# THE RESPONSE OF CEREAL ALEURONE TISSUE TO GIBBERELLIC ACID

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## SUMMARY

A technique was developed for the rapid isolation of large quantities of viable and reproducible wheat aleurone tissue. The tissue consisted of the aleurone layer, the testa-pericarp and a few cells of the starchy endosperm immediately adjacent to the inner surface of the aleurone layer.

A number of wheat varieties were surveyed and one was selected whose aleurone tissue produced only small amounts of α-amylase in the absence of gibberellic acid (GA) but reasonably large amounts in its presence.

The GA-induced production of α-amylase by this tissue was studied as a function of GA concentration, time, pH and addition of various compounds such as sugars, calcium ions and actinomycin D. The response of the tissue to GA was found to be qualitatively similar to that of barley aleurone.

Methods for homogenising and fractionating the tissue into subcellular components were investigated. One soluble and three particulate fractions were obtained by centrifugation. The particulate fractions were heterogeneous but each was enriched with one or two specific organelles.

The distribution of GA-induced hydrolytic enzymes (acid phosphatase, ribonuclease (RN-ase) and amylase) among these fractions was investigated after incubation of the tissue with GA for various times. Only small amounts of acid phosphatase and RN-ase were present in particulate fractions. However, appreciable amounts of α-amylase were found in all three pellets. The nature of the tissue, the homogenization and fractionation techniques, the sensitivity of the enzyme assays and the amounts of enzymes detected, are discussed with respect to the probability of finding particulate enzymes.

Although the results were inconclusive, it was suggested that the GA-induced enzymes might be particulate and that the homogenization and fractionation techniques resulted in rupture of the particles so that only a small proportion remained intact. It was suggested that hormonal control of the synthesis of these enzymes might be analogous to that of animal lysosomes.

## DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

## ACKNOWLEDGMENTS

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## 1. INTRODUCTION

During the germination of cereal seed, gibberellins are secreted by the embryo into the endosperm where they have a marked effect on the aleurone layer. This tissue undergoes several changes in both ultrastructure and chemical composition either as a result of germination or the application of gibberellic acid (GA)*. For example, the aleurone grains (which are thought to be the storage organelles for protein, lipid and phytate) become distended, their contents diminish and often several grains coalesce; the spherosomes (which, in the aleurone tissue of the mature seed surround the aleurone grains and line the plasmalemma) diminish in size, change in shape and staining properties, and eventually disappear. Other membranous structures such as the endoplasmic reticulum, golgi bodies and mitochondria become less distinct and also disappear. Several hydrolytic enzymes increase in concentration and are secreted into the endosperm where they hydrolyse such storage compounds as starch and protein to their constituent sugars and amino acids. These break-down products, in turn, are utilized by the embryo for growth.

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* The symbol GA will always be used to refer to gibberellic acid; other gibberellins will be designated by the symbol $GA_x$ where x is the appropriate integer.

The aleurone tissue is particularly suitable for the study of the mechanism of action of gibberellic acid: firstly, because it is homogeneous and non-meristematic, and secondly, because it responds to the hormone in a specific manner. However, one major difficulty is obtaining large quantities of viable tissue free of other seed materials. In the present investigation a technique for the mass isolation of aleurone tissue was developed. The response of this tissue to GA was then examined.

The experimental work involved:

a) Development of a technique for the mass isolation of aleurone tissue;
b) Characterization of the response of the tissue to GA under various experimental conditions;
c) Fractionation of the tissue into subcellular components;
d) Examination of the distribution of hydrolytic enzymes among subcellular fractions from tissue treated for various times with GA.

## 2. RESPONSE OF ALEURONE TISSUE TO GIBBERELLIC ACID: PREVIOUS WORK

### 2.1 The Aleurone Layer

The aleurone tissue of cereal seed is the outermost layer of cells of the endosperm. In barley it is three cells thick, while in wheat it is only one cell thick (Esau, 1953). It is derived from the triple fusion nucleus of the embryo sac and, thus, is triploid. Although the aleurone layer has the same genetic constitution as the underlying starchy endosperm, it is very different in morphology, chemical composition, metabolism and physiological function. Cells of the starchy endosperm are occupied almost entirely by starch granules and protein bodies (Jennings et al., 1963; MacLeod et al., 1964a) and appear to be metabolically inert at least insofar as they do not respire (Paleg, 1964). Cells of the aleurone layer, on the other hand, possess all of the normal cellular structures associated with active metabolism such as nuclei, mitochondria, endoplasmic reticulum and golgi bodies, as well as aleurone grains and spherosomes which are presumably storage organelles (Buttrose, 1963; Paleg, 1964; Paleg and Hyde, 1964; MacLeod et al., 1964a). These cells respire at a rate comparable with that of other

tissues (Paleg, 1964; Rowsell and Ali Khan, 1966).

## 2.2 Gibberellin: the endosperm mobilizing hormone

The importance of the aleurone layer in germination was recognized as early as the 1890's when it was observed that this tissue secretes enzymes which hydrolyse the storage compounds of the endosperm and that the activity of the aleurone cells is somehow controlled by the embryo (Haberlandt, 1890; Brown and Escombe, 1898). It was not until the 1960's that gibberellins were identified as the controlling compounds secreted by the embryo into the endosperm. The work leading to this discovery has been reviewed by Briggs (1963) and Paleg (1965) and is briefly described below.

In a series of papers Yomo (1958, 1960 a,b,c and d) reported that separated embryos and endosperms of barley had to be cultured together for substantial amounts of amylase activity to develop. He isolated a compound from cultured embryos and from green malt which could replace the embryo in causing the endosperm to develop amylase activity and showed that this compound resembled the gibberellins in chemical properties. Furthermore, a mixture of gibberellins could duplicate the effect of the unknown compound or the embryo on

isolated endosperm. These results were later confirmed by MacLeod and Millar (1962) and Briggs (1963). Yomo concluded that during germination the embryo secretes a gibberellin-like compound into the endosperm where it initiates amylase activity. The same conclusion was reached by Paleg (1960a and b, 1961) who demonstrated that GA over a range of concentrations from $2 \times 10^{-9}$ to $2 \times 10^{-4}$ M caused barley endosperm to release reducing sugars, amylases and protein nitrogen into the surrounding medium.

Since that time, much evidence has accumulated that gibberellins are the endogenous hormones (Brian, 1966). Thus, GA was tentatively identified in germinating barley seed by Yomo (1960d), Radley (1959) and Lazer et al. (1961) and positively identified in immature seed by Jones et al. (1963). MacLeod and Palmer (1966) identified GA in one day old barley seedlings while Yomo (1966) found gibberellin-like substances in isolated barley embryos only after three days of culturing. Finally, Radley (1967) showed that gibberellin-like substances (predominantly $GA_1$) were released from the scutellum of several varieties of barley during at least the first two days of germination. She suggested that the scutellum might contain a pre-

formed gibberellin precursor which is converted to the active form and rapidly translocated to the endosperm during the first 48 hours of germination. At later times the embryo axis appears to produce GA. By dissection of different parts of barley embryos, MacLeod and Palmer (1966) produced evidence that the nodal region of the embryo is the site of production of the hormone (presumably a gibberellin) which brings about endosperm mobilization and α-amylase activity. (This tissue may have been included as part of the scutellum examined by Radley, 1967). Cohen and Paleg (1967) also showed that isolated barley embryos secreted gibberellin-like substances into the surrounding medium, and that these substances induced sugar-release from endosperm isolated from the same batch of seed. Moreover, the quantities of the gibberellin-like substances and the times over which they were secreted were sufficient to account for the mobilization of the endosperm in vivo.

Thus there is little doubt that the gibberellins are the factors which are secreted from the embryo into the endosperm where they bring about hydrolysis of the storage starch and protein.

## 2.3 Identification of the aleurone layer as the target tissue

The site of action of the hormone was eventually localized in the aleurone layer. The starchy endosperm did not respond to GA if the aleurone layer was removed. However, the aleurone layer by itself produced hydrolytic enzymes, particularly α-amylase, in the presence of GA but not in its absence (Macleod and Millar, 1962; Briggs, 1963; Rowsell and Goad, 1963; Paleg, 1964; Varner, 1964; Yomo and Iinuma, 1964a and b). The value of the aleurone tissue as an ideal experimental material for studying the mechanism of action of gibberellic acid was immediately recognized (Paleg, 1965; Varner *et al.*, 1965). This tissue is a homogeneous population of differentiated, non-meristematic, non-photosynthetic cells which responds to the hormone both *in vitro* and *in vivo* in a specific and measurable manner.

## 2.4 Biochemical manifestations of the response of aleurone cells to GA

The initial work on intact but isolated endosperm showed that a whole spectrum of hydrolytic enzymes increased in response to GA. These enzymes were α-amylase (Paleg, 1960), endo-β-glucanase, endo-pentosanase (MacLeod and Millar, 1962), proteinase (Yomo,

1961), endopeptidase (Yomhida and Morimoto, 1963), maltase (Simpson and Naylor, 1962; Briggs, 1963), phosphatase, peroxidase, catalase, transaminase, invertase (Briggs, 1963), ribonuclease (RN-ase) (Varner et al., 1965) and enzymes hydrolysing a variety of other carbohydrates, proteins, dipeptides and glycosides (Briggs, 1963). Nearly all of these enzyme activities have been shown to arise in the aleurone layer (MacLeod and Millar, 1962; Paleg, 1964; Briggs, 1964; Yomo and Iinuma, 1964a and b; Varner, 1964; Chrispeels and Varner, 1967a).

Time-course studies always showed an initial lag period of at least 6 hours before an increase in hydrolytic enzymes was measurable in isolated aleurone layers (MacLeod et al., 1964b; Chrispeels and Varner, 1967a). MacLeod et al. (1964) indicated that endo-β-glucanase was the first enzyme to be initiated, followed by α-amylase which, in turn, was followed by proteinase. Jacobsen and Varner (1967), however, found that α-amylase and proteinase were initiated simultaneously and had very similar release curves. RN-ase production also was initiated at about the same time as the amylase and proteinase but it was not released until some time later.

Experiments with intact endosperm also showed that at least three different parameters of the GA response, sugar release, protein nitrogen release and dry weight loss behaved in the same way with respect to time of development, temperature optimum and various concentrations of GA and inhibitors (Paleg, 1961). This led to the suggestion that GA had one major "trigger" effect which initiated all three responses. Once initiated, the three responses then proceeded at different rates because of differences in $Q_{10}$s. When it became clear that these responses were the result of increases in hydrolytic enzymes, it was suggested that GA might in some way activate already pre-formed enzymes (Paleg, 1960). The presence of an active α-amylase during development of the seed lent support to this hypothesis (Paleg, 1965; Guilbot and Drapon, 1963). However, GA had no direct effect on the activities of at least two of these enzymes in vitro (Paleg, 1960; MacLeod and Millar, 1962) and there were several different enzymes involved in the response.

MacLeod and Millar (1962) suggested a way in which these difficulties could be resolved. They had shown that GA induced an increase in endo-β-glucanase in both intact aleurone layers and homogenates of

aleurone cells from ungerminated grain but not in filtered extracts of endosperm slices. This, coupled with the fact that most of the enzymes known to be affected in the aleurone layer were hydrolytic ones, led them to suggest that the enzymes might be bound in a particle such as the lysosome of animal cells and that GA acts by causing the enzymes to be released from these particles (MacLeod and Millar, 1962). MacLeod et al. (1964b) later ruled out this hypothesis when they failed to isolate a particle which could be stimulated to release enzymes in vitro and when they showed that different enzymes develop at different times whether in intact seed, endosperm or isolated aleurone layers. If these enzymes were all together in a lysosome, it was reasoned that the time course of their release should be identical. (This hypothesis will be reconsidered later).

