a-AMYLASE FROM GIBBERELLIN-INDUCED BARLEY ALEURONE CELLS: PARTIAL CHARACTERIZATION AND EFFECTS OF 5-FLUOROURACIL

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SHIRLEY J. RODAWAY

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This is to certify that the

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Partial Characterization and Effects of 5-Fluorouracil

presented by

Shirley J. Rodaway

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Botany and Plant Pathology

Major professor

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ABSTRACT

α-AMYLASE FROM GIBBERELLIN-INDUCED BARLEY ALEURONE CELLS: PARTIAL CHARACTERIZATION AND EFFECTS OF 5-FLUOROURACIL

By

Shirley J. Rodaway

α-Amylase has been purified from the combined incubation medium and homogenate of de-embryonate grains ("half-seeds") of barley (*Hordeum vulgare* L. cv. Betzes) which have been treated with 1 μM gibberellic acid (GA_3) to induce α-amylase synthesis. The purified enzyme contained all the six isozymes of α-amylase found in the original crude extract. Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis there was only one component by molecular weight, a 41,400 dalton polypeptide. Free calcium, which stabilized the enzyme to heat at pH 8, and free carbohydrate could be removed by chromatographic procedures.

Initially an investigation of the precise time course of the synthesis of α-amylase mRNA and a determination of the half-life of that mRNA were intended. Carlson had reported (Nature New Biol. 237: 39, 1972) that imbibition of aleurone tissue in H_2O for 1 day, followed by incubation in 10^{-4} M 5-fluorouracil (5-FU) for 2 days and then by GA_3 plus 5-FU for one more day led to the synthesis α-amylase which was more thermolabile in the absence of calcium than was α-amylase obtained from aleurone tissue incubated according to the same schedule but without 5-FU. Presence of 5-FU together

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with GA_3 on the last day of incubation only, led to the synthesis of two populations of α-amylase molecules, apparently representing the more and less thermolabile populations of the first two treatments. Carlson concluded that the population of α-amylase molecules which was more heat stable must have been synthesized prior to treatment with GA_3. Carlson proposed that 5-FU was incorporated into α-amylase mRNA during transcription, thus leading to the altered (more thermolabile) population of α-amylase molecules.

In attempting to extend those observations and to justify some of the assumptions which were made, I collected the following data which were consistent with Carlson's conclusions. Metabolites of 5-FU entered the pool of RNA precursors in aleurone tissue because 5-FU at 10^{-2} M inhibited synthesis of rRNA and 4 to 5S RNA by as much as 90%. At the same 5-FU concentration, α-amylase synthesis was only inhibited 10 to 20%. The α-amylase synthesized after 5-FU treatment did not differ from normal α-amylase in isozyme composition and molecular weight. However, some findings were not in agreement with those of Carlson. In my experiments α-amylase could not be purified by the method which he had employed because all of the α-amylase activity was lost during one of the intermediate purification steps. Instead, an alternate, more gentle method was used. α-Amylase prepared by this procedure exhibited thermal denaturation kinetics which were *independent* of the previous incubation of the aleurone with 5-FU. The rate of thermal denaturation was independent of α-amylase concentration, and no degradation of α-amylase occurred as a result of the heat treatment. These results have established that, contrary to the report by Carlson, one cannot use the thermal

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denaturation of α-amylase prepared from 5-FU-treated half-seeds to measure the timing of the synthesis of α-amylase mRNA.

In Section II of this dissertation, a method is described for preparing small amounts (<1 mg) of protein for analysis of its covalently bound sugar content. This method combined SDS-polyacrylamide gel electrophoresis and either aqueous or non-aqueous techniques of carbohydrate hydrolysis followed by gas-chromatographic analysis of the sugars. Using this method, the α-amylase secreted by whole grains of the Himalaya cultivar of barley was found to contain on the average up to 0.5 residues of mannose, glucose, and glucosamine. The amino-acid composition of α-amylase from Himalaya barley grains showed that it contained proportionately more histidine and tyrosine and less glutamate/glutamine than the average protein molecule. A second protein is described which was not synthesized during GA_3-treatment but which had many properties similar to α-amylase, including partial glycosylation.

α-AMYLASE FROM GIBBERELLIN-INDUCED BARLEY ALEURONE CELLS: PARTIAL CHARACTERIZATION AND EFFECTS OF 5-FLUOROURACIL

By

Shirley J. Rodaway

A DISSERTATION

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1977

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ACKNOWLEDGEMENTS

One of the major advantages of doing research at the Plant Research Laboratory is that an informal interchange of ideas and expertise is highly encouraged and we are continually learning as much from each other as we are from our own experiments. For this reason, I find that I must thank the whole of the PRL for philosophies and ideas which no doubt have incorporated themselves into my own attitudes and research.

Hans Kende is especially acknowledged for setting a fine example of how to approach and solve basic problems in plant development, while encouraging independent thinking by his students. He is also acknowledged for teaching us the fine art of not taking ourselves too seriously, while taking our research very seriously.

I thank Andrew Mort for teaching me techniques of sugar and amino acid analysis and Derek Lamport for the use of equipment maintained within his laboratory. In addition, I thank J. E. Varner and P. Filner for valuable discussions concerning work reported in Section I of this dissertation.

Finally, I would like to express my continuing gratitude to L. M. Blakely for encouraging and supporting my early interest in plant physiology which ultimately has led to the completion of this dissertation.

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SECTION I .

INTRO

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ABBREVIATIONS

Acca	a buffer of 1 m$\underline{M}$ acetate, 100 m$\underline{M}$ $CaCl_2$, pH 4.8
AMPS	ammonium persulfate
αA-mRNA	α-amylase messenger RNA
DEAE-cellulose	diethylaminoethyl-cellulose
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid
5-FO	5-fluoroorotic acid
5-FU	5-fluorouracil
GA_3	gibberellic acid
mA	milliamperes
PAS	periodic acid-Schiff reaction
pI	isoelectric point
r-preRNA	precursor to rRNA
SDS	sodium dodecylsulfate
TCA	trichloroacetic acid
TEMED	N,N,N'N'-tetramethylenediamine
TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)aminomethane
Trisca	a buffer of 5 m$\underline{M}$ Tris-HCl, 5 m$\underline{M}$ $CaCl_2$, pH 8.0

SECTION I

INTRODUCTION

Control of Gene Expression in Eukaryotes

In eukaryotes the events which occur between transcription and appearance of a functional protein are separated both in space and time. This is unlike the situation in prokaryotes where the nascent RNA transcript may be engaged in translation even before its transcription is completed. In prokaryotes, gene expression has been found to be regulated at the transcriptional or post-translational levels. In eukaryotes, however, the supply of a specific protein may be controlled either at the level of transcription or at several possible post-transcriptional steps. Because development is a dynamic process, it is easy to see that by varying the rates at which each reaction occurs, or by varying the time spent between reactions, very fine controls can be exerted over the amounts and kinds of proteins present in the cell at any one time. For this reason, the striking effects of hormones in those systems where the amounts and species of proteins change must ultimately result from hormonally induced changes in the rates of such key reactions which lead to the synthesis or activation of protein molecules.

Four levels at which gene expression may be controlled are generally distinguished: the transcriptional, post-transcriptional, translational, and post-translational levels of control. A transcriptional mechanism of control implies that a functional protein is synthesized as soon as the gene is transcribed. Cases of

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post-transcriptional control include processing of the pre-mRNA molecules into potentially active mRNA, transport of mRNA out of the nucleus, unmasking of the mRNA from an inactive to an active form by removal of protein, binding of the mRNA to the appropriate class of ribosomes, and regulating the degradation rate for that particular mRNA. At the transcriptional level, the rates of initiation, elongation, and release of the polypeptide and/or mRNA may be limiting, and even after the encoded amino acids are joined together, that polypeptide may need to be covalently modified or activated by a cofactor as post-translational control mechanisms are effected. It is necessary when studying hormone-induced enzyme synthesis to suspect that any one of these events may be the rate-limiting reaction which is affected by the hormone.

Induction of α-Amylase Synthesis by Gibberellic Acid in Barley Aleurone Layers

The control of gene expression in eukaryotes has been most extensively studied in metazoan systems (Maclean, 1976) and relatively little in higher plants. I have been concerned with the mechanism by which the plant hormone, gibberellic acid (GA_3), induces the synthesis of α-amylase in aleurone cells of mature barley grains. The aleurone cells constitute a homogeneous layer of non-dividing, triploid cells which are committed to the synthesis and secretion of hydrolytic enzymes and to the hydrolysis and release of most of their cell contents to provide substrates for the growth of the germinating embryo (reviewed by Varner and Ho, 1976). The induction of enzyme synthesis and secretion and the release of inorganic ions and carbohydrate is controlled by GA_3. However, the rates of general protein synthesis, of respiration (Varner et al., 1965), and of total RNA

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synthesis (Jacobsen and Zwar, 1974a) are unaffected by the hormone. α-Amylase is the most prominent among the hydrolases whose synthesis is regulated by GA_3; at its maximum rate of synthesis α-amylase represents 40% of the total protein being synthesized by the cell (Varner and Ho, 1976). Before GA_3 is added to isolated aleurone layers, as few as 1 or 2 units of α-amylase may be present within the tissue, while after an 8-hour lag period, the GA_3-treated layers may synthesize and secrete up to 40 units per layer over a period of 16 hr.

An obvious major question with regard to the hormonally-controlled synthesis of α-amylase concerns the level of control at which GA_3 exerts its effect. More specifically, does GA_3 induce the transcription of the α-amylase mRNA (αA-mRNA)? The possibility that GA_3 might exert its control over α-amylase synthesis by affecting the rate of transcription of αA-mRNA must be considered for the following reasons. First, the 8-hour lag period before the appearance of active α-amylase is of sufficient duration to allow time for transcription, processing and translation to occur. Second, it has now been proven that the complete polypeptide portion of the α-amylase molecule is synthesized at the time when the increase in enzyme activity is first detected, thus eliminating the possibility that GA_3 controls α-amylase synthesis at the post-translational level. This conclusion can be drawn from work of Ho (1976), who showed that the buoyant density of α-amylase increased when ^{13}C-amino acids were fed to aleurone layers after the lag phase, when the activity of α-amylase was increasing. Conversely, the addition of ^{13}C-amino acids during the period of the lag phase alone had no effect on the buoyant density of the α-amylase. Earlier, Varner and Chandra (1964) had shown that nearly all of the tryptic peptides prepared from

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α-amylase were labeled when radioactive amino acids were added simultaneously with GA_3, and Filner and Varner (1967) had shown that α-amylase was synthesized *after* GA_3 was added, since the buoyant density of all of the α-amylase was increased when the layers had been incubated in medium containing GA_3 and $H_2{}^{18}O$. In this latter case, the endogenous amino acids became labeled with ^{18}O as they were released from storage proteins by proteolysis, and the ^{18}O-amino acids were incorporated into the nascent α-amylase molecules.

Recent reports indicate that GA_3 affects α-amylase synthesis at the transcriptional level. Higgins et al. (1976) showed that there was an increase in the capacity of total poly(A)-RNA from aleurone layers to direct *in vitro* synthesis of α-amylase following treatment of the tissue with GA_3 for increasing periods of time. Poly(A)-RNA was incubated with ^{35}S-methionine in the wheat germ protein-synthesizing system, and labeled α-amylase was identified by immunoprecipitation. The amount of radioactivity which could be precipitated in the *in vitro* system increased with precisely the same kinetics as the appearance of α-amylase activity *in vivo*. GA_3-treatment of aleurone layers also increased the rate of polyadenylation of RNA, beginning half-way through the lag period (at 4 to 5 hr) and reaching a maximum (a 60% increase) at the end of the lag period (Ho and Varner, 1974; 1975). Within the first 16 hr of GA_3 treatment, the total amount of new poly(A)-RNA species increased 70% over that of the control tissue (Jacobsen and Zwar, 1974a,b).

Furthermore, a number of early observations indicated that some critical species of RNA molecule were synthesized during the lag period in response to GA_3, although some of these conclusions have been subsequently challenged (Jacobsen and Zwar, 1974a,b). These

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observations were based on the use of various inhibitors of RNA synthesis (Varner and Chandra, 1964; Varner et al., 1965; Chandra and Varner, 1965; Varner and Johri, 1967), the analysis of the overall base composition of RNA molecules labeled before and after the hormone was added (Varner et al., 1965), and the increase in the specific activity of the RNA labeled in the presence of the hormone (Chandra and Varner, 1965). Certain inhibitors of RNA synthesis, notably cordycepin (Ho and Varner, 1974) and Actinomycin D (Varner and Chandra, 1964; Goodwin and Carr, 1972), only exerted their inhibitory effects during the lag period before α-amylase synthesis had begun, again *suggesting* that α-amylase synthesis is regulated at the level of transcription or processing of αA-mRNA.

From all of these data it is tempting to conclude that GA_3 exerts a direct effect on the synthesis of the active αA-mRNA molecules. However, no direct proof was presented to show that GA_3 increased the rate of αA-mRNA synthesis. Even though the rate (Ho and Varner, 1974) and amount (Jacobsen and Zwar, 1974) of total poly(A)-RNA synthesis increased in response to GA_3, this did not imply that the specific mRNA for α-amylase was polyadenylated or transcribed during that time. Even the demonstration that some of the poly(A)-RNA contained the α-amylase message, and that the *in vitro* activity of the α-amylase message appeared to increase after GA_3 treatment, did not constitute proof that either the transcription or the processing of αA-mRNA was dependent upon the presence of GA_3 (Higgins et al., 1976). The experiments performed with inhibitors of RNA synthesis may provide "food for thought", but it should be pointed out that cordycepin inhibits total RNA synthesis as well as polyadenylation (Ho and Varner, 1974) and Actinomycin D interferes

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with the morphology of the endoplasmic reticulum (Vigil and Ruddat, 1972). In no case has it been shown that the primary effect of any RNA synthesis inhibitor involved the transcription or processing of α A-mRNA and not some other mRNA species required for the previously synthesized α-amylase message to be expressed.

The following problem still remains to be solved: How can one determine which of the numerous events involving αA-mRNA synthesis and utilization may be affected by GA_3? One approach which would unequivocally show whether GA_3 controls the de-repression of the α-amylase gene would be that used by Axel et al. (1973). In this classic experiment, DNA complementary to hemoglobin mRNA was synthesized using reverse transcriptase, and this DNA was used to test for the presence of a hemoglobin mRNA among the RNA molecules transcribed *in vitro* from isolated reticulocyte chromatin.

Use of 5-Fluorouracil to Study the Transcription of αA-mRNA

A new technique to determine the timing of αA-mRNA synthesis was used by Carlson (1972) in experiments with barley aleurone layers. Barley aleurone tissue was exposed to the base analog 5-fluorouracil (5-FU) either during GA_3-treatment or both prior to and during GA_3-treatment. In some treatments, no 5-FU was given, while in others 5-FU was applied as a "pulse" which was chased by an excess of exogenous uracil. Carlson hypothesized that 5-FU would be incorporated into αA-mRNA if the transcript was being synthesized at the time when 5-FU was in the medium. He also postulated that the presence of 5-FU in the αA-mRNA would manifest itself through an altered amino acid sequence of the α-amylase molecules which were later synthesized in response to GA_3. After the 5-FU and

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GA_3-treatments, Carlson determined the thermal stability of α-amylase obtained at the end of each GA_3 treatment and found that the α-amylase exhibited much greater thermal lability when extracted from aleurone tissue which had been incubated with 5-FU during the period *before* GA_3 addition. The alteration of thermal stability had been taken as indication for changes in the primary structure of α-amylase. From a series of such experiments, Carlson concluded that much of the αA-mRNA must have been synthesized prior to addition of GA_3, and that transcription of the α-amylase gene therefore occurred independently of the action of GA_3.

It was upon Carlson's experiments that I had wished to base the first section of this dissertation. I had proposed to use his technique of short-time treatment with 5-FU followed by chase with uracil to obtain a precise time course for the transcription of the α-amylase gene, beginning at the time when the dry tissue was first imbibed in H_2O. The technique would also have allowed me to estimate the functional lifetime of the αA-mRNA. However, the data which Carlson had published were considered controversial because he had not established the rate at which 5-FU entered the pool of RNA precursors, nor the rate at which added uracil was able to reverse the effects of 5-FU. In addition, he had not established the purity of the α-amylase preparations which he had obtained, nor had he shown that the isozyme compositions of the various α-amylase preparations were the same. Studies of thermal decay kinetics of enzymes may require pure enzyme solutions, and constancy of isozyme composition was necessary because isozymes of α-amylase differ substantially in their stability characteristics (Jacobsen et al., 1970; Frydenberg and Nielsen, 1965). Additional support for Carlson's hypothesis

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would have been provided by showing directly that 5-FU was able to cause random amino acid substitutions in α-amylase and/or that 5-FU was incorporated into poly(A)-RNA.

In the course of my investigations, it became evident that Carlson's conclusions may have been premature. When α-amylase was purified by a method which led to the recovery of all of the α-amylase isozymes at a high level of purity, it was found that incubation of the aleurone tissue with 5-FU had no effect on the thermal stability of the enzyme. This is in contrast to Carlson's observations, according to which the thermal stability of the enzyme could be altered by 5-FU treatment. To examine further the feasibility of this technique, I proceeded to investigate the possibility that the α-amylase which I had purified was unlike the α-amylase originally synthesized and secreted by the aleurone tissue, or that the α-amylase I studied was contaminated by protein, carbohydrate, calcium, protease, etc., or that 5-FU was not utilized by the aleurone tissue in my experiments. In the end, however, none of these possibilities could explain the lack of a 5-FU effect. Based on my data, there appears to be no reason to believe that altered α-amylase is synthesized as a result of treating aleurone layers with 5-FU. At least in this instance, use of this technique appears to be without any practical benefit.*

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*Because of the nature of the following report, it will be necessary to summarize the methods and results of Carlson (1972) so that a comparison may be made to methods which I have employed. Carlson incubated de-embryonated seeds of barley (*Hordeum vulgare* cv. Betzes) in H_2O for one day; following this, he removed (most of) the starchy endosperm and incubated the aleurone tissue in buffer (Chrispeels and Varner, 1967) for two more days. At that point 1 μM GA_3 was added for 24 hr, and after the incubation was terminated, the tissue was homogenized, combined with the medium, and the enzyme

purified by a method developed for pancreatic α-amylase (Loyter and Schramm, 1962). 5-Fluorouracil was added for different periods throughout the incubation period, up to a maximum of 48 hr before and 24 hr after GA_3-addition, and it was implied that 5-FU treatment had no effect on the amount of α-amylase which was synthesized. If 5-FU was removed before the end of the incubation period, it was replaced with 2.5×10^{-4} M uracil. The purity and isozyme composition of the α-amylase preparation were not described. It is possible that β-amylase may have contaminated the final enzyme preparation. Other proteins and carbohydrates were removed from α-amylase by binding the α-amylase to glycogen, passing the α-amylase and glycogen hydrolysis products over a charcoal column (pH 10.0) to remove carbohydrate, precipitating the enzyme with acetone, ether and ethanol. Calcium was removed by dialysis against 1 mM EDTA in the presence of 1 mM hexametaphosphate.

Without 5-FU treatment, the half-life of the enzyme at 72 C was 12 min and with the longest 5-FU treatment the half-life was 5 min [these half-lives were calculated by Chandra (1974) in his re-interpretation of Carlson's results]. α-Amylase from other treatments exhibited a mixture of both elements, leading to biphasic decay kinetics. Carlson's conclusions about the timing of α-amylase mRNA synthesis were based on the shapes of these thermal decay curves.

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MATERIALS AND METHODS

Preparation and Incubation of Barley Half-Seeds

Barley grains (*Hordeum vulgare* L., cv. Betzes) were harvested at Michigan State University in 1968 and were stored at 7 C until 1974, then at 20 C during the course of these experiments. The grains were dehusked by stirring the seeds slowly in 50% H_2SO_4 at room temperature for 35 min. Following this, the seeds were immediately rinsed several times with distilled H_2O, then with double-distilled H_2O, and were then blotted and dried on paper towels. The dehusked seeds were de-embryonated by transsecting the seeds behind the embryo with a scalpel in a plane perpendicular to the seed axis. Seeds prepared in this manner will be called "half-seeds." The half-seeds were also nicked at the distal ends to aid H_2O uptake during imbibition. Half-seeds were surface sterilized in 1% NaOCl for 25 min at room temperature and rinsed 5 times with 4 volumes of distilled H_2O. The half-seeds were incubated for a total of 4.5 days. During the first 24 hr, the half-seeds were incubated in one batch in 100 to 200 ml of bathing solution. The solution was well aerated with air passed first through a cotton filter, then through 2% $KMnO_4$ (as a sterilizing agent) into a gas dispersion tube, the end of which was immersed in the incubation solution. After the first 24 hr, groups of 50 half-seeds were transferred to 125-ml flasks containing 8 ml of 1 m$\underline{M}$ sodium acetate, pH 4.8, and 100 m$\underline{M}$ $CaCl_2$ ("Acca" buffer). Chloramphenicol (0.001%) was added to

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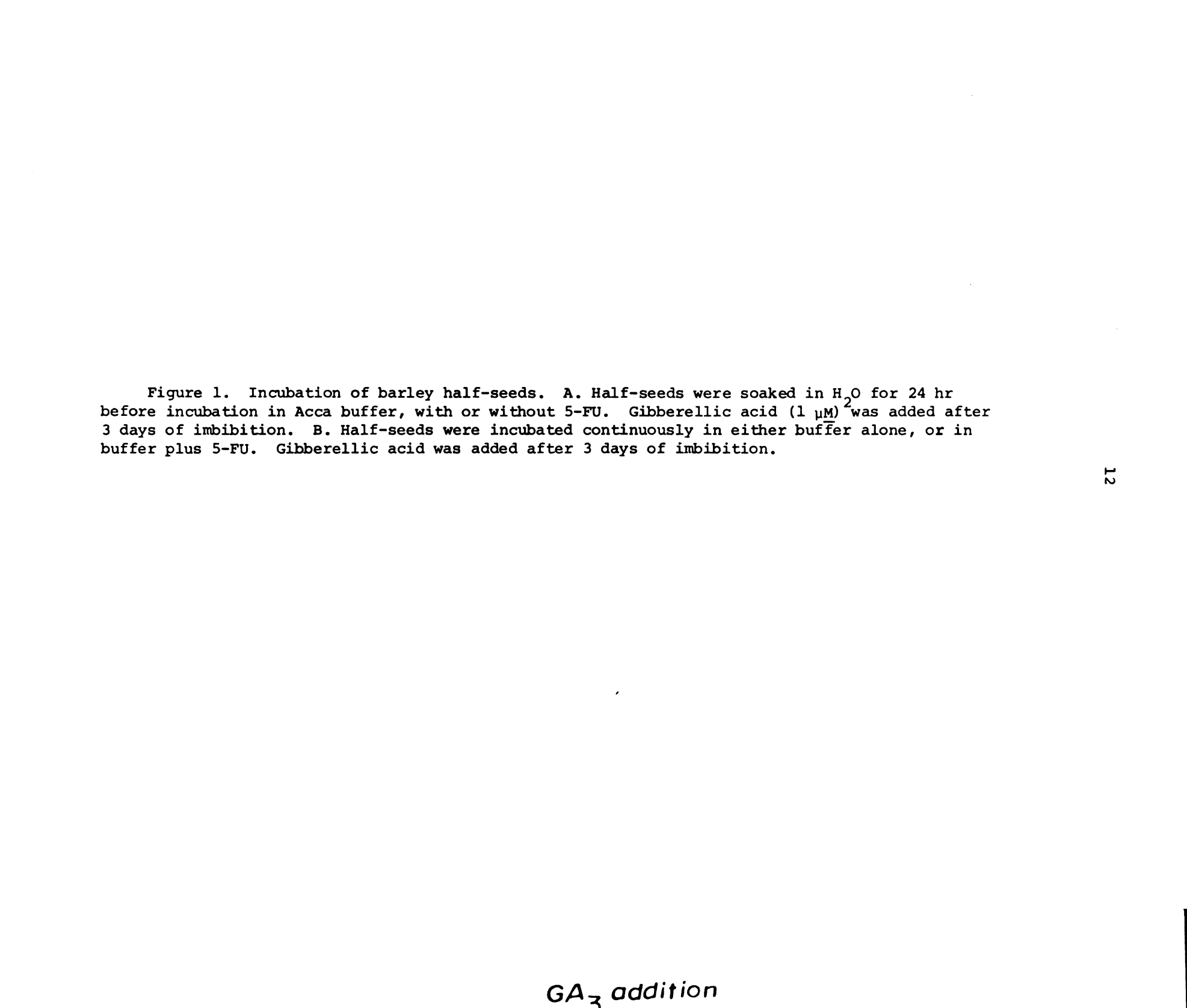
inhibit bacterial growth. The flasks were incubated for 48 hr more on a reciprocating shaker operating at 125 to 150 oscillations per min. Filter-sterilized GA_3 was added to the incubation medium to give a final concentration of 1 μM, and hormone treatment was continued for 36 hr during which time the tissue had synthesized a maximal amount of α-amylase. In treatments with 5-FU, the base analog was solubilized by briefly warming it in Acca buffer at 30-40 C. The solution was filter-sterilized and then added to the incubation medium. Typical incubation regimes are shown in Figure 1A and 1B.

When aleurone layers were used, they were prepared by removing the starchy endosperm with thin spatulas under sterile conditions. It was necessary to incubate half-seeds for a minimum of 2 days (3 days was best) to effectively remove the starchy endosperm and to avoid damaging the aleurone layer in this process. No amylase was released into the medium before the start of the GA_3 treatment.

Homogenization of Tissue

The medium was decanted from flasks containing half-seeds or aleurone layers and set aside. Aleurone layers or half-seeds were homogenized in one of two ways. Large batches of material were ground with one-half of their weight of acid-washed sea sand in an electric mortar (Model MG-2, Torsion Balance Company, Clifton, NJ, USA). After the tissue was homogenized, the mortar was rinsed 3 times with a minimum of Acca buffer. Small batches of 10 to 25 layers or 10 half-seeds were homogenized by hand with a small porcelain mortar and pestle using about 1.4 g of sea sand and 1 ml of Acca buffer. The mortar was then rinsed 3 times with 1 ml of buffer. When the

Figure 1. Incubation of barley half-seeds. A. Half-seeds were soaked in H_2O for 24 hr before incubation in Acca buffer, with or without 5-FU. Gibberellic acid (1 μM) was added after 3 days of imbibition. B. Half-seeds were incubated continuously in either buffer alone, or in buffer plus 5-FU. Gibberellic acid was added after 3 days of imbibition.



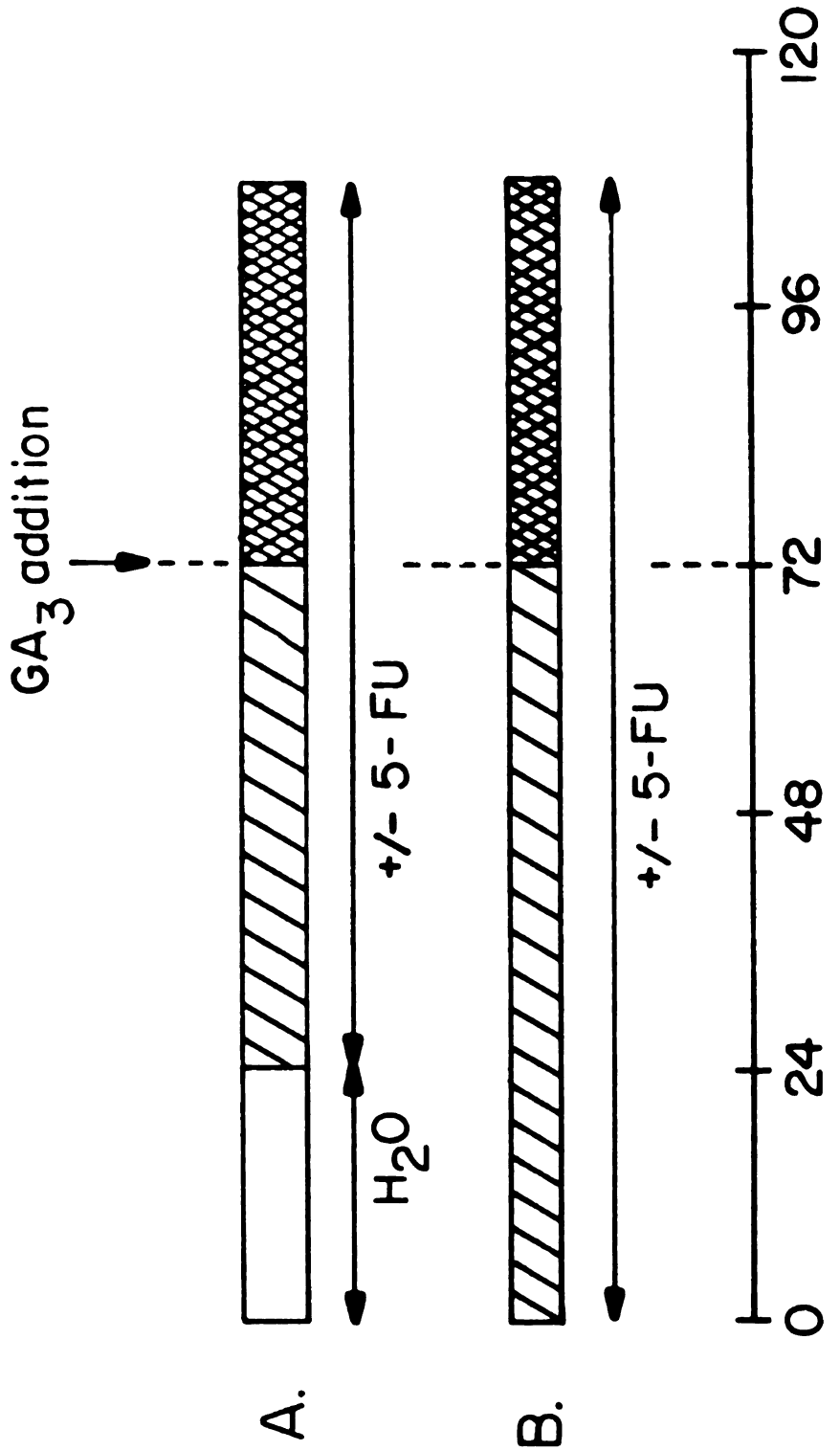


Figure 1

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enzyme was to be purified, the homogenate was combined with the medium. For quantitation of the amount of enzyme synthesized and released, the layers were first washed with 200 m$\underline{M}$ $CaCl_2$ for 10 min, and this rinse was combined with the medium for an estimate of the "released" amylase. If half-seeds were used, the starchy endosperm, medium and the 200 m$\underline{M}$ $CaCl_2$ wash of the stripped aleurone layers were designated as total α-amylase released. Residual enzyme activity in the cell walls and periplasmic space were included as "tissue" amylase along with that actually retained within the cells.

Purification of Calcium-Free α-Amylase (Method I)

Step 1. The combined medium and homogenate from 300 to 400 barley half-seeds was diluted to 200 ml with Acca buffer. This dilution step was necessary to maximize the recovery of α-amylase during steps 2 and 3 (Figure 2), although dilution alone had no effect on enzyme activity at the end of step 1. The Acca buffer was superior to the following buffers for extracting the enzyme: 20 m$\underline{M}$ succinate, pH 4.8, with 0.1 $\underline{M}$ $CaCl_2$ and 0.3 $\underline{M}$ NaCl; 50 m$\underline{M}$ Tris [tris(hydroxymethyl)aminomethane], pH 8.0, with 0.1 $\underline{M}$ $CaCl_2$; and 50 m$\underline{M}$ Tris, pH 8.0, with 0.1 $\underline{M}$ $CaCl_2$ and 0.3 $\underline{M}$ NaCl. In all experiments to be reported, the pH values are those determined at 22 C.

The diluted homogenate was incubated at 25 C for 2 hr with gentle shaking to release most of the α-amylase from its native substrate. The solution, which still contained cell debris, starch grains and sand, was centrifuged at room temperature in an International Clinical Centrifuge at 1300 $\underline{g}$ for 10 min.

Figure 2. Dilution effect on α-amylase recovery during purification. Half-seeds were incubated as in Figure 1A, without 5-FU. The medium and half-seeds were pooled from individual flasks, then redistributed into four groups. Each group was homogenized in a different buffer: (a) 1 mM sodium acetate, 100 mM $CaCl_2$, pH 4.8, o——o; (b) 20 mM succinate buffer, 100 mM $CaCl_2$, 300 mM NaCl, pH 5.3, Δ——Δ; (c) 50 mM tris, 100 mM $CaCl_2$, pH 8.0, □——□ ; or (d) 50 mM tris, 100 mM $CaCl_2$, 300 mM NaCl, pH 8.0, x——x. The ratios of the total extractable activities were 1.3/1.1/1.0/1.0 for a/b/c/d before dilution of the crude enzyme solutions. The enzyme solutions were diluted to between 25 and 150 units per ml. Dilution alone yielded the expected enzyme activities. The pH of the diluted solutions was raised to 8.0 and the solutions heated at 70 C for 15 min. The recovery of α-amylase activity (ordinate) is expressed as the percent of the originally assayed activity *before* the pH change and heating (abscissa) that the enzyme activity *after* such treatments represented. Dilution of the enzyme before the pH change and heating thus allowed more soluble enzyme activity to be recovered than was originally thought to be present.

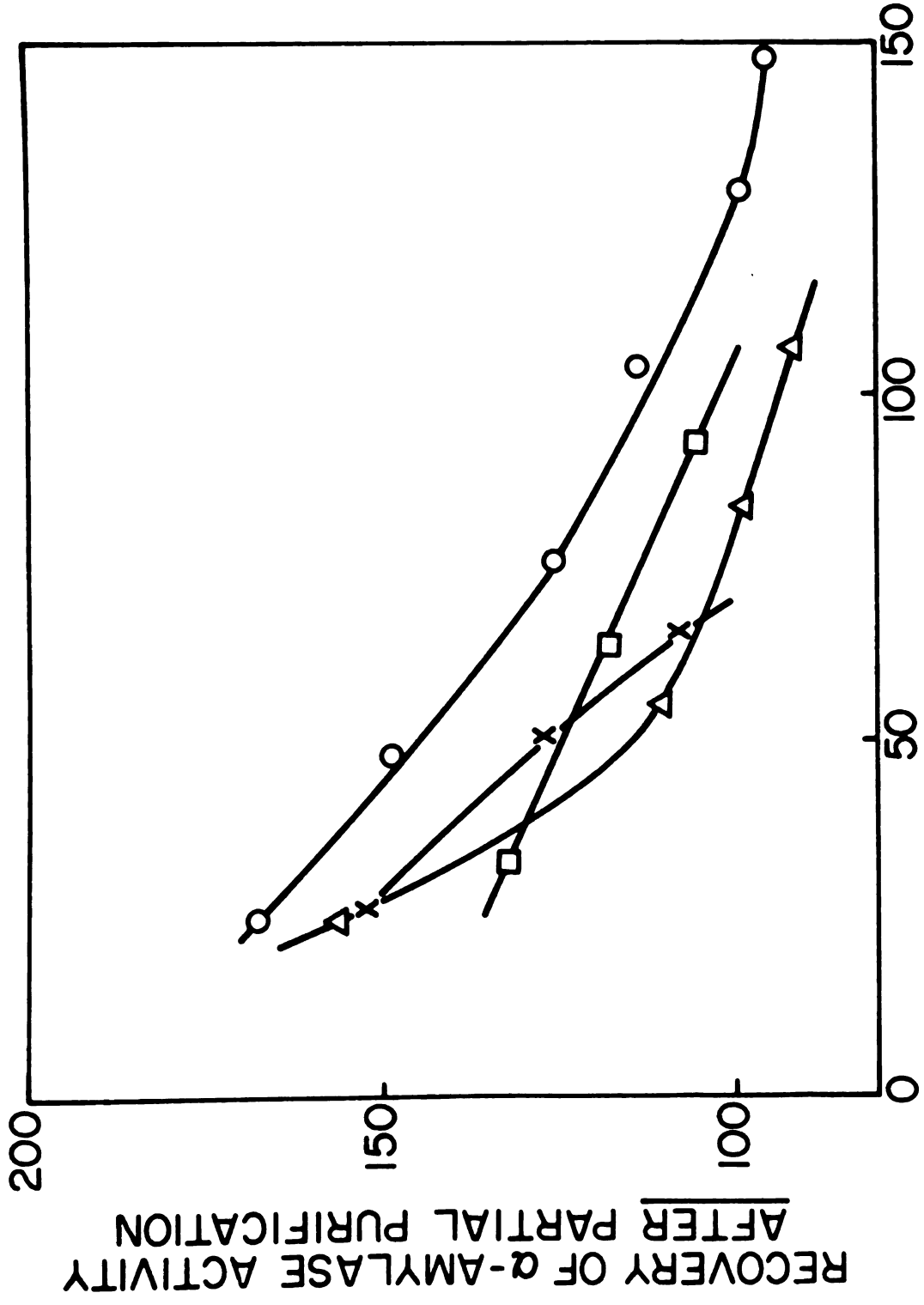
RECOVERY OF α-AMYLASE ACTIVITY
AFTER PARTIAL PURIFICATION
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Figure 2

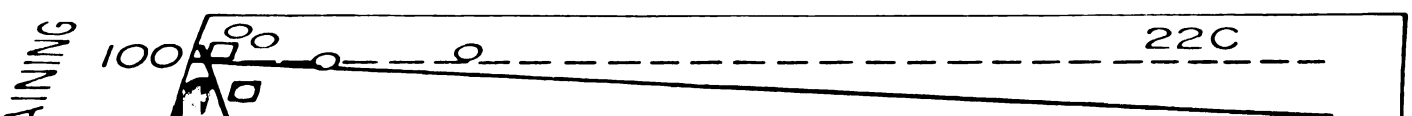
Step 2. The supernatant solution was carefully decanted, and the pH of the solution was changed to 8.0 by dropwise addition of unbuffered 0.1 M Tris base. The final concentration of Tris required was about 18 mM.

