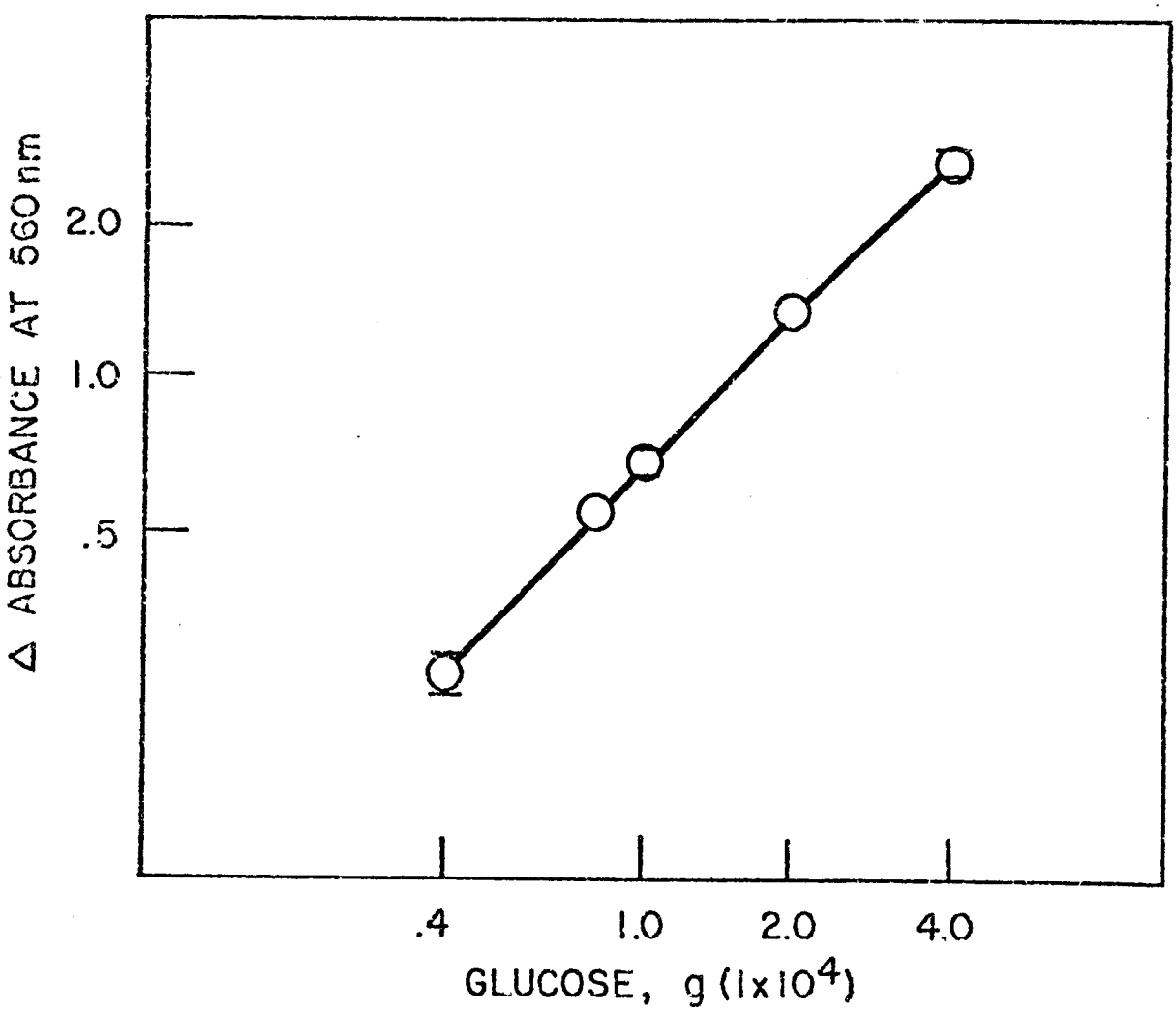


Figure 11. Reducing sugar standard curve.

The measurements of reducing sugars in solution were performed according to the methods of Paleg (1960), Coombe, Cohen, and Paleg (1967), and Nelson (1944). Glucose at the concentrations indicated, served as the standard for the reducing sugar assay. One milliliter of the glucose solution was mixed with 1 ml of Somogyi's alkaline copper reagent (24 g anhydrous bicarbonate, 12 g sodium potassium tartrate, 16 g sodium bicarbonate, 4 g copper sulfate·$5H_2O$, and 180 g anhydrous sodium sulfate in 1 liter of solution) in a test tube. Test tubes were placed in a boiling water bath for exactly 10 minutes, then in cold ammonium molybdate in 450 ml ddH_2O, add 3 g of $Na_2HSO_4 \cdot 7H_2O$ dissolved in 25 ml of H_2O, mix, and place in incubator for 24 to 48 hour) was added to each test tube, the contents of each tube was diluted to 10 ml, mixed thoroughly, and read at 560 nm in a Beckman Model 25 spectrophotometer. The values for the glucose standards were plotted on a log-log grid.

TABLE XIII

MURASHIGE AND SKOOG MEDIUM FORMULATION[a]

Original Formulation Compounds	Mol. wt.	Concentration in Final Medium	
		mg/l	mM
Macro-Salts			
NH_4NO_3	80.1	1,650	20.60
KNO_3	101.1	1,900	18.79
$CaCl_2 \cdot 2H_2O$	147.0	440	2.99
$MgSO_4 \cdot 7H_2O$	264.4	370	1.50
KH_2PO_4	136.1	170	1.25
Micro-Salts			
H_3BO_3	61.8	6.2	0.100
$MnSO_4 \cdot 4H_2O$	223.0	22.3	0.100
$ZnSO_4 \cdot 4H_2O$	233.4	8.6	0.037
KI	166.0	0.83	0.005
$Na_2MoO_4 \cdot H_2O$	242.0	0.25	0.001
$CuSO_4 \cdot 5H_2O$	249.6	0.025	0.0001
$CoCl_2 \cdot 6H_2O$	237.9	0.025	0.0001
Chelated Iron			
Na_2EDTA	372.3	37.3	0.10
$FeSO_4 \cdot 7H_2O$	277.9	27.8	0.10
Organics			
Sucrose	342.3	30,000	87.64

[a]Adaptation from Murashige and Skoog (1962).

VITA

Gary L. Fowler was born in Findlay, Ohio, on April 30, 1955. He graduated from Findlay Senior High School in June 1973. In September of the same year, he entered Miami University in Oxford, Ohio, and received his Bachelor of Science degree in May 1977.

The following September, he began study towards a Master of Science Degree in the Department of Botany at The University of Tennessee, Knoxville. This was made possible through a Graduate Research Assistantship at The University of Tennessee/Department of Energy-Comparative Animal Research Laboratory in Oak Ridge, Tennessee.