## 2.5 Control of protein synthesis

More recent experiments have suggested that at least some of the enzymes involved in the GA response are synthesized de novo. Accordingly, enzyme production or manifestations of it were prevented by respiration (Briggs, 1963; Varner, 1964; MacLeod, 1963), and protein synthesis inhibitors (Briggs, 1963;

Paleg, 1964; Varner and Shidlovsky, 1963). In addition, the incorporation of radioactive amino acids (from a $^{14}C$-algal protein hydrolysate) into the proteins which were secreted into the incubation medium by endosperm pieces was stimulated by GA, and this increased incorporation was completely prevented by dl-p-fluorophenylalanine (18 mM) (Briggs, 1963). Varner and co-workers confirmed and extended these observations by showing that l-phenylalanine-$^{14}C$ was incorporated into protein by barley half-seed both in the presence and in the absence of GA, but that the proportion of the $^{14}C$-protein secreted into the medium was much greater when GA was present (Varner and Ram Chandra, 1964). Less than 40% of this GA-induced secreted protein was precipitated by heating to 70°C for 20 minutes (α-amylase and some of the other hydrolases are heat stable up to about 70°C). When the medium was chromatogrammed on DEAE-cellulose, α-amylase activity was associated with one of the radioactive peaks (Varner et al., 1965). After incorporation of labelled leucine, alanine, proline and threonine, the radioactive amylase was purified and subjected to proteolysis. All but two of the resulting peptides were radioactive (Varner and Ram Chandra, 1964). This appeared to be very good evidence for the de novo synthesis of α-amylase.

However, the labelled amino acids were diluted by hydrolysis of endogenous protein, and so it was not certain that all of the amylase produced was synthesized de novo. Filner and Varner (1967) overcame this difficulty with a very elegant experiment in which they incubated aleurone layers with GA in the presence of either $H_2{}^{18}O$ or a mixture of $H_2{}^{16}O$ and $^3$H-lysine. The α-amylase formed in the presence of $^3$H-lysine was highly purified and then traces of it (not enough to be measured by enzyme activity) were mixed with crude α-amylase formed in the presence of $H_2{}^{18}O$ and subjected to isopycnic centrifugation in a cesium chloride density gradient. The $^{18}$O-α-amylase was located in the density gradient by measuring enzyme activity while the $^{16}$O-α-amylase was located by measuring radioactivity. It was found that the band of α-amylase activity corresponding to the $^{18}$O-enzyme was displaced toward regions of higher density. This demonstrated unequivocally that virtually all of the enzyme was synthesized de novo. Jacobsen and Varner (1967) demonstrated in the same way that GA-induced proteinase was also synthesized de novo. Since the induction of some of the other enzymes (e.g. RN-ase and endo-β-glucanase) by GA is inhibited by protein synthesis inhibitors, it is possible that they too are synthesized de novo.

## 2.6 Derepression of a gene hypothesis

The question that now arises is: How does GA control this protein synthesis? According to current theory, the protein molecule is constructed on an RNA-template (messenger RNA or m-RNA) which in turn is synthesized in the nucleus on a complementary DNA-template (the gene). If a protein is absent from a system, the gene which codes for that protein may be repressed (or "turned off"). If that protein appears in the system in response to a certain stimulus, then that stimulus may be considered to have derepressed the gene, thereby allowing the appropriate messenger RNA to be synthesized (Karlson, 1963). Perhaps this is what happens in the aleurone tissue. Little α-amylase can be detected in aleurone cells of ungerminated seed but it is synthesized de novo during both germination and treatment of the cells with GA in vitro. Hence, GA may derepress the gene which codes for α-amylase. This hypothesis may be tested by determining whether specific m-RNA synthesis is a necessary prerequisite for synthesis of the enzyme. The problem has been approached in two ways: (a) by specifically inhibiting RNA synthesis without impairing the protein synthesizing apparatus, and (b) by direct measurement of RNA

synthesis in the presence or absence of GA.

Two kinds of inhibitors have been used:

a) actinomycin D, an antibiotic which prevents synthesis of RNA molecules by binding to the guanine residues of the DNA molecule and altering its structure, thus blocking RNA polymerase activity (Goldberg et al., 1963; Cerami et al., 1967), and

b) the purine and pyrimidine base analogues, 8-azaadenine, 8-azaguanine, 6-azaguanine, 6-methylpurine, 6-bromouracil, 2-thiouracil, 5-fluorouracil, thiocytosine and 5-azacytidine, which either compete with the natural purine and pyrimidine bases for incorporation into the newly synthesized RNA molecules resulting in formation of altered protein molecules (Mahler and Cordes, 1966), or act as antimetabolites preventing the conversion of the purines or pyrimidines to their nucleotides (Ross, 1964).

2.61 Experiments with half-seed

Paleg (1964) showed that actinomycin D (5 to 500 µg/ml), 2-thiouracil ($10^{-5}$ to $10^{-3}$M) and 5-fluorouracil ($10^{-5}$ to $10^{-3}$M) had no effect on the GA-induced sugar-release from isolated barley endosperm and concluded that RNA synthesis is not involved in the GA

response. Varner (1964) also showed that if the inhibitors azaguanine, thiocytosine, thiouracil and actinomycin D were added to pre-incubated half-seed either at the same time as, or 24 hours before GA, there was no inhibition of α-amylase formation. However, if the inhibitors were added to the dry half-seed at the beginning of the pre-incubation period, α-amylase formation was reversibly inhibited. Varner concluded that some RNA synthesis must occur before the aleurone cells can respond to GA but it was not clear whether this synthesis took place before or after the addition of GA. The possibility existed that in the pre-incubated seed the inhibitors did not reach the aleurone layers but were sequestered or degraded in the starchy endosperm. This was ruled out when Varner *et al.* (1965) showed that the incorporation of $^{32}P$ into RNA of the aleurone layers was inhibited by 50% while there was no effect on α-amylase formation.

These experiments indicate that some RNA synthesis takes place during the imbibition period. However, this synthesis is probably independent of exogenous GA because the half-seed, after an initial imbibition period in water, can respond to the hormone in the presence of the RNA-synthesis inhibitors. Furthermore, there is no indication that the RNA

synthesised is a messenger since all kinds of RNA (messenger, transfer and ribosomal) are DNA-dependent (Mahler and Cordes, 1966), and r-RNA synthesis is much more sensitive to actinomycin D than is m-RNA synthesis (Reich and Goldberg, 1964).

Nevertheless, Ram Chandra and Varner (1965) demonstrated an effect of GA on RNA synthesis during imbibition. They incubated dry half-seed with 2-$^{14}$C uridine, 8-$^{14}$C adenosine or $^{32}$P in the presence or absence of GA and measured incorporation into RNA. From 8 to 24 hours GA enhanced the incorporation whereas from 24 to 48 hours it caused a reduction in incorporation. The RNA formed had a higher specific activity than that of the controls and there was some indication of a GA-induced conversion of adenosine to guanylic acid. α-Amylase, measured simultaneously, increased in parallel with the RNA up to 24 hours but continued to increase after the incorporation into RNA started to fall off. (The decrease in RNA synthesis after 24 hours could be due to dilution by endogenous nucleotides produced by RN-ase activity in the same way as $^{14}$C-amino acids were diluted out by endogenous proteolysis).

Actinomycin D (12.5 μg/ml) and 5-bromouracil ($10^{-3}$ M) had no effect on incorporation into RNA in the

absence of GA up to 12 hours (possibly indicating a stable m-RNA polysome complex) but inhibited from 12 to 24 hours (perhaps because of increased turnover of ribosomes and all classes of RNA due to increased synthetic activity). On the other hand, the inhibitors completely prevented the GA-induced incorporation after both 12 and 24 hours. At the same time, there was only a partial inhibition of α-amylase production: 22% after 12 hours and 51% after 24 hours. Once again the GA-induced RNA synthesis did not precede the initiation of α-amylase synthesis but increased in parallel with it. Some of this RNA synthesis appears to be necessary for α-amylase synthesis to continue at its maximum rate which again may be an indication of increased turnover of all RNA fractions due to increased synthetic activity.

### 2.62 Experiments with isolated aleurone tissue

Inhibitor experiments with isolated aleurone layers seem to substantiate this conclusion (i.e. RNA synthesis must continue during α-amylase synthesis), but the effect of various inhibitors is extremely complex (Chrispeels and Varner, 1967a and b). 6-Methyl-purine and actinomycin D are two potent inhibitors of RNA synthesis but their effects on the GA-induced

α-amylase synthesis are very different.

When 6-methylpurine (0.1 mM) was added to aleurone layers at the same time as GA, α-amylase synthesis was inhibited by 90%; when it was added 4 hours after GA the inhibition was 70% and 8 hours after, 45%. Higher concentrations resulted in larger inhibitions at these times. In another type of experiment aleurone layers were first incubated in GA until the maximum rate of α-amylase synthesis was reached and then the GA was rinsed away and the layers were further incubated with or without GA plus various inhibitors. When the layers were further incubated without GA, the rate of enzyme production fell off progressively indicating a necessity for a continuous supply of GA. When GA was restored to the system the enzyme synthesis immediately returned to its previous maximum rate. When 6-methylpurine (5 mM) was added with the second lot of GA, α-amylase synthesis was inhibited after a short lag period of 2½ to 3 hours. At the same time $^{14}$C-uridine incorporation was inhibited by 65-70%. It would appear from these experiments that α-amylase synthesis is dependent upon continued RNA synthesis. Other inhibitors which act in the same way are abscisic acid (a natural plant hormone), 8-azaguanine and 5-azacytidine (Chrispeels and Varner, 1967b).

The conclusions based on experiments with actinomycin D are entirely different. When actinomycin D (100 µg/ml) was added to aleurone layers at the same time as GA, α-amylase synthesis was inhibited by 58%; when it was added 4 hours after GA, the inhibition was 41%, and 8 hours after, only 9.8%. The inhibition of $^{14}$C-uridine incorporation into RNA when the inhibitor was added after 8 hours was more than 66%. Thus, it would appear that RNA synthesis is only necessary during the first few hours of treatment with the hormone and that continued RNA synthesis is not necessary for further enzyme synthesis. Similarly, when GA was removed from the system as described above, there was very little effect of actinomycin D on synthesis of the enzyme when GA was restored. Chrispeels and Varner (1967b) have concluded from the experiments with these two inhibitors that a specific RNA fraction must be continuously synthesized in order that α-amylase synthesis may proceed, and that synthesis of this specific RNA is sensitive to inhibition by 6-methylpurine but not by actinomycin D.