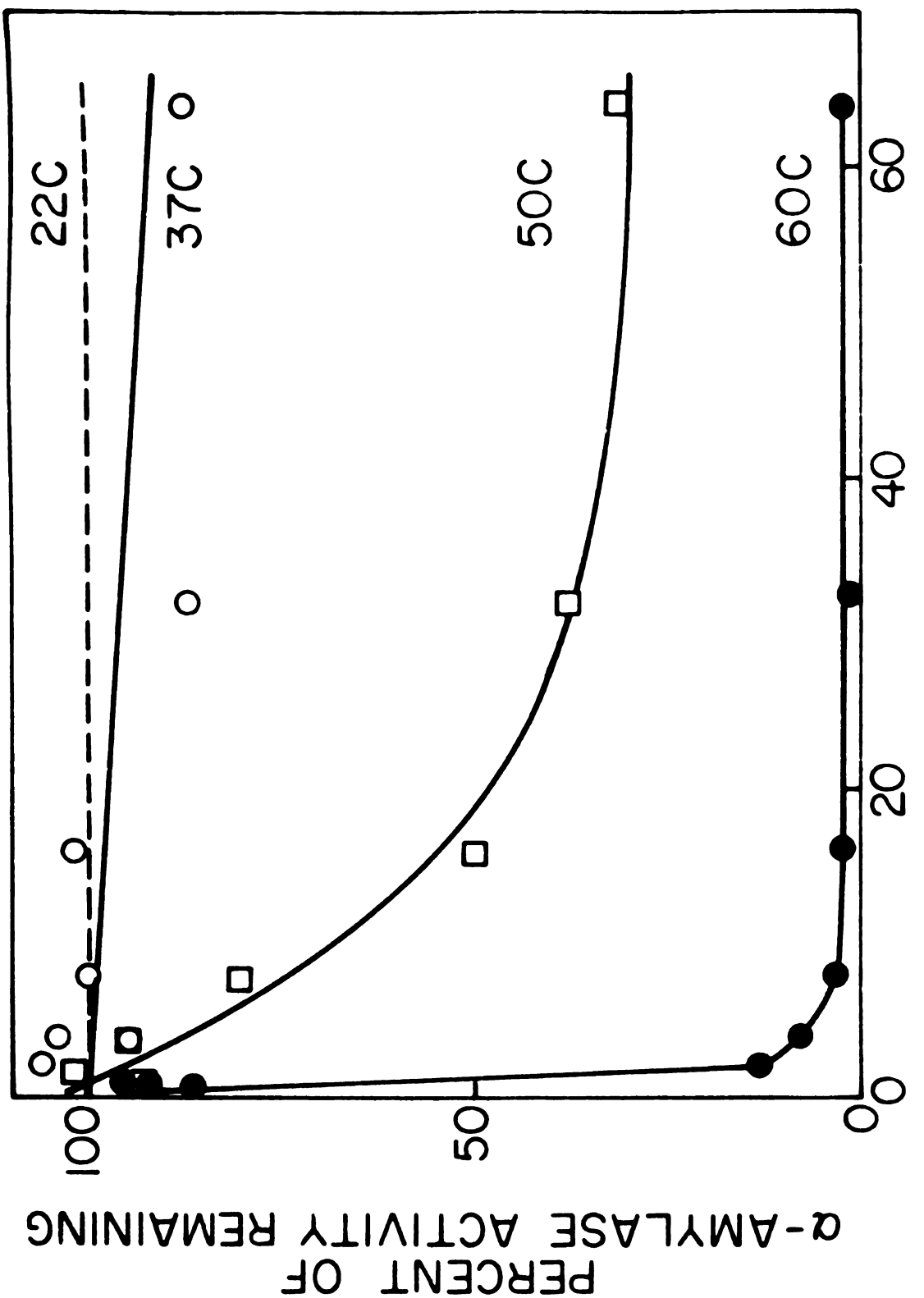
Step 3. The solution was poured into a 1500-ml Erlenmeyer flask equipped with a thermometer. The flask was incubated in a water bath set at 80 to 85 C while the temperature of the solution in the flask was maintained at 70 C. Five minutes were required to heat the solution in the flask to 70 C. For the next 10 min, the solution was maintained at 70 C ± 1 C by either removing the flask to cool it or by returning it to the bath to warm it. This heating procedure was employed to inactivate β-amylases (Kneen et al., 1943; Schwimmer, 1947; Schwimmer and Balls, 1949). During this heat treatment, α-amylase retained its activity since the presence of calcium at pH 8.0 prevented the denaturation of the enzyme. At pH 4.8 (the original pH of the medium), both α- and β-amylase activities were eliminated at higher temperatures (Figure 3). After the solution was heated, it was rapidly cooled by immersing the flask into an ice bath. The heat-precipitated proteins and carbohydrates were removed by centrifugation at room temperature.

The crude preparation contained both α-amylase and β-amylase; however, no simple method was available to determine how much of the total amylolytic activity was contributed by either of those enzymes.

Step 4. The clear supernatant solution was cooled to 7 C and ice-cold absolute ethanol was added dropwise to give a final concentration of 40%. This step was preparatory to the precipitation of

Figure 3. Effect of increasing temperatures on the amylase activity of the crude homogenate. α-Amylase was extracted from half-seeds which had been incubated as in Figure 1A. After brief centrifugation, the clear solution was heated to the indicated temperatures. The original solutions contained about 200 units per ml of amylase activity in Acca buffer at pH 4.8. The temperatures were 22 C, dashed line; 37 C, O; 50 C, □ ; and 60 C, ●.

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glycogen in step 5. Thirty percent ethanol was as effective as 40% ethanol in precipitating the glycogen-amylase complex. The ethanolic mixture was chilled for at least 60 min, centrifuged at 5800 g for 10 min, and the small pellet was discarded. No activity could be recovered from the pellet, although a part of the α-amylase activity was lost during this step.

Step 5. Glycogen was used to specifically precipitate the active α-amylase as a glycogen-amylase complex. The glycogen was first deproteinized (Loyter and Schramm, 1962). To this end, oyster glycogen (2%) was centrifuged at 11,000 g for 10 min, and 5 g of Dowex 1-X8 and 5 g of AG 50WX2 were added to each 100 ml of supernatant. After 30 min of stirring, the beads were removed by centrifugation and the ion exchange step was repeated with new resin. The final glycogen concentration was theoretically 1.6% (Loyter and Schramm, 1962). No traces of protein remained in the glycogen solution after this procedure was followed.

A small amount of glycogen solution was added to the stirred ethanolic solution, and the mixture was stirred in the cold for 15 to 60 min. A ratio of 1 µl of glycogen solution to 7 units of α-amylase enzyme (to be defined later) was used. The small glycogen-α-amylase precipitate was pelleted by centrifugation at 4000 g for 10 min. The supernatant fraction was removed, and 1 ml of a solution containing 5 mM tris, pH 8.0, and 5 mM $CaCl_2$ ("Trisca" buffer) was used to dissolve the pellet. The enzyme solution was removed, and a second 1 ml of Trisca was used to rinse the area in the centrifuge vial where the pellet had been. The two solutions were combined and incubated at 37 C for 3 hr. During this time, the α-amylase digested

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part of the glycogen. Some of the glycogen was no longer soluble and was subsequently pelleted by centrifugation at 3000 g for 10 min.

Step 6. The enzyme solution contained glucose polymers (as a result of the glycogen precipitation) and some arabinose-containing polymers. Dialysis tubing specified as retaining molecules of nominal molecular-weights of 6000-8000 daltons also retained the glucose and arabinose. Therefore, a column of DEAE-cellulose was used to remove this carbohydrate. The column material (Cellex D, Bio-Rad, Richmond, CA, USA) was purified essentially as suggested by the manufacturer. Cellex D (25 g) was suspended in 1 liter of a solution containing 0.25 M NaCl and 0.25 M NaOH, followed by two resuspensions in distilled H_2O, one resuspension in 1 liter of 0.25 M HCl, five resuspensions in distilled H_2O and five resuspensions in Trisca. After each resuspension the column material was left to settle for 15 to 30 min. The DEAE-cellulose was then stored overnight in Trisca. The buffer was decanted and the DEAE-cellulose preparation was stored at 4 C in fresh Trisca buffer.

Chromatography was performed at room temperature using a column 1.7 cm I.D. filled to a height of 10-12 cm with DEAE-cellulose. A pump on the outlet side restricted the flow to 0.5 ml per min. The freshly poured column was washed for 2 hr with Trisca buffer, then the α-amylase solution from step 5 (2 ml) was added to the column, and the wash was continued with 90-100 ml of buffer as 3-ml fractions were collected. All of the neutral carbohydrate, as detected using the anthrone reagent (see "Carbohydrate Assays") was removed in the first column volume. The α-amylase was eluted using a gradient of 0 to 0.5 M NaCl in 300 ml of Trisca buffer. α-Amylase activity eluted

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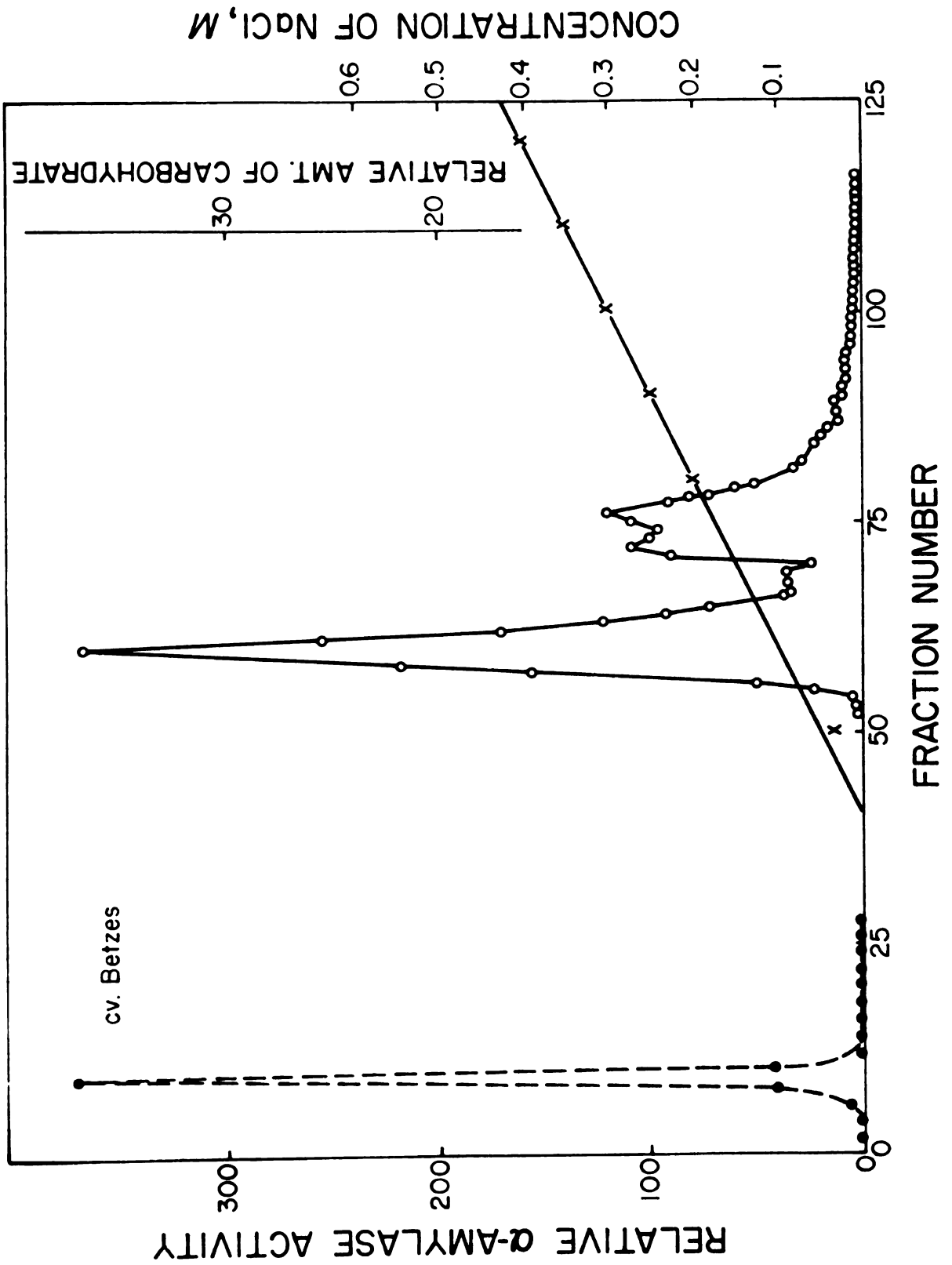
in two peaks, the first peak (I) at about 0.085 M NaCl and the second (II) at about 0.150 M NaCl (Figure 4).

Step 7. The fractions containing α-amylase were pooled and concentrated to between 1 and 2 ml. Before concentration, the α-amylase was present at less than 10 μg/ml. Lyophilization resulted in loss of all of the enzyme activity as did concentrating the dilute enzyme with Amicon filters (Amicon Corporation, Lexington, MA, USA). The best method for concentrating the enzyme involved the removal of the solvent across a dialysis membrane. Dialyapor A membrane tubing for dialysis was boiled 4 times in a solution containing 2% sodium bicarbonate and 0.03% EDTA (ethylenediaminetetraacetic acid), and one final time in distilled H_2O. Extractive dialysis was performed using one of the following methods: (i) air drying of a suspended, filled tube with the aid of a fan, followed by dialysis in Trisca buffer; (ii) placing dry Aquacide II (Calbiochem), a carboxymethyl cellulose, on the outside of the bag; or (iii) placing dry Sephadex G-200 on the outside of the bag. This last procedure was the most satisfactory. Air-drying was time-consuming and crystals of NaCl and $CaCl_2$ formed during the up-to-50-fold concentration. The use of Aquacide II induced a pH increase of 0.4 units as the DEAE-cellulose eluate was concentrated.

Concentration of α-amylase was performed at room temperature. The concentrated protein was dialyzed overnight in the cold against two 2-liter changes of Trisca buffer.

Step 8. Calcium was removed from the enzyme solution by desalting at 7 C on a Sephadex G-25 (coarse) column equilibrated in 5 mM Tris and 1 mM EGTA at pH 8.0. No free calcium above the limit of

Figure 4. DEAE-cellulose chromatography of α-amylase from Betzes barley. Step 6 of the purification of α-amylase. α-Amylase activity, O; carbohydrate (by anthrone test), ●; and NaCl concentration (by refractive index), x. In this case, α-amylase activity and carbohydrate are given in arbitrary units.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detectability (5×10^{-8} M) could be recovered from the column. Fractions (0.73 ml) were collected from the 1.6 x 40 cm column at a rate of 0.7 ml/min. In some experiments, the calcium was removed by passing the sample over a Bio-Gel P-30 column equilibrated in 5 mM Tris, pH 8.0 with 1 mM EGTA. This step replaced the Sephadex G-25 column chromatography. The column volume was 1.6 x 40 cm, and the flow rate was 0.2 ml per min. Because of the decreased stability of the α-amylase upon removal of calcium, the enzyme was maintained at 2-7 C during this and subsequent steps. The active fractions from the void volume of the column were pooled and concentrated using Aquacide II or Sephadex G-200 placed on the outside of a dialysis bag containing the enzyme solution. The presence of either Aquacide II or Sephadex G-200 changed the pH of the solution inside of the bag. Aquacide II had the greatest effect, changing the pH from pH 8.0 to as low as pH 6.6, presumably by differential mobilities or ion exchange of EDTA and Tris across the membrane. Sephadex had a lesser effect, decreasing the pH from pH 8.0 to pH 7.6. Most experiments were performed using Aquacide II, but in later experiments the Sephadex material was used.

Partial Purification of α-Amylase (Method II)

α-Amylase was also partially purified using methods which closely approximated those of Loyter and Schramm (1962) for purifying pancreatic amylase. This purification procedure had been used by Carlson without modifications (1972). Because of the nature of barley α-amylase and the medium into which it was released, certain modifications of the Loyter and Schramm method were necessary.

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The medium and homogenate from barley aleurone layers, which had been incubated in Acca buffer, were combined and centrifuged to remove cell debris. The supernatant fraction was brought to pH 6.9 by the addition of 0.5 M K_2HPO_4. A voluminous precipitate formed composed primarily of calcium phosphate salts which precipitated at pH 5.6 to 5.8. The solution was chilled at 4 C and centrifuged at 1300 g for 8 min. Ice-cold, absolute ethanol was added until its concentration was 40%. The mixture was stirred for an additional 60 min and then centrifuged at 20,000 g for 20 min.

Glycogen was added in three steps. First, glycogen was added to the solution at pH 6.9 using 1 µl of a 1.6% solution per 7 units of α-amylase (units are defined later). After 15 min, 0.2 M phosphate buffer, pH 8.0, was added to give a final buffer concentration of 10 mM. Ethanol was immediately added to bring the ethanol concentration back to 40%. A second volume of glycogen was added, equivalent to the first. After 15 min, the precipitate was pelleted by centrifugation. The pellet was drained and was washed with 5 ml of 10 mM phosphate buffer, pH 8.0, in 40% ethanol. After a second centrifugation at 4000 g for 5 min, the pellet was resuspended in 1 ml of 20 mM phosphate buffer, pH 6.9, containing 3 mM $CaCl_2$. This latter solution contained a fine precipitate of calcium phosphate. The centrifuge vial was rinsed a second time with 1 ml of the pH 6.9 buffer. The solutions were combined and incubated for 2.5 hr at 37 C.

After the glycogen and α-amylase were solubilized, NH_4OH (0.2 N) was used to increase the pH of the solution to 8.4 ± 0.1. The solutions were centrifuged at 6000 g for 5 min at room temperature to remove the insoluble carbohydrate.

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The method of Loyter and Schramm (1962) requires a rapid change of buffer after the preceding steps, because the sample must be at pH 10.0 during subsequent chromatography on a charcoal column. α-Amylase was somewhat unstable at pH 10.0 (Figure 5). After 3 hr at room temperature, 40% of the activity was lost. Loyter and Schramm apparently precipitated pancreatic amylase at its isoelectric point, pH 7.0, then dissolved the precipitate in pH 10.0 buffer made of 10 m$\underline{M}$ glycine and NaOH. This spontaneous precipitation did not occur with barley α-amylase at the final concentration of enzyme obtained prior to this step (about 320 enzyme units/ml). Preparative isoelectric focusing of barley α-amylase, in fact, segregated barley α-amylase into two peaks of activity, one with a pI of about 5.0 and the second with a pI of about 6.1 (data not shown). Thus, barley α-amylase would not be expected to precipitate at pH 7.0, and it did not.

However, in order to approximate the conditions to which Carlson must have exposed his α-amylase solutions by following the procedure of Loyter and Schramm, the following adaptation was made. Two samples of α-amylase were processed differently at this step. Sample A: The pH of the first sample was adjusted from pH 8.4 to pH 7.0 by the addition of 0.2 $\underline{M}$ acetic acid. No precipitation resulted after a 60-min incubation at 2 C. This meant that the buffer could not quickly be exchanged with one at pH 10.0 by dissolving the precipitate in the new buffer. Instead, 0.1 $\underline{M}$ glycine-NaOH, pH 10.0, was added to the solution to give a concentration of 10 m$\underline{M}$, and the pH of the solution was adjusted to 10.0 with NH_4OH. Sample B: After first changing the pH of the solution to 7.0 and chilling it for 60 min, the second sample was dialyzed at room temperature against 10 m$\underline{M}$ glycine-NaOH, pH 10.0, containing 5 m$\underline{M}$ $CaCl_2$. The 2 liters of external buffer were changed every 20 min.

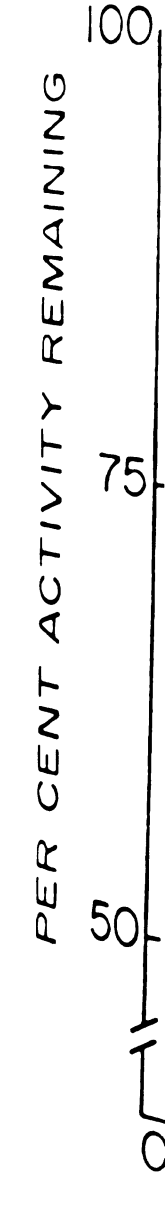
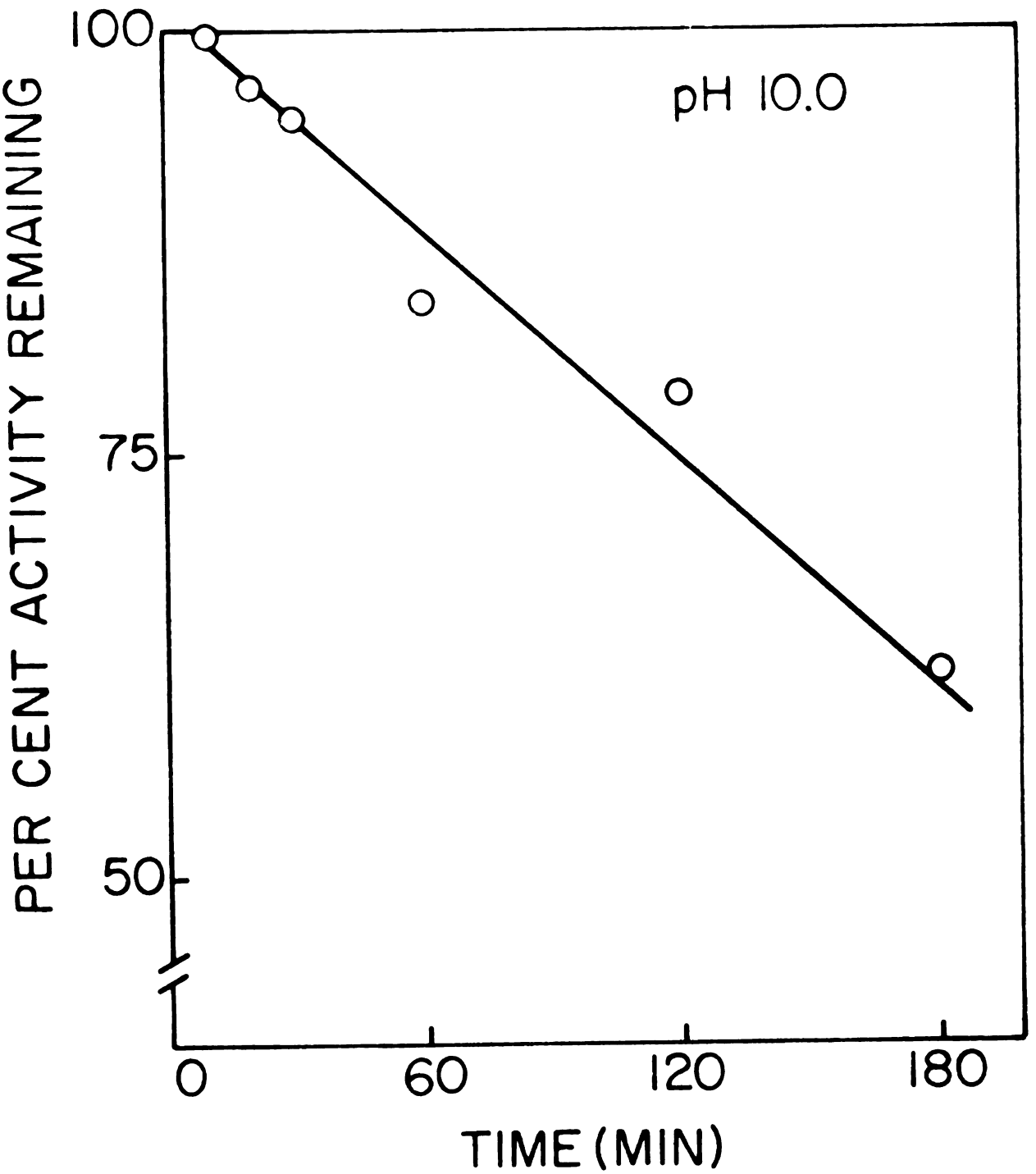


Figure 5. Stability of α-amylase at pH 10.0. α-Amylase was purified by Method II (Loyter and Schramm procedure). The enzyme was precipitated by the addition of glycogen, then solubilized by incubation of the glycogen/amylase complex at 37 C for 2.5 hr. The pH of the solution was adjusted by the addition of glycine-NaOH buffer (pH 10) until a concentration of 10 m$\underline{M}$ was reached, followed by titration of the solution to pH 10.0 by the addition of 1N NH_4OH. The activity was determined at intervals thereafter. The initial α-amylase concentration was 69.1 units per ml. The incubation was performed at room temperature.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After three changes (total time 1 hr), the pH of the dialysate was only 8.5. However, much of the phosphate had been removed. Calcium phosphate was removed from the dialysate by centrifugation and the supernatant solution was titrated to pH 10.0 with NH_4OH.

The charcoal-celite columns were prepared from a mixture of charcoal (activated Darco G-60, Matheson, Coleman, and Bell, Cincinnati, OH, USA) and celite 545 as suggested by Loyter and Schramm (1962), except that instead of washing the particles on a Buchler funnel, the particles were suspended and left to settle so that the fine particles could be removed. The final buffer contained 10 m$\underline{M}$ glycine-NaOH, pH 10.0. The column material was equilibrated in this solution over a period of 3 weeks. The charcoal used was kindly supplied by Dr. P. Carlson.

α-Amylase Assay

α-Amylase was measured by the method of Varner and Mense (1972). Starch substrate was prepared by suspending 150 mg of potato starch (Nutritional Biochemical Corporation) in 100 ml of a solution containing 50 m$\underline{M}$ KH_2PO_4 and 10 m$\underline{M}$ $CaCl_2$. This solution was boiled for 1 min, cooled, and centrifuged at 12,000 $\underline{g}$ for 15 min at 0-5 C. The supernatant solution was separated from the gelatinous pellet and diluted with 1.5 volumes of H_2O. The iodine solution was prepared by diluting 0.3 ml of iodine stock solution (6 g KI and 0.6 g I_2 in 100 ml H_2O) with 100 ml of 0.05 N HCl. This acidified solution was sensitive to air and light and had to be freshly prepared every 2 hr. The α-amylase was diluted to 1.0 ml with H_2O, and 0.5 ml of starch solution was added at room temperature. After a suitable time interval, 1.0 ml of the acidified iodine reagent was added, thus

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stopping the hydrolysis reaction. The absorbance of the blue reaction product was measured spectrophotometrically at 620 nm.

Certain features of the assay are worthy of note. First, the reaction of iodine with starch was only linear at $\underline{A}_{620}$ from zero to 0.950 absorbancy units (Figure 6). Thus, the starch should be diluted enough to yield a starch-iodine product which has an absorbancy below this value. Second, the enzyme assay is not linear, especially when more than 70% of the starch has been hydrolyzed (Figure 7). For this latter reason, the enzyme concentration or incubation time was varied so that the final starch-iodine complex would exhibit between 30 and 70% of the possible color development.

One enzyme unit is defined as that activity of enzyme which elicits a change of one unit in absorbance at 620 nm in one minute. A convenient formula for calculation is the following:

$$\text{number of enzyme units} = (1 - \frac{a}{b})(\frac{V}{v \times t}) = \frac{\Delta \underline{A}_{620}}{\text{min}}$$

where a = absorbance of the starch-iodine complex after hydrolysis

b = absorbance of the starch-iodine complex before hydrolysis (a and b are different samples, of course)

V = total volume of enzyme (ml)

v = sample volume of enzyme assayed (ml)

t = time allowed for hydrolysis in minutes.

α-Amylase was assayed in four replicates, except that the fractions resulting from column chromatography were usually assayed only once.

The presence of β-amylase would cause an over-estimate of the supposed α-amylase activity. However, the starch substrate contained a substantial amount of polymer which was inaccessible to β-amylase alone. Pure β-amylase from sweet potato (Sigma type IB with a

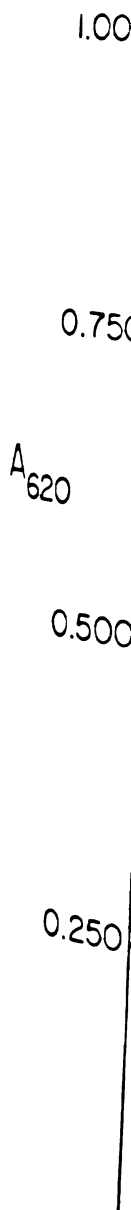
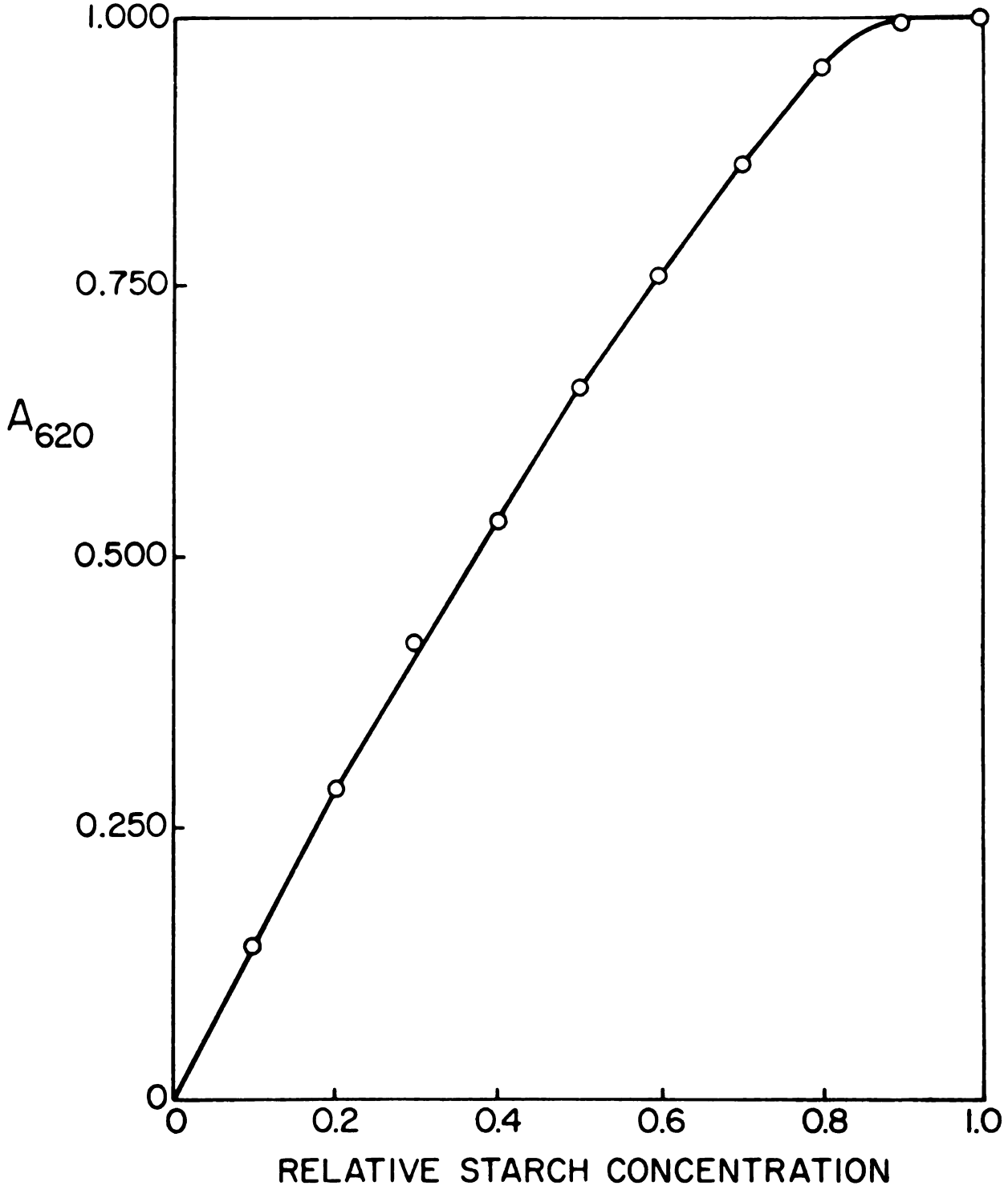


Figure 6. Effect of starch concentration on the development of the starch-iodine complex. The starch was boiled, centrifuged, then diluted to various concentrations. The standard iodine solution was added and the absorbance at 620 nm was determined.


1.000
0.750
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A_{620}
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RELATIVE STARCH CONCENTRATION

Figure 6

Figure 7. Hydrolysis of starch by α-amylase: reaction of the products with iodine. α-Amylase was incubated with the starch solution for increasing amounts of time, whereupon the iodine solution was added. The amount of α-amylase used in the assay was 0.107 units. The dotted area designates the region of $\Delta\underline{A}_{620}$ within which the α-amylase assays were considered for calculation.

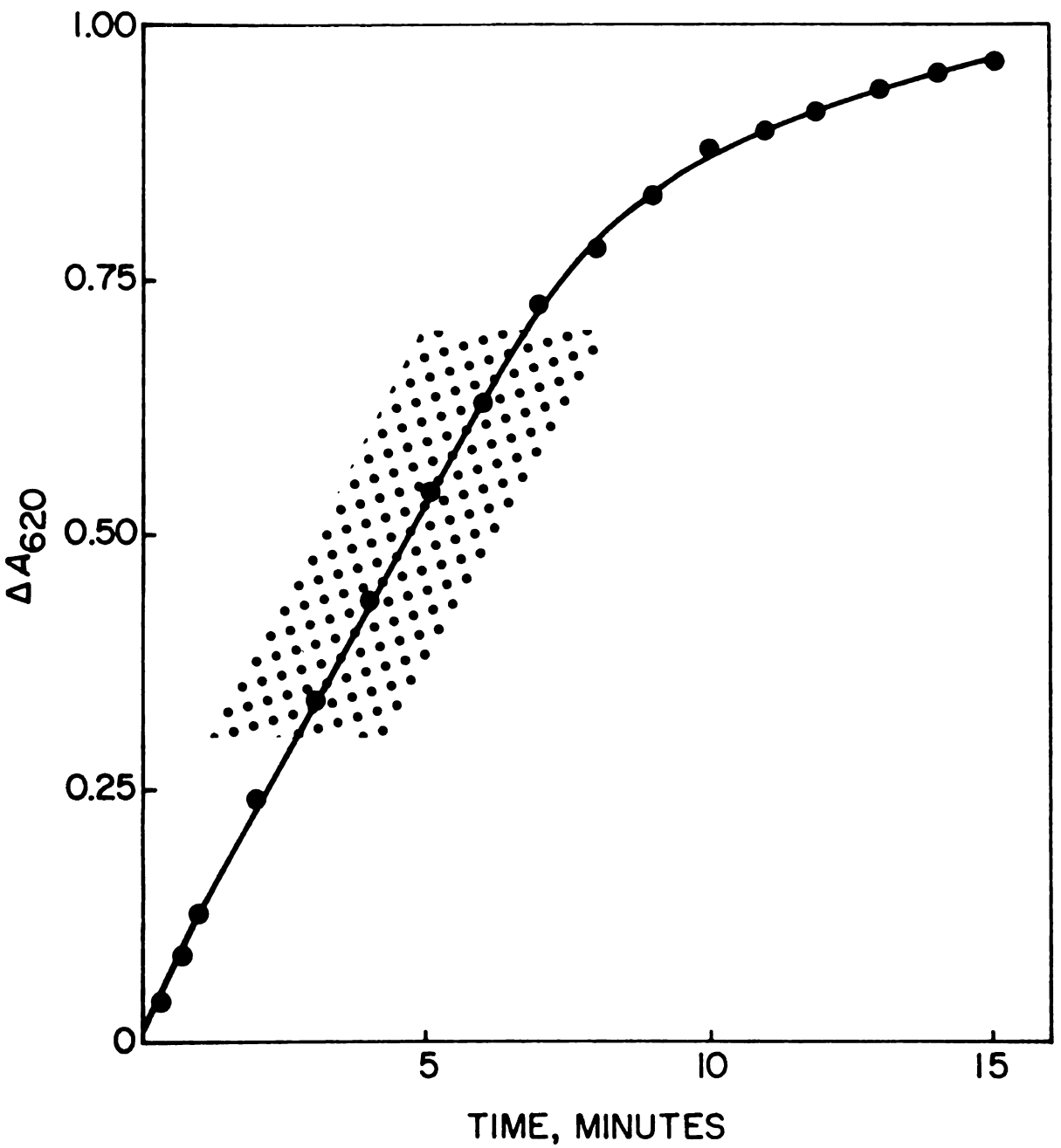


Figure 7

specific activity of 785 units per ml by the maltose assay) could only hydrolyze the starch solution to a ΔA_{620} of 0.350 (Figure 8).

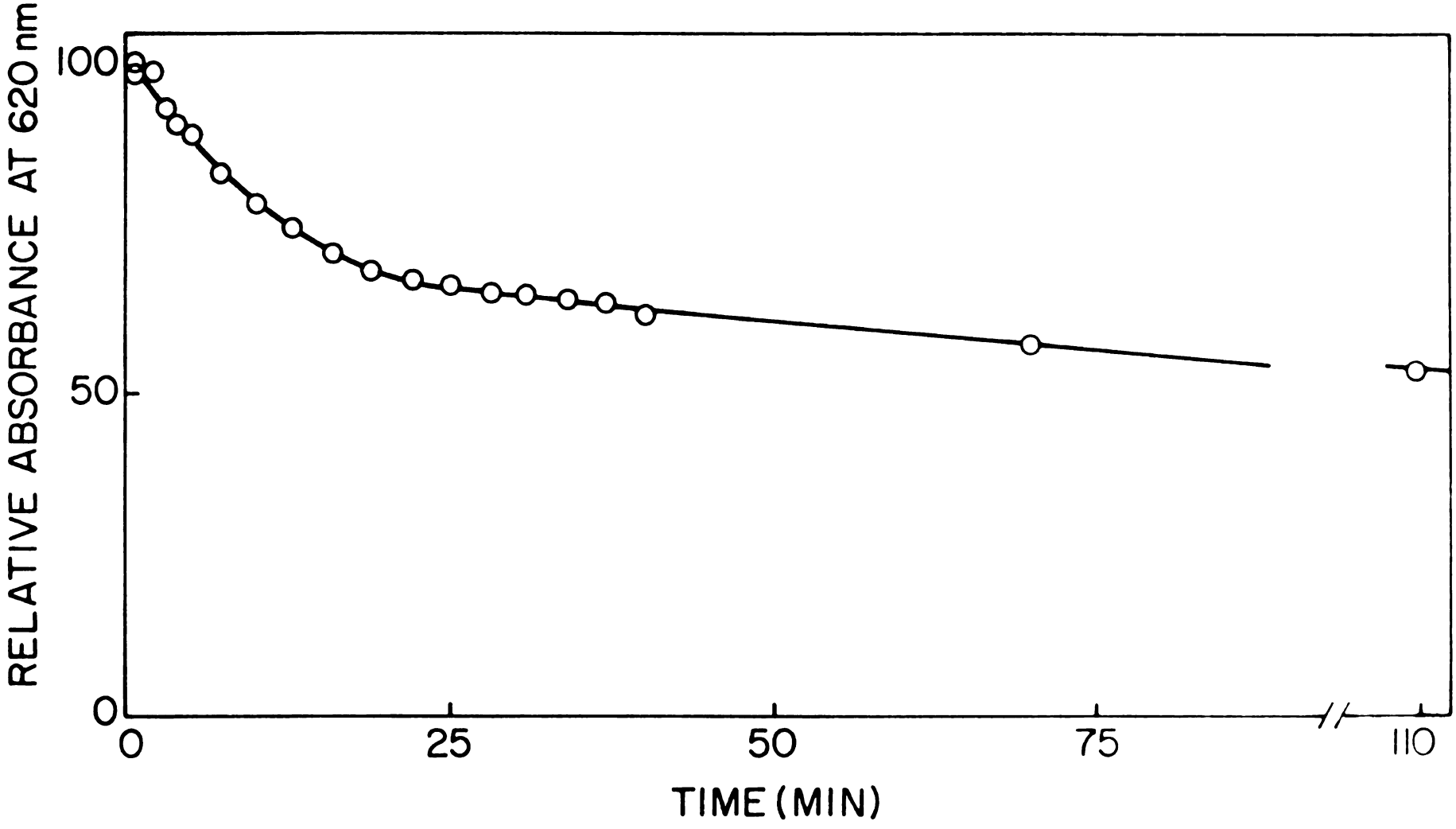
Protein Assays

Protein was assayed according to the procedure of Lowry et al. (1951) as described by Layne (1957). In some cases, samples contained a high concentration of amino acids and other interfering substances. In these instances, the following procedure was adopted. Portions of each sample (10 to 20 µl) were spotted onto cellulose thin-layer plates of 0.1 mm thick cellulose MN300 (Brinkman Instruments, Westbury, NY, USA), and thin-layer chromatography was performed in n-butanol, acetic acid, and H_2O (4:1:1 v/v). The protein remaining at the origin was assayed by scraping 0.5 by 1.0 cm sections from the plate. NaOH (0.1 N, 100 µl) was added to the powder, and after 10 min, 900 µl of a solution containing 2% $NaCO_3$, 0.02% sodium potassium tartrate, and 0.01% $CuSO_4 \cdot 5H_2O$ was added with rapid mixing. The usual procedure (Layne, 1957) was then followed, and the samples were assayed spectrophotometrically at 750 nm. Bovine serum albumin (BSA, Sigma type VI) was used as a standard. It was assumed that all proteins in these crude mixtures were solubilized after chromatography and processing. However, pure BSA was not easily released from the cellulose. Instead, scrapings of the cellulose MN300 plates from areas devoid of protein or ninhydrin-positive material were used as blanks and soluble BSA was assayed in the presence of the cellulose after the scrapings had been suspended in the first reaction mixture.

Carbohydrate Assays

The presence of carbohydrate was determined using the anthrone reagent (Sigma Chemical Company, St. Louis, MO, USA). This reagent

Figure 8. Hydrolysis of starch by sweet potato β-amylase. At zero time β-amylase (0.5 mg) was mixed with 100 ml of the starch solution and aliquots (1.5 ml) were added to the iodine solution (1 ml) after the appropriate times of incubation. The absorbance at 620 nm was measured and the percent change was recorded.



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(200 mg) was dissolved in 100 ml of concentrated H_2SO_4. The test solution (0.5 ml) and the dissolved reagent (1.0 ml) were mixed thoroughly. The mixture was boiled for 10 min, cooled, and the absorbance measured spectrophotometrically at 620 nm. Individual sugars were identified as their alditol acetate derivatives by gas chromatography (Jones and Albersheim, 1972).

Calcium Assay

Free calcium was determined by complexing the ion with glyoxal-bis(2-hydroxyanil). The method for qualitative identification of calcium (Feigl, 1958) was modified in the following manner to permit quantitative measurements. To a 0.5 to 1.0 ml test solution were added sequentially, with mixing at each addition, 50 µl of 1 N HCl (or sufficient HCl to drop the pH to equal to or less than pH 7.0), 200 µl of a saturated solution (up to 1%) of glyoxal-bis(2-hydroxyanil) in ethanol, 100 µl of 10% NaOH, and 100 µl of 10% Na_2CO_3. The aqueous solution was extracted with 3 ml of chloroform, and the absorbance of the chloroform solution was determined at 560 nm. At this wavelength, a calcium-free solution showed very little absorbance. The limit of detection for the spectrophotometric assay was 5 µg (in 3 ml) or about 1.6×10^{-6} M of calcium.

Thermal Denaturation

Calcium-free α-amylase was diluted to between 200 and 500 units per ml with 5 mM Tris, pH 8.0, containing 1 mM EGTA. The volume of enzyme was usually between 600 and 800 µl. The zero-time sample was usually assayed six times to determine the initial α-amylase concentration. The sample, placed in a tightly sealed, 3.5-ml vial, was heated in a small waterbath (Aloe Scientific, St. Louis, MO, USA),

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and after various times the vial was briefly opened so that samples could be rapidly mixed with one-half of their volume of 1 M $CaCl_2$ which had previously been pipetted into small vials placed on ice. Each heat-treated sample was assayed three or four times for the amount of α-amylase activity remaining.

Separation of Proteins by Polyacrylamide Gel Electrophoresis

Proteins were separated by electrophoresis on polyacrylamide gels by one of three methods.

Fairbanks (et al.) Method

Usually, the method of Fairbanks et al. (1971) was used, with some modifications. The running buffer consisted of 40 mM Tris base, 20 mM sodium acetate, 2 mM EDTA, and 1% SDS. The pH of the running buffer was adjusted to pH 7.4 with acetic acid before SDS was added. The gels consisted of 5.6% acrylamide, 0.21% bis-acrylamide, 10% glycerol, and the running buffer. Polymerization required 24.2 µl of TEMED and 65 mg of ammonium persulfate (AMPS) per 100 ml of gel solution. The gel solution was distributed to glass tubes (6 x 150 mm) which had been silanized with 1% dichlorodimethyl silane (v/v) in toluene for 1 hr and rinsed thoroughly with methanol. Each gel tube contained about 3.5 ml of gel solution, and the gels were about 10 cm long. When the polymerizing gels were layered with isobutanol, a very flat gel surface resulted. Polymerization was complete within 45 min, after which the isobutanol was washed with running buffer from the gel surfaces.

The protein samples were dialyzed if necessary to reduce the salt concentration. Two volumes of protein were then added to one volume of a boiling solution of 5% SDS and 12.5% β-mercaptoethanol, and

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boiling was continued for 10 min. The samples were cooled and one additional volume of a buffer-tracker dye solution containing 40% glycerol, 40 mM Tris, 2 mM EDTA, and 0.02% pyronin Y was added. The pH of the buffer-tracker solution had first been adjusted to 8.0 with HCl. The ratio of the volumes of the solution of protein, denaturants and buffer were thus 2:1:1, respectively.

The protein samples, each of which contained a maximum of 10 units of α-amylase or 100 μg of a mixture of proteins, were layered onto the gel surface under the running buffer of the upper chamber. The portions of the gel tubes which contained the polyacrylamide were immersed in the buffer of the lower chamber to facilitate cooling since the electrophoretic runs were performed at room temperature. The direction of electrophoresis was toward the anode using 5 mA per gel for 3 hr. After electrophoresis, gels were removed from the glass tubes using a jet of H_2O from a 2-inch, 25-gauge needle inserted between the gel and the glass tubing.

The gels were stained for protein with 0.1% Coomassie Brilliant Blue R in 50% methanol and 7.5% acetic acid. Pre-fixing was unnecessary. After 8-16 hr in the staining solution, excess stain was removed by several incubations in 5% methanol and 7.5% acetic acid at 60 C. The stained gels were scanned at 560 nm using a Beckman DUR monochrometer equipped with a Gilford linear transporter, or the mobilities relative to pyronin Y were determined with a ruler. India ink, injected with a needle, was used to mark the dye position before the gel was stained. Poor staining could be remedied by repeating the staining procedure or could be prevented by staining the gels in large volumes (50 ml per gel) of staining solution.

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The molecular weight of α-amylase was determined by comparison to those of several marker proteins. These were bovine serum albumin (68,000), ovalbumin (43,000), horse liver alcohol dehydrogenase (41,000), aldolase (40,000), yeast alcohol dehydrogenase (37,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), chymotrypsinogen (25,700) and cytochrome c (12,400). The molecular weights in parentheses were taken from Weber and Osborne (1969) and had been determined by polyacrylamide gel electrophoresis. The molecular weight of cytochrome c was given by the distributor. All marker proteins were obtained from Sigma.

Laemmli Method

The method of Laemmli (1970) was also used for electrophoresis of protein samples. Samples were prepared by adding crystalline urea to a boiling solution containing 0.125 M Tris base, 0.119 N HCl, 4% SDS, 0.0002% bromophenol blue, and 10% mercaptoethanol to give a final concentration of 8 molal urea. One volume of protein was added to this with rapid mixing, and the solution was immediately returned to the boiling water bath for 10 min.

Ingle Method

A third method used a gel system based on that of Ingle (1968), using non-denaturing conditions. The running buffer was 5 mM Tris and 5 mM glycine at pH 8.9. The gels contained buffer, 7.5% acrylamide, and 0.15% bis-acrylamide, with 50 µl TEMED and 7.5 mg of aqueous AMPS per 100 ml of gel solution. A H_2O overlay was used to cover the gels during polymerization. After 1 hr of pre-electrophoresis at 7 C and 2.5 mA per gel, the enzyme samples, which contained 8% sucrose, and 0.008% bromophenol blue, were layered onto the gel surfaces.

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Electrophoresis was continued in the cold at 2.5 mA per gel for 50 min. The gels were removed from the tubes and either stained with Coomassie blue or were examined for enzymatic activity. These unstained gels were sliced at room temperature into 2-mm sections (approximately) which were placed onto petri plates containing 1% agar, 0.5% soluble potato starch, 20 m$\underline{M}$ potassium phosphate buffer (pH 5.6), and 1 m$\underline{M}$ $CaCl_2$. After 20 hr at 25 C in the dark, the gel slices were removed, and the plates were flooded with acidified I_2KI solution. The diameter of the clear circles was used to indicate the relative α-amylase activity.

Isozyme Separation by Agar Gel Electrophoresis

Amylase isozymes were separated by electrophoresis in agar gel slabs (Jacobsen et al., 1970). The gels were formed from 1% Difco purified agar dissolved in the running buffer, 4 m$\underline{M}$ sodium phosphate, pH 7.55. The samples were each placed in one of eight individual, 1-cm-long slits arranged across the plexiglass tray holding the gel. The dimensions of the tray were 17.5 cm x 19.5 cm x 0.3 cm. The slits were arranged parallel to and 6 cm away from the narrower (17.5 cm) side of the plate. Up to 8 µl of solution containing a total of 0.3 units of α-amylase were placed on 2.5 x 10 mm wicks of Whatman No. 2 filter paper and these were gently inserted into the slits. Thin sponges (duPont cellulose sponge cloth) were used as wicks for the buffer during the electrophoresis. The edge of one sponge cloth was placed over the shorter end of the gel to within 2 mm of the samples. This was the anode end of the gel. The second cloth was placed 3.5 cm from the cathode end but was underlain with a single layer of dialysis membrane (4.0 cm wide) which helped prevent

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^{32}P-Labeling and Isolation of Ribonucleic Acid

One hundred half-seeds were incubated for 24 hr in aerated H_2O, followed by 36 hr in Acca buffer, pH 4.8. Inorganic ^{32}P, at 70.3 μCi per ml or 560 μCi per flask of 50 half-seeds, was added to the medium for a further 12 hr. After labeling, the half-seeds were placed on paper towels and aleurone layers were prepared. Each layer was immediately rinsed in 50 m$\underline{M}$ PO_4, blotted, and dropped into a tube partially filled with liquid nitrogen. The frozen layers were lyophilized overnight and ground to a powder in the presence of additional liquid nitrogen. Ribonucleic acid was extracted from the

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aleurone powder by a method which was based on that of Bellamy and Ralph (1968). The powder was homogenized with sea sand using equal volumes of (a) 1% SDS with 200 µg/ml sodium heparin and (b) a H_2O-saturated solution containing 80 g phenol, 10 ml of m-cresol and 0.01% 8-hydroxyquinoline. The mixture was again stirred and then centrifuged in a clinical centrifuge to separate the phases. The phenol phase was extracted a second time with fresh SDS solution. The two resulting aqueous upper phases were combined, and 2 volumes of ice-cold ethanol were added. The solution was chilled for 1 hr, and the precipitate pelleted by centrifugation. The pellet was dissolved at room temperature in 5 ml of 0.1 M sodium acetate, pH 5.5, containing 0.5% SDS and 0.1% sodium heparin. CTA-bromide (1 ml of 1% w/v) was added dropwise, and the solution was placed in ice for 30 min. The precipitate was collected by centrifugation for 10 min at 13,300 g, and the pellet was washed and recentrifuged three times with cold 70% ethanol containing 0.1 M sodium acetate, pH 5.5. The washed pellet was resuspended in 0.33 M sodium acetate, pH 5.5, and reprecipitated with two volumes of cold ethanol. The final pellet was dissolved in 0.3 ml of electrophoresis buffer. This method extracted rRNA and tRNA, but poly(A)-RNA species were probably left behind.

The RNA was separated electrophoretically using the method of Loening (1967). The electrophoresis buffer included 40 mM Tris, 33 mM sodium acetate, 2 mM disodium EDTA·$2H_2O$ and 0.4% SDS. The pH was adjusted to 7.8 with glacial acetic acid. The gels contained 2.5% acrylamide, 0.125% bis-acrylamide, 82 µl of TEMED per 100 ml of solution, and 0.8% ammonium persulfate. Plexiglass tubing (6 mm x 120 mm) was fitted at one end with a small piece of Tygon tubing so

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that the opening of the tube was reduced to 1 to 2 mm. This prevented the gels from sliding out of the gel tubes. The gel solution was added to a depth of 9-10 cm and was immediately over-layered with 4 mm of H_2O. Polymerization was complete within 30 to 45 min. The gels were pre-treated by electrophoresis without samples for 1 hr at 5 mA per gel. Afterwards, samples were prepared by mixing one $\underline{A}_{260}$ unit of RNA with ribonuclease-free sucrose (8%) and were layered onto the tops of the gels, and electrophoresis was allowed to proceed for 105 min at 5 mA per gel. Following this, the gels were removed from the tubes and were incubated in distilled H_2O for 1 hr. The unstained gels were scanned spectrophotometrically at 260 nm. They were then lain at a slight angle in foil troughs with rubber plugs at the lower end and were frozen over dry ice. The frozen gels were sliced into 0.7 or 1.0 mm slices with a Mickle Gel Slicer. A Packard Tri-Carb liquid scintillation spectrometer, model 544, was used to determine the radioactivity. The scintillation fluid contained 0.5 g PBBO and 8 g of butyl-PBO per liter of toluene.

RESULTS

Response of Half-Seeds of Betzes Barley to GA_3

Half-seeds of Betzes barley contained no detectable α-amylase activity after a three-day imbibition period on moistened sand. When 10^{-6} M GA_3 was added and the half-seeds were shaken in a liquid medium, α-amylase activity increased sharply after a lag period of about 8 hr (Figure 9). Ninety-five percent of the enzyme was released into the medium or the starchy endosperm. Only a low level of α-amylase was produced when no hormone was added. One half-seed of Betzes barley each synthesized between 10 and 15 units of amylase activity in a period of 40 hr.

Aleurone layers prepared from half-seeds of Betzes barley synthesized 5.0 to 5.6 units of α-amylase, again after a lag period of about 8 hr (Figure 10). However, about 30% of this activity remained associated with the tissue, even after the layers were rinsed with 0.2 M $CaCl_2$. 5-Fluorouracil at a concentration of 10^{-3} M did not affect the lag time for α-amylase synthesis and the amount of α-amylase synthesized by or released from the aleurone layer (Figure 10). Less than 0.12 units of enzyme were synthesized without the addition of GA_3.

Despite the fact that the Betzes barley seed had been stored (at 7 C) for five years before these studies were begun, the seeds were completely viable when placed on moist filter paper. The

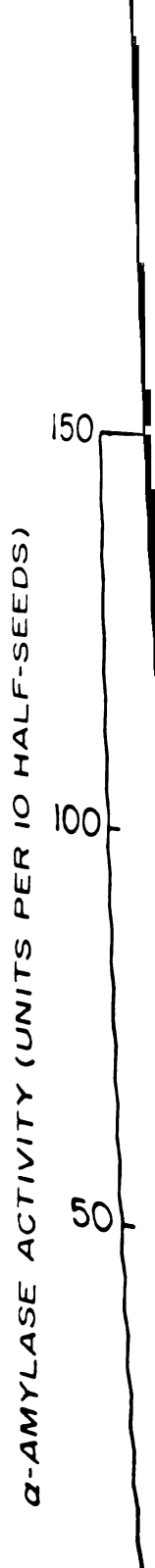

Figure 9. Synthesis and release of amylase by half-seeds of Betzes barley in response to GA_3. Amylase activity was measured in the medium plus starchy endosperm ("released") and in the homogenate of the aleurone layers ("tissue"). Solid lines, 1 μM GA_3; dashed lines, no GA_3. Released, O; tissue, ●.

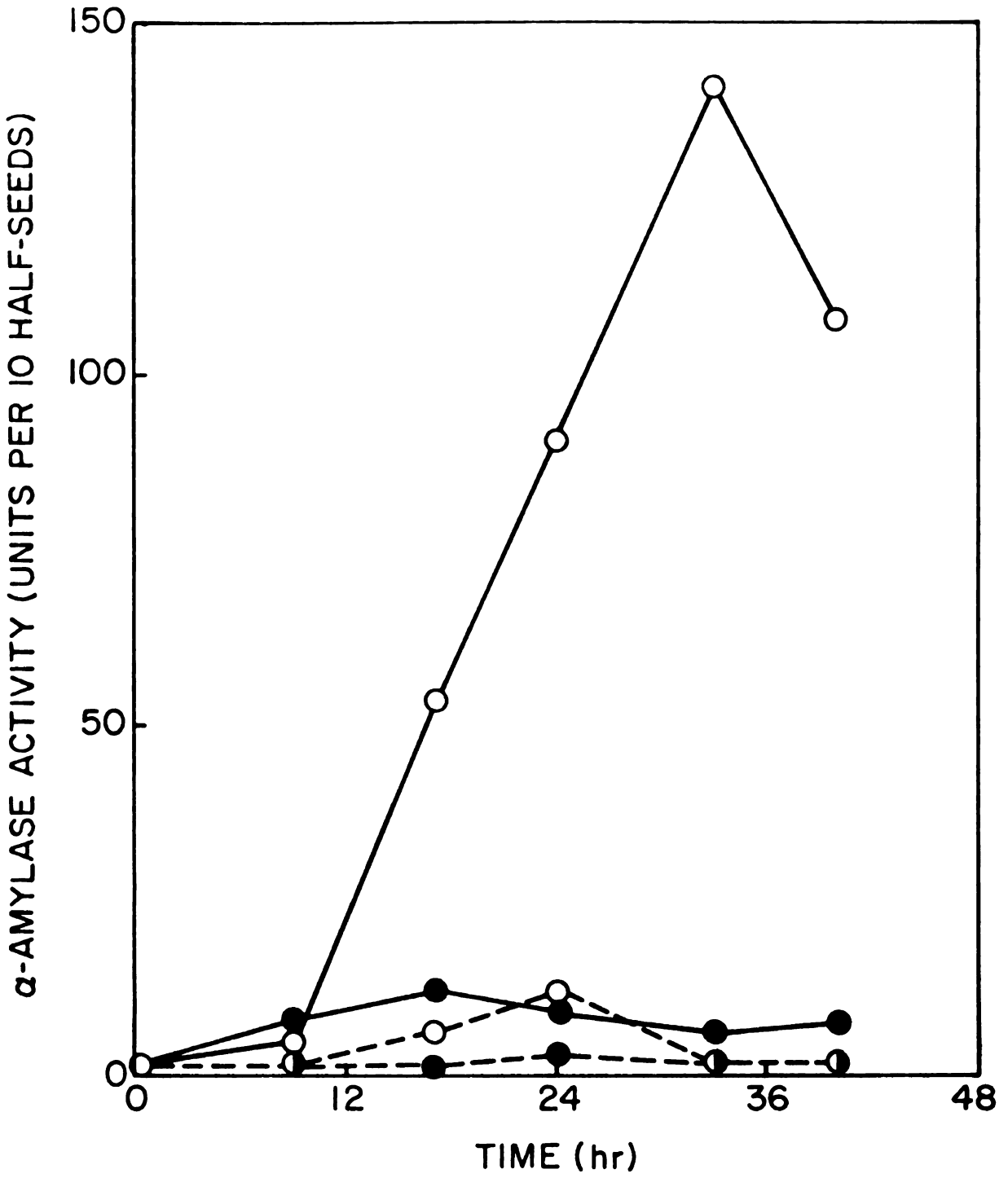
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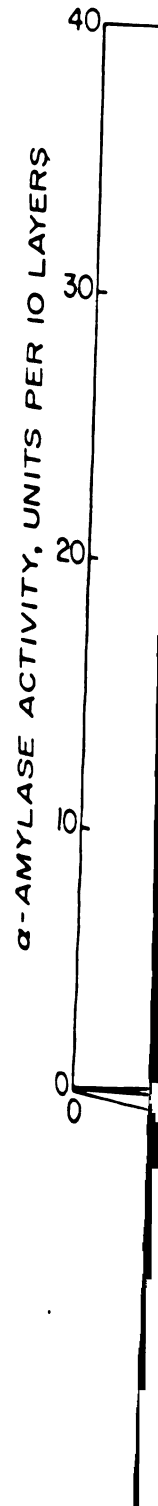
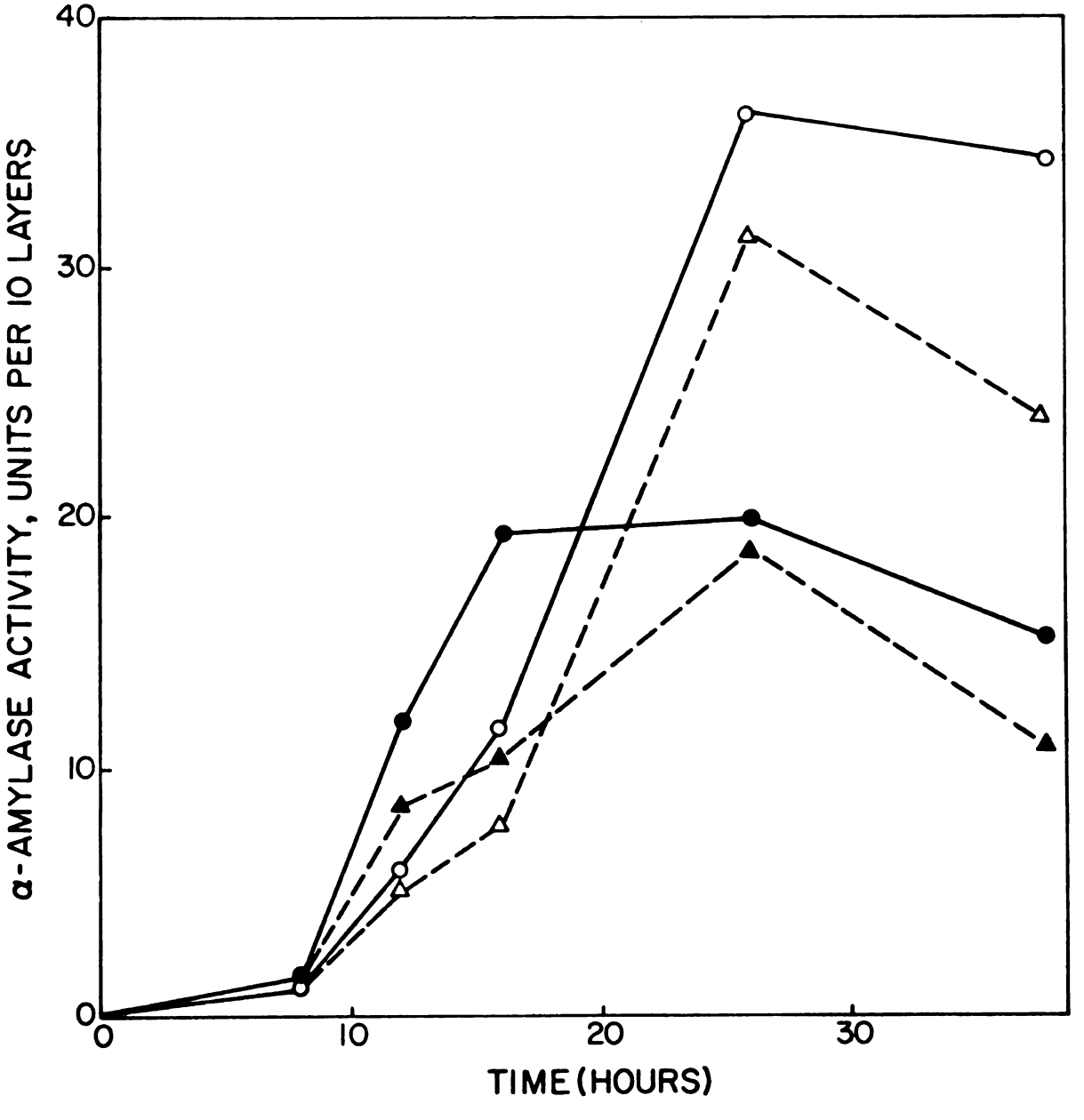
Figure 9

Figure 10. Synthesis and release of α-amylase from aleurone layers of Betzes barley in response to GA_3; effect of 10^{-3} M 5-FU. The layers were prepared from half-seeds which had been pre-incubated for 3 days, with or without 5-FU. Fresh medium (with or without 5-FU) was added with 1 μM GA_3 at zero time. The aleurone layers were rinsed with 0.2 M $CaCl_2$ in 1 mM sodium acetate, pH 4.8 before homogenization, and the rinse was added to the medium. No 5-FU, solid lines; 1 mM 5-FU, dashed line. Released, open symbols; tissue, solid symbols.

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dehusking with acid decreased the viability by 30%, presumably due to an effect of the acid on the superficially located embryo.

Effect of 5-FU on α-Amylase Synthesis

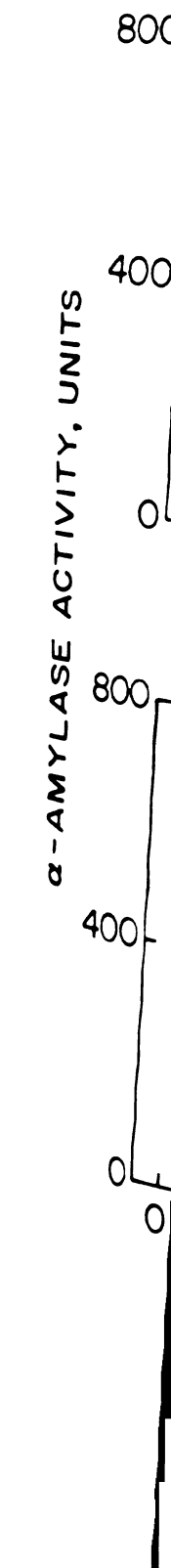
Fluorouracil did not inhibit the synthesis of α-amylase by more than 20% at concentrations of up to 10^{-2} M 5-FU (Figure 11). This result strongly indicates that 5-FU does not affect normal cellular metabolism required for the synthesis of α-amylase.

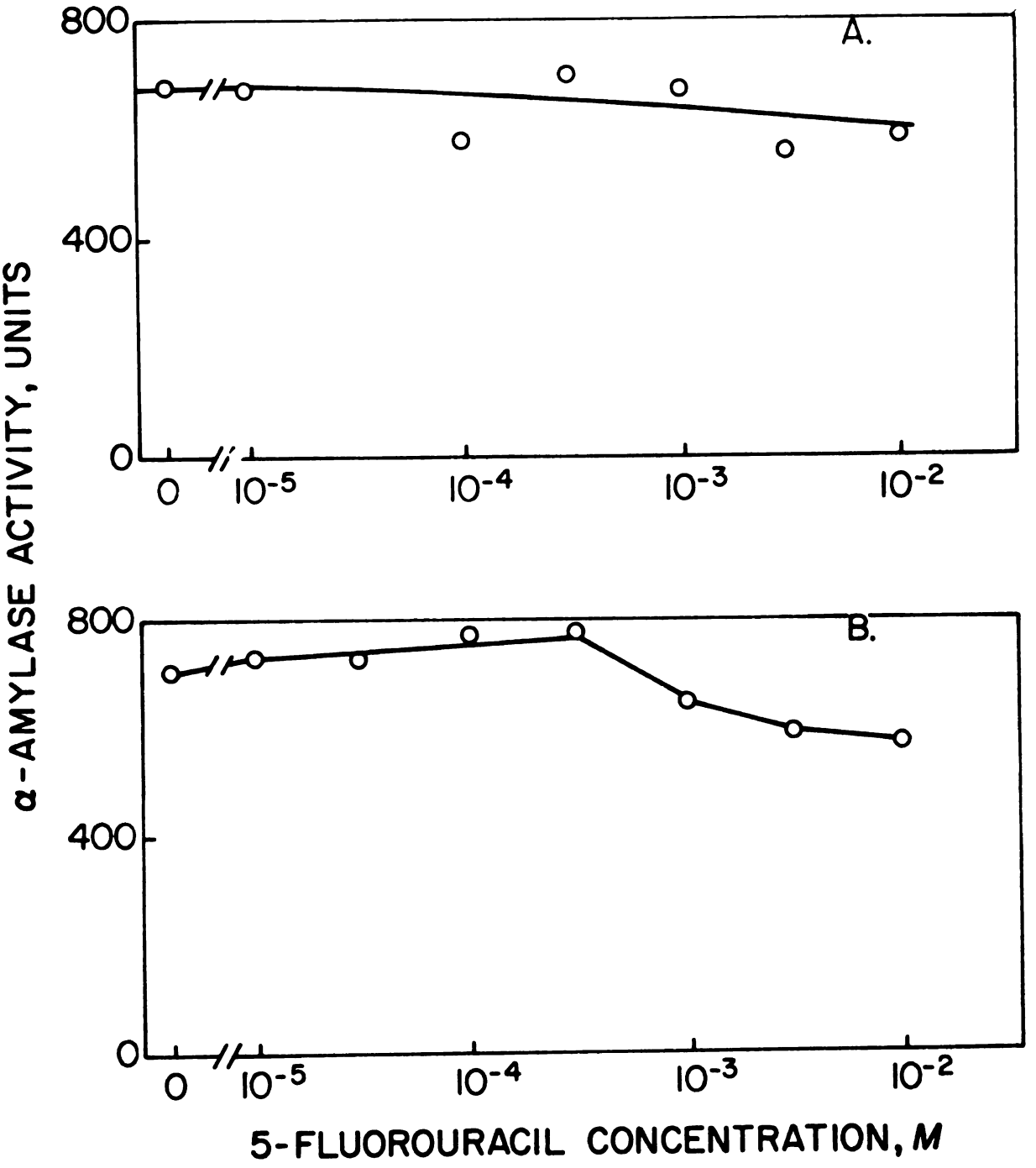
Effect of 5-FU on RNA Synthesis

The lack of an inhibitory effect of 5-FU on α-amylase synthesis might have been due to insufficient uptake of this drug by the tissue, thus precluding competition of endogenously formed 5-FU ribosyl-triphosphate (5-FURTP) with UTP during RNA synthesis. This possibility was examined by measuring the effect of 5-FU on rRNA synthesis in the half-seeds. It has been well documented in animal cells (Wilkinson et al., 197; Hadjiolova et al., 1973; Wilkinson and Pitot, 1973) that 5-FU inhibits the processing of precursors of rRNA and thus the formation of new rRNA molecules. In soybean, 5-FU also inhibits the appearance of rRNA (Key, 1966). An effect of 5-FU on rRNA synthesis in the barley half-seeds would indicate that 5-FU was taken up by the aleurone tissue and that it competed with the endogenous pools of uracil metabolites.