What then is the effect of actinomycin D? Chrispeels and Varner (1967a) showed that the secretion of α-amylase by aleurone layers is much more sensitive

to this inhibitor than is its synthesis - secretion was appreciably inhibited at 25 µg/ml while synthesis was only depressed by about 7%. Furthermore, when actinomycin D (100 µg/ml) was added 7 hours after GA there was virtually no inhibition of synthesis but a complete inhibition of release. Experiments with RN-ase clarified this effect on secretion. RN-ase increased in response to GA but was retained in the tissue until 24 hours, after which it was released into the medium. When actinomycin D was added at the same time as GA, there was no effect on RN-ase production in the first 24 hours, but there was almost complete inhibition of both synthesis and release in the second 24 hours. Cycloheximide (an inhibitor of protein synthesis) completely prevented release of the enzyme when added at or before 24 hours but had no effect on release when added after 28 hours. Similarly, actinomycin D added during the first 26 hours inhibited release but had no effect when added after 26 hours. This indicated that synthesis of a specific protein and a specific RNA may be involved in the development of the release mechanism and that this may take place between 20 and 28 hours after addition of GA. The suggestion was made that synthesis and release (at least for RN-ase) may be intimately related and that some of the observed effects of actinomycin D on synthesis may simply be a

consequence of inhibition of secretion.

All of these studies with inhibitors must be dealt with cautiously for the following reasons.

a) Inhibition effects do not indicate the site of action of a hormone since the process which is inhibited may be necessary for manifestation of the hormonal effect but may be remote from the site of action.

b) Inhibitors may have multiple sites of action:
   (i) actinomycin D which was originally thought to inhibit m-RNA synthesis has now been shown to be a more potent inhibitor of r-RNA synthesis (Reich and Goldberg, 1964);
   (ii) cycloheximide which was used initially as an inhibitor of cytoplasmic amino acid incorporation is also a potent inhibitor of synthesis or maturation of ribosomes (Holland, 1963; Tamaoki and Mueller, 1963 and 1965; Waters and Dure, 1966).

c) Many inhibitors may be non-specific metabolic poisons e.g. at high concentrations actinomycin D causes break-down of m-RNA in the cytoplasm, disintegration of structural elements and disturbance of glucose metabolism (Honig and Rabinovitz, 1964).

For these reasons the effect of inhibitors alone is not sufficient to pinpoint the site of action of the hormone. The work with high concentrations of actinomycin D is particularly suspect as indicated in (c) above. However, inhibitor studies may point to involvement of a particular biochemical system and, if followed up by suitable analyses, can prove to be a valuable tool. In the case of the aleurone tissue, inhibitor studies indicated that RNA synthesis might be involved in the GA response. If the hormone acts at the level of RNA synthesis, it should be possible to demonstrate

a) either an increased synthesis of RNA or an alteration in the kind of RNA being formed, and
b) that these changes occur before synthesis of the specific proteins.

Attempts to show this with the aleurone layer, thus far, have failed. GA did not stimulate incorporation of $^{14}$C-uridine into the RNA of isolated aleurone layers (Ram Chandra et al., 1967). Furthermore, the enhanced incorporation of $^{14}$C-precursors into the RNA of unimbibed half-seed did not precede the initiation of new protein synthesis (Ram Chandra and Varner, 1965).

Work with other tissues, on the other hand, does

indicate a possible effect of the hormone at the level of RNA synthesis. When GA was added during the isolation of nuclei from dwarf pea shoots, RNA synthesis was enhanced and new kinds of RNA were produced (Johri and Varner, 1968). GA also stimulated incorporation of 2-$^{14}$C uracil and $^{32}$P into RNA of coconut milk nuclei (Roychoudry and Sen, 1965) but there was no evidence that these were new kinds of RNA. The dwarf pea stem nuclei are from developing, differentiating cells, whereas the aleurone cells are neither developing nor differentiating. Various kinds of cells may respond differently to the hormone and the tissue itself, in addition to the hormone, may render specificity.

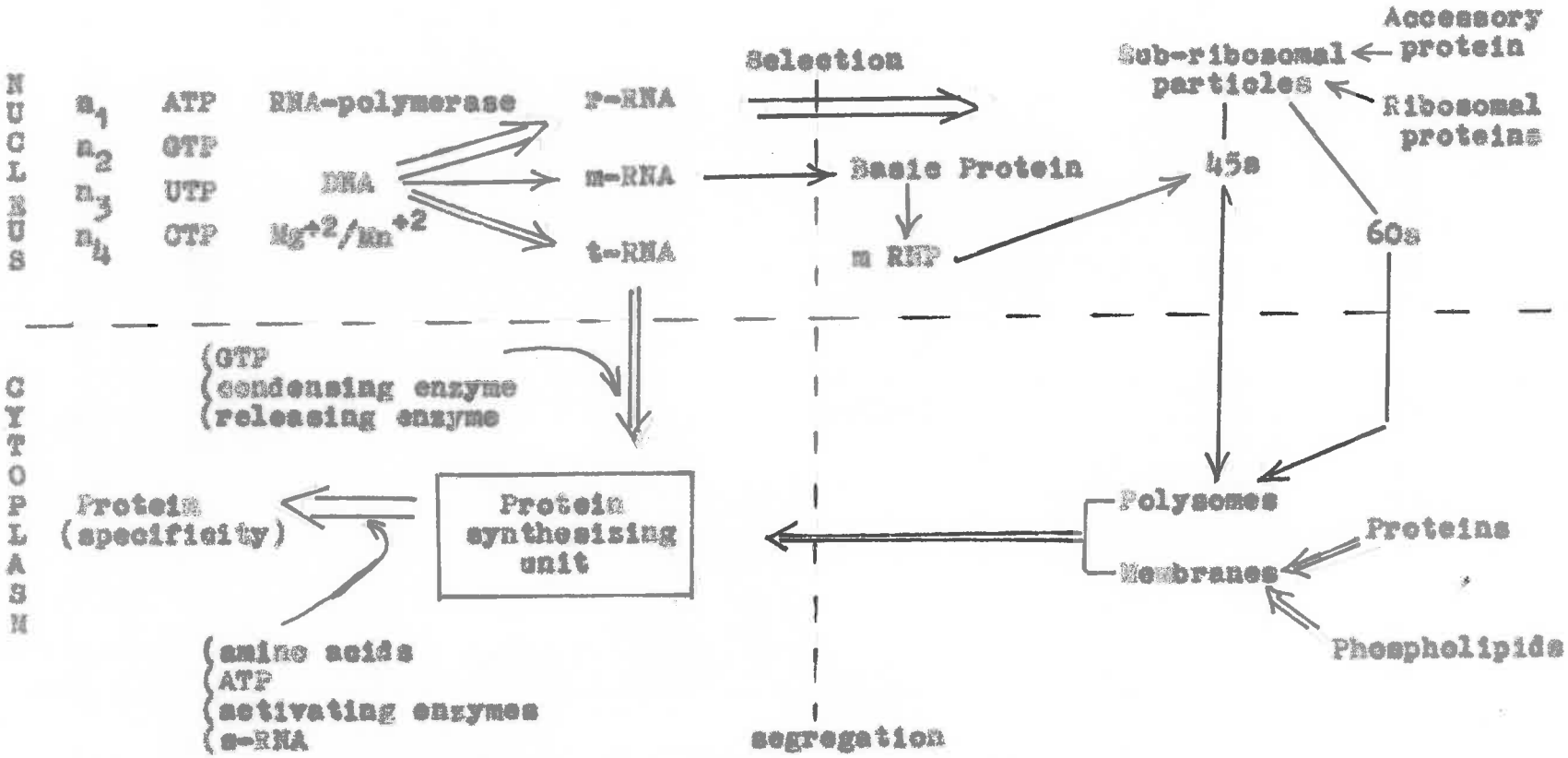
Since RN-ase increases during both pre-incubation of the half-seed in water (perhaps in response to small amounts of endogenous gibberellins present in the aleurone layers) and during GA treatment of the aleurone layers (Chrispeels and Varner, 1967a), it is possible that the requirement for continued RNA synthesis is a function of the more rapid turnover of RNA caused by this enzyme rather than a direct effect of GA. Alternatively, the increase in protein synthesis itself may result in more rapid turnover of any or all of the RNA fractions resulting in the need for continued re-

synthesis. This alternative is supported by the experiments with unimbibed half-seed in which both RNA and protein synthesis began simultaneously.

## 2.7 Stable m-RNA hypothesis

Scheme 1 modified from J.R. Tata (1968) indicates possible points of hormonal regulation during protein synthesis. In the previous section only one of these possibilities has been considered - the selection of m-RNA - and a lack of supporting evidence for the necessity of any kind of RNA synthesis to precede protein synthesis has been found. Indeed the initial idea of derepression of a gene arose from the assumption that only one or a few enzymes are synthesized in response to GA (Varner and Ram Chandra, 1964). However, as noted previously, a great many unrelated enzymes increase in response to the hormone. This complicates the simple derepression theory because one would then have to postulate either that all of these enzymes are coded on the same operon or that the hormone selectively derepresses a large number of different genes.

If protein synthesis can proceed without previous RNA synthesis, the implication is that m-RNA, ribosomes and t-RNA are already present in the cell in a relatively stable form and that they are prevented




Scheme showing some of the processes regulating protein synthesis in nucleated cells and their response to the growth promoting stimulus of hormones as modified from J.R. Tata, Nature 219: 336, 1968.

SCHEME 1

from synthesizing proteins until the system is activated by addition of the hormone.

## 2.71 Evidence for stable m-RNA

Experiments with peanut cotyledons, wheat embryos and cotton seed embryos point to the presence of stable m-RNA molecules in ungerminated seed. Microsomal preparations from unimbibed peanut cotyledons or wheat embryos were unable to incorporate $^{14}$C-amino acids into protein whereas those from imbibed seed (1-4 days) were able to do so (Marcus and Feeley, 1964). The lesion in the unimbibed system was not in the soluble enzymes or t-RNA but in the microsomes. A shift from monosomes to polysomes during imbibition suggested that the unimbibed system was inactive because of a spatial separation of m-RNA from the ribosomes (Marcus and Feeley, 1965; Marcus et al., 1966). Since actinomycin D did not prevent activation during imbibition, the need for synthesis of new m-RNA was ruled out (but see cautions on use of inhibitors, section 2.62). Activation of ribosomes from unimbibed seed was achieved in vitro by pre-incubation of the isolated ribosomes either with unfractionated homogenate or with the recombined 20,000 g pellet, 105,000 g pellet and 105,000 g supernatant (Marcus and Feeley, 1966).

These experiments suggest that stable m-RNA is present in the unimbibed seed and combines with monosomes to form polysomes during imbibition.

Waters and Dure (1964) also produced evidence that there is stable m-RNA in cotton seed embryos. However, in contrast to the findings of Marcus and Feeley for peanut cotyledons and wheat embryos, they found no shift from monosomes to polysomes during imbibition. The number of polysomes relative to free monomeric ribosomes was as high in mature embryos as at any subsequent time during germination (Dure and Waters, 1965).

More recently Chen et al. (1968) have confirmed the hypothesis that a stable m-RNA is present in unimbibed wheat embryos and that it is activated during imbibition. These workers showed that there was little $^{32}P$ incorporation into RNA during the first 24 hours of germination but considerable incorporation from 24 to 48 hours. Total RNA extracted from both unimbibed embryos and embryos germinated for 48 hours showed template activity when added to a cell free system of ribosomes from Escherichia coli. Hybridisation experiments with $DNA_s$ in which RNA from dry embryos was allowed to compete with that from embryos

germinated for 24 and 48 hours, showed that the m-RNA was identical in all three kinds of embryo. However, embryos germinated for 72 hours contained some different m-RNA species.