Half-seeds were incubated for 2.5 days in H_2O or buffer, followed by incubation with $^{32}PO_4$ (inorganic) for 12 hr. 5-Fluorouracil was either absent, or present at 10^{-4} M or 10^{-2} M for all but the first 24 hr of incubation. After incubation, the starch was mechanically removed, RNA was isolated from the aleurone layers, purified, and applied to polyacrylamide gels for electrophoresis. The purified RNA

Figure 11. 5-Fluorouracil concentration and α-amylase synthesis. A. Half-seeds of Betzes barley were incubated as in Figure 1A. B. Half-seeds of Betzes barley were incubated as in Figure 1B. For each treatment, triplicate flasks with 25 half-seeds each were incubated.

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separated into peaks of 28S, 18S, and 4 to 5S RNA, which is characteristic of eukaryotic RNA (Figure 12); the Svedberg units were assigned by analogy to those determined for other plant nucleic acids (Zwar and Jacobsen, 1972).

The RNA preparation contained 4 or 5% DNA, and the ratio of $\underline{A}_{260}$ to $\underline{A}_{280}$ was 1.88. The amounts of labeled phosphate incorporated into aleurone RNA at various concentrations of 5-FU are shown in Table 1. The incorporation of ^{32}P into RNA was clearly inhibited by 5-FU. When one $\underline{A}_{260}$ unit of each RNA preparation was subjected to electrophoresis on polyacrylamide gels, a decrease in the incorporation of ^{32}P into each major RNA species was evident (Figure 13) in response to increasing concentrations of 5-FU. The specific radioactivities of each 28S, 18S or 4 to 5S species were calculated by dividing the total radioactivity in each peak by the total absorbancy of each peak after scanning the gels spectrophotometrically at 260 nm. These specific radioactivities are shown relative to that of labeled RNA purified from tissue incubated without 5-FU (Figure 14).

The synthesis of new molecules of the major RNA species was strongly inhibited by 10^{-2} $\underline{M}$ 5-FU, with 10^{-4} $\underline{M}$ 5-FU eliciting a lesser effect. Incorporation of radioactivity into 5 to 18S RNA was insufficient to detect any effect on synthesis of putative mRNA species. However, one can conclude that sufficient 5-FU entered the aleurone tissue to inhibit both rRNA and tRNA synthesis, and that α-amylase synthesis occurred even in the presence of 5-FU.

Purification of α-Amylase

The thermal stability of a protein is measured most reliably when the protein preparation is free from other contaminating molecules.

Figure 12. SDS-Polyacrylamide gel electrophoresis of RNA from barley aleurone layers. Aleurone layers were removed from half-seeds which had been incubated for 24 hr in H_2O, followed by 24 hr in buffer, and 12 hr in buffer plus $^{32}PO_4$. One $\underline{A}_{260}$ unit (about 41.7 µg) of RNA was applied to the gel. A small peak of DNA also entered the gel.

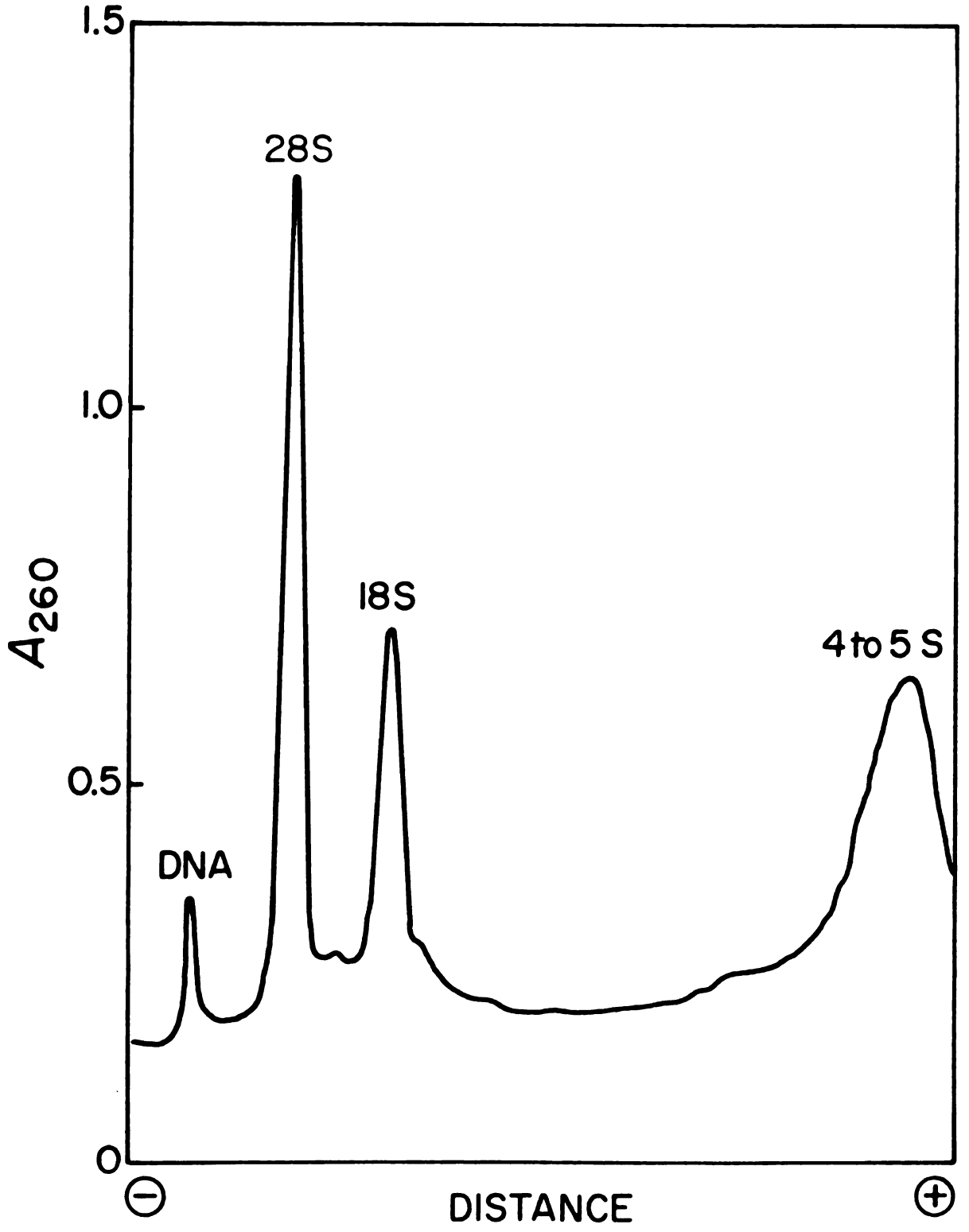

Figure 12

Table 1. Incorporation of ^{32}P into RNA by aleurone layers of Betzes barley

Treatment[a]	^{32}P in Medium (cpm per flask)	Specific Activity (cpm per µg RNA)	Relative Specific Activity %
no 5-FU	7.1×10^8	2884	100.0
10^{-4} M 5-FU	7.1×10^8	1524	52.8
10^{-2} M 5-FU	7.1×10^8	616	21.4

[a]Half-seeds of Betzes barley were incubated as in Figure 12. After incubation, the starchy endosperm was removed and RNA was extracted from the aleurone layers.

Figure 13. Incorporation of ^{32}P into RNA species from aleurone tissue of Betzes barley: Effect of 5-FU. One $\underline{A}_{260}$ unit (about 41.7 µg) was applied per gel. After electrophoresis on polyacrylamide gels, each gel was sliced, and the radioactivity per slice was determined. A. No 5-FU, 0.7 mm slices. B. 10^{-4} $\underline{M}$ 5-FU, 1.0 mm slices. C. 10^{-2} $\underline{M}$ 5-FU, 1.0 mm slices. Notice that the slices from (A) are thinner and thus have relatively fewer cpm per slice. The total radioactivity (cpm) in each peak is printed next to the peak.

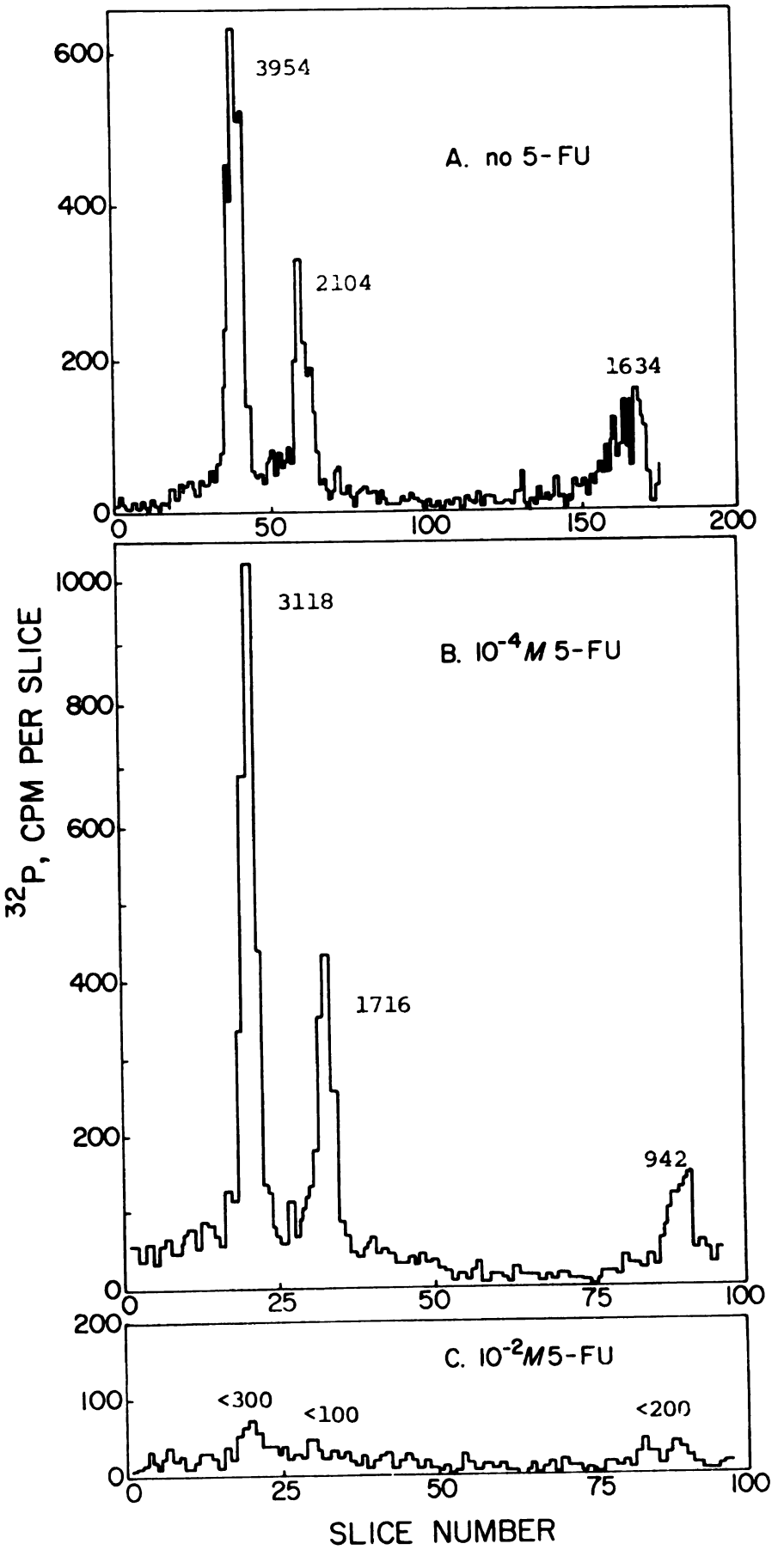
600
400
200
0
3954
2104
1634
A. no 5-FU
0
50
100
150
200
1000
800
600
400
200
0
3118
1716
942
B. $10^{-4}M$ 5-FU
0
25
50
75
100
200
100
0
<300
<100
<200
C. $10^{-2}M$5-FU
0
25
50
75
100
^{32}P, CPM PER SLICE
SLICE NUMBER

Figure 13

Figure 14. Specific activities of the RNA species after treatment of half-seeds with 5-FU. The relative specific activity is the ratio of radioactivity (cpm) of ^{32}P per $\underline{A}_{260}$ peak, relative to the specific activity of the RNA species from control (no 5-FU) tissue.

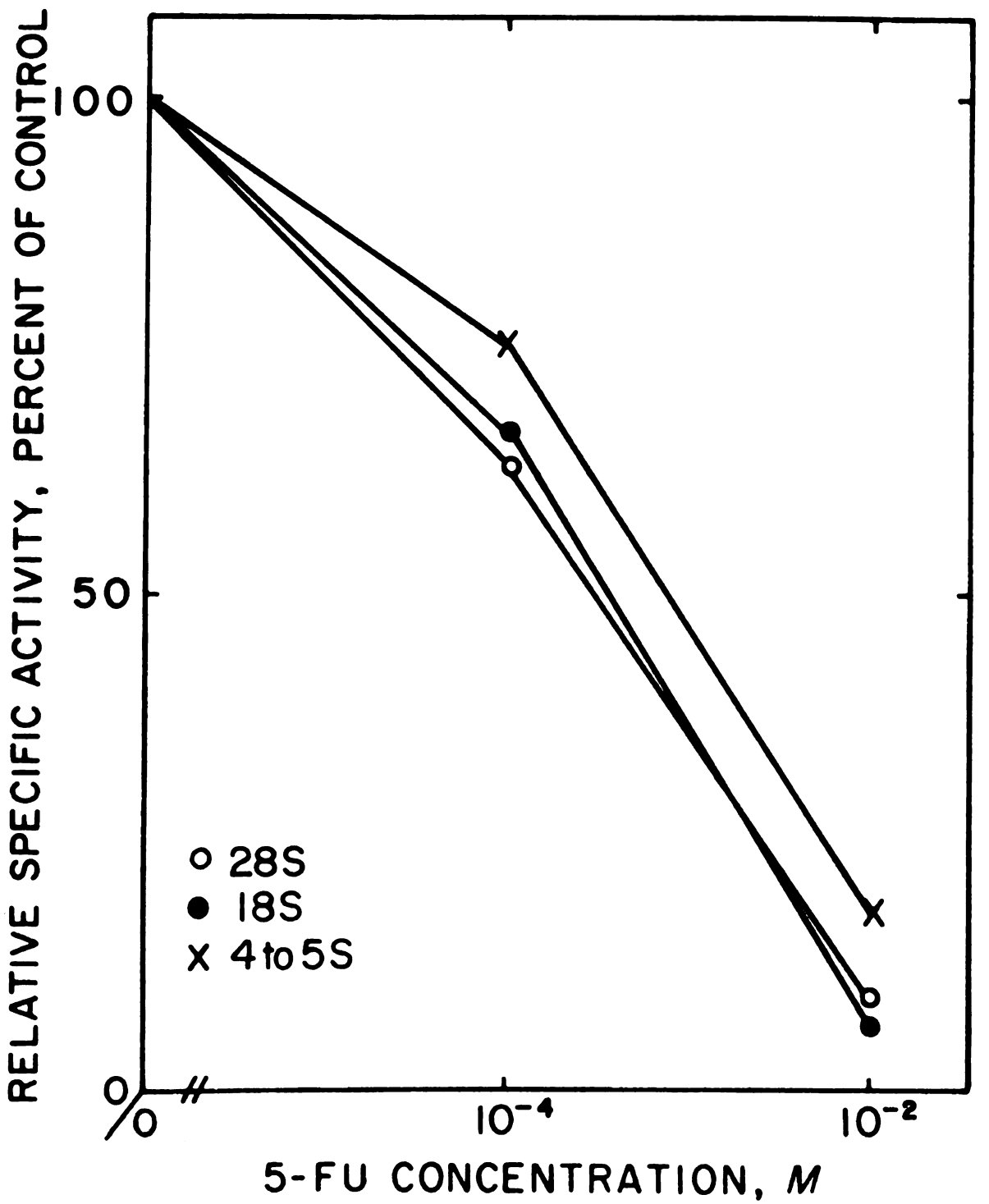
RELATIVE SPECIFIC ACTIVITY, PERCENT OF CONTROL
100
50
0
0
10⁻⁴
10⁻²
5-FU CONCENTRATION, M
o 28S
● 18S
x 4to5S

Figure 14

The reason for this is that 5-FU treatment of the aleurone tissue may have affected the synthesis and/or release of compounds other than α-amylase which may have resulted in there being different kinds and levels of contaminants in the enzyme solutions at the time the thermal stability was determined. Certain of the contaminants may have either affected the stability of the α-amylase or have also utilized the starch substrate. Some examples of substances which could interfere with the determination of the thermal stability of α-amylase, and the levels of which may have been especially affected by 5-FU treatment, are β-amylases, proteases, and relatively high concentrations of proteins or of polysaccharides.

β-Amylase. The β-amylases become activated by proteases which are synthesized and released along with α-amylase (Jacobsen and Varner, 1967). β-Amylases are exo-hydrolases while α-amylases are endo-hydrolases. Since calcium stablizes α-amylase against thermal inactivation (Kneen et al., 1943), all of the calcium must be removed from the enzyme. In the *absence* of calcium, *both* α-amylase and β-amylase are unstable to heating. Thus, β-amylases would definitely interfere with determinations of the heat stability of α-amylase.

Proteases. Even trace amounts of protease could increase the apparent rate of enzyme inactivation during heating. If isozymes of α-amylase were differentially "inactivated" by proteases, multi-phasic inactivation kinetics could be produced.

Other proteins. Carbohydrases, in addition to β-amylase, which utilize soluble starch as substrate, might also cause the appearance of altered heat stability of α-amylase which would manifest itself

through multi-phasic decay kinetics. Contamination by large amounts of protein (on the order of 1% w/v or more of the test solution) could decrease the activity of H_2O in the solution and thus decrease the rate of thermal inactivation. Such proteins could also bind to either the α-amylase or the starch substrate, or they could interfere with the renaturation of the α-amylase during cooling of the enzyme solution.

Polysaccharides. In the aleurone system, there are many sources of polysaccharide contamination, including the cell walls of the aleurone and starchy endosperm, the starch grains, and the glycogen used to precipitate the α-amylase. Because enzymes bound to their substrates are frequently more stable than when they are free, the presence of carbohydrate polymers could affect the stability of the α-amylase during heating.

Removal of β-Amylase

There are two ways to avoid the contamination of α-amylase by β-amylase. One would be to remove all of the starchy endosperm from the aleurone layer before placing the layer into medium which contained GA_3. For the starchy endosperm to be efficiently removed, it must have been "softened" during a suitable hydration period of at least 2 days of incubation in an aqueous medium. Although Carlson (1972) indicated he had used aleurone layers for his studies, I found that I could not prepare clean aleurone layers by incubating half-seeds for only 24 hr in H_2O as he had. Therefore, I used half-seeds, heavily contaminated with β-amylase, as a source of the α-amylase, but I employed a different method to eliminate that contaminant. β-Amylases are inactivated by heating the extract of the half-seeds

at 70 C for 15 min at pH 8.0, α-amylases are stable to this procedure, as long as the medium contains calcium. Therefore, a heat treatment was used to inactivate β-amylases. Following this, β-amylases should no longer bind to glycogen, permitting separation of the two amylases in the glycogen precipitation step.

Electrophoretic Homogeneity on SDS-Polyacrylamide Gels

1. α-Amylase was purified using Method I and was subjected to polyacrylamide gel electrophoresis using the method of Fairbanks et al. (1971) with modifications as indicated. This method leads to the separation of proteins according to their molecular weight. When purified α-amylase was subjected to electrophoresis, a single band of protein appeared following staining with Coomassie Brilliant Blue R (Figure 15). When compared to proteins of known molecular weights, this protein was calculated to have a molecular weight of about 41,400 daltons (Figure 16).

In some instances, prolonged storage (several months) of the stained gels in the de-staining solution led to the staining of a trace protein component with a molecular weight of about 21,000 daltons, or roughly one-half the molecular weight of α-amylase. The presence or absence of 5-FU in the incubation medium of the half-seeds had no effect on the presence or absence of this minor component on the gels. Data obtained in the second part of this dissertation indicated that the minor protein component did not derive from α-amylase. This contaminant represented less than 3% of the protein on the gels where it eventually became visible.

Figure 15. SDS-Polyacrylamide gel electrophoresis of purified α-amylase. The protein was applied to a polyacrylamide gel, and electrophoresis was performed according to the method of Fairbanks et al. (1971). The gel was scanned at $\underline{A}_{560}$ after staining with Coomassie Brilliant Blue R.

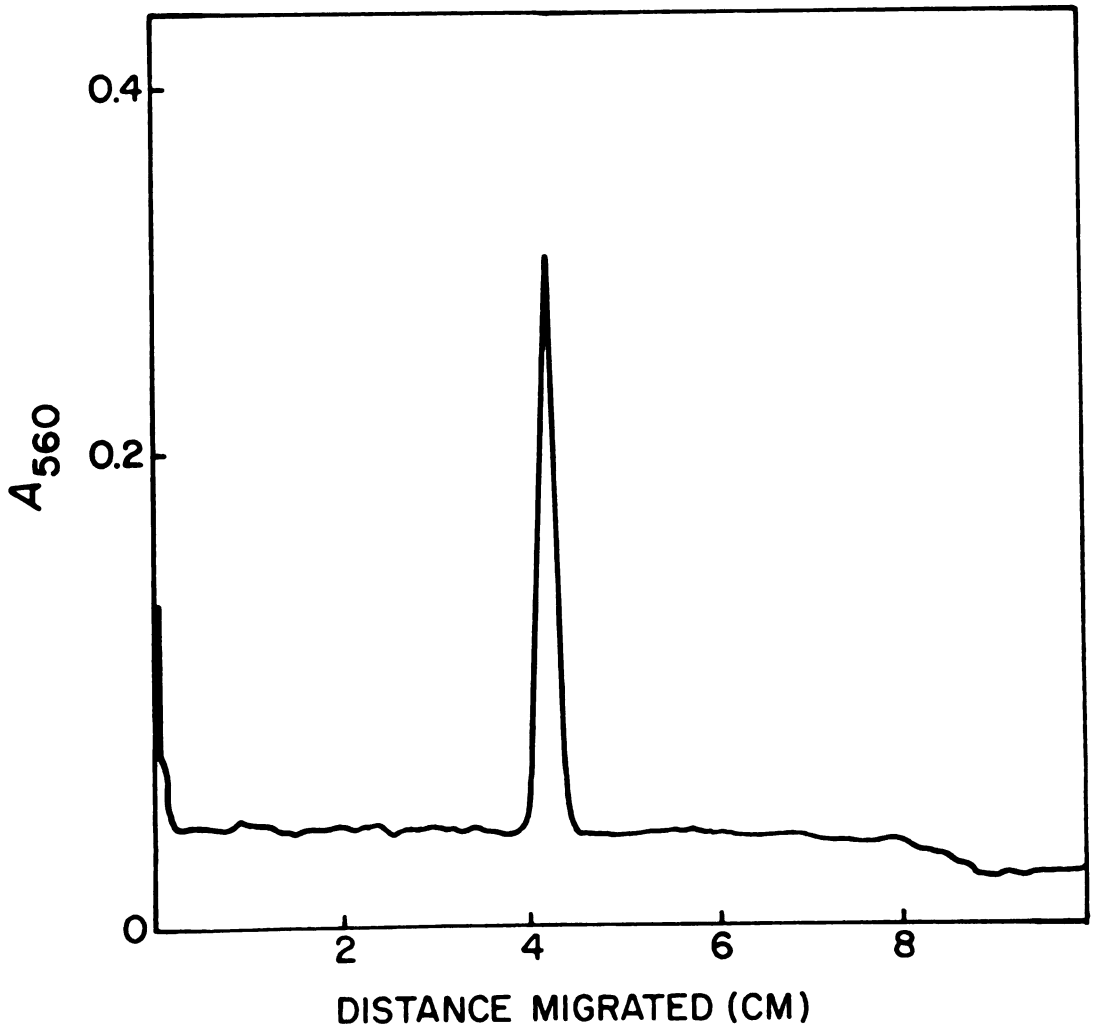

Figure 15

2. When the procedure of Laemmli (1970) was used for electrophoresis of denatured α-amylase on polyacrylamide gels, two additional bands of protein were evident (Figure 17). By comparison, crude malt α-amylase from Sigma was low in α-amylolytic activity and contained very little of the band of α-amylase protein. In the latter case, two bands of protein predominated, which had mobilities similar to those of the "additional" bands of protein mentioned above (Figure 17).

Since the electrophoresis of the purified α-amylase by Fairbanks' method had not led to the separation of these two additional proteins, and since the Fairbanks method uses strong denaturing conditions which are designed to severely inhibit proteolysis, it appeared that the two additional proteins may have been derived from hydrolysis of α-amylase. This is supported by the observation that no proteins from the purified α-amylase were retarded by a column of Bio-Gel P-30 which theoretically should have retarded these smaller proteins. The origin of these two additional protein bands was not investigated further. If these bands were produced as a result of proteolysis during the denaturation of protein prior to electrophoresis, this could have meant that a trace amount of protease was present in the final α-amylase preparations. However, as will be discussed later, no hydrolysis of the α-amylase occurred in the absence of SDS when calcium-free α-amylase was heated to 60 C in the thermal denaturation experiments.

3. If the purified protein was electrophoresed on polyacrylamide gels prepared by the method of Ingle (1968), five bands of protein could be separated (Figure 18). This method separates "native" proteins and leads one to conclude that the α-amylases, which have

Figure 16. Mobilities of protein standards in polyacrylamide gels prepared as in Figure 15. The mobility of α-amylase under these conditions was 0.556, relative to Pyronin Y. The standards used were: 1) bovine serum albumin, 2) ovalbumin, 3) horse liver alcohol dehydrogenase, 4) aldolase, 5) yeast alcohol dehydrogenase, 6) glyceraldehyde-3-phosphate dehydrogenase, 7) chymotrypsinogen, and 8) cytochrome c from horse heart.

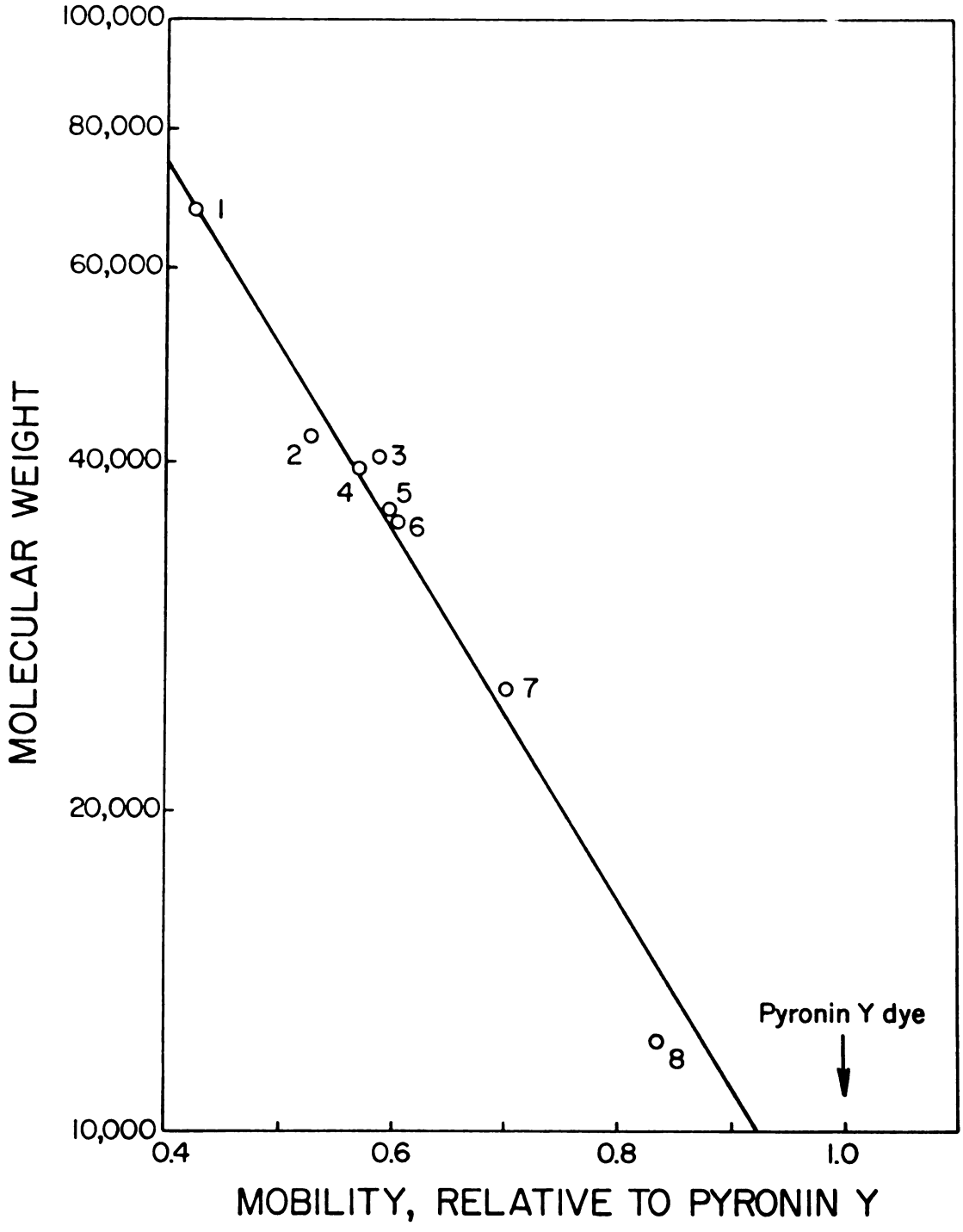
100,000
80,000
60,000
40,000
20,000
10,000
MOLECULAR WEIGHT
1
2
3
4
5
6
7
8
Pyronin Y dye
0.4
0.6
0.8
1.0
MOBILITY, RELATIVE TO PYRONIN Y

Figure 16

Figure 17. Electrophoresis of α-amylase using the method of Laemmli (1970). Protein samples were prepared by adding first 8 M urea and then the protein to a boiling solution of SDS, mercaptoethanol, and buffer. The solid line represents purified α-amylase from Betzes barley half-seeds, while the dashed line represents the soluble protein in barley malt amylase from Sigma. The gels were stained for protein and were scanned at 560 nm.

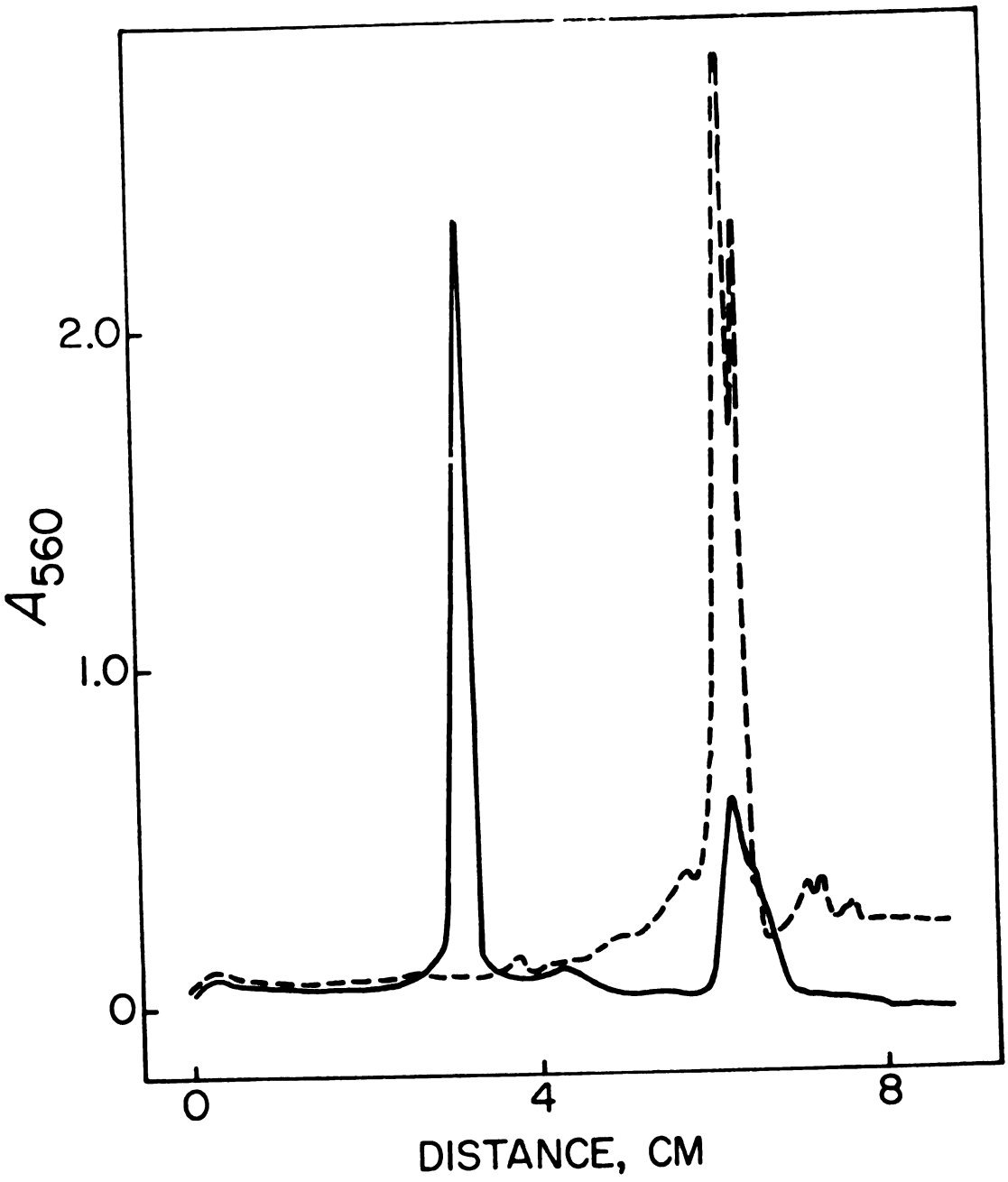

Figure 17

Figure 18. Polyacrylamide gel electrophoresis of α-amylase without SDS. Half-seeds were incubated as in Figure 1B, then the α-amylase was purified and the protein components were separated by electrophoresis using non-denaturing conditions. The amount of protein applied to each gel varied. A. No 5-FU. B. 10^{-4} M 5-FU. C. 10^{-2} M 5-FU.