From this work it would appear that no new m-RNA is synthesized in wheat embryos for at least the first 24 hours of imbibition although active protein synthesis occurs during this time (Marcus et al., 1966). From 24 to 48 hours more of the m-RNA species which are already present in the dry seed are actively synthesized. It is not until 72 hours that different kinds of m-RNA begin to appear.

2.72 Implications of stable m-RNA in the aleurone layer

From the foregoing, it is clear that stable forms of m-RNA exist in plant tissues and, consequently, that new m-RNA synthesis need not be a prerequisite to new enzyme synthesis. This appears to be particularly true in seeds during the early stages of germination.

It is therefore necessary to consider ways in which GA can control protein synthesis other than through an effect on m-RNA synthesis. Studies of changes in ultrastructure of aleurone cells in response to GA and considerations of the enzymes involved in the

GA-induced responses will be discussed since they offer important clues about potential control mechanisms.

## 2.8 Cytological manifestations of the response of aleurone cells to GA

### 2.81 Response of isolated barley aleurone tissue to GA

Using the electron microscope, Paleg and Hyde (1964) examined dry barley aleurone tissue, imbibed tissue and tissue incubated for various times with water or GA. Only two kinds of organelles were obvious in dry, untreated aleurone cells fixed with $KMnO_4$:

a) large aleurone grains comprised of a single electron dense body (about 1μ diameter) and two or more electron transparent bodies (1μ or less in diameter) embedded in a matrix which was surrounded by a unit membrane, and

b) smaller membrane-bound spherical bodies (about 0.2μ diameter) called spherosomes which were usually electron transparent but sometimes electron dense. These spherosomes were positioned around the periphery of the aleurone grains and along the cell wall.

Hydration of the tissue for 24 to 48 hours increased the clarity of the cytoplasmic bodies and

membranous structures especially mitochondria, golgi bodies and plastids. Aleurone grains were relatively unaltered. However, the spherosomes became more irregular in shape and were usually more electron dense, particularly around their peripheries. Very few other changes took place with further incubation in water.

After 8 hours in GA the spherosomes became a little larger, less electron dense and were no longer as regularly distributed along the cell wall. The membranes of the aleurone grains became extended and the grains increased in size. There was some indication that aleurone grain membranes formed projections towards the spherosomes. At 18 hours the aleurone grains were very irregular in shape and some grains had begun to coalesce. The electron transparent inclusions within the aleurone grains also became extended and fragmented. At 24 hours the aleurone grains were extremely irregular in shape with large extensions of the membranes resembling endoplasmic reticulum (ER) profiles. The electron transparent inclusions had virtually disappeared although there seemed to be little change in the electron dense bodies. By 42 hours the aleurone grains of the control tissue were becoming slightly irregular and there appeared to be

fewer spherosomes particularly around the aleurone grains. In the GA-treated tissue at 42 hours the spherosomes had virtually all disappeared, aleurone grains had fused into large vacuoles filling almost the whole cell and there was extensive corrosion of the cell wall. However, there were still normal mitochondria and golgi bodies present outside the vacuoles particularly near the cell walls.

In summary, the most obvious early changes in the GA-treated aleurone cells were the alteration and dissolution of spherosomes and aleurone grains. Unfortunately, the endoplasmic reticulum and ribosomes were not well preserved in these studies and so nothing can be said about changes in these constituents.

## 2.82 Changes in barley aleurone tissue during germination

Van der Wb and Nieuwdorp (1967) studied changes which occurred in aleurone cells during normal germination of barley seed at 11°C. The changes under these conditions were much slower presumably because of decreased rates of imbibition and transport of endogenous gibberellin to the target tissue. The spherosomes and aleurone grains underwent alterations quite similar to those obtained *in vitro* except that the aleurone grains did not appear to coalesce with each other. When the

contents of the aleurone grains seemed to be very much depleted (17 days), spherosomes were still present although fewer in number. The dense globoids in the aleurone grains, although still present at the end of 17 days, were somewhat altered in shape in contrast to virtually no change in the appearance of these bodies in the *in vitro* system. The ER which was not seen in cells from seeds imbibed for only 24 hours increased in amount during germination and formed parallel rough lamellar stacks which were numerous by 8 days. Fragments of the ER were often found associated with spherosomes while other regions showed numerous terminal swellings especially in the region of the aleurone grains. These terminal swellings appeared to be pinched off from the ER to form spherical vesicles of medium electron density. In some instances ER and both intact and collapsed vesicles appeared to be associated with the plasmalemma. The cell wall was extremely corroded by about 10 days.

One other change which was observed as germination proceeded was an increase in number and size of the cristae of the mitochondria particularly at 8 to 10 days, followed by a reduction in number and size by 17 to 19 days.

## 2.83 Implications of the ultrastructural changes

Depletion of the contents of both spherosomes and aleurone grains during germination or GA treatment is consistent with their postulated functions as storage bodies for lipids, proteins and phosphorus (Van der Eb and Nieuwdorp, 1967). Changes in mitochondria suggest an increased energy metabolism which would be necessary to maintain the enhanced synthetic activity. Proliferation of the ER and formation of terminal vesicles which become detached, first in the region of the aleurone grains and spherosomes and later throughout the cell, suggests an increased protein synthesis coupled with formation of protein containing vesicles which appear to be discharged at the plasmalemma particularly at the time when cell wall corrosion becomes extensive. These vesicles could be the vehicles for accumulation, transport and secretion of the newly synthesised GA-induced hydrolytic enzymes.

## 2.9 Enzymes involved in the GA response

The activities of several enzymes are enhanced when GA is applied to the aleurone cells (section 2.4). These GA-induced enzymes are as follows:-

| | |
|---|---|
| α-amylase | endo-β-glucanase |
| RN-ase | endopentosanase |
| acid phosphatase | invertase |
| proteinase | peroxidase |
| endopeptidase | catalase |
| dipeptidase | transaminase |
| maltase | |

The majority of these enzymes are acid hydrolases and in animal cells many of them are localised in lysosomes. Two types of plant organelles have been suggested as potential plant lysosomes, aleurone grains (Yatsu and Jacks, 1967) and spherosomes or vacuoles (Bals, 1966; Holcomb et al., 1967; Matile, 1968). Both of these kinds of organelles are present in the aleurone cells and, as described in section 2.8, undergo considerable modification during GA treatment.

If the enzymes listed above are located in membrane-bound organelles, it is possible that the control of additional enzyme synthesis is similar to that suggested by de Duve and Wattiaux (1966). They postulated that the release of lysosomal enzymes in or from animal cells may act as a stimulus for the synthesis of more lysosomes, and that this phenomenon may be under hormonal control (also Gahan, 1967). Indeed, the whole process of synthesis, transport and release

of secretory granules by pancreatic cells appears to be hormonally controlled (Hokin, 1968; Beams and Kessel, 1968). The formation of vesicles and the apparent discharge of these at the plasmalemma suggests that a similar process may occur in the aleurone cells of germinating barley seed.

In summary, the following events must take place in the aleurone cells during germination or GA-treatment (although not necessarily in the sequence indicated):

(1) Initiation of hydrolysis of storage lipid, protein and phosphate and transport of the hydrolysates to sites of energy, membrane and protein metabolism;

(2) Proliferation of ER and mitochondrial membranes;

(3) Enhancement of respiration and protein synthesis;

(4) Synthesis, packaging and export of secretory proteins.

None of the GA-induced enzymes has been localized within the aleurone cells. The ultimate aim of the present investigation was to determine whether or not any of these enzymes are membrane bound. To do this, it was first necessary to develop a method for the mass isolation of aleurone tissue. This tissue was then

fractionated into subcellular components, and the association of some of the GA-induced enzymes with these fractions was studied.

## 3. EXPERIMENTAL AND RESULTS

### 3.1 Isolation of aleurone tissue

In theory, the aleurone layer is an ideal tissue with which to study the mechanism of action of GA. In practice, it is far from ideal.

The first major difficulty is the separation of this tissue from the rest of the seed, particularly from the underlying starchy endosperm. Previously, three methods have been used: a) hand-peeling half-seed which have been pre-incubated in water (Paleg, 1964; Varner and Ram Chandra, 1964), b) digesting the starchy endosperm with a mixture of hydrolytic enzymes obtained from *Trichoderma viride* (Yomo and Iinuma, 1964b) and c) grinding soaked half-seed by hand in a glass homogenizer (MacLeod *et al.*, 1964b). The first method is time-consuming and entails much handling of the tissue which, in turn, increases the danger of microbial contamination. The second method requires prolonged incubation of the tissue with hydrolytic enzymes which may also affect the aleurone cells. The third method may also result in damage to the tissue. Large quantities of tissue cannot be isolated easily by any of these methods. Therefore, the first step in the present study was to investigate ways of obtaining large quantities of viable aleurone tissue relatively quickly.

## 3.11 Response of wheat mill bran to GA

Most of the previous work on the GA-induced response of cereal aleurone tissue has been done with barley. However, the aleurone layer of barley is three cells thick and the cells sometimes vary considerably from one layer to another (Paleg and Hyde, 1964). The aleurone layer of wheat, on the other hand, is only one cell thick and, thus, should be much more uniform in response to the hormone. In addition, the proteins of wheat endosperm are more water-soluble than those of barley endosperm and, therefore, should be more easily rinsed away from the aleurone layer. Finally, there is a certain amount of experimental evidence that the aleurone tissue of at least some wheat varieties responds to GA in a manner analagous to that of barley (Paleg et al., 1962; Rowsell and Goad, 1962 and 1963; Rowsell et al., 1966).

As a first step wheat mill bran was investigated as a potential experimental material. A wheat bran fraction can be obtained which contains the aleurone layer and comprises 14-16% of the grain. This can be secured in large quantities by a milling process which separates it from most of the starchy endosperm (Hlynka, 1964). Stimulation of α-amylase production was chosen as the criterion for the GA response.

Dry seed of the wheat variety, Wollongong, were cut in half with a razor blade and the embryos discarded. The half-seed were soaked for 24 hours in a quantity of water sufficient to bring the moisture content to 17-19%. These imbibed half-seed were milled in a roller-type flour mill with the first break at $^{1}/_{20,000}$ inch, the second at $^{1}/_{30,000}$ inch and the third with rollers touching. The milled material was sieved for 5 minutes and the bran fraction, which was retained on the largest mesh sieve, recovered. This fraction represented 16% of the initial weight of the half-seed and consisted of small pieces (about 2 mm x 2 mm) of aleurone layer with adhering testa-pericarp and some starchy endosperm. The whole milling and sieving process was completed in only 15 minutes.

Four samples of mill bran were weighed into 100 ml erlenmeyer flasks and incubated in a shaking water bath at 30°C with either 5 ml of water or 5 ml of GA (100 µg/ml). Of the two samples incubated with GA, one weighed 133 mg and the other 269 mg. Similar amounts of tissue were incubated with water. After 24 hours the ambient solutions were decanted and α-amylase activity was measured by the procedure of Paleg (1960b).

The reaction mixture contained:

| | |
|---|---|
| Starch (soluble potato, 1%) | 1.5 ml |
| Acetate buffer (0.01 M, pH 4.6) | 0.5 ml |
| Enzyme and water | 1.0 ml |
| Total volume | 3.0 ml |

The starch was prepared by boiling one gram of soluble potato starch (BDH, AR) in 100 ml of water for 1 minute, cooling and centrifuging. The assay was carried out at 37°C; 0.2 ml aliquots of the reaction mixture were taken at suitable times and added to 0.1 ml of an IKI solution (0.2% iodine in 2% potassium iodide) and immediately diluted to 10 ml with water. The optical density was measured at 600 nm on a Unicam SP350 spectrophotometer. The enzyme solutions were made 5 millimolar with calcium acetate and heated to 70°C for 20 minutes to destroy β-amylase before assaying. Enzyme activity is expressed as the change in the percent of the initial optical density (OD) at 600 nm per hour per amount of tissue in the sample.