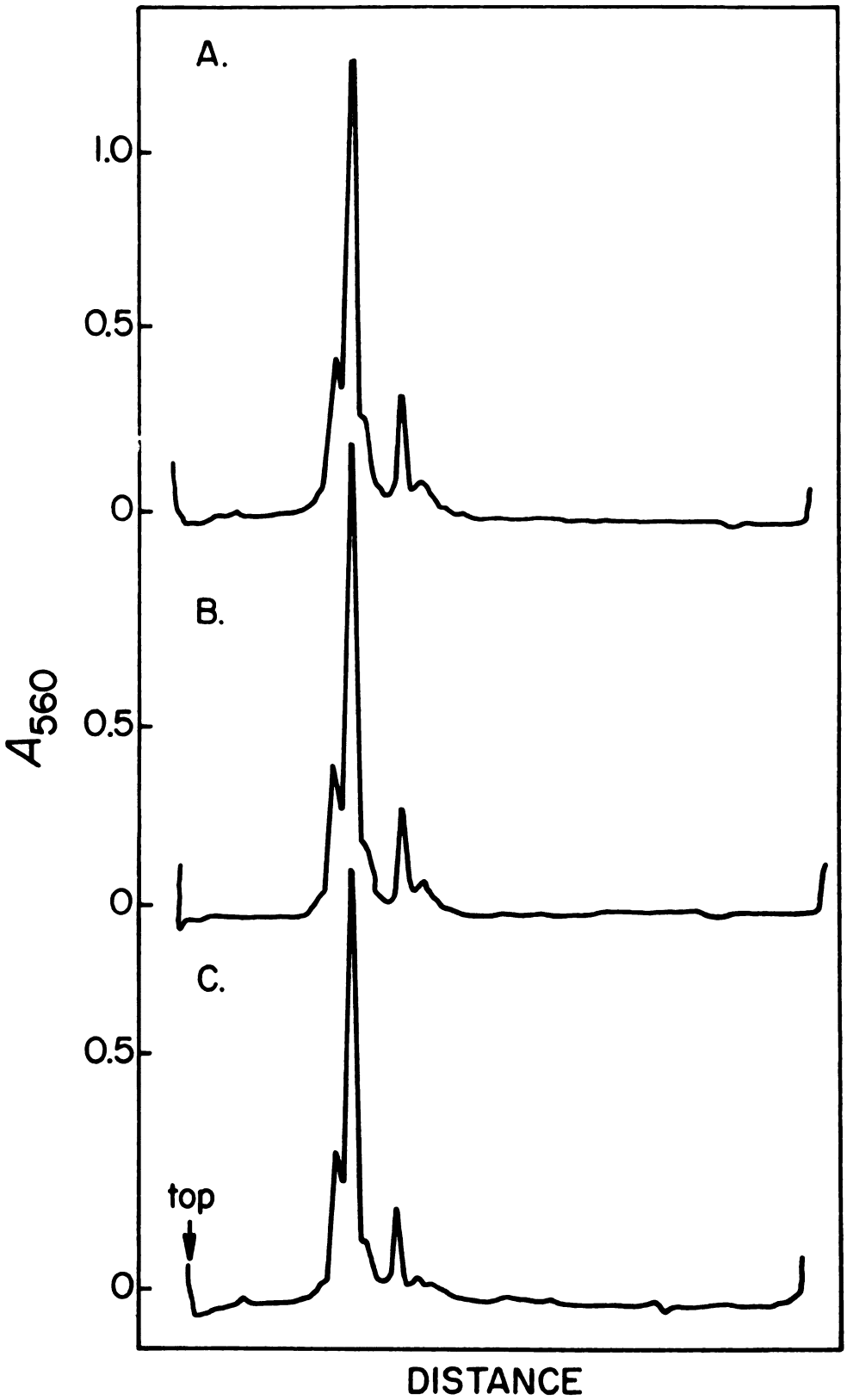

Figure 18

approximately the same molecular weight, differ in their net charges. The amylase in these polyacrylamide gels was still active after electrophoresis, as was shown by the following test. The gels were sliced with a razor blade, and each slice was placed in a petri plate containing a mixture of starch and agar. After 20 hr, the gel slices were removed, and the plates were flooded with an I_2/KI solution which turned the plates blue except for where α-amylase had diffused into the starch-agar and had hydrolyzed the starch. The region of the polyacrylamide gel which contained protein also contained α-amylase activity. However, the isozymes were not sufficiently separated to test each protein band individually for α-amylase activity.

Removal of Carbohydrate

After the α-amylase-glycogen complex was resuspended and incubated in Trisca buffer, it was no longer pelletable by centrifugation at 6000 *g* for 10 min. However, the glycogen had probably not been completely hydrolyzed by the bound α-amylase. In later experiments with α-amylase from Himalaya barley seeds as little as 10% ethanol again precipitated the α-amylase from the supernatant solution as a carbohydrate-α-amylase complex, but 5% ethanol did not. The hydrolysis products of glycogen could not be removed from the solution by dialysis. Interestingly, glucose was not the only sugar residue present in this mixture. Gas-chromatographic analysis established the presence of arabinose residues, which may have originated from the aleurone cell walls (Jones and Albersheim, 1972), in addition to the glucose residues.

The neutral carbohydrate polymers were removed by column chromatography using DEAE-cellulose. The carbohydrate was eluted from the

column in the first column volume of Trisca buffer. Following this, the enzyme was eluted with a gradient of sodium chloride in Trisca (Figure 4). The fractions with α-amylolytic activity did not react with the anthrone reagent.

The Loyter and Schramm Purification Procedure

Carlson (1972) purified barley α-amylase by the method of Loyter and Schramm (1962) designed for the purification of pancreatic α-amylase.* I attempted to use this procedure so that Carlson's and my methods of purification could be compared. A minor modification was necessary in the early stages of purification, because the barley α-amylase was secreted into a medium containing $CaCl_2$ which led to precipitation of the phosphate buffer which Loyter and Schramm had used during homogenization of their tissue. The addition of 0.012 M phosphate buffer (pH 6.9), as required by Loyter and Schramm, was therefore insufficient to change the pH of the homogenate. Instead, dibasic potassium phosphate was used to titrate the homogenate to

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*An outline of the procedure of Loyter and Schramm (1962) is included here for comparison to the methods employed in this dissertation. Commercial pancreatin (9 g) was homogenized in 40% ethanol containing 12 mM phosphate buffer, pH 7.9, 4 mM NaCl, and 3 mM $CaCl_2$, and the precipitate was removed. The pH was changed to 8.0, glycogen was added to bind the α-amylase, and the amylase-glycogen complex was pelleted by centrifugation. The pellet was resuspended in pH 6.9 buffer and incubated for 1 hr at 25 C. A small precipitate could be removed by centrifugation at pH 8.5 at 20 C. The α-amylase was precipitated at pH 7.0 by incubating the solution at 0 C for 30 min. The enzyme precipitate was resuspended in glycine-NaOH buffer (pH 10.0) and passed down a column of charcoal and celite which adsorbed contaminating carbohydrates. The eluted α-amylase was precipitated by acetone followed by ethanol-ether at -10 C. The enzyme was resuspended in distilled H_2O, the pH was changed to 6.5 with NH_4OH, and a lipid precipitate was removed. After a second acetone and ethanol-ether precipitation, a phosphate-$CaCl_2$ buffer (pH 6.8) was added and the enzyme, at a protein concentration of 3%, crystallized spontaneously within 2 days.

pH 6.9. This resulted in the formation of a large precipitate of calcium phosphate before pH 6.9 was attained. Certain problems arose later in the procedure for which I could find no solution, however. These problems involved (i) the difficulty of precipitating α-amylase from the resuspended glycogen-amylase mixture, (ii) the lability of the α-amylase in the pH-10 buffer used during chromatography on the charcoal-celite column, and (iii) the difficulty of removing α-amylase from the charcoal-celite column. With regard to point (i), the α-amylase which I had prepared could not be precipitated by adjusting the pH of the digested glycogen-amylase mixture to 7.0 and by placing the solution at 0 C for 30 min. Even when left at 0 C overnight, the α-amylase which I had prepared did not precipitate. This presented certain difficulties in rapidly changing the buffering solution to the glycine-NaOH buffer (pH 10.0). As can be seen in Figure 6, α-amylase was quite unstable at pH 10.0. Forty percent of the enzyme activity was lost after 3 hr of incubation at that pH. The purified α-amylase was split into two samples, and the pH of each was adjusted to 10.0 by slightly different means.

Sample A (see Materials and Methods) was applied to a short charcoal-celite column (1.8 cm wide x 1.4 cm long) immediately after its pH had been changed to 10.0. This sample contained 320 units of activity. An attempt to remove α-amylase from the charcoal column was made by the further addition of glycine-NaOH buffer (10 m$\underline{M}$, pH 10.0) to the column. Fractions of 2 ml were collected at a flow rate of 1 ml min^{-1}. After five fractions were collected, the pH of each fraction was lowered to 7.0 by the addition of 0.2 $\underline{M}$ acetic acid. Although Loyter and Schramm (1962) reported that 70% of the pancreatic α-amylase could be removed in the second and third column volumes of

buffer, the barley α-amylase was not removed by 50 column volumes. Ovalbumin was then added to the column in an attempt to displace competitively the α-amylase from the charcoal, but again no enzyme was eluted.

Sample B (420 units) was applied to an even smaller column containing 0.5 ml of charcoal-celite. Again no α-amylase could be recovered, although 200 ml of buffer were used to wash the column. Increasing the ionic strength of the buffer 10-fold or adding Triton X-100 to the elution buffer were equally ineffective.

These results precluded the possibility of a direct comparison between α-amylase as prepared by Carlson and α-amylase prepared by the method which I have reported (Method I). The α-amylase could not be precipitated at 0 C at pH 7.0, it was unstable at pH 10.0, the pH of the elution buffer from the charcoal column, and it could not be removed from the charcoal column.

It is not altogether clear that the α-amylase purified by Carlson was a pure preparation of α-amylase. Although he indicated that aleurone layers were used for his studies, there is reason to believe that these layers were not completely free of starchy endosperm. It is likely that his final enzyme preparation was contaminated by proteins originating from the starchy endosperm (such as β-amylase) which would bind to glycogen.

In one experiment, aleurone layers were isolated after 3 days of incubation on moist sea sand (Chrispeels and Varner, 1967). The layers were incubated for 24 hr on a shaker in Acca medium containing 1 $\mu\underline{M}$ GA_3. Following this the α-amylase was purified by the Loyter and Schramm method as far as the glycogen precipitation step and subsequent incubation to release the α-amylase. This preparation was

subjected to electrophoresis on polyacrylamide gels (Fairbanks method) and was found to contain only one protein band. Thus if Carlson *was* able to obtain very clean aleurone layers, it was possible that his enzyme preparation had been fairly pure. The purity of his final α-amylase preparation must therefore have depended on his ability to remove all of the starchy endosperm. Because I found it impossible to carry out the entire Loyter and Schramm purification procedure, I could not determine which of the α-amylase isozymes would have been released in the final steps of that method.

Yield of α-Amylase During Purification

The final recovery of α-amylase activity was 20-25% of the total activity in the original solution. A flow diagram is included to indicate the fate of the enzyme during the purification procedure (Figure 19). The major loss of activity occurred because of inactivation of some of the enzyme by the addition of ethanol. Some of the α-amylase was also not precipitated by glycogen, but much of that activity could be precipitated by addition of more glycogen.

The two glycogen-amylase hydrolysates were combined, and the solution was applied to a DEAE-cellulose column. Two peaks of activity were eluted, the first containing 22% and the second 10% of the original activity. In order to determine the effect of removing the calcium from the enzyme in each peak, the fractions constituting each peak were concentrated and chromatographed separately on a column of Sephadex G-25 equilibrated in 1 mM EGTA and 5 mM Tris at pH 8.0. The solutions were recovered with yields of 19% and 9%, respectively, and a combined yield of 28%. Normally, the α-amylase-containing fractions were combined after the DEAE-cellulose chromatography step.

Figure 19. Flow diagram for the purification of α-amylase. The α-amylase was purified by Method I. Some additional steps are included to test procedures during which the enzyme activity might be lost. The numbers under many of the entries refer to the percent of the original amylase activity which was represented in that fraction. Footnotes are included under the diagram.

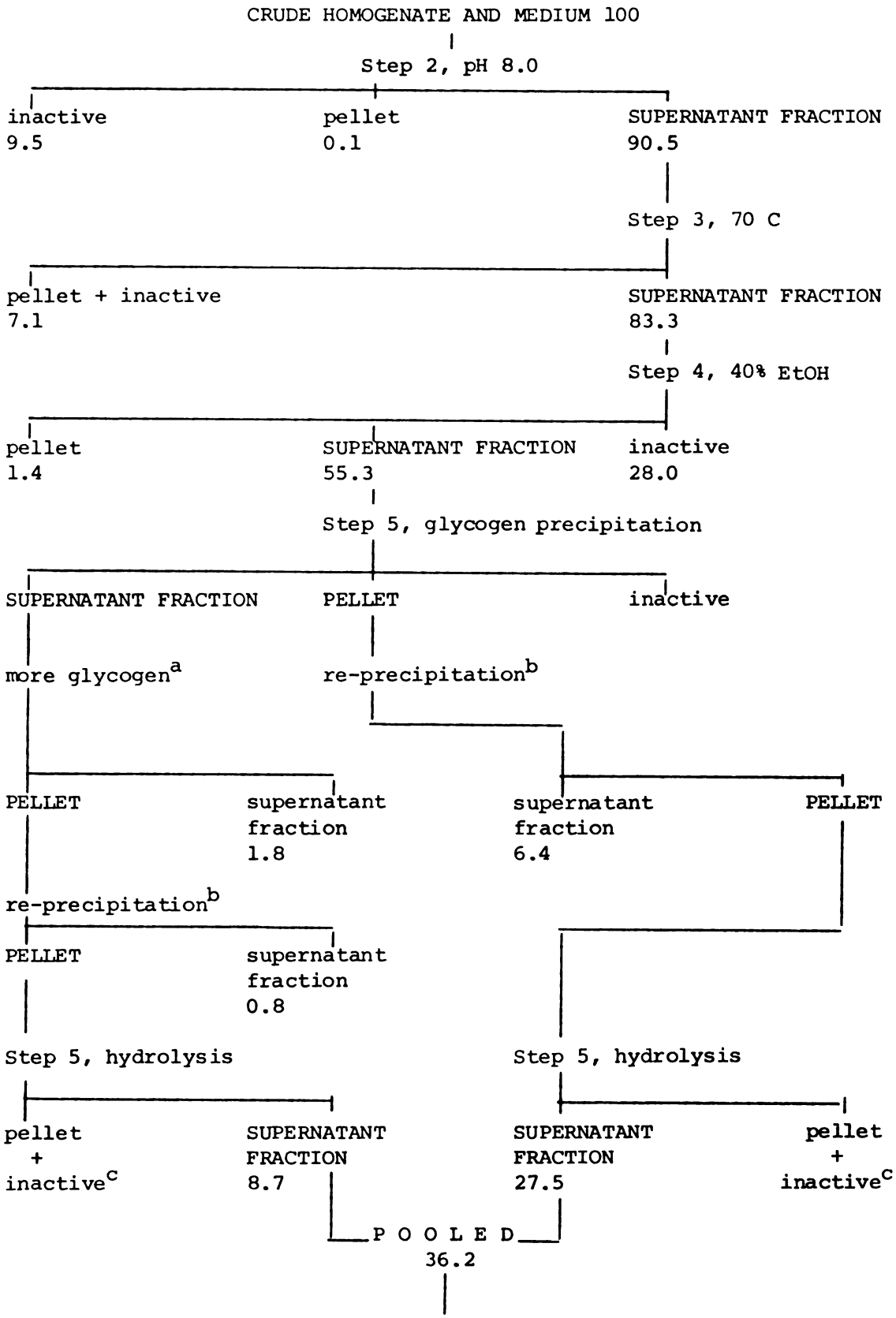
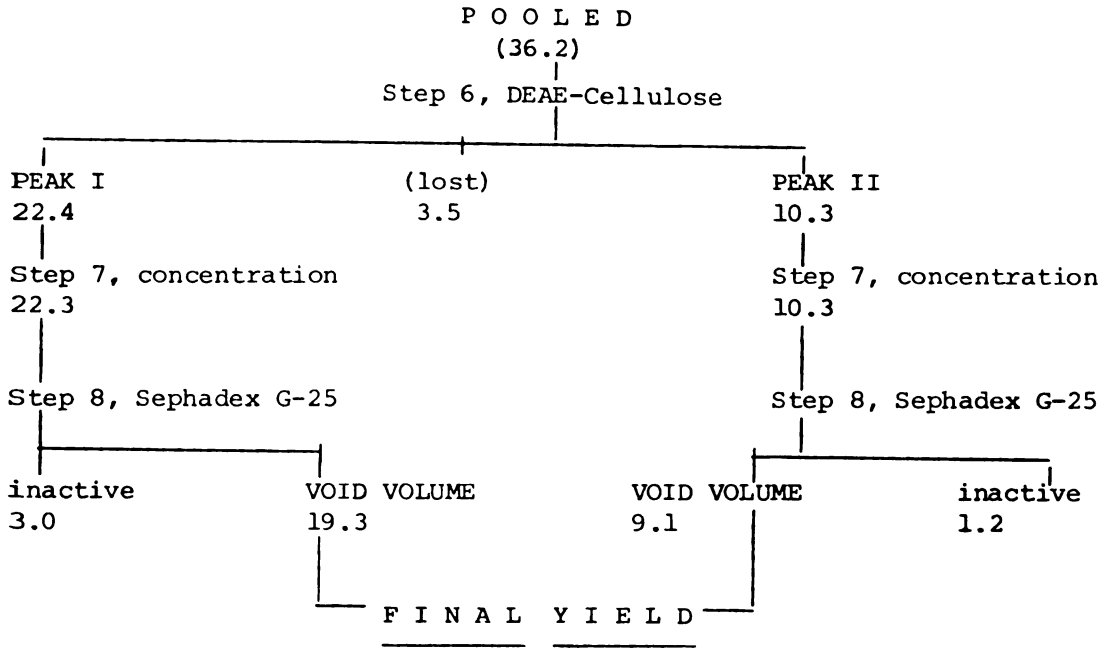

CRUDE HOMOGENATE AND MEDIUM 100
Step 2, pH 8.0
inactive
9.5
pellet
0.1
SUPERNATANT FRACTION
90.5
Step 3, 70 C
pellet + inactive
7.1
SUPERNATANT FRACTION
83.3
Step 4, 40% EtOH
pellet
1.4
SUPERNATANT FRACTION
55.3
inactive
28.0
Step 5, glycogen precipitation
SUPERNATANT FRACTION
PELLET
inactive
more glycogen[a]
re-precipitation[b]
PELLET
supernatant fraction
1.8
supernatant fraction
6.4
PELLET
re-precipitation[b]
PELLET
supernatant fraction
0.8
Step 5, hydrolysis
Step 5, hydrolysis
pellet + inactive[c]
SUPERNATANT FRACTION
8.7
SUPERNATANT FRACTION
27.5
pellet + inactive[c]
POOLED
36.2

Figure 19



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[a]An equivalent amount of glycogen (as at Step 5) was added a second time.

[b]The glycogen/ amylase complex was suspended in Trisca for 1 to 2 min, then ethanol was added until it reached a concentration of 40%.

[c]Together, these must account for 10.0% of the total.

[d]Aquacide II was used.

Figure 19, continued

Isozyme Recovery

It was important to establish whether any of the α-amylase isozymes might have been lost during the purification of the enzyme. α-Amylase was purified from Betzes barley, following Method I, and the isozymes were separated by electrophoresis on agar slab gels (Figure 20). The presence of the various isozymes was monitored at each step during the purification. At each step the pattern of isozymes was essentially unchanged, except that electrophoresis of the original solution did not separate the isozymes as clearly as did electrophoresis of the less crude preparations.

The preparations routinely examined were (a) the original, crude homogenate, (b) the solution after titration to pH 8.0, (c) the solution after heat treatment at 70 C for 15 min, (d) the solution after DEAE-cellulose chromatography, and occasionally (e) the solution after the removal of calcium.

The α-amylase activity on the agar gels separated into six bands. The active regions were those which remained clear after incubation of the slab with a starch solution prior to staining the gel with I_2KI. The original solution also contained three or four additional bands which moved more rapidly toward the cathode and which were suspected to be β-amylases on the basis of their incomplete hydrolysis of the starch substrate, their disappearance after heat treatment at 70 C, and the fact that their mobilities were close to those of β-amylases from Himalaya barley (Jacobsen et al., 1970).

The α-amylases of peak I from the DEAE-cellulose chromatography migrated the farthest and those of Peak II migrated more slowly during the electrophoresis.

Figure 20. Separation of α-amylase on agar slab gels. After electrophoresis, the gels were stained for α-amylase activity. Top: diagrammatic sketch; zones 1-6 are α-amylases, zones 7-10 are believed to be β-amylases. (A) original mixture of homogenate plus medium, (B) purified α-amylase. Bottom: photographs of actual slab gels; I. The original mixture of medium and homogenate, left to right are alternating pairs of control and 1 m$\underline{M}$ 5-FU treatments at increasing protein concentrations; II. After pH 8.0 treatment, with better resolution of zones 7 to 10; left to right are control (at two concentrations) and 1 m$\underline{M}$ 5-FU (two concentrations); III. Purified α-amylase, left to right are four samples of control then four of 1 m$\underline{M}$ 5-FU. The control is without 5-FU treatment.

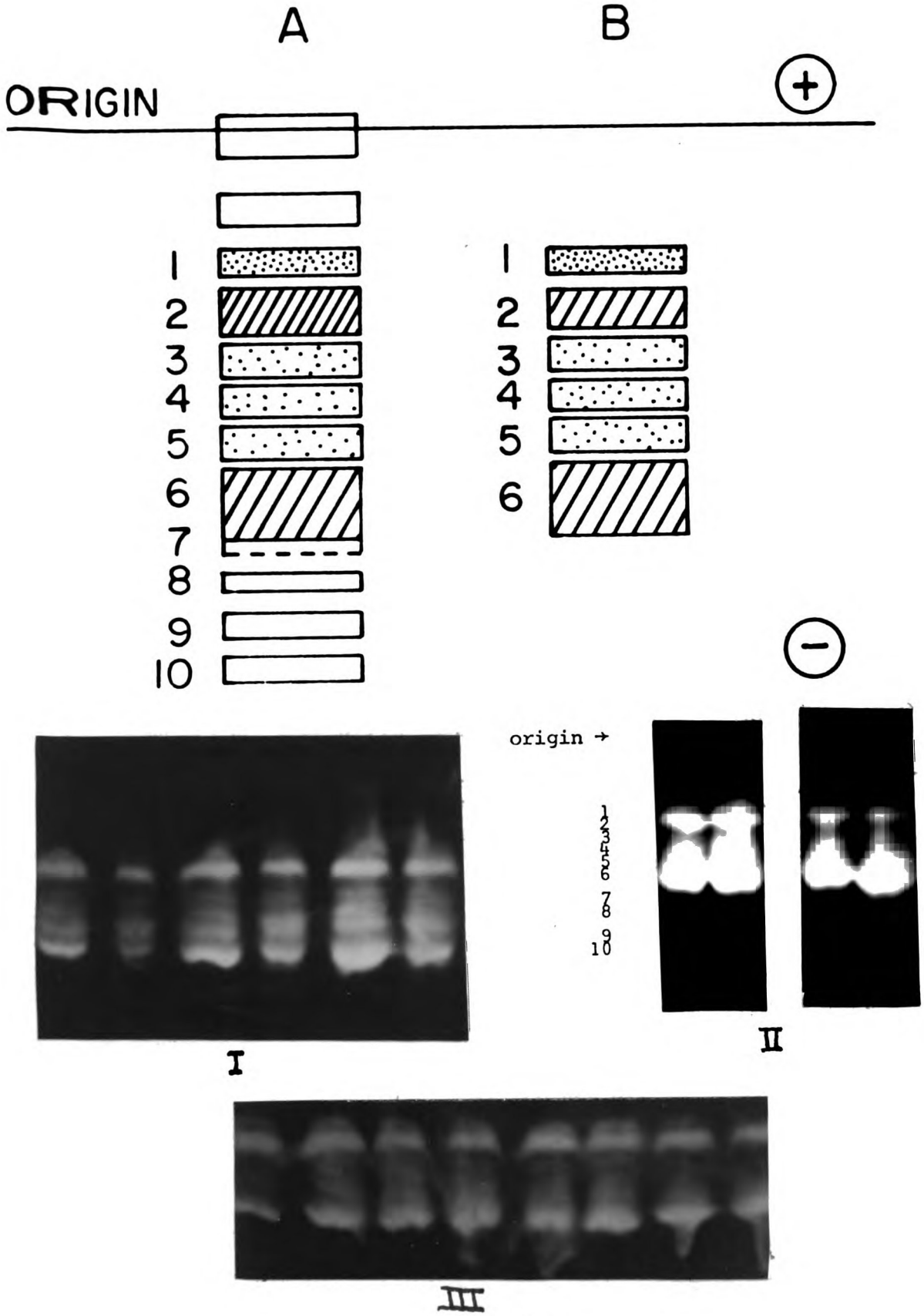

Figure 20

Effects of 5-FU on Molecular Weight and Isozyme Content of α-Amylase

Molecular Weight

Half-seeds were imbibed with concentrations of 5-FU between 10^{-5} and 10^{-2} M, after which 10^{-6} M GA_3 was added to the 5-FU-containing solutions for 36 hr. The α-amylases which were subsequently purified had the same molecular weights, and no enzyme with altered molecular weight was generated.

Isozyme Composition

Half-seeds were imbibed as in Figure 1A or 1B using zero, 10^{-4} M, or 10^{-3} M 5-FU. The isozymes were separated in agar slab gels. Because two of the isozymes predominated in the mixture, several different dilutions of the α-amylase had to be examined for the real pattern of isozymes to be understood. 5-Fluorouracil was found to have no effect on the pattern of isozymes present in the crude homogenate, in the solutions at subsequent purification steps, or in the final solutions after the removal of calcium (Figure 20).

In addition the amylase was separated into isozymes and stained for protein with Coomassie Brilliant Blue R after electrophoresis on acrylamide gels according to Ingle's method (Figure 18). In one case the half-seeds were incubated without 5-FU and in the others in the presence of 10^{-4} M or 10^{-2} M 5-FU. The relative optical densities of the protein bands appeared to be identical despite the 5-FU treatment. This is of some significance because the isozyme activities on the agar gels only present a qualitative measure of the relative enzyme activities, while staining with Coomassie Blue dye indicates in a more quantitative way that the amounts of protein in each band

maintained the same ratio, despite the 5-FU treatment. This supports the conclusion that 5-FU had no effect on the composition of isozymes which were recovered after purification of the α-amylase.

Recovery of Activity and Specific Activity of α-Amylase During Purification

Half-seeds were incubated as in Figure 1B in buffer without 5-FU or with 5-FU at concentrations between 10^{-5} and 10^{-2} M. These concentrations of 5-FU had had little effect on the amount of α-amylase which was synthesized by the half-seeds (Figure 11B). The homogenate and medium from each of the 5-FU treatments were pooled and the recovery and specific activity of the α-amylase was determined at each purification step. The amount of α-amylase activity recovered from each 5-FU treatment at each purification step is shown in Figure 21. Thus, the percent recovery after glycogen precipitation, for example, has been calculated as the activity *after* precipitation divided by the activity *before* the precipitation step. 5-Fluorouracil had little effect on the recovery of α-amylase during the purification of the enzyme. Changing the pH of the original homogenate to pH 8.0 may have the greatest differential effect on the α-amylase activities. However, because only one sample (of 75 half-seeds) was used for each determination, it is difficult to conclude whether these differences were significant.

The effect of the 5-FU treatments on the specific activities of the enzyme is shown in Figure 22. It should be recalled that, except for the case of the most purified α-amylase, differences in specific activity may be based on differences in the amount of contaminating protein present rather than in any changes in the amount of α-amylase activity recovered. At concentrations of 5-FU greater than 10^{-3} M,

Figure 21. Effect of 5-FU treatment on the recovery of α-amylase activity during purification. The percent recovery of α-amylase at each individual stage in the purification (ordinate) was calculated as the activity at the end of the step divided by the activity at the end of the previous step multiplied by 100. The original solutions were those collected from the material represented in Figure 11B.

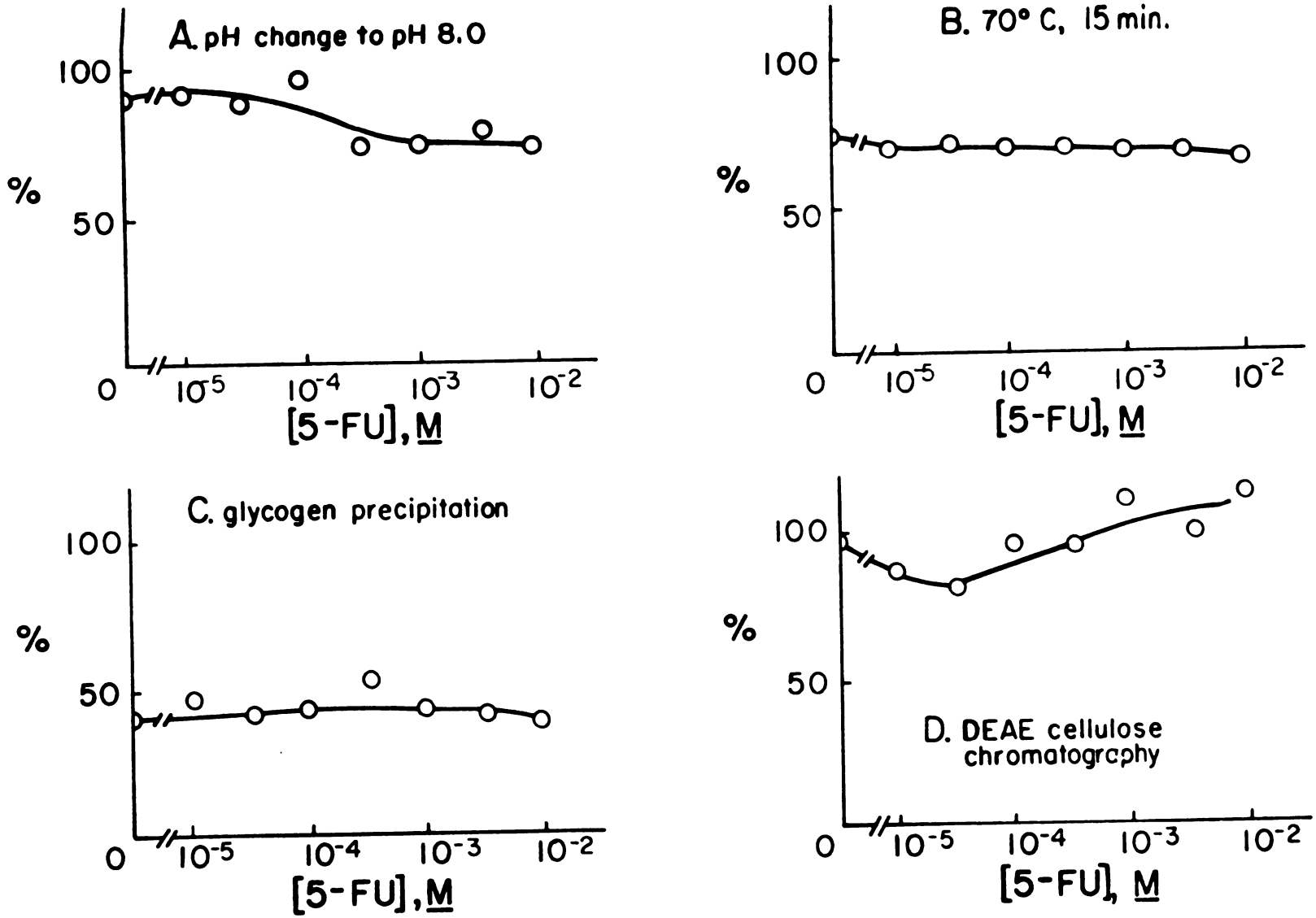
A. pH change to pH 8.0
100
%
50
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
[5-FU], M
B. 70° C, 15 min.
100
%
50
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
[5-FU], M
C. glycogen precipitation
100
%
50
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
[5-FU], M
100
%
50
D. DEAE cellulose chromatography
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
[5-FU], M

Figure 21

Figure 22. Specific activities of α-amylase at each stage of purification. The α-amylase collected from the half-seeds represented in Figure 11B was purified and the specific activity of the α-amylase was determined as a function of the stage of purification. BSA was used as a protein standard. For the first three curves, the protein sample was chromatographed on a cellulose thin layer plate before the protein assay was performed. Crude homogenate and medium, Δ; after pH change to pH 8.0, x; after 70 C treatment, ●; after glycogen precipitation and hydrolysis, □; after DEAE-cellulose chromatography, O.

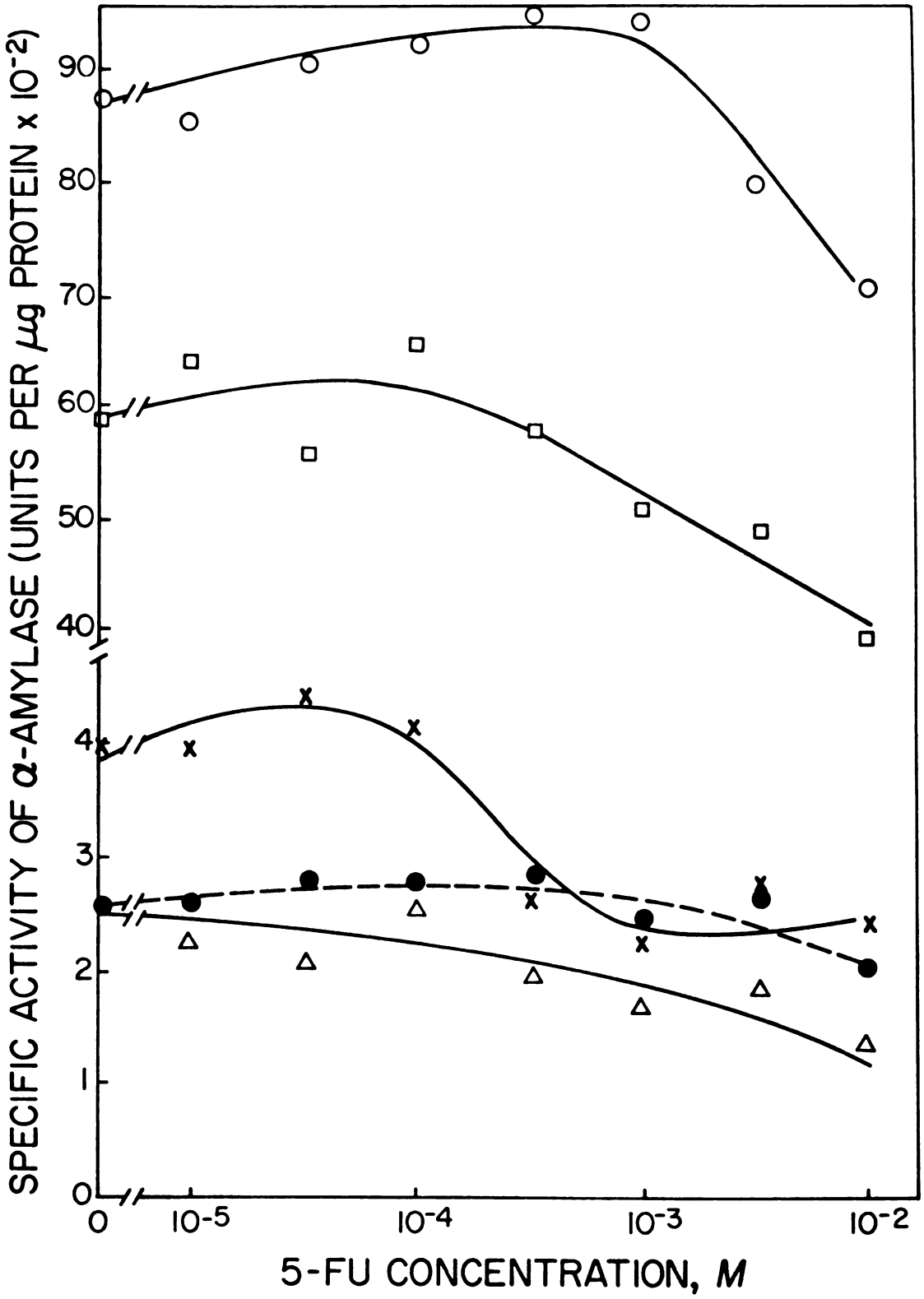
SPECIFIC ACTIVITY OF α-AMYLASE (UNITS PER μg PROTEIN x 10-2)
90
80
70
60
50
40
4
3
2
1
0
0
10-5
10-4
10-3
10-2
5-FU CONCENTRATION, M

Figure 22

the final specific activity was less than that of the control α-amylase. Again, this may or may not be significant, but it may correlate with the lower recovery of α-amylase after the change of pH to 8.0 as seen in Figure 21, or the small decrease in the amount of α-amylase synthesized at the higher concentrations of 5-FU. By the methods used to separate and identify the α-amylase isozymes, however, there was no indication that such differences, if real, were caused by the loss of a particular isozyme.