No α-amylase activity was detected in any of the ambient solutions. Furthermore, electrophoretic examination of the ambient solutions substantiated the conclusion that GA had little, if any, effect on wheat aleurone obtained in this way. (The results of these electrophoresis experiments, and a brief comparison with

the GA-induced changes in protein and enzyme spectrum of barley aleurone are shown in Appendix 1.)

3.12 Response of hand-peeled wheat aleurone tissue to GA

The lack of responsiveness of wheat aleurone obtained as a mill bran fraction could have been due either to the rather severe milling technique, or to an intrinsic inability of isolated wheat aleurone tissue of this variety to react to GA. To determine the reason, hand-peeled aleurone layers were tested for their α-amylase- producing capacity in the presence and absence of GA.

Whole seed of the variety, Wollongong, were sterilised by soaking at 30°C for two hours in a freshly prepared solution of calcium hypochlorite (5 g calcium hypochlorite shaken for 10 minutes in 100 ml of water and filtered) followed by rinsing ten times with 10 ml sterilised water (distilled deionized water autoclaved for 20 minutes at 2 atmospheres absolute pressure). Both ends of the seed were excised and the embryo-halves discarded. The cut seed were rinsed again with sterilized water, placed on filter paper in petri dishes and incubated in water at 30°C for 16 hours. (About 50 half-seed were incubated with 10 ml of water in a 9 cm petri dish.) The soaked half-seed were slit with a

razor blade along both sides as near the crease as possible, and the aleurone layer plus testa-pericarp was peeled off with a pair of forceps. The isolated aleurone layers were then shaken for 30 minutes in a water bath at 30°C to rinse off adhering starch granules. After a final rinse with sterilized water, the layers were placed in 100 ml glass-stoppered erlenmeyer flasks with either water or GA (100 μg/ml) and shaken at 30°C for 24 hours. All solutions and instruments used in this isolation procedure were sterilized. The cutting and peeling of the seed was carried out in a small chamber which had been sterilized by fumigating with propylene glycol.

Twenty-five aleurone layers were placed in each of four 100 ml erlenmeyer flasks with either 2.5 ml of GA (100 μg/ml) or 2.5 ml of water. The flasks were incubated in a shaking water bath at 30°C for 24 hours. The ambient solutions were decanted, diluted with an equal amount of $10^{-2}$ M calcium acetate and stored in the cold until required. The tissues were rinsed with ice-cold water, blotted on filter paper, ground with acid-washed sand in a mortar and pestle in 2.5 ml of $5 \times 10^{-3}$ M calcium acetate and centrifuged for 15 minutes at 20,000 g. α-Amylase activity was measured in both ambient solutions and extracts as described in section 3.11.

The results are shown in Table 3.12-1.

TABLE 3.12-1

Production of α-amylase by hand-peeled wheat aleurone tissue. Variety: Wollongong. Incubation medium: water or GA (100 μg/ml). Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 600 nm per hour per total volume of sample.

| Treatment | α-amylase activity[a] | | |
|---|---|---|---|
| | Ambients | Extracts | Total |
| Water | 124 | 0 | 124 |
| GA | 239 | 157 | 396 |

[a] Each value is the mean of two determinations on two different samples.

Both ambient solutions and extracts of tissue treated with GA contained considerably more α-amylase than the water controls. This indicated unequivocably that wheat aleurone does indeed have the capacity to respond to GA in a manner apparently identical to that of barley aleurone, and that the mill bran fraction tested in section 3.11 must have been too severely damaged to produce the response.

### 3.13 Development of a rapid method for the mass isolation of viable wheat aleurone tissue

It is noteworthy, as mentioned earlier, that the proteins of wheat endosperm are relatively more water-soluble than those of barley and, therefore, may be more readily dissolved away from the aleurone layer. For example, only 16 hours were necessary to soften wheat half-seed for hand-peeling whereas 72 hours were required for barley.

Graham *et al.* (1963) used a "roller-mill" technique for squeezing the soft endosperm of immature wheat grains out of the seed coats. The seed were rolled between two rotating surfaces, resulting in rupture of the tough outer seed coat and squeezing out of the soft inner endosperm. Residual pieces of endosperm were washed away by the addition of a buffer solution. This technique was tried with mature wheat in the hope that the half-seed could be softened sufficiently for the starchy endosperm to be squeezed from the outer layers and rinsed away with water.

#### 3.131 Procedure

A roller-mill consisting of an outer screw-top glass jar (diameter 12 cm) and an inner polythene bottle

filled with sand (diameter 7 cm, weight 1169 g) was rotated by mounting on a set of motor-driven rollers (Figure 3.131-1). A variable-speed motor was used initially so that the speed of rotation could be altered as desired.

The method depends upon a) softening the starchy endosperm to such a degree that it will be squeezed out of the tough outer layers of the seed when it is crushed between the two bottles, and b) rinsing away the remaining starchy endosperm. Three variables were found to affect the degree to which the aleurone layers could be freed of starchy endosperm:

a) time of imbibition of the half-seed;
b) addition of suitable amounts of water at appropriate time intervals during the rolling process;
c) speed of rotation of the mill.

The following are some observations which led to the final standardised method.

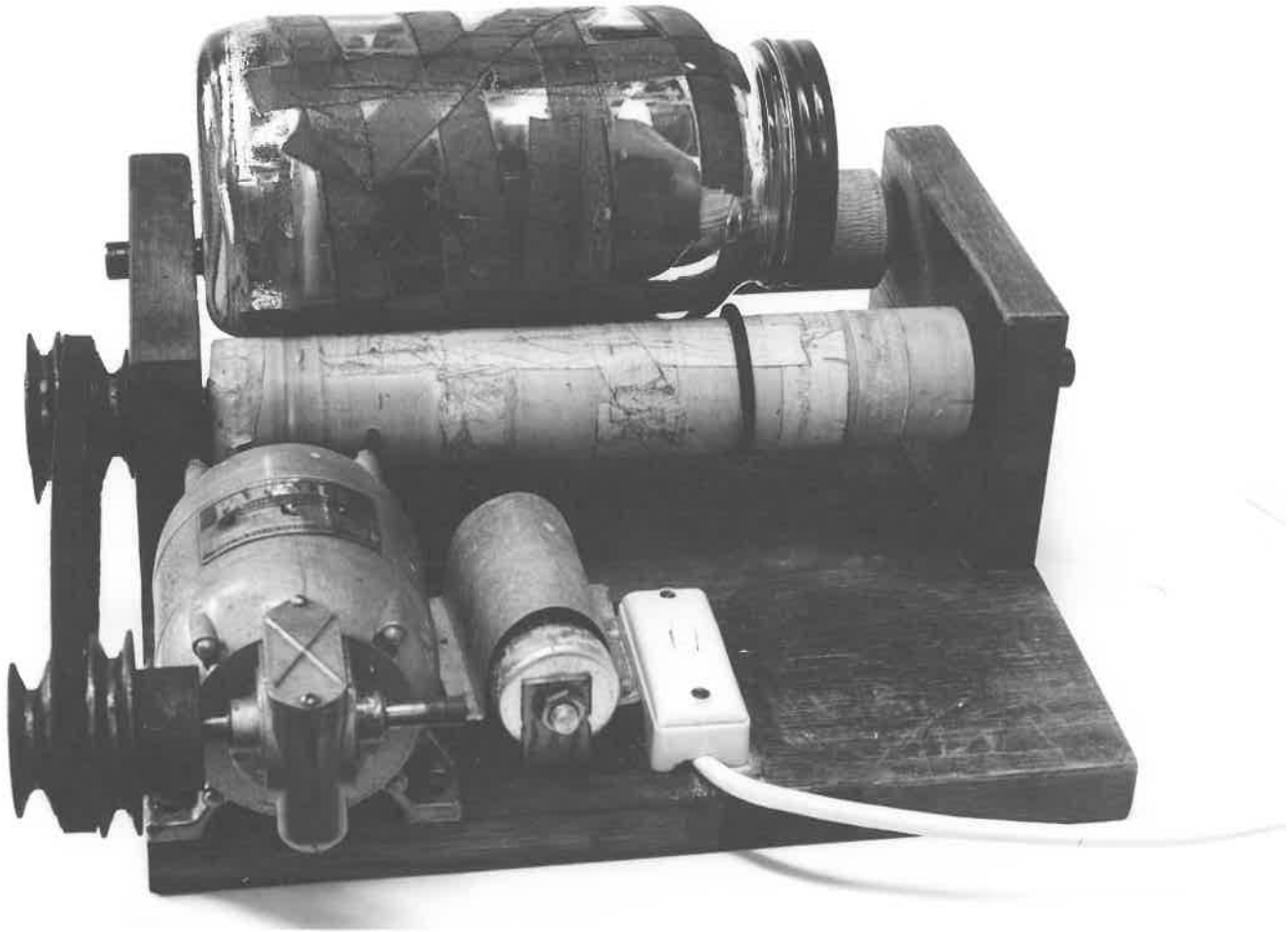
a) Time of imbibition

Half-seed, imbibed at 30°C for various periods of time after the initial 2 hour sterilization period, were placed in the roller-mill and rolled for 30 to 45 minutes with the addition of 10 ml aliquots of water at

FIGURE 3.131-1

The roller-mill; constant-speed motor (left fore-ground); large-mouthed glass bottle (top); polythene bottle filled with sand (inside glass bottle).

Top



suitable time intervals. At the end of the rolling procedure the contents of the mill were poured onto a sieve and the starchy endosperm rinsed away with water. The aleurone layers attached to the testa-periarp remained on the sieve. They were stained with IKI (0.2% iodine in 2% potassium iodide) and the amount of starch adhering to them observed. It was found that half-seed soaked for 22 to 24 hours yielded the cleanest tissue. After shorter times of imbibition (12 and 16.5 hours), the starchy endosperm immediately adjacent to the aleurone layer was not softened enough and could not be removed. After longer times (26 hours), the endosperm was too soft and formed a glutinous dough which adhered tenaciously to the aleurone layer. Therefore, 24 hours after the initial two hour sterilisation period was selected as the standard imbibition time.

b) Addition of water during rolling

If too much water was added initially to the roller-mill, the half-seed simply floated behind the inner polythene bottle and were not crushed between it and the glass jar. If too little water was added initially, the half-seed were crushed between the two bottles and the endosperm became sticky and doughy, adhered to the glass jar and was difficult to rinse away. The final

procedure adopted was as follows: for 15 g of half-seed, 10 ml of water were added initially and every 5 minutes thereafter for a total of 30 minutes. The tissue was then rinsed on a sieve with 1.5 litres of water, replaced in the mill and rolled for a further 5 minutes with the addition of 10 ml of water every 1.5 minutes. This was followed by a second rinse on the sieve with another litre of water.

c) Speed of rotation of the roller mill

The same difficulties were encountered for too high or too low a speed of rotation as for the addition of too much or too little water. Furthermore, the tissue was damaged if the speed was too high. The optimum speed was about 50 rpm. The variable-speed motor was then replaced by an appropriate constant-speed motor.