The Thermal Denaturation Process: General Aspects

Effect of Calcium

When calcium was present in the crude homogenate, the enzyme was not inactivated by heating it at 70 C for 15 min if the pH of the solution had been adjusted to 8.0. The purified enzyme was also stabilized by the presence of calcium in the solution (Figure 23). If calcium was removed from the solution by passing the solution over a Bio-Gel P-30 column equilibrated in 1 m$\underline{M}$ EGTA and 5 m$\underline{M}$ Tris (pH 8.0), the α-amylase lost 99% of its activity after incubation for 20 min at 54 C. However, addition of calcium to a final concentration of 0.33 $\underline{M}$ prevented the inactivation of the enzyme during the 40-min test period. For this reason, the thermal denaturation process could be stopped by the addition of the heated sample to one-half of that volume of 1 $\underline{M}$ $CaCl_2$ in an ice-chilled vial. The high concentration of $CaCl_2$ did not itself inactivate the α-amylase but had the added advantage of rapidly binding to any EGTA present in the final solution. Since the thermal inactivation process exhibited log-linear decay kinetics over as much as four decades, I

Figure 23. Effect of calcium on the rate of thermal denaturation of purified α-amylase. α-Amylase was heated at 54 C in the presence or absence of 0.33 M $CaCl_2$. The α-amylase was purified by Method I. Calcium was removed with the aid of a Bio-Gel P-30 column equilibrated in 1 mM EGTA with 5 mM Tris, pH 8.0, and the sample was concentrated using Aquacide II. Control (no calcium), at 224 units per ml, ●; 0.33 M $CaCl_2$, at 335 units per ml, O.

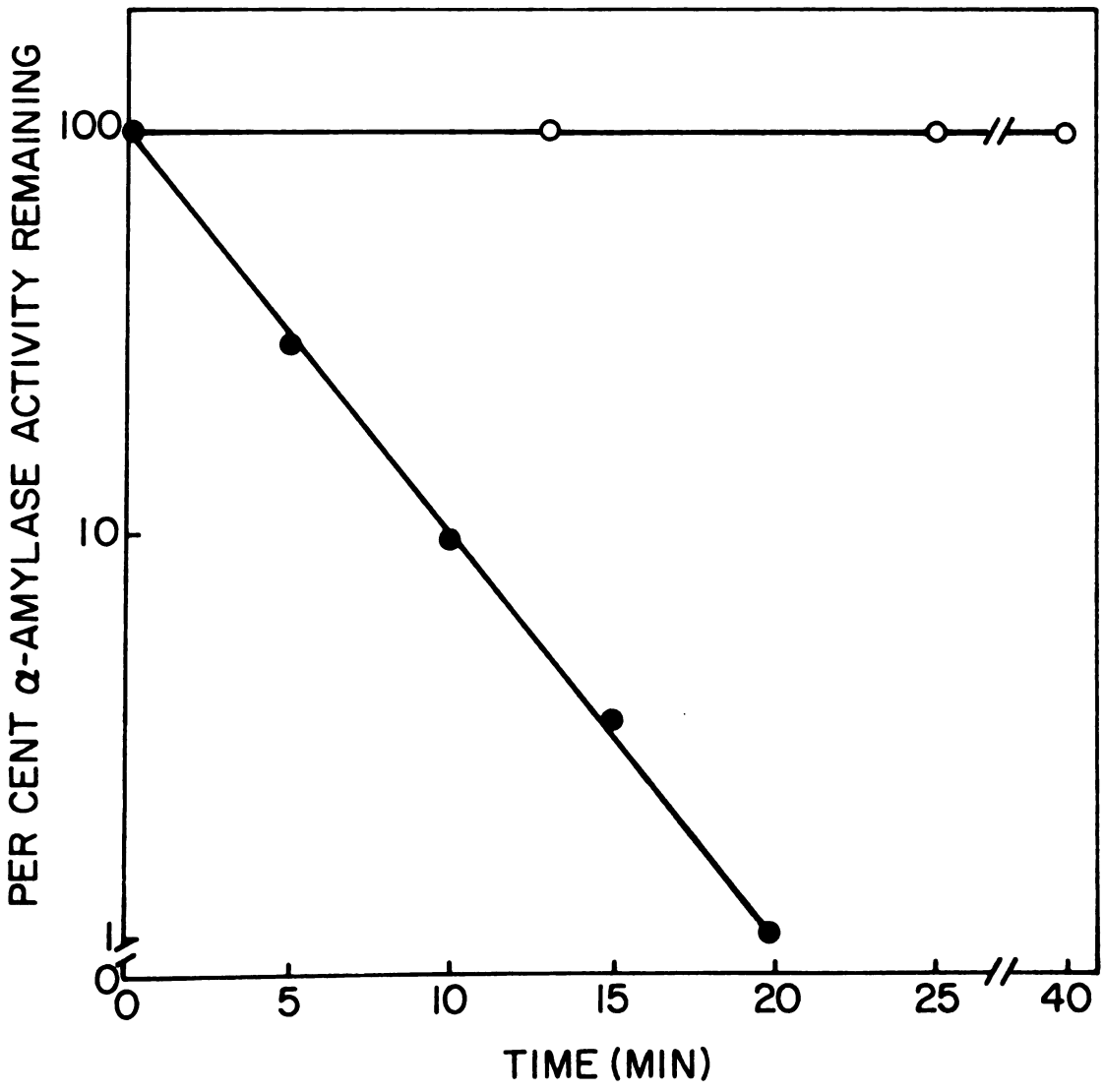
PER CENT α-AMYLASE ACTIVITY REMAINING
100
10
1
0
0
5
10
15
20
25
40
TIME (MIN)

Figure 23

have assumed that calcium was effectively removed from the α-amylase by the EGTA de-salting column.

Effect of Protein Concentration

The rate of thermal inactivation of α-amylase should be independent of the concentration of the enzyme during the heat treatment at low concentrations of enzyme. If the decay rate was dependent on the concentration of enzyme, one would suspect that a second, "non-amylase" component was affecting the denaturation process. Table 2 records the effect of diluting the enzyme solution with buffer on the amount of α-amylase activity remaining after 5 min of heating at 56 C. In the range of 50 to 500 enzyme units per ml, no significant effect of protein concentration on the degree of thermal inactivation was observed.

Test for Protease Activity

The removal of calcium from the α-amylase also makes the enzyme highly susceptible to hydrolysis by protease. Thus, it was necessary to examine whether α-amylase was hydrolyzed by traces of proteases present during the thermal denaturation treatment. If proteolysis had occurred, the apparent inactivation rate would be dependent on the activity of the protease and not the structure of the α-amylase. Samples of calcium-free α-amylase were subjected to SDS-polyacrylamide gel electrophoresis (Fairbanks' method). The samples were examined before heating, or after 99% of the enzyme activity had been destroyed. The electrophoretic properties of the protein remained unchanged, indicating that no hydrolysis of the α-amylase had occurred during heat inactivation, and that the inactivation rate must have been

Table 2. Effect of protein concentration on the thermal denaturation of α-amylase

Treatment[a]	Initial Concentration, units/ ml	Activity Remaining, %
no 5-FU	503	9.01
	402	8.31
	302	9.07
	201	8.66
	101	8.89
	50	8.76
10^{-4} M 5-FU	493	8.97
	394	8.71
	296	9.14
	197	8.93
	98.6	9.16
	49.3	8.63

[a]Half-seeds were incubated as in Figure 1A. After calcium was removed from the purified α-amylase, the enzyme was heated at 56 C for 5 min, and the remaining activity was determined.

dependent on heat-induced structural changes in the α-amylase molecules themselves.

Effect of 5-FU on the Thermal Stability of α-Amylase

The kinetics of thermal inactivation of α-amylase from tissues incubated either in the presence or in the absence of 5-FU are shown in Figure 24. For Figure 24A, half-seeds were incubated as in Figure 1A using 10^{-4} M 5-FU. These conditions were the same as those under which Carlson obtained α-amylase with the maximum decrease in thermal stability. It is clear that 5-FU had no effect on the thermal stability of the α-amylase. A second such experiment is shown in Figure 24B. This experiment differs from that of 24A in that a higher denaturation temperature (60 C as compared to 54 C) was used. In Figure 24C, a similar experiment is shown, except that the concentration of 5-FU had been raised to 10^{-2} M. In none of these experiments had the treatment of the half-seeds with 5-FU affected the thermal stability of the α-amylase. These results disagree with those reported by Carlson. Closer examination of Figure 24C shows that the denaturation rates of both the control and the 5-FU samples was more rapid than those of a comparable experiment shown in Figure 24B. The Aquacide II which was used to concentrate the samples was later found to have decreased the pH of the enzyme solutions, resulting in more rapid rates of thermal decay.

Thermal stability determinations were performed six times, using six separate preparations of α-amylases from 5-FU-treated half-seeds. Along with each α-amylase sample from 5-FU-treated tissue, a sample of α-amylase from control tissue was purified in parallel.

Figure 24. Thermal denaturation of α-amylase from 5-FU-treated half-seeds. A. α-Amylase was purified from half-seeds which had been incubated as in Figure 1A. In the concentration steps, Aquacide II was utilized. The denaturation temperature was 54 C and the 5-FU concentration was 10^{-4} M. B. α-Amylase was prepared from half-seeds as in (A) above, except that two glycogen precipitation steps were performed, and the α-amylase was pooled from these. The denaturation temperature was 60 C and the 5-FU concentration was 10^{-4} M. C. α-Amylase was obtained as in (A) above. Thermal denaturation was performed at 60 C. The 5-FU concentration used for this experiment was 10^{-2} M. Control (no 5-FU), O; 5-FU-treated, ●.

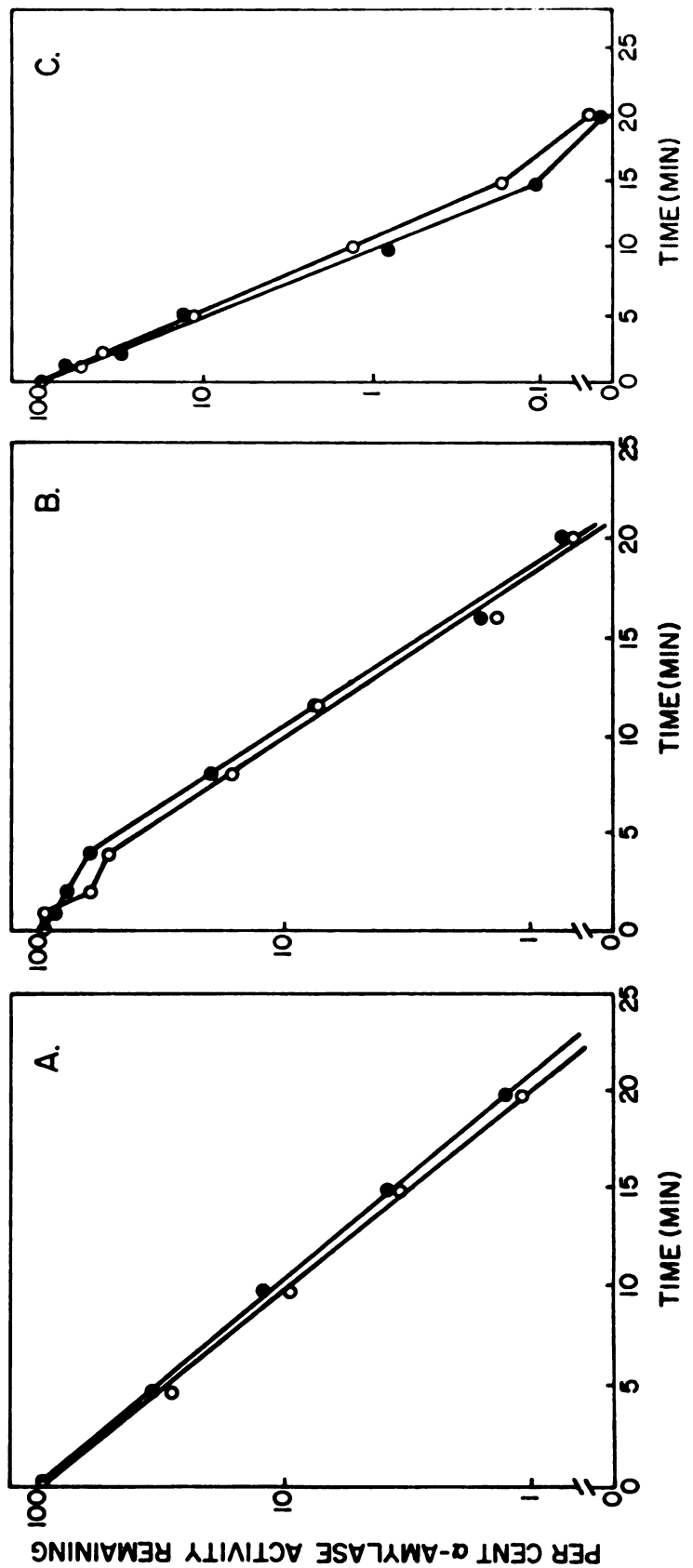
PER CENT α-AMYLASE ACTIVITY REMAINING
A.
B.
C.
100
10
1
0.1
0
5
10
15
20
25
TIME (MIN)
TIME(MIN)

Figure 24

The three experiments above lead to the conclusion that the α-amylase from 5-FU-treated half-seeds was not significantly different from that of control half-seeds. Such experiments have been performed six times using α-amylases prepared from different batches of half-seeds. It should be recalled, however, that the incubation regime for these experiments was that shown in Figure 1A; i.e., the half-seeds were imbibed for 24 hr in water prior to addition of 5-FU. It may be argued that the seeds which I used were able to synthesize all of their α-amylase mRNA during that first day of incubation, while those which Carlson had used did not synthesize α-amylase mRNA in the course of the first day of incubation without 5-FU. In a final attempt to decrease the thermal stability of α-amylase by treatment with 5-FU, I isolated α-amylase from half-seeds which had been incubated continuously in 10^{-4} M 5-FU, as in Figure 1B. The α-amylase was purified, calcium was removed, and the enzyme was heated to 54 C. Again, the thermal inactivation kinetics were similar, although the slope of the denaturation curve was slightly less steep in the case of the α-amylase from 5-FU-treated half-seeds. The order of the curves was different than the order which was reported by Carlson, where the α-amylase from 5-FU-treated half-seeds was less stable than normal. The reason for the slight difference in α-amylase as seen in Figure 25 is not known.

Figure 25. Thermal denaturation of α-amylase: continuous incubation in 10^{-4} M 5-FU. Half-seeds were incubated as in Figure 1B and concentration of the α-amylase was achieved using Aquacide II. The denaturation temperature was 54 C. Control (no 5-FU), O; 5-FU treated, ●.

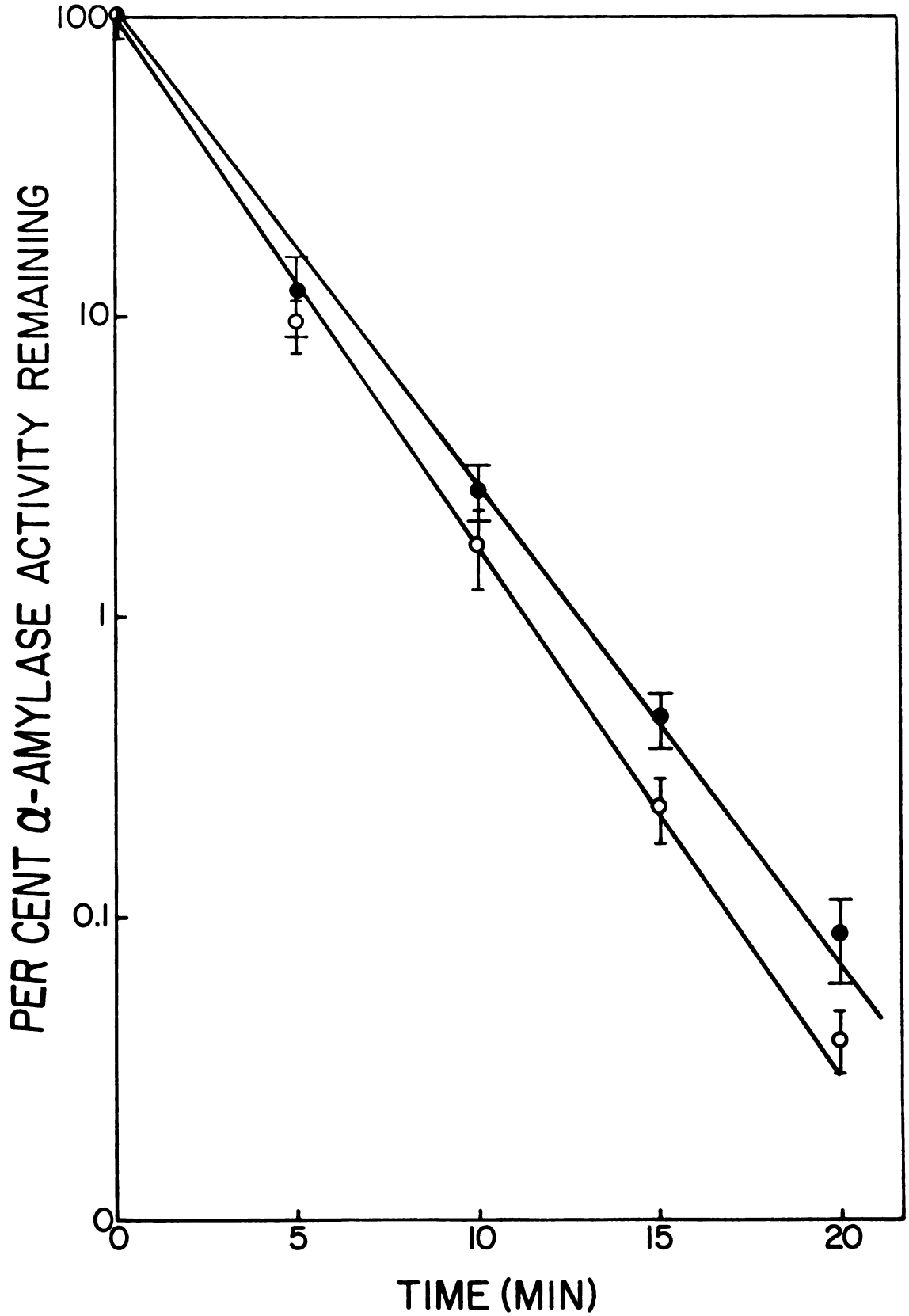
100
10
1
0.1
0
PER CENT α-AMYLASE ACTIVITY REMAINING
0
5
10
15
20
TIME (MIN)

Figure 25

DISCUSSION

Suitability of the α-Amylase Preparation for Studies of Thermal Inactivation Kinetics

The report by Carlson that α-amylase was rendered heat labile by the inclusion of 5-FU in the incubation medium for barley aleurone tissue (Carlson, 1972) has not been supported by this study. If 5-FU was able to affect the synthesis of proteins other than or in addition to α-amylase, artifacts could have been introduced which could have affected the analysis of the thermal stability characteristics of this isozymic enzyme system. Certain isozymes which may have been altered as a result of 5-FU treatment could have been lost during the purification of the α-amylase. This could have produced artificial differences in the kinetics of thermal inactivation if the isozymes had had different thermal stability properties. Alternatively, the co-purification of a second factor, which was present in different amounts in each solution of "purified" α-amylase, could have influenced the kinetics of thermal inactivation. It was therefore necessary to determine as closely as possible that isozymes of α-amylase had not been lost during the purification and that no factors had co-purified with the enzyme which would have affected its thermal stability.

The α-amylase synthesized by Betzes barley aleurone tissue was found to be heterogeneous, six isozyme species having been separated. During electrophoresis on SDS-polyacrylamide gels, the isozymes

co-migrated as one band of protein corresponding to a molecular weight of 41,400 daltons. A faint trace of a second component of 21,000 daltons was sometimes visible after the stained SDS-gels were stored for a long time. In the absence of calcium at least 99% of the purified enzyme was heat labile while in the presence of calcium the enzyme was stable to the same heat treatment (Figure 23).

The isozyme composition of the α-amylase was monitored throughout the purification process and was found to be unaltered as a result of purification or removal of calcium from the enzyme. The base analog 5-FU, which had little or no effect on α-amylase synthesis or secretion by the aleurone tissue, had no effect on the isozyme composition of the α-amylase, its molecular weight, or its purity.

Thus, the purification method which I selected for obtaining α-amylase must be considered to be at least as effective as that which was employed by Carlson (1972), although Carlson did not characterize the α-amylase purified by his procedures, and a direct comparison of the properties of our preparations cannot be made.

I was unable to purify α-amylase using the method which Carlson had employed (Loyter and Schramm, 1962). The α-amylase did not precipitate spontaneously in the cold at pH 7.0, it was unstable at pH 10.0, the pH of the charcoal column chromatography, and it bound irreversibly to or was inactivated by the charcoal. Although the α-amylase had to be purified by an alternative procedure, attempts were made to simulate Carlson's incubation conditions. Thus, the half-seeds were soaked by a batch method for one day before they were incubated in flasks on a shaker, and Betzes barley seeds were used instead of the more commonly studied Himalaya variety. In addition,

the aleurone tissue was homogenized and the homogenate was added to the medium (plus the extract of the starchy endosperm) before purification was begun.

Because Carlson did not heat-treat his original extract in the presence of calcium, it is probable that other proteins such as β-amylases were present in his preparations of purified α-amylase. This suggestion is made because aleurone layers cannot be scraped clean of starchy endosperm after one day of incubation in H_2O or buffer. Such proteins originating from the endosperm may have appeared to represent a second population of molecules with the capability of hydrolyzing starch, or they may have affected the rate of inactivation of the calcium-free α-amylase during the thermal denaturation experiments.

The identification of the isozymes of α-amylase which retained their activity after purification and removal of calcium was not a trivial requirement. Jacobsen et al. (1970) have shown that there are two types of α-amylases synthesized by Himalaya barley. One type was strongly affected by GA_3-treatment (a 12.1-fold increase), was unstable at pH 3.7 and bound calcium only weakly. The second type of α-amylase also produced amylose from starch, but it was stable at pH 3.7 and it tightly bound calcium. Unlike the first group, the second group of isozymes was less affected by GA_3, being increased only 2.6-fold by GA_3-treatment.

The α-amylases of Betzes barley undoubtedly exhibit such polymorphic behavior as well, leading Chandra (1974) to presume that Carlson (1972) may have only been able to remove the calcium from the isozymes which weakly bound the ion. The incomplete removal of calcium may have generated biphasic decay curves. Alternatively,

the calcium-free α-amylases in Carlson's solutions may have been partially inactivated before the thermal denaturation experiments were begun, or the charcoal-binding step may have selected for certain isozymes, again giving rise to a series of biphasic decay curves. It is easy to see that the thermal decay kinetics could depend on the relative proportions of the active isozymes which were present in each solution if traces of calcium were present.

To establish further that the α-amylases which I had purified were of sufficient purity for the thermal denaturation experiments, the thermal inactivation reaction was shown to occur as if it was a monomolecular reaction since the concentration of α-amylase, at concentrations of protein below 50 μg (BSA equivalents) per ml, had no effect upon the rate of the inactivation. In addition, the inactivation of α-amylase during heating was not due to proteolysis.

From the evidence described in this study no conclusion can be drawn concerning the timing of α-amylase mRNA synthesis. This is contrary to the conclusion of Carlson (1972) who, based on similar experiments with 5-FU, proposed that the α-amylase in RNA was produced both in the presence and absence of GA_3.

Use of 5-FU to Time the Synthesis of α-Amylase mRNA

Since treatment of the half-seeds with 5-FU did not cause the formation of a more thermolabile population of α-amylase molecules, one cannot make any conclusions concerning the timing of α-amylase mRNA synthesis in barley aleurone layers from the data which have been presented here. α-Amylase mRNA may have been produced before imbibition or, equally as likely, 5-FU may have been incorporated into nascent mRNA during the course of these experiments; but the

presence of substitutions of 5-FU in the mRNA may not have led to amino acid substitutions in the α-amylase.

5-Fluorouracil was also found to inhibit the synthesis of rRNA and tRNA in barley aleurone tissue without affecting the synthesis or release of α-amylase in response to GA_3. The synthesis of new species of rRNA and tRNA is therefore not required for the synthesis or release of α-amylase. These results agree with those of Jacobsen and Zwar (1974) that 15 m$\underline{M}$ 5-FU inhibited the synthesis of rRNA (90%) and tRNA (over 50%) by barley aleurone tissue (cv. Himalaya) during the first 16 hr of GA_3-treatment with no concomitant inhibition of α-amylase synthesis. In a rat liver-system ^{14}C-5-fluoroorotate (5-FO) was used as a more efficient precursor of 5-fluorouracil ribosylmonophosphate in RNA than 5-FU. The ^{14}C-5-FO was incorporated into 45S RNA (r-preRNA) but not into 28S or 18S RNA (Wilkinson et al., 1971). In addition, 5-FU was shown to have no effect on the transcription or methylation of 45S RNA in rat liver (Wilkinson and Pitot, 1973). Therefore, 5-FU apparently affects the last steps of rRNA processing rather than inhibiting the synthesis of the original r-preRNA transcript.

Radioactively labeled 5-FU is also incorporated into polysomal mRNA of HeLa cells (Gleason and Fraenkel-Conrat, 1976), TMV-RNA in tobacco leaves (Mandel, 1969), and D-RNA from soybean (Key, 1966). In fact, the use of 5-FO has been proposed as a method to label mammalian mRNA specifically (Wilkinson et al., 1971). In barley aleurone tissue, 1 m$\underline{M}$ 5-FU had no effect on the labeling of 5S to 14S RNA by ^{3}H-adenosine (Zwar and Jacobsen, 1972), indicating that 5-FU does not inhibit the synthesis of mRNA in this system.

No conclusive evidence has been obtained from studies of eukaryotes which would prove that the incorporation of 5-FU is responsible for the substitution of amino acids in the polypeptides of specific proteins. It has been hypothesized that the substitution of fluorine in the 5-position of uracil affects the ratio of the keto and enol forms of the pyrmidine by decreasing the pK of the pyrimidine in RNA *in vivo*, since poly(U) has a pK of 9.8 while poly(5-FU) has a pK of 8.1 (Mandel, 1969). The protein-synthesizing capacity of poly(U) and poly(5-FU) has been examined *in vitro* with a protein-synthesizing system from wheat germ. Poly(5-FU) was translated less efficiently than poly(U), but the 5-FU polymer was read exclusively as if it were a poly(U) message (Mandel, 1969). In contrast, the substitution of 5-FC in poly(C) elicited some unexpected changes in the amino-acid composition of the resultant polypeptides, indicating that 5-FC may have occasionally been read as U, and it was hypothesized that the infrequent converstion of 5-FU to 5-FC *in vivo* causes the mutagenic effects currently attributed to 5-FU (Gleason and Fraenkel-Conrat, 1976).

In some instances, treatment of bacterial cultures with 5-FU has produced phenocopies of the bacteria or of the bacteriophage infecting them. Such cases have provided the only unambiguous demonstrations that 5-FU can alter the phenotype of an organism by being incorporated into messenger or messenger-like RNA. Growth of *Escherichia coli* on 5-FU, for example, was effective in reversing the phenotypes of 17 out of 339 mutants of T_4 bacteriophage, the original mutations of which had arisen by the change of a single base in the viral RNA (Champe and Benzer, 1962). 5-Fluorouracil was also able to induce amber (nonsense) mutations in the RNA coding for

the coat protein of R17 bacteriophage, and these mutations could be suppressed if the 5-FU-mutants were subsequently grown on suppressor strains of the host (Tooze and Weber, 1967). Thus, a precedent for the induction of phenocopies by 5-FU treatment has been established in bacterial viruses, and the absence of such examples in eukaryotes may simply derive from the lack of well-defined eukaryotic systems in which the hypothesis can be tested. The results summarized here indicate that the induction of α-amylase synthesis by GA_3 in barley aleurone tissue does not provide a suitable eukaryotic system for such studies.

SECTION II

INTRODUCTION

At least two carbohydrases are involved in the hydrolysis of starch reserves in the endosperm of barley grains. These are the α-amylases (E.C. 3.2.1.1) and the β-amylases (E.C. 3.2.1.2). The α-amylases randomly cleave the α-1,4-glucosyl linkages and are thus "endo"-enzymes. The reducing ends of their hydrolysis products are in the alpha configuration. α-Amylases are synthesized by the living aleurone layer in response to gibberellin, which originates in the germinating embryo (McLeod and Palmer, 1967). The embryo can be replaced by exogenous gibberellic acid (GA_3) which stimulates the synthesis and/or release of a number of hydrolases, including α-amylase, from isolated aleurone tissue (Yomo and Varner, 1966). α-Amylase constitutes the major protein synthesized in response to GA_3.

The β-amylases, on the other hand, cleave maltose (disaccharide) units sequentially from the non-reducing ends of α-1,4-glucans. They are thus "exo"-enzymes, yielding maltose units which are in the beta configuration. In wheat grains, β-amylases are synthesized during the maturation of the grain, but remain inactive until the embryo germinates. The β-amylases can be activated prematurely by incubation of the starchy endosperm with proteolytic enzymes or sulfhydryl reagents (Rowsell and Goad, 1962); in the germinating grain it seems likely that the proteases, which are synthesized by the aleurone tissue, are responsible for activation of β-amylases.

The barley aleurone system has been chosen as a model system for studying the action of gibberellic acid (Varner, 1964 and references therein). Since the synthesis and secretion of α-amylase are major events which are controlled by GA_3, it would be useful to know the primary structure of the α-amylases from barley grains. Because many glycoproteins are destined for secretion or are sequestered from the ground cytoplasm of the cell (Winterburn and Phelps, 1972), I was particularly interested in determining whether the α-amylases were also glycoproteins. Perhaps the presence of a carbohydrate moiety could partially account for the remarkable stability of α-amylases to protease (Jacobsen and Varner, 1967) and heat (Kneen et al., 1943) in the presence of calcium. Such a study is complicated by the fact that the α-amylases from different varieties of barley grains are electrophoretically heterogeneous (Jacobsen et al., 1970; Frydenberg and Nielsen, 1965; Bøg-Hansen and Daussant, 1974; Tanaka and Akayawa, 1970) and that at least two antigenically different types of α-amylase are produced in those systems where antibody specificity has been studied (Jacobsen and Knox, 1973; Daussant et al., 1974). With this underlying heterogeneity in mind, the average amino acid and carbohydrate composition of the α-amylase secreted by barley grains has been analyzed and is described in the following.

MATERIALS AND METHODS

Incubation of Grains for α-Amylase Purification

Barley seeds (*Hordeum vulgare* L. cv. Himalaya) were obtained from the Agronomy Club at Washington State University in Pullman, Washington, USA. One hundred sixty grams of the grains were surface sterilized by partially evacuating them for 30 min in 1% sodium hypochlorite in a stoppered flask which was attached to a water aspirator. The grains were rinsed several times with sterile, distilled H_2O until 2 liters of H_2O had been used. The grains were distributed into five 1-liter flasks containing 200 ml of incubation medium each, and were incubated for 4.5 days in the dark. The barley grains were aerated by shaking the flasks on a rotary shaker. The incubation medium contained 10 m$\underline{M}$ succinate buffer (pH 5.3), 20 m$\underline{M}$ $CaCl_2$, 0.1% chloramphenicol, and 10 μ$\underline{M}$ GA_3. At the end of the incubation period, some roots had emerged from the seed, and the maximum length of these was about 6 mm.

Purification of α-Amylase

After incubation of the grains, the medium was collected by decantation. The grains were further incubated for 30 min with an additional 100 ml per flask of 1 m$\underline{M}$ sodium acetate buffer, pH 4.8, with 200 m$\underline{M}$ $CaCl_2$. The high concentration of $CaCl_2$ was used to wash adsorbed α-amylase from the starchy endosperm and cell walls of the aleurone layer (Varner and Mense, 1972). This wash was added to the

original medium. The grains were subsequently pressed with a pestle in a mortar to remove the starchy endosperm from the aleurone layer. The aleurone and extruded starch were then combined with the medium and wash, and 1 M $CaCl_2$ was added to yield a final concentration of 200 mM. The aleurone, starch, medium, and wash were incubated for another 2 hr at room temperature on a reciprocating shaker. At this point, the extract contained intact aleurone tissue, whole embryos, and the diluted components of the starchy endosperm. The extract was filtered through cheesecloth and then through glass wool, and the filtrate was centrifuged at 5900 g for 20 min. The supernatant solution contained about 200,000 units of α-amylase activity (see Materials and Methods of Part I of this dissertation for a description of the assay of α-amylase and the definition of α-amylase units).

The cloudy supernatant solution was adjusted to pH 8.0 by the addition of 0.1 M tris base, and was centrifuged at 5900 g for 30 min in a refrigerated centrifuge (0 to 4 C). The resultant supernatant fraction was heated in two 2-liter flasks to 70 C. The total heating time was 20 min, about 7 min being required to reach 70 C. At 53 C, a precipitate of denatured protein began to form. After heating, the precipitate was removed by centrifugation at 5900 g for 30 min. Following this treatment, about 150,000 units of α-amylase remained in the supernatant solution.

Cold ethanol was added to the soluble fraction to give a final concentration of 40%, and this solution was stored in the cold overnight. The mixture was subsequently centrifuged at 6900 g for 30 min, and the pellet was discarded. Deproteinized glycogen (1.6%) was added to the cold supernatant fraction in the proportion of 1 µl per 7 units of α-amylase. At the end of 18 min of constant stirring,

the solution was centrifuged at 5900 g for 30 min. The supernatant solution was removed, and the pellets were suspended in a buffer containing 5 mM tris, pH 8.0, and 5 mM $CaCl_2$. A total of 12 ml were used to collect the α-amylase-glycogen complex.

Preparation of ^{14}C-Labeled α-Amylase from Aleurone Layers

Aleurone layers (200) were separated from barley grains which had been de-embryonated and imbibed under sterile conditions on sand for 3 days (Chrispeels and Varner, 1967). The aleurone layers were transferred to four 125-ml flasks containing 8 ml each of 20 mM succinate buffer (pH 5.3), 0.2 M $CaCl_2$, 0.1% chloramphenicol, 1 μM GA_3, and 50 μCi ^{14}C-(U)L-leucine (286 mCi/mmole). The aleurone layers were incubated at 25 C for 24 hr on a reciprocating shaker. The medium was decanted, and diluted to 50 ml with 0.2 M $CaCl_2$. The pH was adjusted to 8.0 with 0.1 M tris base, and the solution was heated to 70 C for 20 min. The solution was centrifuged at 13,000 g for 5 min, and the precipitate was discarded. Cold ethanol was added to a final concentration of 35%, and after 1 hr of incubation in the cold the solution was centrifuged at 13,000 g for 10 min. One milliliter of 1.6% glycogen was added, and the glycogen-amylase complex was removed by a second centrifugation. The pellet was resuspended in 1 ml of 5 mM tris-HCl buffer (pH 8.0) with 5 mM $CaCl_2$. Fifteen hundred units of α-amylase were recovered.

Incubation of Grains on Agar

In order to estimate the time required for barley grains to synthesize and release a maximal amount of α-amylase, the grains were incubated on agar for different periods of time, and the distribution of α-amylases in the tissues was determined. Grains were nicked with

a scalpel blade at the end distal to the embryo, surface sterilized with 1% sodium hypochlorite, and rinsed with sterile distilled H_2O. The grains were placed on 2% agar and were incubated at 27 C in the dark. At the designated times, grains were slit open and the starchy endosperm tissue was removed and ground in 1 ml of 0.2 M $CaCl_2$. To this homogenate was added four 1-ml H_2O washes of the aleurone layers and two 1-ml H_2O washes of the portions of the embryo still in the seeds. All of the amylase collected by this method was termed "released" amylase. The agar was not assayed for α-amylase activity. The H_2O-washed aleurone layers were briefly rinsed three times in 10 ml of distilled H_2O and were incubated twice successively for 5 min in 2-ml portions of 0.2 M $CaCl_2$. The α-amylase thus released was termed "cell-wall" amylase. The aleurone layers were further incubated for 10 min in 1 mM HCl to inactivate the remaining α-amylase, were rinsed three times in 10 ml of distilled H_2O, and the layers were homogenized with a mortar and pestle and acid-washed sea sand in 1 ml of 1 mM sodium acetate buffer, pH 4.8, containing 0.2 M $CaCl_2$. Three 1-ml washes (same buffer solution) of the mortar and pestle were added to the homogenate. This amylase activity was termed "tissue" amylase.

Polyacrylamide Gel Electrophoresis

Proteins were separated electrophoretically by SDS-polyacrylamide gel electrophoresis. The method of Fairbanks et al. (1971) was used with the slight modifications described in Part I of this dissertation. For analytical separations, 8 to 10 units of α-amylase activity were used per gel. For preparative separations, about 100 units of α-amylase or up to 400 µg of total proteins were added per gel. The

large amount of protein present in the preparative gels caused the proteins to have increased mobility. Thus, attempts to localize proteins in unstained gels on the basis of the relative mobility of the proteins after analytical separation was not reliable. Instead, the proteins in preparative gels were localized directly by a careful visual examination made possible by the refractile nature of the protein in the gel at the leading edge of the protein band. Protein was eluted from the gels in the following way. Portions of the gels which contained the protein in question were excised and all gel pieces containing the same protein band were placed in line in an empty gel tube. Up to three segments were placed in each tube. In order to exclude bubbles, a drop of electrophoresis buffer was placed between the gel pieces. The gel pieces were pushed to the very bottom of the gel tube. A 10-cm piece of dialysis tubing was closed at one end with a dialysis bag clip (National Scientific, Cleveland, OH, USA), 1 ml of electrophoresis buffer was pipetted into it, and the gel tube was gently slid to the bottom of the dialysis bag. A rubber band was used to fasten the upper portion of the bag to the gel tube. The rubber band was wrapped around the bag and tube about 3 cm above the closed end of the dialysis bag. Buffer was added to the lower reservoir of the electrophoresis apparatus to a height equal to that of the buffer in the dialysis bag when the tubes were in place. Next, buffer was added to the upper reservoir, and 50 µl of the buffer-tracker solution was placed on top of each gel. The tracker dye was pyronin Y. Up to 100 volts were applied to the total system for twice the time required for the tracker to be eluted from the end of the gel. (The mobility of α-amylase relative to the tracker dye is greater than 0.5).

Gels were stained for protein using Coomassie Brilliant Blue R250 and were scanned at 560 nm. Radioactivity in the gels was determined by cutting the gels into 1-cm pieces, drying the pieces under a heat lamp, and combusting them using a Packard Tri-Carb Sample Oxidizer (Model 306).

Staining of Gels for Carbohydrate

The SDS-polyacrylamide gels were stained for carbohydrate by the method of Fairbanks (1971) using a periodate-Schiff reaction or by the method of Eckhardt et al. (1976) which is based on the transfer of a dansyl group from dansyl hydrazine to periodate-oxidized carbohydrates. The gels which were stained with the Schiff reagent were scanned spectrophotometrically at 560 nm. Gels which were exposed to the dansyl hydrazine were photographed using a 350 nm light source, a Whatman 2D filter and Kodak "Kodalith" film. The light source (four black-light tubes) was held 12 to 14 inches from the gels and the film was exposed for 10 min at f/5.6.

Isoelectric Focusing

Isoelectric focusing was accomplished in a preparative column containing 110 ml of liquid. A gradient was formed in the column by mixing 48.5 g glycerol, 14.25 g H_2O and 2.25 ml of a 40% stock of Ampholines (LKB) with a solution of 0.75 ml of the Ampholine stock plus the sample in a total volume of 55 ml (H_2O was used to adjust the volume). The sample initially contained 22,000 units of α-amylase activity; 21% of that activity was recovered after focusing. The column was cooled at 8 C, the initial voltage was 600 volts, and 2.5 days were allowed for focusing to be completed. The pH of each fraction subsequently collected was measured by equilibrating the

tubes in an ice-bath and measuring the pH after 1 min of electrode immersion in each sample.

Ammonium-Sulfate Fractionation

Protein was fractionated by precipitation with ammonium sulfate (Mann Research Laboratories, special enzyme grade) at 0 C. After a suitable incubation period, precipitates were removed by centrifugation at 0 to 2 C for 30 min at 29,000 g. Before determination of α-amylase activity or protein content, the pellets were dissolved in 1 ml of Trisca buffer (5 mM tris buffer, pH 8.0, and 5 mM $CaCl_2$) and dialyzed for 40 hr against cold (about 7 C) Trisca.

Amino Acid Analysis

Protein was eluted electrophoretically from preparative SDS-polyacrylamide gels. The protein solution was carefully removed from the dialysis bag with a Pasteur pipette and transferred to a pre-weighed test tube (Pyrex, 13 x 100 mm). The dialysis bag was rinsed once with a minimum volume (100 µl) of distilled H_2O, and the rinse was added to the test tube. Trichloroacetic acid (TCA) was added from a 100% (w/v) stock solution (i.e., 100 g TCA made up to 100 ml with H_2O) to a final concentration of 20%. At 15% TCA, a precipitate formed but 20% TCA was used to insure that all of the protein was precipitated. After 60 min at room temperature, the solution was centrifuged using an International Clinical Centrifuge at low speed (a setting of '2' indicating 500 rpm). The supernatant solution was removed with a Pasteur pipette, but not completely, and 1 ml of 95% ethanol (re-distilled) was added to the precipitate. After 15 min at room temperature, the centrifugation was repeated. The pellet was not suspended after addition of ethanol, but was washed once more

with 1 ml of 95% ethanol and then three times with 4 ml each of 95% ethanol. After each rinse, the sample was centrifuged. This procedure removed SDS, the electrophoresis buffer, and TCA. The final pellet was carefully dried under a stream of nitrogen. The pellet, upon drying, separated from the test tube and could be weighed on a sensitive balance to give an estimate of the maximum weight of protein present in the pellet. The pellet was carefully returned to the test tube to which 0.6 ml of constant boiling HCl was added. The tube was flushed with nitrogen for 1 min without placing the needle attached to the nitrogen line directly into the acid solution. The tube was quickly sealed with the aid of a gas-oxygen torch and incubated in a heating block at 107 C for 18 hr. Thereafter, the tube was cooled to room temperature and cracked open. The constant boiling HCl was evaporated to dryness at 50 C under a stream of nitrogen. The residue was resuspended in 200 to 500 µl of 0.01 N HCl, and was frozen or was used directly for amino acid analysis. Amino acid analysis was performed using a Technicon system (Technicon Corporation, Tarrytown, NY, USA) as modified by Lamport (1969). The Chromo-Beads ion exchange column (Type C-2, 0.6 x 70 cm) was eluted under pressure with buffer which was applied as a gradient of increasing pH and ionic strenth. The column effluent was monitored at 570 nm and 440 nm, after it was mixed with ninhydrin reagent. For best results 60 to 100 µg of protein hydrolysate were applied to each column per determination. Three or four determinations were made for each protein analyzed. The amounts of each amino acid were determined relative to 100 nmoles of norleucine. The values were corrected by comparison to the color intensities of known amounts of standard

amino acids recovered from the columns. Tryptophan was destroyed by this procedure and yields of cysteine were not reliable. Glutamine was converted to glutamate and asparagine to aspartate.

Carbohydrate Analysis

Sugars in glycoproteins were identified and quantitated by gas chromatography. A glass column packed with a support of Gas Chrom-Q which was coated with SE 30 (Applied Science Laboratories, Inc., State College, PA, USA) was used to separate sugars as their methyl-glycosides after trimethylsilylation (Bhatti et al., 1970). The following sugars were used as standards: arabinose, rhamnose, fucose, xylose, mannose, galactose, glucose, N-acetyl glucosamine, N-acetyl galactosamine, and sialic acid. The internal standard was mannitol at 100 nmoles per sample. The detection limit was about 1 nmole of sugar per sample in 25 µl of the trimethylsilylation reaction mixture. About 1 mg of α-amylase and 0.24 mg of band-2 protein (the weight being based on the amino acid composition) were used per sample in these carbohydrate determinations.

Two methods were used to cleave the carbohydrate moiety of the glycoproteins into monosaccharides. The first method was non-aqueous, using dry methanol containing 1.5 M HCl (Chambers and Clamp, 1971). Mannitol (100 nmole) was added to the test sample, and the sample was dried *in vacuo* for 12 hr over P_2O_5. One milliliter of dry methanolic HCl was added to the sample, and the vial or test tube was flushed with N_2 for 1 min. The vial or tube was sealed, and the mixture was heated at 80 C for 12 hr. If vials were used, the paper liners of the caps were replaced by teflon liners. After methanolysis, the solution was cooled, the container opened, and the HCl neutralized by

cautious addition of solid silver carbonate. When gas was no longer evolved upon further addition of silver carbonate, 100 µl of dry acetic anhydride was added, the vial was closed again and incubated for 6 hr. The liquid was removed and placed in a 15-ml centrifuge tube. The solid was resuspended two times in 1 ml of dry methanol and was removed by low-speed centrifugation. The rinses were added to the centrifuge tube which was centrifuged at 13,300 g for 20 min. The supernatant solution was subsequently evaporated at 45 C under a stream of nitrogen. The sample was dried under vacuum over P_2O_5 for 6 to 10 hr. The trimethylsilylating reagent (25 µl) was added, and after 45 min, the samples were analyzed on a Perkin-Elmer 900 gas chromatograph. The silylation reagent, containing pyridine, trimethylchlorosilane, and hexamethyldisilazane (5:1:1, v/v/v), was stored up to 72 hr and was centrifuged before use. After derivatization, the sample was diluted with dry ethyl acetate, if required.

The second method was designed to allow the cleavage of the carbohydrate into monosaccharide units to occur while the glycoprotein was still in the polyacrylamide gel. The gels were fixed for 48 hr in 50% methanol containing 5% acetic acid. Several changes of about 500 ml of fixing solution per gel were used. Portions of the gel which contained the precipitated protein were excised with a razor blade, and up to 1.25 cm^3 of gel was placed in a test tube (Pyrex, 13 x 100 mm). The gel pieces were subsequently minced up using a dissecting tool with an arrow-shaped tip. Two volumes of 3 N trifluoroacetic acid (TFA) were added to one volume of gel so that the concentration of TFA in the total aqueous phase was about 2 N. Mannitol was added as an internal standard and the tubes were sealed with a gas-oxygen torch. The sealed tubes were incubated at

121 C for 60 min. The tubes were cooled, the aqueous phase was transferred to a new tube, and the H_2O and TFA were removed by a stream of nitrogen while the tube was maintained at 45 C. The gel material, which had been left behind, was meanwhile rinsed with a small volume of H_2O which was subsequently combined with the first aqueous phase which was being evaporated. The residues were dried *in vacuo* for 7 to 10 hr over P_2O_5. The monosaccharides released by this aqueous procedure were then processed as in the first method, beginning at the step where dry methanolic-HCl was added to the sample.

Glassware and Dialysis Tubing

Glassware used for carbohydrate and amino-acid analyses were cleaned with Dichrol (Scientific Products, McGaw Park, IL, USA) followed by detergent, acetone, and methanol. Every attempt was made to avoid contamination of the samples by dust. Dialysis tubing (Dialyapor A, molecular weight cutoff 6000 to 8000, National Scientific Company) was boiled once in 2% sodium bicarbonate, three times in 2% sodium bicarbonate plus 0.03% EDTA, and once in distilled H_2O. The dialysis tubing was rinsed and stored in 50% glycerol with 10% ethanol.

Source of Chemicals

Chemicals used for carbohydrate analysis were obtained from sources suggested by Bhatti et al. (1970) or Chambers and Clamp (1971). Those for the dansyl hydrazine reaction were as suggested by Eckhardt (1976). Reagents for electrophoresis included SDS from Pierce (Rockford, IL), acrylamide from Ames (Miles Laboratories, Elkhart, IN), bis-acrylamide, TEMED, and ammonium persulfate from

Bio-Rad (Richmond, CA). Basic fuchsin was obtained from Allied Chemical Corporation (New York, NY). Ovalbumin, BSA, Coomassie Brilliant Blue R, and chymotrypsinogen were obtained from Sigma Chemical Company (St. Louis, MO), while lysozyme (muramidase) was obtained from Worthington Biochemicals (Freehold, NJ). Isoelectric focusing was performed using ampholines from LKB (Rockville, MD).

RESULTS

Synthesis of α-Amylase by Barley Grains

A time course of the synthesis and release of amylolytic activity by germinating Himalaya barley grains is shown in Figure 26. The amylase activity increased for 5 days, after which time the starchy endosperm was completely solubilized. After one day of imbibition, 87% of the α-amylase was released from the aleurone cells, and after 5 days, 99% of the α-amylase which had been synthesized had been released from the cells.

Partial Purification of α-Amylase

Based on the results of the time course experiment, grains were incubated for 4.5 days, and the secreted α-amylase was collected and purified. The grains were continuously incubated in buffer and 10 μM GA_3 to insure that the maximum amount of α-amylase was synthesized. α-Amylase was bound to glycogen in the presence of 40% ethanol and was precipitated as a glycogen-amylase complex. The precipitate was subsequently solubilized in Trisca buffer at room temperature. Most of the α-amylase and glycogen were solubilized within the time required to collect the precipitates from the centrifuge bottles (Figure 27). The α-amylase solubilized during zero to 45 min of incubation in Trisca were pooled. The solubilized α-amylase was precipitated a second time by 30% ethanol, and the pellet was solubilized again in Trisca. This α-amylase was then precipitated

Figure 26. Synthesis and release of α-amylase by germinating barley grains. Grains were allowed to germinate on agar in the dark. α-Amylase in: tissue, □ ; cell wall, ■ ; released, ●; total, O.

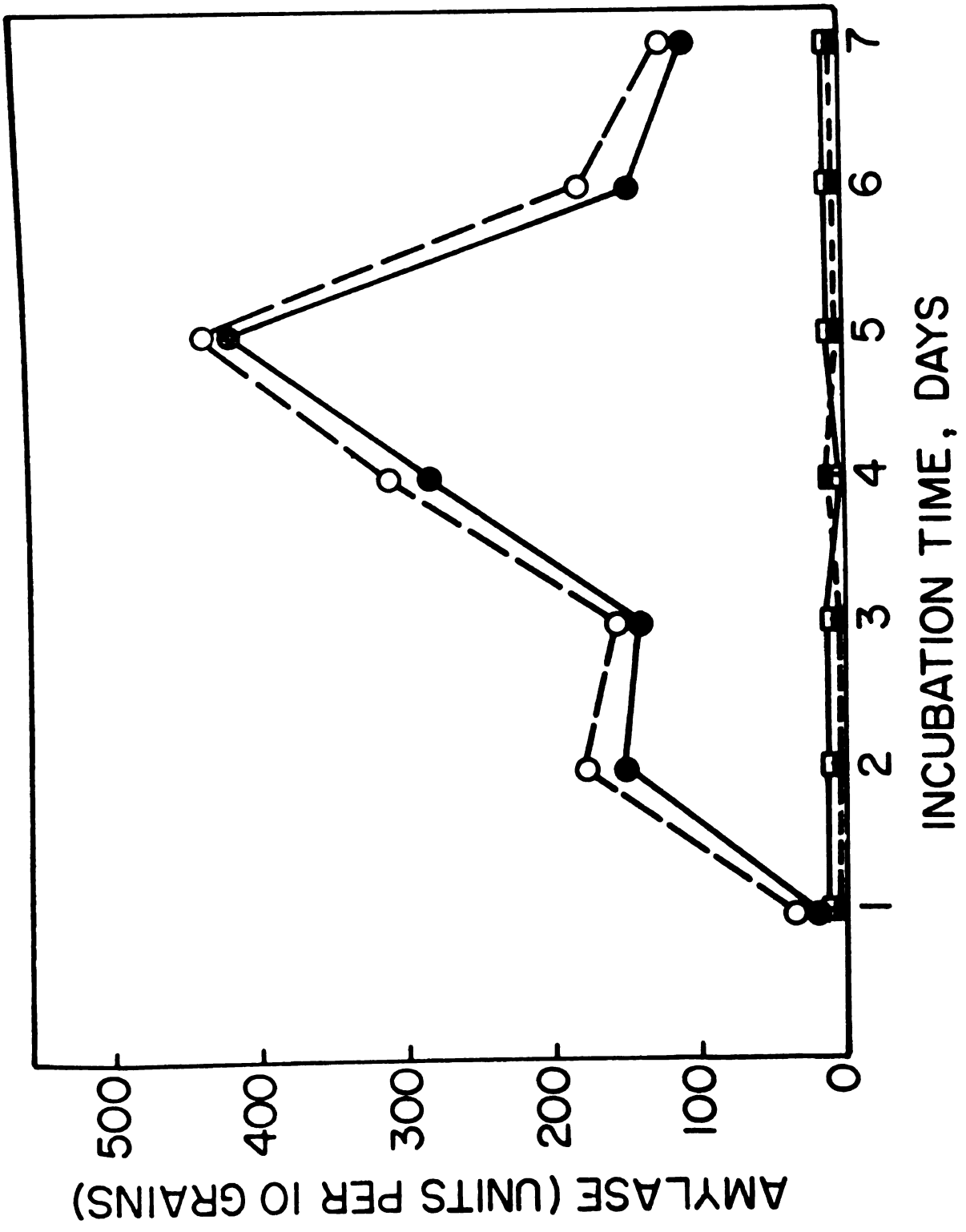
AMYLASE (UNITS PER 10 GRAINS)
500
400
300
200
100
0
1
2
3
4
5
6
7
INCUBATION TIME, DAYS

Figure 26

Figure 27. Solubilization of α-amylase after glycogen precipitation. The glycogen-amylase complex was resuspended in buffer three times in a row and the amount of α-amylase released into the buffer after each resuspension was determined. Bar 1: α-amylase released during the first resuspension period (15 min). Bar 2: additional α-amylase released during a second resuspension period (30 min). Bar 3: further α-amylase released after a third resuspension period (60 min). Incubations were at room temperature and were followed by centrifugation to separate soluble and insoluble α-amylase/glycogen complexes.

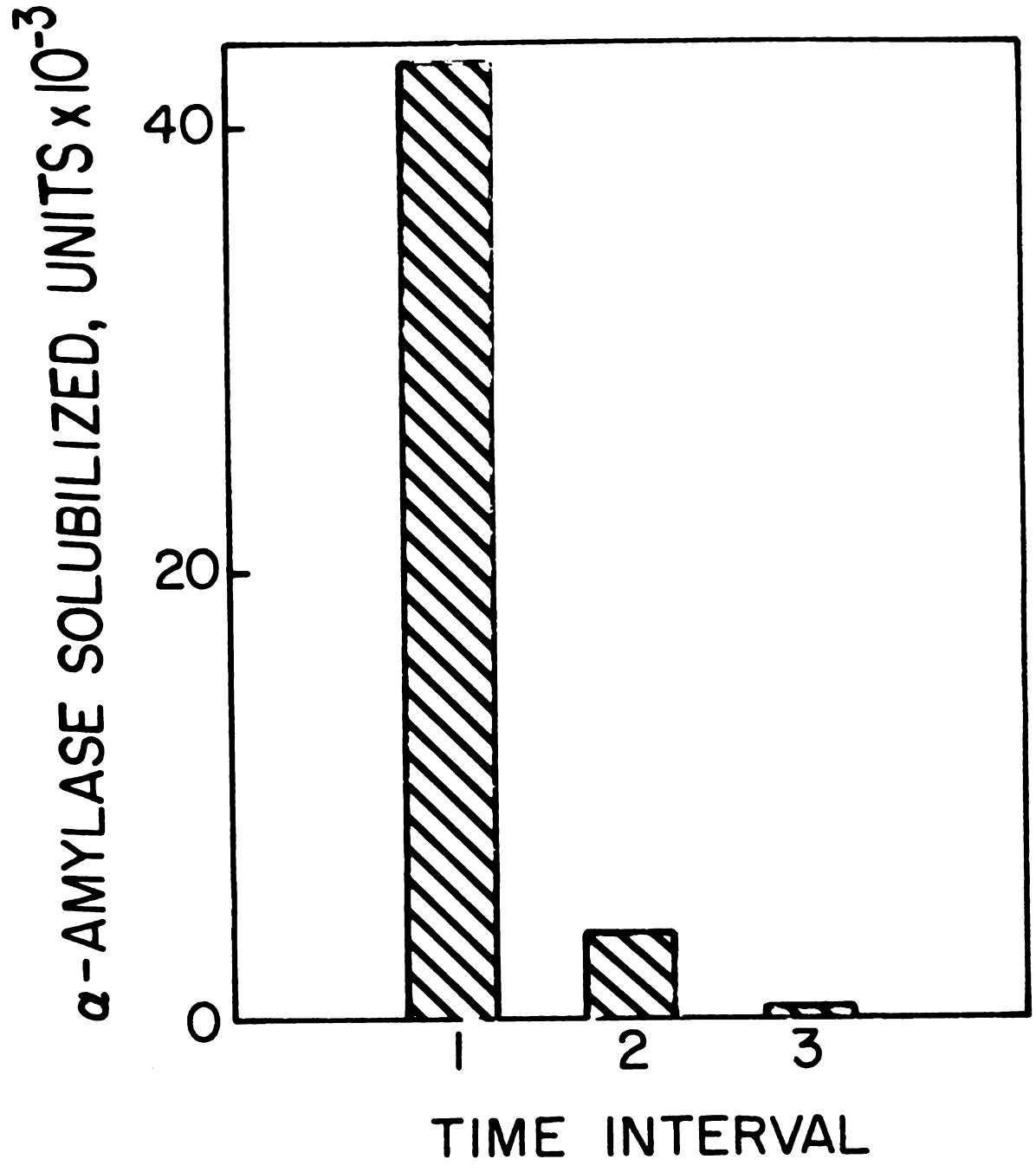

Figure 27

with 20%, 10%, and finally 5% ethanol in the same fashion. This produced the somewhat surprising result that most of the α-amylase was precipitated by concentrations of ethanol as low as 10% (Figure 28). The α-amylase must have remained attached to the glycogen throughout these manipulations, and the solubilization of the glycogen-amylase complex in Trisca buffer must not have occurred solely as a result of hydrolysis of the glycogen to limit dextrins as had been suggested by others (Loyter and Schramm, 1962).

Purity of the α-Amylase Solution

The α-amylase was tested for purity after solubilization of the glycogen-amylase complex using SDS-polyacrylamide gel electrophoresis (Figure 29). Two protein bands were seen after staining the gels with Coomassie Brilliant Blue R. The first of these was α-amylase, with a molecular weight of between 41,000 and 42,000 daltons, and accounted for 73% of the total staining intensity of the two bands. The second protein ("band-2") constituted 27% of the applied protein and had a molecular weight of 20,500 daltons. The approximate molar ratio of the two was thus 1.35 molecules of α-amylase to one of the smaller component.

Attempts to Remove the 20,500 Dalton Protein (Band-2)

Several attempts were made to remove the band-2 protein from the α-amylase preparation. However, this proved to be a difficult undertaking. Because of the differences in the molecular weights of the two proteins, it was anticipated that they would be separable by exclusion chromatography on Bio-Gel P-60. However, only one peak of protein was eluted from the column. Six fractions taken from different regions across the peak were tested for the presence of the

Figure 28. Precipitation of the solubilized glycogen-amylase complex by ethanol. The α-amylase fractions represented by Figure 27 were combined, then re-precipitated by the addition of cold 30% ethanol. This new precipitate was solubilized by addition of cold Trisca buffer, then re-precipitated with 20% ethanol. This procedure was repeated with 10% ethanol, then with 5% ethanol. The amount of α-amylase which was precipitated each time is shown. All procedures were performed at 0-2 C.

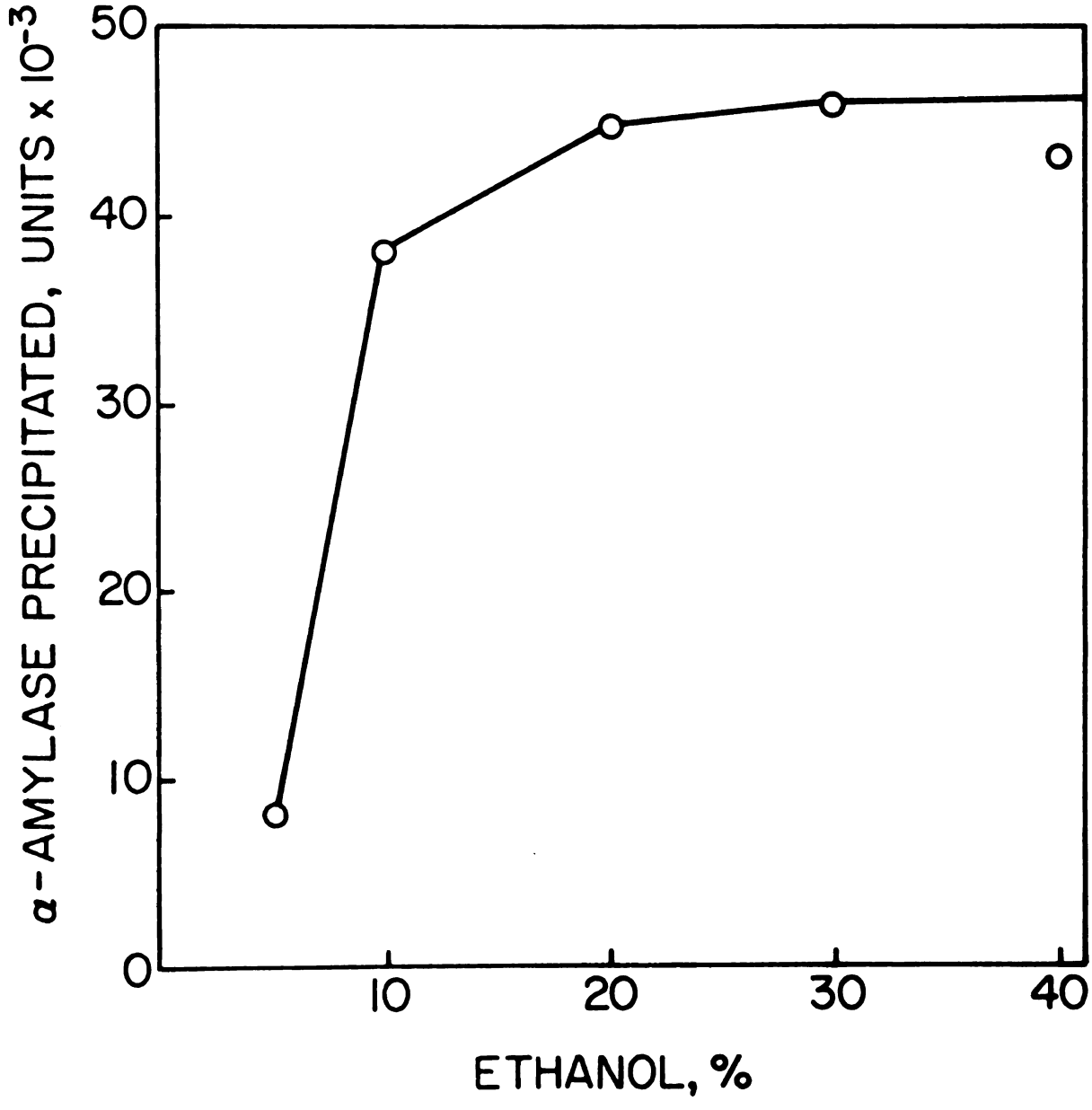
α-AMYLASE PRECIPITATED, UNITS x 10⁻³
50
40
30
20
10
0
10
20
30
40
ETHANOL, %

Figure 28

Figure 29. Purity of the α-amylase after precipitation by glycogen. SDS-polyacrylamide gels were stained with Coomassie Brilliant Blue R250 and were scanned at 560 nm. Proteins secreted by barley grains, solid line; from isolated aleurone tissue, dotted line. The histogram represents the radioactivity associated with 1-cm sections of gel which contained the α-amylase from isolated aleurone tissue.

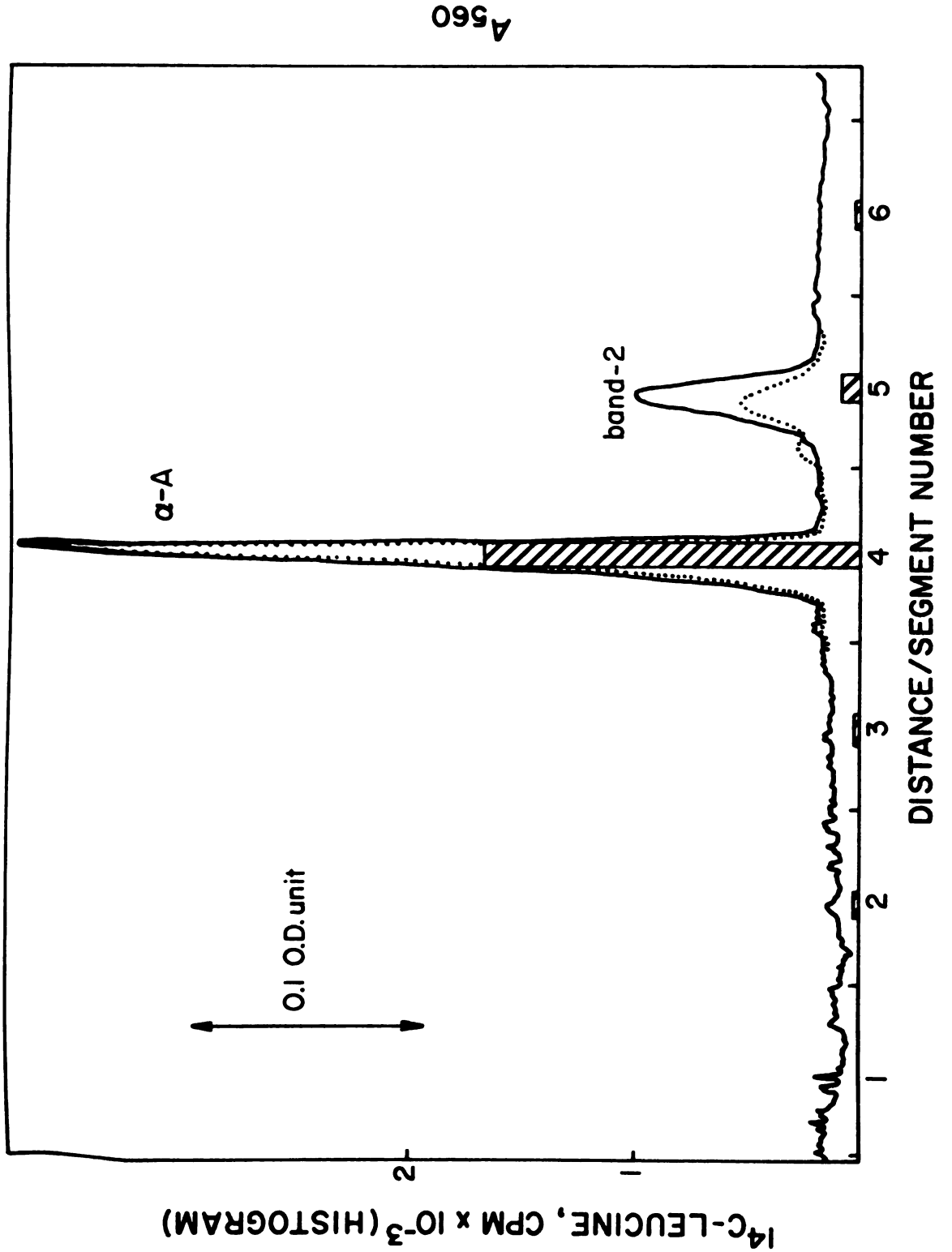
A_{560}
α-A
band-2
0.1 O.D.unit
DISTANCE/SEGMENT NUMBER
^{14}C-LEUCINE, CPM x 10^{-3} (HISTOGRAM)

Figure 29

two proteins. Only the sample taken from the trailing edge of the peak was pure α-amylase. This suggested that in its native state the protein of band-2 contained two of the 20,500 dalton peptides and thus had a molecular weight about equal to that of α-amylase. As very little pure α-amylase was obtained by this method, use of this method as a preparative technique was not feasible.

Since the native proteins could not be separated on the basis of their molecular weights, an attempt was made to separate the two by preparative isoelectric focusing (Figure 30). Two components were resolved, one with a pI of 5.0 and the second with a pI of 6.8, based on the regions of α-amylase activity in the column. The first component (pI 5.0) was pure α-amylase. The second (pI 6.8) included both α-amylase and the second protein in fraction IIc and IId. [In actuality, the true pI of the second component lay between pH 5.5 and pH 6.8 based on the amounts of protein (assayed after dialysis of the fractions) present in each region. Some of the α-amylase had been inactivated at the region of its true pI.] From this it appeared that the α-amylase and the band-2 protein could not be separated by differences in net charge of the molecules.

In addition, the contaminating protein could not be removed by differential ammonium-sulfate precipitation. Most of the α-amylase (95%) was precipitated by ammonium sulfate at 20 to 40% of saturation (Table 3). A small amount of α-amylase activity (5%) precipitated when the ammonium sulfate concentration was at 50 to 60% of saturation. The α-amylase from the first precipitate contained the band-2 contaminant. That from the second precipitation (50 to 60%) was very nearly pure α-amylase, with only a faint trace of the second protein being present. The first precipitate (20 to 40% saturation of

Figure 30. Isoelectric focusing of α-amylase. A mixture of α-amylase and band-2 protein was focused at 8 C using a gradient of pH 5 to 8 in glycerol. After focusing, the indicated fractions were pooled, dialyzed, concentrated (Millipore immersible molecular separation) and analyzed on SDS-polyacrylamide gels. Fraction IIb contained the most protein. Fractions I and IIa contained pure α-amylase. The gels are not shown.