The final standardized procedure is as follows:

1) Cut seed in half with razor blade and discard embryo-half.
2) Transfer 5 g of half-seed to a 100 ml glass-stoppered erlenmeyer flask. Surface-sterilize half-seed for 2 hours at 30°C in 10 ml of 5% calcium hypochlorite and one drop of detergent.

3) Rinse half-seed 10 times with 10 ml of water using an automatic syringe. Flame mouth of flask and tip of syringe after each operation.

4) Finally, add 10 ml of water, flame mouth of flask, and slurry water and half-seed into a 9 cm petri dish enclosed within a 15 cm petri dish.

5) Incubate half-seed in the petri dishes for 24 hours at 30°C.

6) Transfer the inner petri dish containing the half-seed to a sterile cabinet containing previously sterilized equipment.
All subsequent procedures are carried out inside the sterile cabinet (Figure 3.131-2).

7) Rinse half-seed 3 times with water.

8) Transfer half-seed to roller-mill.

9) Roll at 50 rpm adding 10 ml water initially and every 5 minutes for a total of 30 minutes.

10) Rinse away starchy endosperm through a sieve (aperture: 2 x 2 mm) with 1.5 litres of water.

11) Replace aleurone tissue in roller-mill.

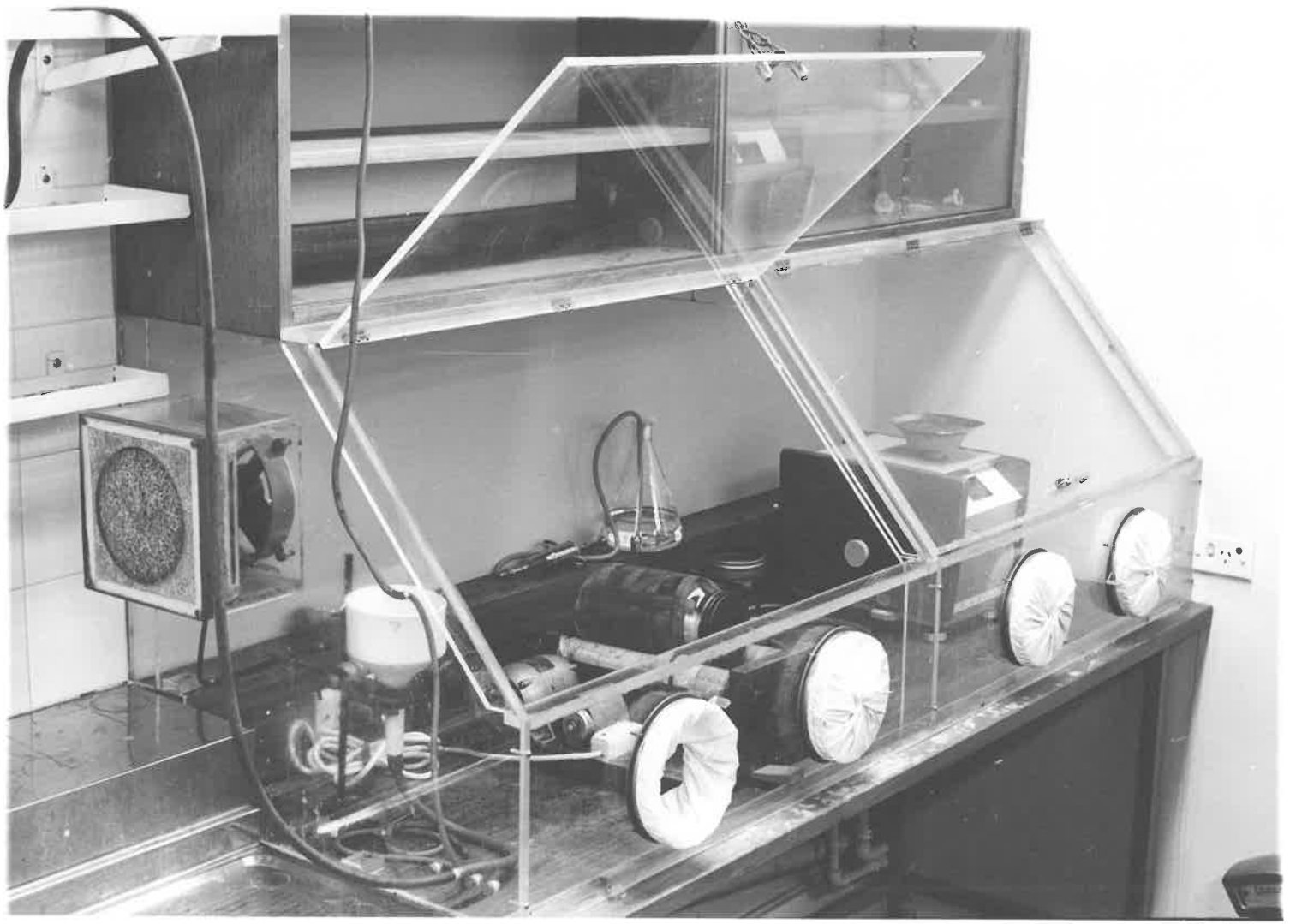
12) Roll for a further 5 minutes adding 10 ml water every 1.5 minutes.

13) Rinse tissue on sieve with an additional 1 litre of water.

14) Remove excess moisture by placing the aleurone tissue

FIGURE 3.131-2

The sterile cabinet: a plexiglass cabinet with top-opening doors and four arm-holes. Connection tube to suction pump (outside, far left); connection tube to sterile water reservoir (outside, left); buchner funnel (inside, far left); roller-mill (left of centre); bunsen burner (hidden behind roller-mill) and container of propylene glycol for fumigation (right of roller-mill); top pan balance (far right); flask of sterilized water with automatic syringe (back centre).



between layers of nylon gauze sandwiched between a single layer of filter paper and several outer layers of absorbent tissue in a 15 cm petri dish. Blot for 5 minutes.

15) Weigh out required amounts of tissue and place in incubation flasks or petri dishes.

16) Add appropriate test solutions with sterilized pipettes or syringes.

17) Incubate at 30°C for desired times.

3.132 Maintenance of sterility

It is obvious from the procedure outlined above that the tissue could become contaminated with micro-organisms at almost every step especially if all procedures are carried out in the open laboratory. It was necessary, therefore, to sterilize all solutions and equipment and to carry out most of the procedures inside a sterile cabinet as indicated in step 6 and shown in Figure 3.131-2.

The precautions taken to control microbial contamination were as follows:

Step 2: Each five gram quantity of half-seed was soaked for 2 hours at 30°C in 10 ml of calcium hypochlorite (5 g calcium hypochlorite shaken in 100 ml of water for

ten minutes and filtered) to which was added 1 drop of a detergent, Teepol (Shell Chemicals, Australia Pty.Ltd.).

Step 3: The surface sterilized seed were then rinsed 10 times with 10 ml aliquots of sterilized water using a sterilized 10 ml automatic syringe and flaming both the tip of the syringe and the mouth of the flask after each rinse. If this procedure was carried out in the laboratory, the bench was wiped with 70% ethanol and the area over the bench sprayed with 70% ethanol to settle dust and spores before opening the flasks.

Steps 3 and 4: Finally, 10 ml of sterilized water were added to the flask, the mouth of the flask flamed and the half-seed quickly slurried into a sterilized 9 cm petri dish enclosed in a larger sterilized 15 cm petri dish.

Step 6: A few hours before the isolation was to take place, all equipment to be used for milling was autoclaved and placed in the cabinet which was then sterilized by fumigating with propylene glycol. The 9 cm petri dishes containing the imbibed half-seed were transferred to the cabinet, leaving behind the outer 15 cm petri dishes. Hands and arms were thoroughly scrubbed with detergent and 70% ethanol before they were placed in the cabinet.

Steps 7-15: These procedures were all carried out inside the sterile cabinet. The only piece of equipment that

could not be autoclaved was the polythene bottle of sand and this was stored in 70% ethanol and wiped dry with sterilized tissue paper before placing in the roller-mill. Water was added during the rolling process with a sterilized automatic syringe fitted with a hypodermic needle which could be inserted into a small aperture in the roller-bottle top. All test solutions to be used were either autoclaved or filtered through sterilized Millipore filters (pore size 0.22μ).

Microbial contamination was determined at various times throughout the procedure in several different experiments by plating aliquots of solutions and pieces of tissue or half-seed onto nutrient agar. Results of a typical experiment are shown below.

One ml aliquots of solutions, or pieces of tissue or half-seed were plated on nutrient agar (1.0% agar, 0.1% Difco yeast extract, 0.1% peptone in tap water) and incubated at 30°C for 48 hours. Bacterial and fungal colonies were then counted. The results are shown in Table 3.132-1.

TABLE 3.132-1

Microbial contamination of half-seed, aleurone tissue and incubation solutions at various stages of the isolation procedure.

| | Sample | Bacterial Colonies | Fungal Colonies |
|---|---|---|---|
| Solutions at end of 24 hour imbibition period | 1 | 7 | 0 |
| | 2 | 2 | 5 |
| | 3 | 5 | 0 |
| Half-seed at end of 24 hour imbibition period. Three half-seed plated for each sample | 1 | 0 | 2 |
| | 2 | 0 | 0 |
| | 3 | 1 | 0 |
| Ambient solutions after 9 hours incubation | Water | 63 | 0 |
| | *GA | 635 | 0 |
| | Water | 13 | 0 |
| | **GA | 15 | 0 |
| Ambient solutions after 36 hours incubation | Water | 18 | 0 |
| | GA | 29 | 0 |
| Aleurone layers after*** 24 hours incubation. Six pieces from 6 different flasks plated. | Water | 0 | 0 |
| | " | 0 | 0 |
| | " | 0 | 0 |
| | GA | 0 | 0 |
| | " | 0 | 0 |
| | " | 0 | 0 |

* GA prepared with sterilized water but not filtered through Millipore.

** GA filtered through Millipore.

*** After incubation of the aleurone tissue on the agar plates for 96 hours, two pieces showed fungal and one bacterial growth.

Thus, the bacterial and fungal counts were extremely low indicating that the procedure was adequate for the maintenance of reasonable sterility.

3.133 Reproducibility

Two criteria have been used to determine the reproducibility of the isolation procedure. Firstly, the fresh weight to dry weight ratios were used to reflect any differences in the tissue from sample to sample. Secondly, the amount of contamination by starchy endosperm was estimated by measuring the amount of starch present in the isolated tissue.

a) Fresh weight to dry weight ratio

Samples of tissue were taken from three separate isolations carried out on different days. The fresh weight of the samples was determined and the tissue was then frozen on dry ice and lyophilized to a constant weight. The results are found in Table 3.133-1.

TABLE 3.133-1

The relationship of the fresh weight of the aleurone tissue to its dry weight.

| Sample | Fresh weight (FW) (g) | Dry weight (DW) (g) | Ratio FW/DW |
|---|---|---|---|
| 1 | 10.5 | 2.6 | 4.0 |
| 2 | 8.0 | 2.0 | 4.0 |
| 3 | 13.0 | 3.2 | 4.1 |

The fresh weight to dry weight ratio was almost constant from one batch of tissue to another, as would be expected for tissue prepared by a reproducible method.

b) Starch content

Samples of tissue from several separate isolations were lyophilised and then the starch content determined according to the method of Pucher *et al*. (1948) as modified by Jenner (1967) using methods of McCready and Hassid (1943) and McCready *et al*. (1950).