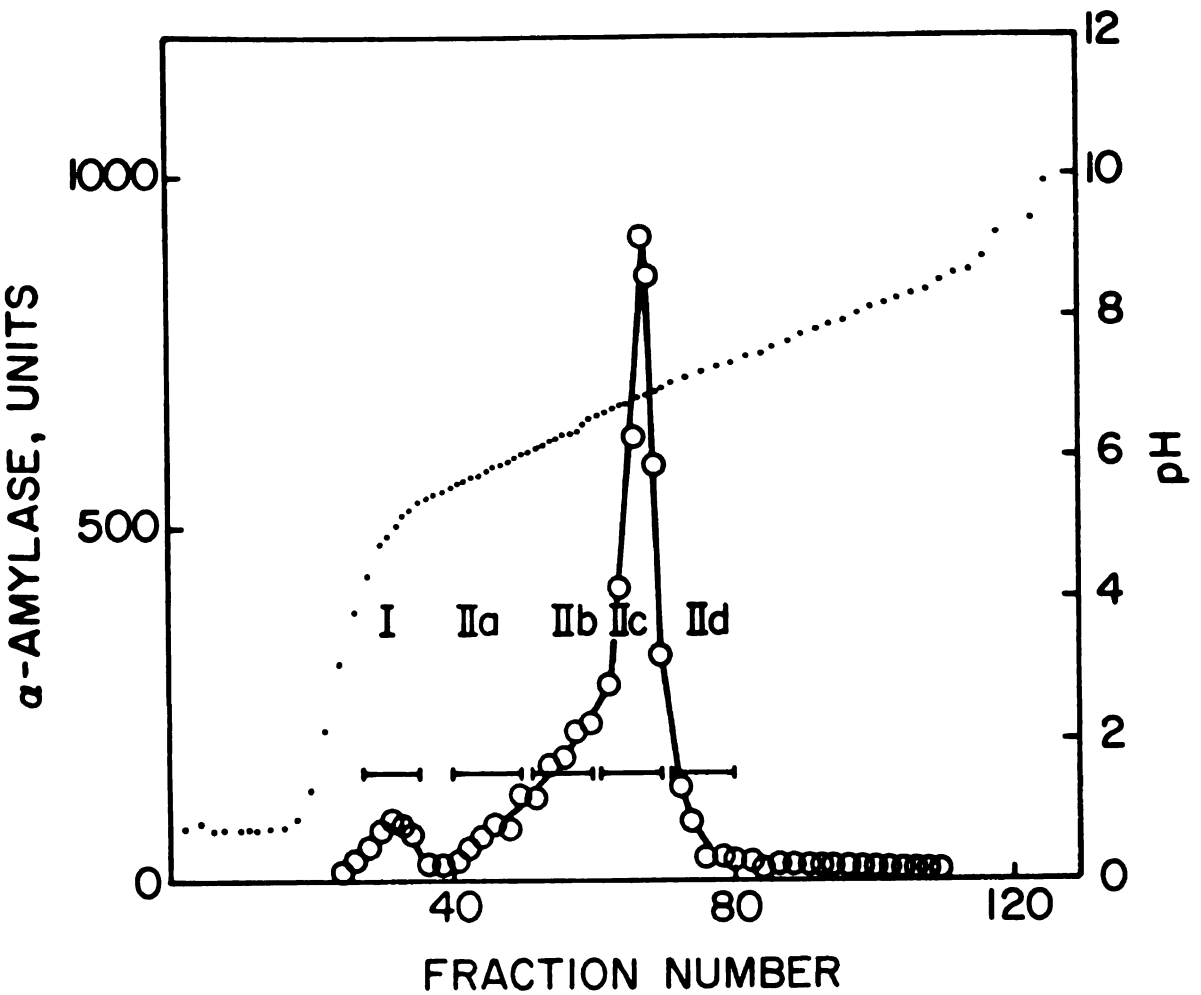
α-AMYLASE, UNITS
1000
500
0
pH
12
10
8
6
4
2
0
I
IIa
IIb
IIc
IId
40
80
120
FRACTION NUMBER

Figure 30

Table 3. Ammonium-sulfate precipitation of purified α-amylase

$(NH_4)_2SO_4$,[a] % of saturation	Time at 0 C	α-Amylase, units of activity	Protein, μg[b]
0 to 20%	1 hr	0	--
20 to 40% I[c]	1 hr	23,402	15,360
20 to 40% II[d]	3 hr	1533	1290
40 to 50%	10 hr	17.4	--
50 to 60%	1 hr	1341	1703
60 to 80%[c]	72 hr	<1	--

[a]At 0 C.

[b]Measured as bovine serum albumin equivalents.

[c]Some non-proteinaceous crystals formed during dialysis.

[d]Incubation of the supernatant fraction from the first ammonium-sulfate precipitation was continued for a further 3 hr.

ammonium sulfate) was thereafter designated as αA-40I, and was kept separate from the second precipitate (50 to 60% saturation), which was designated as αA-60.

The band-2 protein had roughly the same molecular weight as α-amylase (in its native state) and the same net charge. It also precipitated with the bulk of the α-amylase during ammonium sulfate precipitation. Furthermore, the protein also bound to glycogen and was not precipitated by the 70 C heat treatment which was a part of the purification procedure. The band-2 protein was also rendered soluble to the same extent as was α-amylase during the solubilization of the glycogen-amylase pellet (Figure 27), and it precipitated in a constant ratio to α-amylase during the repeated ethanol precipitations at different ethanol concentrations (Figure 28). However, it did not appear that the presence of this peptide was necessary for α-amylase to be active since fractions which contained only the 41,000 to 42,000 dalton protein were fully active, based on the expected specific activity of α-amylase.

No method for the separation of active α-amylase isozymes from the band-2 protein was found. Therefore, the two proteins were separated by preparative SDS-polyacrylamide gel electrophoresis.

Amino-Acid Composition of α-Amylase and the Band-2 Protein

The two proteins, α-amylase and band-2, were characterized on the basis of their amino-acid compositions. The α-amylase, it should be recalled, was composed of four α-amylase isozymes. The average amino-acid composition of this mixture of α-amylases is shown in Table 4. For reference, the ratio of each of the amino acids to isoleucine was determined. The amino acid composition of the 20,500

Table 4. Amino acid composition of barley α-amylase

Amino Acid	Number of Residues, normalized to 1.0 of isoleucine
ala	1.93 ± 0.015[a]
gly	2.69 ± 0.044
val	1.30 ± 0.021
thr	0.93 ± 0.006
ser	0.81 ± 0.010
leu	1.47 ± 0.006
ilu	1.00
pro	1.14 ± 0.044
met	0.38 ± 0.010
asp	2.86 ± 0.051
phe	0.98 ± 0.026
glu	1.54 ± 0.012
lys	1.29 ± 0.010
tyr	0.83 ± 0.021
arg	0.97 ± 0.023
his	0.94 ± 0.104

[a]Standard deviation, based on four replicates.

dalton protein is shown in Table 5. The relative ratio of the individual amino acids in α-amylase and the band-2 protein was also determined. If the two proteins had the same amino acid composition, the values determined in the last column of Table 5 would have been identical. The variation in the ratios suggests that the band-2 protein is different from the average α-amylase molecule. Cysteine was detected in both proteins, but the amount was not quantitated.

The amino acid composition of barley α-amylase was compared to that of wheat α-amylase (Tkachuk and Kruger, 1974) in Table 6. The percent of the weight of α-amylase which was represented by each amino acid was determined, assuming that the tryptophan and cysteine compositions of the barley enzyme were the same as those of wheat. The average number of residues of each amino acid present in barley α-amylase was determined using this same assumption. Barley α-amylase differed from that of wheat α-amylase by being noticeably poorer in glutamate/glutamine, serine and proline, while being somewhat richer in aspartate/asparagine, lysine, and phenylalanine.

Glycosylation of α-Amylase and the Band-2 Protein

Mitchell (1972) reported that barley α-amylase was a glycoprotein, although the mole percent of each sugar residue had not been determined. Preliminary experiments indicated that my barley α-amylase preparations, which still were contaminated by the 20,500 dalton protein, contained a variety of sugars including arabinose, xylose, glucose, and small amounts of mannose. These samples had been prepared after the solubilized glycogen-amylase complexes had been incubated at room temperature, then passed over a column of DEAE-cellulose to remove the neutral sugars and polysaccharides.

Table 5. Amino acid composition of the band-2 protein

Amino Acid	Number of Residues, Normalized to 1.0 of ilu	Ratio of Band-2 to α-Amylase[a]
ala	3.08 ± 0.078[b]	1.60
gly	3.85 ± 0.014	1.43
val	1.89 ± 0.014	1.45
thr	1.70 ± 0.035	1.83
ser	2.10 ± 0.092	2.59
leu	2.32 ± 0.049	1.58
ilu	1.00	1.00
pro	2.96 ± 0.113	2.36
met	0.30 ± 0.071	0.79
asp	3.47 ± 0.085	1.21
phe	1.46 ± 0.212	1.49
glu	2.62 ± 0.156	1.70
lys	1.48 ± 0.071	1.14
tyr	1.44 ± 0.495	1.73
arg	2.36 ± 0.071	2.43
his	1.94 ± 0.134	2.06

[a]Normalized to a ratio of 1.0 for ilu.

[b]Standard deviation, based on three replicates.

Table 6. Comparison of amino acids in barley and wheat α-amylases

Amino Acid	Weight Percent		Ratio,	Number of Residues	
	barley[a]	wheat	barley: wheat[b]	barley[c]	wheat[d]
ala	5.68	5.42	1.05	33.2	33.0
gly	6.27	5.55	1.13	46.2	42.0
val	5.27	5.68	0.93	22.3	24.8
thr	4.10	4.76	0.86	16.0	20.4
ser	2.89	4.11	0.70	13.9	20.4
leu	6.94	7.16	0.97	25.3	27.4
ilu	4.22	5.21	0.81	17.2	19.9
pro	4.43	5.86	0.76	19.6	26.1
met	2.12	1.88	1.13	6.5	6.2
asp	13.42	11.00	1.22	49.1	41.5
phe	6.08	4.87	1.25	16.8	14.3
glu	8.16	12.20	0.67	26.5	41.0
lys	6.97	5.56	1.25	22.2	18.8
tyr	5.70	6.05	0.94	14.3	12.7
arg	6.35	6.25	1.02	16.7	17.3
his	4.66	4.06	1.15	16.2	12.8
cys	--[e]	1.04	--	--	2.2
trp	--[e]	4.75	--	--	11.0

[a]Adjusted, as if barley α-amylase contained the same weight percent of cys and trp as did wheat α-amylase.

[b]Calculated from weight percent.

[c]Per molecule of 41,400 daltons.

[d]Per molecule of 43,300 daltons.

[e]Not determined.

Even when the α-amylase was fully inactivated by incubation at pH 3 and then dialyzed extensively, the dialysate still contained these sugars, although their amounts in different samples varied. Because α-amylase is known to bind tenaciously to its substrate (Fischer and Stein, 1960), even during its crystallization, I had concluded that the α-amylase would have to be denatured before it was analyzed for the presence of sugars. However, while the dialysate of the pH 3-denatured α-amylase contained sugars, there was no proof that these were covalently bound to the protein. Perhaps the sugars were not removed by dialysis, although they should have been released from the protein during denaturation.

The two proteins, α-amylase and band-2, could only be separated by SDS-polyacrylamide gel electrophoresis. This procedure was also well suited to the removal of carbohydrate polymers which were not covalently attached to the α-amylase molecule. Since SDS treatment converts proteins into more or less linear molecules, it would be nearly impossible for any non-covalent association of carbohydrate and protein to be maintained during electrophoresis. Unattached carbohydrate would be left behind at the top of the gel. α-Amylase from the first precipitation by ammonium sulfate (20 to 40%) was denatured and subjected to SDS-polyacrylamide electrophoresis, and the number of sugar residues per molecule of α-amylase was determined by gas chromatography (Table 7). The sugars were identified as their trimethylsilylated methyl glycosides. The gas-chromatographic method was chosen because each monosaccharide present in the final solution yielded two or more peaks after gas chromatography, thus producing a "fingerprint" characteristic of that particular sugar, which aided in its identification.

Table 7. Residues of sugar per molecule of α-amylase

$(NH_4)_2SO_4$ Fraction	Method of Analysis[a]	Mannose		Glucose		N-Acetylglucosamine	
		minimum[b]	maximum[c]	minimum	maximum	minimum	maximum
20 to 40% I	A	0.29	0.43	--[d]	--[d]	0	0
20 to 40% I	B	0.33	0.49	0.36	0.54	0.31	0.45
50 to 60%	C	0.31	0.47	0.16	0.24	0.11	0.17

[a]A. In situ hydrolysis in the polyacrylamide gels.

B. Protein eluted from the gels by electrophoresis.

C. Protein dialyzed against 10 mM glycine buffer, pH 3.0.

[b]Based on an approximation of the weight of protein used in the determination.

[c]Based on the number of nmoles of each amino acid (except trp or cys) in a similar sample of α-amylase.

[d]Values could not be determined due to a high background of glucose in the gel.

α-Amylase was removed from the gels by electrophoresis, precipitated with TCA, and treated with methanolic HCl to release the sugar residues. Small amounts of mannose, glucose, and N-acetylglucosamine were identified (Table 7, line 2). The sugar residues were also hydrolyzed without removing the protein from the gel. The electrophoresis buffer was removed from the "fixed" gel and sugar residues were solubilized by an aqueous hydrolysis technique using trifluoroacetic acid. In this case (Table 7, line 1) mannose was detected, but not N-acetylglucosamine. A high concentration of glucose in the gels interfered with the glucose determination. The glucose was also present in gels which had not been loaded with protein. The α-amylase which was precipitated at 50 to 60% of ammonium sulfate saturation was nearly free of the band-2 protein and was therefore examined without being subjected to electrophoresis. Instead, it was denatured at pH 3 and then dialyzed. The entire sample was used for one sugar determination. Again, mannose, glucose and N-acetylglucosamine were found (Table 7, line 3).

In all of these cases, a constant number of mannose residues was detected, ranging from 0.3 to 0.5 residues per molecule of α-amylase, depending on the method used to determine the amount of protein which was analyzed. The amount of glucose varied, contributing up to 0.5 residues per molecule. N-Acetyl glucosamine was present (except after trifluoroacetic acid treatment of the α-amylase in the gels), again contributing up to 0.5 residues per molecule. It therefore appears that all of the α-amylase molecules are not glycosylated, but some of them may be.

For comparison, the number of sugar residues per molecule of the band-2 protein was determined (Table 8). No mannose was detected, but

Table 8. Residues of sugar per molecule of band-2 protein

$(NH_4)_2SO_4$ Fraction	Method of Analysis[a]	Glucose		N-Acetylglucosamine	
		minimum[b]	maximum[c]	minimum	maximum
20 to 40% I	A	--[d]	--[d]	0.04	0.06
	B	0.38	0.65	0.14	0.25

[a,b,c,d] Refer to Table 7.

up to 0.6 residues of glucose and 0.25 residues of N-acetylglucosamine per molecule were found.

Three unidentified peaks appeared on the chromatograms after gas chromatography of the cleavage products of proteins which had been subjected to electrophoresis. These peaks did not correspond to the positions of derivatized sugar residues which have been found in glycoproteins. They had retention times relative to mannitol of 0.20, 0.42, and 0.92 (proportional to retention times obtained by Bhatti et al., 1970). The three compounds appear to have originated from the SDS used for electrophoresis, since SDS from the manufacturer's bottle gave the same peaks, which were probably due to the presence of contaminants in the SDS. The unidentified compounds remained tightly bound to the proteins even after the proteins were eluted from the gels and precipitated by TCA or after the proteins were fixed in the gels and the gel buffer removed by repeated washings of the gels. The presence of tris also produced peaks in the chromatogram, so that tris also had to be removed from the protein samples before they were processed.

To insure that the procedures which were used did not prematurely remove the monosaccharides from the glycoproteins, ovalbumin was subjected to electrophoresis and treated in the same ways as α-amylase and the band-2 protein had been. After electrophoresis, the eluted ovalbumin exhibited the same ratio of mannose to N-acetylglucosamine as did ovalbumin which had been simply dissolved in H_2O. The ovalbumin samples which had been subjected to electrophoresis or had been mixed with the electrophoresis buffer but not subjected to electrophoresis also carried along the unidentified components (originating from SDS)

which appeared on the chromatograms, although ovalbumin is known to contain only mannose and N-acetylglucosamine as sugars.

In the analyses of Tables 7 and 8 a maximum and a minimum number of sugar residues per molecule are given. The numbers of sugar residues per molecule were determined by two different methods, giving rise to these maximum and minimum designations. The stock solution of α-amylase was split into two fractions, one being used for sugar analysis and one being used to determine the number of protein molecules present in the sample. This latter was determined from either the weight of the protein pellet prior to analysis of its amino-acid content or from the calculated weight of protein derived from the actual amino-acid content after hydrolysis of the protein (no correction was made for tryptophan or cysteine in this calculation). The number of protein molecules per sample (based on molecular weights of 41,400 for α-amylase and 20,500 for band-2) was greater in the case of the measured weight than in the case where the weight was calculated from the amino-acid content. This may have been due to the presence of solvent in the incompletely dried protein pellet. Using the measured weight of protein per sample, the minimum number of sugars per molecule was calculated, and using the calculated weight of protein, the maximum number of sugar residues per molecule was calculated.

The true molar ratio of α-amylase to the band-2 protein in αA-40 I was 2.25 based on the quantitation of amino acids (except tryptophan and cysteine) after those precipitates were hydrolyzed by acid. This compares to a staining ratio of the proteins on the gels of about 2.72. An estimate of the conversion factor for the Lowry determination based on BSA as a standard, to the true amount of

α-amylase was calculated, based on an estimated loss of 5.8% of the weight of the amylase by destruction of tryptophan and cysteine. Since the Lowry determination was based on a mixture of α-amylase and band-2 protein, the conversion factor which I have estimated would be different if the α-amylase used for the Lowry assay had been completely pure. The conversion factor thus determined was that 1 unit from the Lowry determination was equal to 0.638 units of protein as determined from the amino-acid composition of the protein.

Reactions with Carbohydrate Stains

The periodic acid-Schiff (PAS) test is the conventional method for identifying glycoproteins in polyacrylamide gels. Carbon-carbon bonds with vicinal hydroxyl groups are cleaved in the presence of periodate. The product, a dialdehyde, is reduced by the Schiff reagent, resulting in a rose-red color. α-Amylase and the band-2 protein were tested for the presence of a PAS-positive carbohydrate moiety. Ovalbumin, which gives a weak PAS-positive reaction, was used as a control. Bovine serum albumin and chymotrypsinogen were used as non-glycosylated control proteins. The intensities of the positive reaction products of α-amylase and band-2 were compared to those of ovalbumin in Figure 31. The ovalbumin was added three times at 45-min intervals during electrophoresis, first 75 μg being added, then 25 μg, and finally 7.5 μg, the latter amount producing a very faint staining reaction. Seventy-two units of α-amylase were applied to the gel, the profile of which is shown at the top of Figure 31. Although the ovalbumin was stained within 48 hr by the reaction, α-amylase and the second, band-2 protein, were slower to react, requiring one to three weeks for the color to appear. The proteins

Figure 31. Periodate-Schiff (PAS) staining of α-amylase in polyacrylamide gels. Top scan: A solution containing 72 units of α-amylase was applied to the gel; the major peak is α-amylase and the minor peak is the band-2 protein. Bottom scan: Ovalbumin was added in three stages. First 75 µg (1) were added, later 25 µg (2) and, even later, 7.5 µg (3). The absorbance was measured at 560 nm. There were no other peaks of protein in the gels.

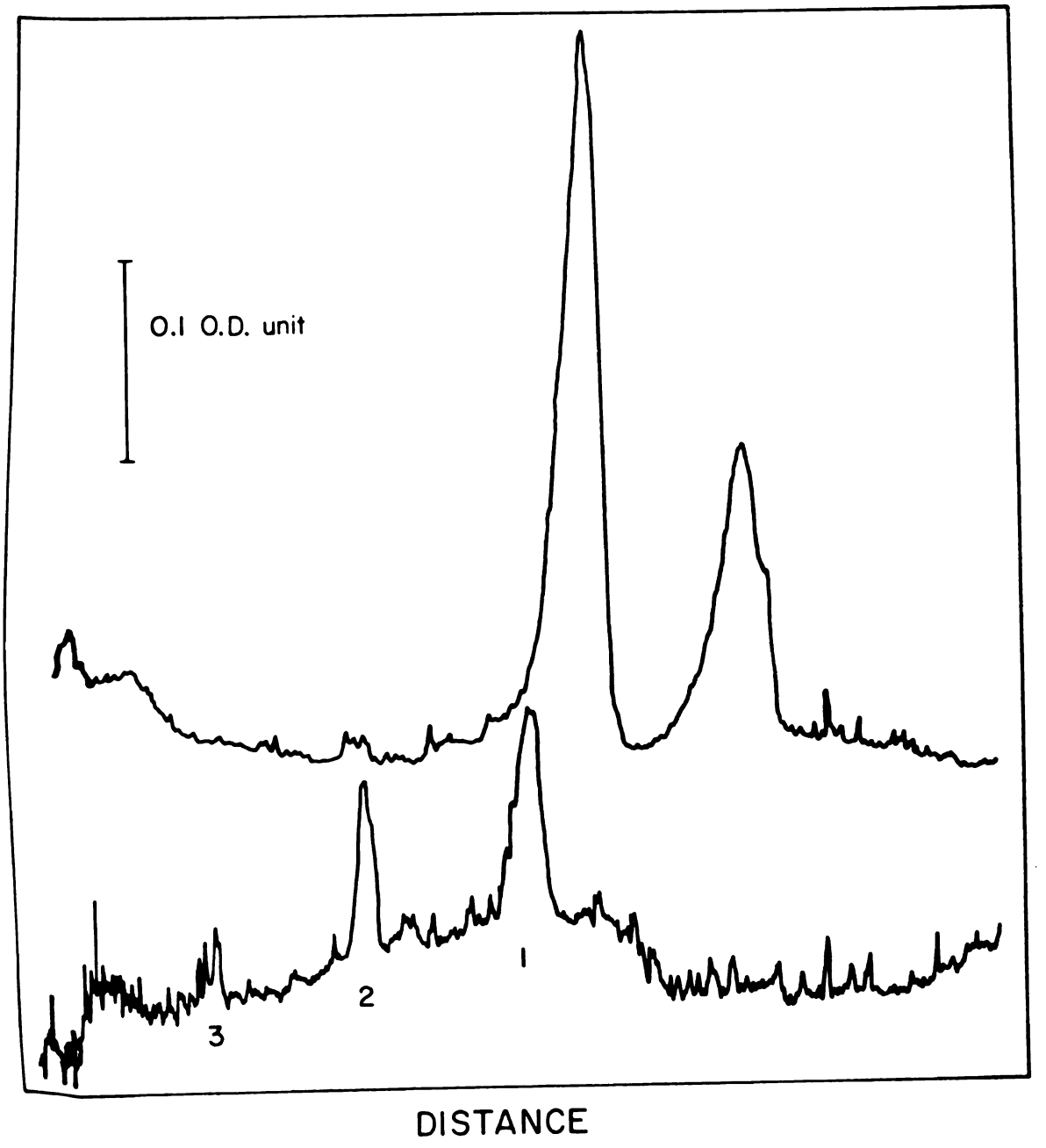

Figure 31

were not run on the same set of gels, however. If the color reaction, in the case of α-amylase, occurred because of a secondary reaction of the protein with the periodate or Schiff reagent, it is interesting that neither bovine serum albumin nor chymotrypsinogen ever developed colored products, when as much as 75 μg of each protein was present per band on similarly prepared gels.

A second procedure was used to test whether the α-amylase was a glycoprotein. After the proteins in the gels were subjected to periodate oxidation, reaction of the oxidized glycoproteins with dansyl hydrazine followed by sodium borohydride caused the formation of a stable, fluorescent product. The fluorescence was detected under long-wavelength ultraviolet light (about 350 nm). Both α-amylase and the band-2 protein were stained by this procedure (Figure 32), as was ovalbumin. Following the procedure of Eckhardt (1976), proteins which had not been subjected to periodate oxidation were used as controls. α-Amylase which had not been oxidized was weakly fluorescent, while the periodate-oxidized material was considerably more fluorescent. The fluorescent products were visible as soon as the excess dansyl hydrazine was leached out of the gel. The staining reactions of ovalbumin (a glycoprotein with a small carbohydrate moiety) and lysozyme are included for comparison. The reaction of lysozyme is noteworthy since this non-glycosylated protein also gave a positive reaction with the dansyl hydrazine, indicating that proteins other than glycoproteins may react with the stain. The band-2 protein reacted with dansyl hydrazine in a manner analogous to that of α-amylase.

Figure 32. Periodate-dansyl hydrazine staining of α-amylase in polyacrylamide gels. Left to right: (1) α-amylase (100 µg) without periodate oxidation step; (2) α-amylase plus band-2 protein at a total of 10 µg per gel with periodate oxidation (PO), (3) α-amylase and band-2 protein at 35 µg per gel with PO, and (4) α-amylase and band-2 protein at 100 µg per gel with PO; (5) and (6) ovalbumin, three bands in the same gel by sequential additions of 100 µg (bottom band), 35 µg (middle band), 10 µg (faint upper band), (5) is the control with no periodate oxidation step and (6) is the gel treated with periodate; (7) and (8) are lysozyme with the same 3 bands per gel as with ovalbumin, (7) is without the periodate step and (8) is periodate-treated. The white arrow points to the position of the marker dye, pyronin Y.

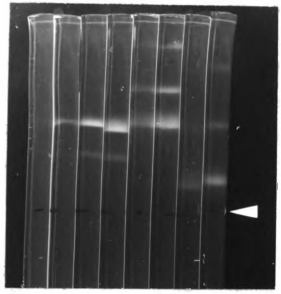

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Figure 32

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Origin of the Band-2 Protein

Since band-2 protein could not be removed from preparations of α-amylase except by SDS-polyacrylamide gel electrophoresis, the origin of the band-2 protein was investigated. Aleurone layers were isolated from imbibed, de-embryonated seeds, and were treated with 1 μM GA_3 for 24 hr. The released α-amylase was then purified in a manner analogous to the purification of α-amylase from the whole grains. In the glycogen precipitate of released enzyme, the ratio of the α-amylase to band-2 on a mole per mole basis increased to 3.34 (Figure 29). The ratio of the two proteins which were isolated from de-embryonated seeds was the same as that of the proteins isolated from whole barley grains, namely 1.35.

The protein synthesized and released by the aleurone layers was labeled with ^{14}C-leucine to test whether band-2 was synthesized during the period of GA_3-treatment. Less than 3% of the label which bound to glycogen was found to be associated with this region of the gel, while the rest of the label was associated with the α-amylase band (Figure 29). These results suggest that the band-2 protein was not synthesized in response to GA_3 and was thus probably not derived from GA_3-induced α-amylase. A major part of the band-2 protein appeared to be associated with the starchy endosperm at the time GA_3 treatment was begun, and its release to the starchy endosperm was apparently not dependent upon GA_3 treatment.

DISCUSSION

Four isozymes of α-amylase are synthesized and secreted by the aleurone tissue of Himalaya barley. These isozymes can be separated by electrophoresis on slab gels of 1% agar (Jacobsen et al., 1970). The isozymes must all have about the same molecular weight of 41,400 daltons, as has been determined by electrophoresis on SDS-polyacrylamide gels, where the isozymes move as one band of protein. I have now investigated the primary structure of α-amylase with respect to its amino acid composition and extent of glycosylation.

Amino-Acid Composition

The "average protein" molecule (see Varner, 1969) consists of different numbers of each amino-acid residue per molecule, that is, of different mole percents of each amino-acid. The "average protein" appears to be rich in glutamate/glutamine, rich in aspartate/asparagine, and rich in alanine and glycine residues. These amino-acids have in common the fact that there is a guanine in the first position of the codon which codes for them. On the other hand, the "average protein" is poor in the aromatic amino-acids phenylalanine, tryptophan, tyrosine, and histidine, as well as the sulfur amino-acids methionine and cysteine. These amino-acids are more likely to participate directly in catalytic or recognition functions of the protein. α-Amylase differs from the "average protein molecule" by having a higher relative proportion of histidine and tyrosine and a lower

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relative proportion of glutamate/glutamine (Table 9). α-Amylase from barley is rather similar to that from wheat (Tkachuk and Kruger, 1974), the barley α-amylases being somewhat richer in aspartate/asparagine, phenylalanine and lysine (when the ratio of weight percents of the amino acids are compared) and somewhat poorer in glutamate/glutamine, proline, and serine (Table 6).

By knowing the molecular weight of a protein, the nanomoles of each amino acid which are present in a given sample of the enzyme, and the weight of protein in that sample as determined by the method of Lowry (Layne, 1957), one can obtain a correction factor which will convert the estimated weight of protein, as determined by the Lowry method (Layne, 1957),to the true weight of protein which is present in the sample. Using a protein preparation that contained 80% by weight of the total protein as α-amylase, I calculated that 1 mg of protein, as estimated by the Lowry method using bovine serum albumin as a standard (Layne, 1957), was equal to 0.63 mg of actual α-amylase.

Glycosylation

The α-amylase which had been secreted by GA_3-treated barley grains also appeared to be glycosylated, although to a limited extent. α-Amylase which was subjected to electrophoresis on SDS-polyacrylamide gels could be stained by the periodic acid-Schiff reaction (Figure 31) and by the periodic acid-dansyl hydrazine reaction (Figure 32). This indicated that at least some of the α-amylase molecules were glycosylated.

Because electrophoresis of a protein on SDS-polyacrylamide gels should remove any non-covalently bound carbohydrate, such an

Table

Amino Acid
lys
his
arg
asp
thr
ser
glu
pro
gly
ala
val
met
ilu
leu
tyr
phe

Table 9. Comparison of α-amylase to an "average protein" molecule[a]

Amino Acid	Mole Percent		Ratio,
	α-amylase	average protein	α-amylase/ average protein
lys	6.3	5.9	1.1
his	3.9	1.8	2.2
arg	4.7	4.9	0.96
asp	13.4	9.7	1.4
thr	4.7	4.8	0.98
ser	3.8	6.0	0.63
glu	7.3	12.7	0.57
pro	5.3	6.2	0.85
gly	12.7	12.6	1.0
ala	9.2	9.6	0.96
val	6.1	5.9	1.0
met	1.9	1.8	1.1
ilu	4.8	6.0	0.80
leu	7.1	6.0	1.2
tyr	4.0	2.3	1.7
phe	4.8	3.7	1.3

[a]See Varner, 1969.

electrophoretic pre-treatment appears to be a useful procedure for preparing small quantities of protein (i.e., on the order of 1 mg or less) for carbohydrate analysis. Any protein which is boiled in SDS and mercaptoethanol should release non-covalently bound carbohydrate, and the carbohydrate should be left behind at the gel/buffer interface as the denatured protein enters the gel. The protein can subsequently be removed from the gel and subjected to carbohydrate analysis, or it can be fixed in the gel and be analyzed "*in situ*" after the electrophoresis buffer has been washed out of the gel. This eliminates the objection that even purified proteins may be non-covalently associated with carbohydrates or may retain carbohydrate at their substrate binding site.

Quantitative analysis of the number of sugar residues in α-amylase was used to show that, on the average, less than one residue each of mannose, glucose, and N-acetylglucosamine were present per average molecule of α-amylase.

On the average, the total population of α-amylase molcules contained by weight a *maximum* of 0.7% carbohydrate. This is much less than the 3% found by Mitchell (1972), who had identified glucose, galactose, mannose, xylose, fucose, and glucosamine in his purified preparations of α-amylase. Galactose, xylose and fucose were absent from the α-amylase which I had prepared by electrophoresis. In comparison to the glycosylation of barley α-amylase, wheat α-amylase is reportedly carbohydrate-free (Tkachuk and Kruger, 1974) and sorghum α-amylase has been reported to contain 2% carbohydrate (Fischer and Stein, 1960). The mannose, glucose, and N-acetylglucosamine residues which were identified after electrophoretic purification of the α-amylase are believed to be covalently attached to the protein on

the assumption that non-covalently bound carbohydrate residues would have been removed from the protein during electrophoresis in the presence of SDS.

Since each sugar residue is present in a ratio of less than one residue per molecule of protein, the molecules of α-amylase must be heterogeneous with respect to their extent of glycosylation. This heterogeneity could originate during the synthesis of α-amylase such that the molecules of certain isozymes are glycosylated with different sugars, or to different extents. On the other hand, the heterogeneity might have arisen from the hydrolytic activity of enzymes which were present outside of the aleurone cytoplasm and which might have removed sugars from glycoproteins. That is, the α-amylase might have been more extensively glycosylated at the time of its synthesis and might have been de-glycosylated at some later time. Because α-amylase is probably synthesized on the endoplasmic reticulum (Chen and Jones, 1974; Jones and Chen, 1976), the protein may originally have been glycosylated by enzymes found within the lumen of the endoplasmic reticulum, although such enzymes have yet to be characterized in the barley aleurone system.

It would be useful to separate the isozymes of α-amylase and determine in a qualitative way, at least, which of the isozymes were glycosylated. To this end, I attempted to resolve the isozymes of α-amylase on an isoelectric focusing polyacrylamide gel, using a pH gradient prepared by a mixture of 3 parts of Ampholines (LKB) in the range of pH 5 to 8 and 2 parts of Ampholines in the range of pH 3 to 10. However, I could only resolve two protein components, a sharp band which contained most of the protein and a very diffuse band at

a lower pI which contained less protein, as measured by Coomassie Brilliant Blue R staining. This degree of resolution would have been inadequate for analytical purposes. However, this result was interesting in another way. It suggested that while the α-amylase isozymes had essentially identical molecular weights and only separated into two groups on the basis of their isoelectric points, there must have been some feature(s) of their interaction with the agar gels which allowed clear resolution of all four isozymes in that electrophoretic system (Jacobsen et al., 1970). Unfortunately, agar itself is a polysaccharide and contains sugars which would interfere with glycoprotein analysis. Therefore, another method of preparative isozyme separation, besides electrophoresis on agar, must be found before the isozymes may be individually analyzed.

To test the possibility that α-amylase is more extensively glycosylated before it is secreted, the enzyme could be isolated from the particulate fraction of barley (Firn, 1975) for determination of its carbohydrate composition. This method of isolation would insure that the α-amylase was not exposed to extracellular enzymes during homogenization. Of course, this latter suggestion assumes a vesiculate mode of α-amylase secretion, which is disputed by experiments of Jones (1972). Some use could also be made of the general observation (e.g., Vigil and Ruddat, 1972) that Actinomycin D interferes with secretion of α-amylase, since Actinomycin D could be used to inhibit secretion and thus to increase the amount of α-amylase in the tissue at any one time. Such an analysis, however, was beyond the scope of the present study.

An alternative explanation for the low amount of sugars which were found in α-amylase, and the fact that less than one residue of

a given sugar was present per α-amylase molecule, was that the results might have been artifactual. Two sorts of artifacts may have occurred. First, the single band of protein which included the four isozymes of α-amylase may also have included a second kind of protein which was glycosylated, while α-amylase was not. At present, there seems to be no reasonable way of testing this possibility. Second, some carbohydrate may have become attached (non-covalently) to α-amylase during the electrophoresis and subsequent stages of protein preparation for carbohydrate analysis, although there was no real reason to expect this. There is also the possibility that the periodate-staining reactions were positive, even though the proteins were not truly glycosylated (Figures 31 and 32). Some treatments of proteins, including α-amylase, with periodic acid (40 mM, 7 C, 105 hr) lead to extensive cleavage of the polypeptide (unpublished results; see also Clamp and Hough, 1965), and it may be possible that these fragments react with either the Schiff reagent or dansyl hydrazine in the subsequent staining reactions. If such reactions had occurred, however, they would have had to have been dependent on the peculiarities of the α-amylase molecule because, except in the case of lysozyme, control proteins which are known to be non-glycosylated did not react with the chromagens to form either colored or fluorescent products as a specific result of periodate oxidation.

The Band-2 Protein

A second protein, the band-2 protein, co-purified with α-amylase up to the step when the proteins were separated by preparative electrophoresis. The presence of the second protein was unexpected since the use of a similar method for the purification of α-amylase

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from de-embryonated grains of Betzes barley (in Section I of this dissertation) yielded a preparation which, in most instances, was free of this contaminant, although faint traces of a protein of about the same molecular weight (21,000 daltons) could be seen on some of the SDS-polyacrylamide gels. Interestingly, Tkachuk and Kruger (1974) also found a minor protein contaminant of about the same molecular weight in their preparations of wheat α-amylase. The band-2 protein may have been present at a much lower concentration in the Betzes barley grains or, for some unknown reason, a similar protein from Betzes barley grains may not have been bound to the glycogen in the final stages of purification.

The band-2 protein was similar to α-amylase in several ways: it had a similar isoelectric point and molecular weight (in its native state), it bound tightly to glycogen, and it precipitated at the same concentration of ammonium sulfate. Much of the band-2 protein was removed by scraping away the starchy endosperm prior to GA_3-treatment. Since the protein did not become labeled during GA_3-treatment, it must have been synthesized prior to that time. The protein could conceivably be an α-amylase which derived from the immature seed, or it may have been some other carbohydrase or perhaps a lectin, which also bound to glycogen. Most probably, the protein was localized in the periplasmic space, the cell wall and/or the residual starchy endosperm at the time GA_3 was added to the isolated aleurone layers, because Jacobsen and Knox (1974) reported that no unlabeled proteins with molecular weight less than 40,000 daltons were released after GA_3-treatment. A notable feature of the composition of the band-2 protein was that it also appeared to be only partially glycosylated. This supports the contention that cleavage

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of sugar residues from the glycoprotein may occur either upon secretion or at some subsequent time. However, alternate explanations for the low amount of carbohydrate per molecule of band-2 protein as discussed regarding α-amylase may also apply here.

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