Samples (about 200 mg) of the dried material as well as samples of standard potato starch (about 50 mg) were heated with water for 15 minutes in a boiling water bath, cooled, and the starch extracted with perchloric acid. After clarification of these extracts

by centrifugation, starch was precipitated from the supernatants with iodine (Fuchor et al., 1948). The iodine complex was decomposed with alcoholic sodium hydroxide and the starch dissolved by heating in 0.5 N NaOH (McCready and Hassid, 1943). The starch content was determined with anthrone (McCready et al., 1950).

The results are presented in Table 3.133-2.

TABLE 3.133-2

Starch analysis of aleurone tissue

| Sample | Starch content (mg/250 mg DW) | % DW | % FW |
|---|---|---|---|
| 1 | 19.9 | 7.96 | 1.99 |
| 2 | 23.7 | 9.49 | 2.37 |
| 3 | 21.8 | 8.72 | 2.18 |
| 4 | 20.5 | 8.20 | 2.05 |
| 5 | 24.8 | 9.92 | 2.48 |
| 6 | 22.6 | 9.05 | 2.26 |
| Average | 22.7 | 9.08 | 2.27 |

The starch content varied from about 2.0 to 2.5% of the fresh weight indicating that the isolation method yields a tissue which is reproducible insofar as contamination from the starchy endosperm is concerned. The value 8-10% of the dry weight is comparable with the starch content of mill bran (Hlynka, 1964).

3.134 Response of the aleurone tissue to GA

Two varieties of wheat - an unclassified 'Soft'

variety and Wollongong - were used during development of the isolation method. The α-amylase produced by the aleurone tissue of these varieties isolated in various ways and incubated with water or GA was measured. The results of some of these measurements are recorded in Table 3.134-1.

The aleurone tissue of both varieties responds to GA by producing α-amylase. However, the varieties differ mainly in the amount of amylase produced in the absence of GA. Thus, the aleurone tissue of Wollongong produces more enzyme in the absence of exogenous GA than does that of the 'Soft' variety. In addition, the 'Soft' variety appears to secrete a greater proportion of the α-amylase into the medium.

3.135 Conclusions

The procedure outlined in section 3.131 is relatively rapid, maintains reasonable sterility, and yields large amounts of a viable and reproducible tissue. This tissue consists of the aleurone layer, the testa-pericarp and some cells of the starchy endosperm. However, since the aleurone cells are the only ones which react to GA, it will be referred to as "aleurone tissue".

The variable amounts of amylase produced by

TABLE 3.134-1

Production of α-amylase by wheat aleurone tissue isolated by various methods. Varieties: 'Soft' and Wollengong. Incubation medium: water or GA (100 µg/ml). Temperature: 30°C. Enzyme activity: change in the percent initial OD at 600 nm per hour per g fresh weight.

| Variety | Time of Imbibition of half-seed (hours) | Time of[x] rolling (minutes) | Time of incubation of aleurone tissue (hours) | Incubation medium | α-amylase activity | | |
|---|---|---|---|---|---|---|---|
| | | | | | Ambients | Extracts[xx] | Total |
| 'Soft' | 12 | 30 + 10 | 26 | Water | 90 | 82 | 171 |
| | | | | GA | 736 | 609 | 1345 |
| 'Soft' | 24 | 45 + 10 | 24 | Water | 10 | 3 | 13 |
| | | | | GA | 747 | 514 | 1261 |
| Wollengong | 21.5 | 30 + 30 | 25.5 | Water | 214 | 81 | 295 |
| | | | | GA | 214 | 544 | 758 |
| Wollengong | 24 | 45 + 10 | 24 | Water | 33 | 129 | 163 |
| | | | | GA | 657 | 1050 | 1707 |

[x] The first number represents the time of rolling before the first rinse; the second number represents the time of rolling after the first rinse.

[xx] Prepared by homogenizing each 1 g sample with 5 ml of calcium acetate ($5 \times 10^{-3}$M) in a motor-driven Kontes ground-glass homogenizer.

the two varieties, both in the presence and in the absence of GA, demonstrated the need for an examination of other wheat varieties before one could be chosen for the remainder of the experimental programme.

## 3.14 Survey of a number of varieties of wheat for their response to GA

The following ten varieties were selected and examined for the response of their aleurone tissue to GA:

| | |
|---|---|
| Olympic | Mengavi |
| Crete | Gamenya |
| Javelin | Persia |
| Montana | Warrigo |
| Heron | Gabo |

Aleurone tissue was isolated from each variety as described in section 3.13. Samples of one gram each were incubated with 5 ml of either water or GA (100 µg/ml) for 22 hours at 30°C.

The α-amylase activity of both the ambient solutions and the extracts of the tissues was determined as described in section 3.11. Results were calculated from slopes of the % initial OD versus time curves for each sample and are presented in Table 3.14-1.

The ideal variety would produce little or no α-amylase when incubated with water but substantial

TABLE 3.14-1

Production of α-amylase by aleurone tissue of a number of wheat varieties. Incubation medium: water or GA (100 µg/ml). Temperature: 30°C. Time: 22 hours. Enzyme activity: change in percent initial OD at 600 nm per hour per g fresh weight.

| Variety | Water Controls | | | GA | | |
|---|---|---|---|---|---|---|
| | Ambients | Extracts | Total | Ambients | Extracts[xx] | Total |
| Olympic[x] | 0 | 3 | 3 | 955 | 364 | 1319 |
| Crete[x] | 185 | 44 | 210 | 3859 | 1839 | 5698 |
| Javelin | 0 | 0 | 0 | 843 | 243 | 1086 |
| Montana[x] | 0 | 8 | 8 | 847 | 442 | 1289 |
| Heron | 0 | 4 | 4 | 53 | 100 | 153 |
| Mongavi | 0 | 4 | 4 | 28 | 70 | 98 |
| Gamenya | 0 | 42 | 42 | 27 | 28 | 55 |
| Persia | 21 | 34 | 56 | 1736 | 1468 | 3204 |
| Warrigo | 1212 | 115 | 1327 | 2032 | 646 | 2678 |
| Gabo | 1290 | 192 | 1482 | 1611 | 627 | 2238 |

[x] Average of three determinations; others, one determination.

[xx] Prepared as described in section 3.134.

amounts when incubated with GA. Olympic and Javelin were the most suitable in these two respects. Although Crete showed a much greater response to GA, it produced considerable amounts of the enzyme in the water controls. Olympic was more readily obtainable than Javelin and so it was chosen for all subsequent experiments.

## 3.2 Characterization of the GA response

In previous work with barley endosperm and barley aleurone tissue, it has been shown that the GA response varies with changing GA concentration, time, pH and addition of such substances as calcium ions, sugars and inhibitors of protein and RNA synthesis (see section 2). The effect of these variables on the response of wheat (variety Olympic) aleurone tissue to GA is reported below. The aleurone tissue was always isolated according to the method of section 3.13.

Seed was stored in a constant temperature room (20°C) sealed in plastic bags. The moisture content was 11.1%. Germination was tested periodically and was always over 95%.

### 3.21 The production of α-amylase by wheat aleurone tissue in response to different concentrations of GA

Samples of aleurone tissue (1 g each) were

incubated with 5 ml of various concentrations of GA for 24 hours at 30°C. At the end of that time α-amylase was measured in the ambient solutions and extracts of the tissue. Extracts were prepared by grinding the tissue with 5 ml of calcium acetate ($5 \times 10^{-3}$ M) in a motor-driven ground glass homogenizer (Kontes Glass Co., Vineland, New Jersey). The results are shown in Table 3.21-1.

TABLE 3.21-1

Production of α-amylase by wheat aleurone tissue in response to various concentrations of GA. Variety: Olympic. Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 600 nm per hour per g fresh weight.

| GA concentration | | α-amylase activity | | |
|---|---|---|---|---|
| µg/ml | Molarity | Ambient | Extract | Total |
| 0 | 0 | 0 | 0 | 0 |
| 0.01 | $2.89 \times 10^{-8}$ | 0 | 20 | 20 |
| 0.1 | $2.89 \times 10^{-7}$ | 191 | 230 | 421 |
| 0.2 | $5.78 \times 10^{-7}$ | 1,657 | 536 | 2,193 |
| 1.0 | $2.89 \times 10^{-6}$ | 1,660 | 270 | 1,930 |
| 2.0 | $5.78 \times 10^{-6}$ | 2,366 | 618 | 2,984 |
| 20.0 | $5.78 \times 10^{-5}$ | 2,503 | 984 | 3,487 |
| 100.0 | $2.89 \times 10^{-4}$ | 3,531 | 1,647 | 5,178 |
| 200.0 | $5.78 \times 10^{-4}$ | 4,278 | 1,169 | 5,447 |

In general, the amount of amylase in both ambient solutions and extracts increased with increasing

GA concentration. There did not appear to be any real maximum. A concentration of 100 μg/ml was chosen for subsequent experiments unless otherwise stated.

## 3.22 Time course of production of α-amylase

Samples of aleurone tissue (1 g each) were incubated with 5 ml of GA (100 μg/ml) for various times at 30°C. At the end of these times the α-amylase activity was measured in both ambient solutions and extracts of the tissue. The results are shown in Figure 3.22-1.

α-Amylase was first detected in the extracts at 16 hours and in the ambient solutions at 18 hours. There was a gradual increase in both extracts and ambient solutions until about 22 hours, after which the increase was much more rapid. From 20 to 24 hours there was more amylase in the ambient solutions than in the extracts.

## 3.23 The effect of pH

Thus far, the aleurone layers have been incubated only with water or GA solutions. Since GA is weakly acidic, it is conceivable that the pH of the solutions may vary during the time of incubation and thus may

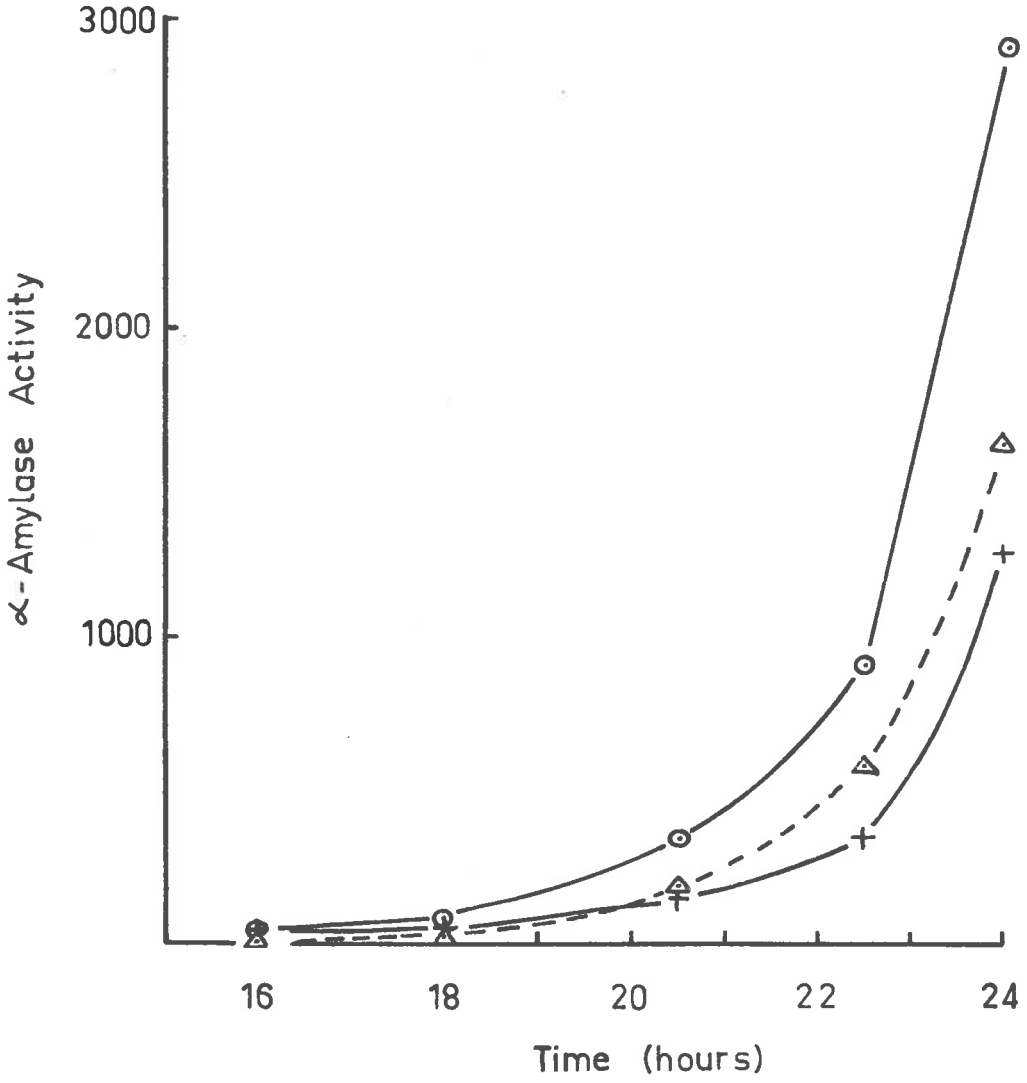
FIGURE 3.22-1

Time course of production of α-amylase by wheat aleurone tissue. Variety: Olympic. Incubation medium: GA (100 μg/ml). Temperature: 30°C. Enzyme activity: change in percent initial OD at 600 nm per hour per g fresh weight.

+ extracts;

△ ambient solutions;

⊙ total.


3000
2000
1000
α-Amylase Activity
16
18
20
22
24
Time (hours)

affect the α-amylase response. Therefore, aleurone tissue was incubated with various buffers in the presence or absence of GA. The α-amylase in the ambient solutions and extracts of the tissues was measured at the end of 24 hours.

3.231 Citrate-phosphate buffer

Aleurone tissue was isolated as usual and 1 g samples were incubated for 24 hours at 30°C in 5 ml of solution containing GA or GA plus $2.5 \times 10^{-3}$M citrate-phosphate buffer at various pH's. The pH of the solutions was measured both before and after the incubation period. The α-amylase activity was measured in both ambient solutions and extracts as described previously. The results are shown in Tables 3.231-1 and -2 and Figures 3.231-1 and -2.

The amount of α-amylase produced by the buffered tissue was generally much lower than that produced by the unbuffered tissue (Table 3.231-1). In the buffered tissue the highest amount of the enzyme was produced at the lowest pH and decreased as the pH increased. Above pH 4.5 there was only a small amount of enzyme produced and this changed only slightly with increasing pH. Below pH 4.5 the amount of enzyme increased very sharply down to pH 2.6 (Figure 3.231-1).

TABLE 3.231-1

Production of α-amylase by wheat aleurone tissue at various pH's. Variety: Olympic. Incubation medium: citrate-phosphate buffer, $2.5 \times 10^{-3}$ M; GA, 50 µg/ml. Temperature: 30°C. Time 24 hours. Enzyme activity: change in percent initial OD at 600 nm per hour per g fresh weight

| Initial pH | Final pH | Change in pH | α-amylase activity | | |
|---|---|---|---|---|---|
| | | | Ambient | Extract | Total |
| 4.0* | 4.8 | + 0.8 | 418 | 815 | 1,233 |
| 2.6 | 3.6 | + 1.0 | 32 | 179 | 211 |
| 3.0 | 3.7 | + 0.7 | 26 | 104 | 130 |
| 3.5 | 3.8 | + 0.3 | 17 | 120 | 137 |
| 4.0 | 3.9 | - 0.1 | 18 | 77 | 95 |
| 4.5 | 4.5 | 0.0 | 23 | 44 | 67 |
| 5.0 | 4.3 | - 0.7 | 17 | 64 | 81 |
| 6.0 | 4.7 | - 1.3 | 17 | 44 | 61 |
| 7.0 | 6.6 | - 0.4 | 23 | 16 | 39 |

* GA unbuffered

TABLE 3.231-2

Production of α-amylase by wheat aleurone tissue at various pH's. Variety: Olympic. Incubation medium: citrate-phosphate buffer, $2.5 \times 10^{-3}$ M; GA, 100 µg/ml. Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 600 nm per hour per g fresh weight.

| Initial pH | Final pH | Change in pH | α-amylase activity | | |
|---|---|---|---|---|---|
| | | | Ambient | Extract | Total |
| 4.0* | 4.1 | + 0.1 | 22 | 652 | 674 |
| 2.6 | 3.6 | + 1.0 | 23 | 637 | 659 |
| 3.0 | 3.6 | + 0.6 | 22 | 577 | 593 |
| 3.5 | 3.7 | + 0.2 | 16 | 295 | 316 |
| 4.0 | 3.9 | - 0.1 | 21 | 190 | 289 |
| 4.5 | 4.3 | - 0.2 | 19 | 123 | 142 |
| 5.0 | 4.2 | - 0.8 | 19 | 98 | 117 |
| 6.0 | 4.9 | - 1.1 | 19 | 110 | 129 |
| 7.0 | 6.7 | - 0.3 | 23 | 43 | 66 |

* GA unbuffered

FIGURE 3.231-1

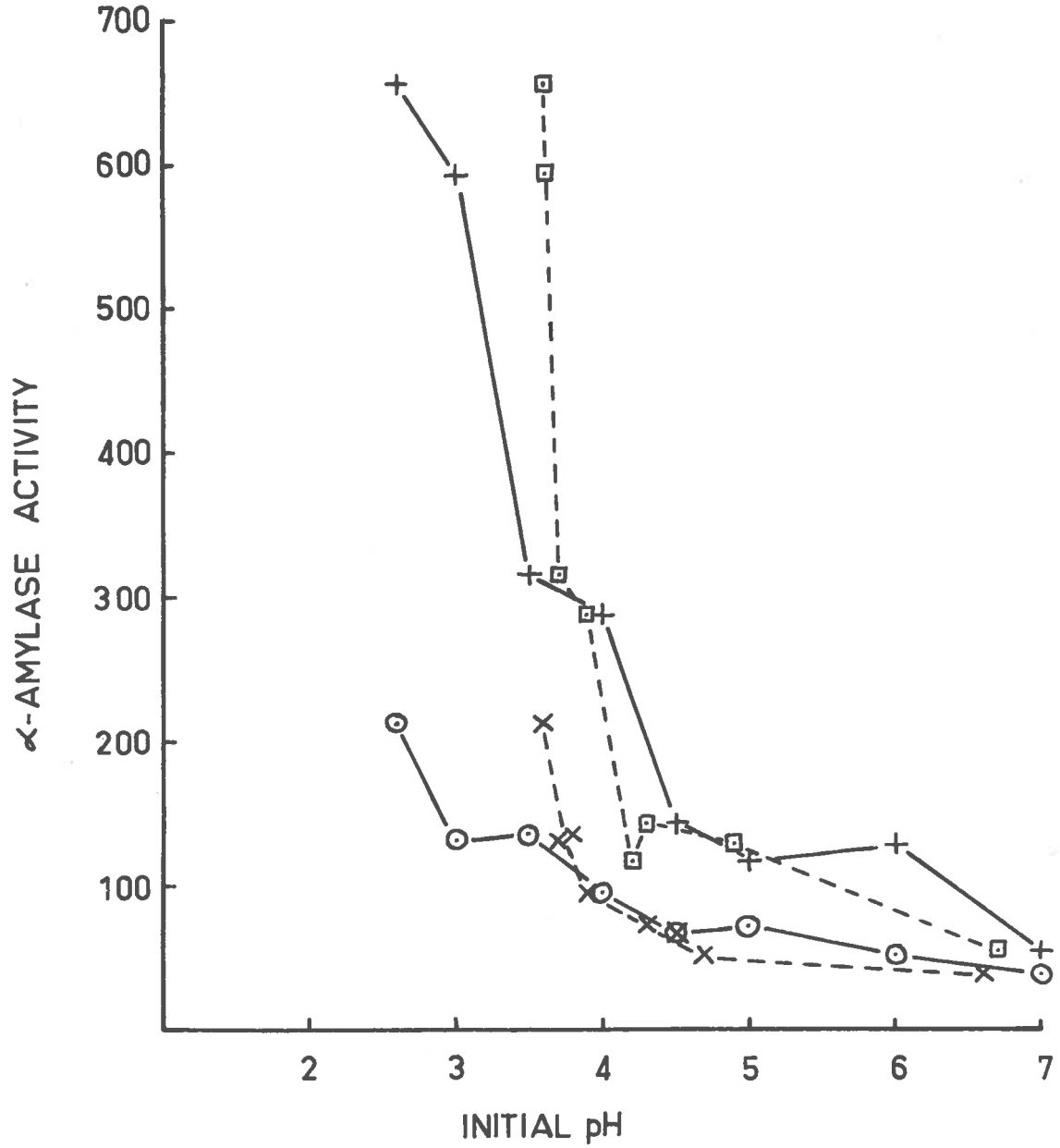
Production of α-amylase by wheat aleurone tissue at various pH's. Variety: Olympic. Incubation medium: citrate-phosphate buffer, $2.5 \times 10^{-3}$ M; GA, 50 or 100 µg/ml. Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 600 nm per hour per g fresh weight.

+ GA (100 µg/ml) plotted against initial pH;

⊡ GA (100 µg/ml) plotted against final pH;

⊙ GA (50 µg/ml) plotted against initial pH;

× GA (50 µg/ml) plotted against final pH.


700
600
500
400
300
200
100
α-AMYLASE ACTIVITY
2
3
4
5
6
7
INITIAL pH

The pH of the solutions shifted during the incubation period. As shown in Figure 3.231-2, the smallest changes occurred between pH 4.0 and 4.5 indicating a natural tendency for the pH to adjust to this value, probably due to the buffering capacity of amino acids leached from, or released by the tissue.

The optimum pH for the response appeared to be below 3.5 (Figure 3.231-1). However, there was very little α-amylase activity in any of the ambient solutions when buffer was present (Tables 3.231-1 and -2). This may reflect an effect of the buffer on either secretion or stability of the enzyme in solution. This latter explanation may be particularly relevant below pH 3.5 where α-amylase is known to be unstable. Moreover, the citrate-phosphate buffer has the capacity to chelate $Ca^{++}$ ions which are essential for activity and stability of the enzyme.

### 3.232 Maleic acid

The enhanced production of α-amylase by aleurone tissue incubated at low pH was investigated further by using maleic acid. The production of α-amylase in both ambient solutions and extracts was measured at various maleic acid concentrations both in the presence

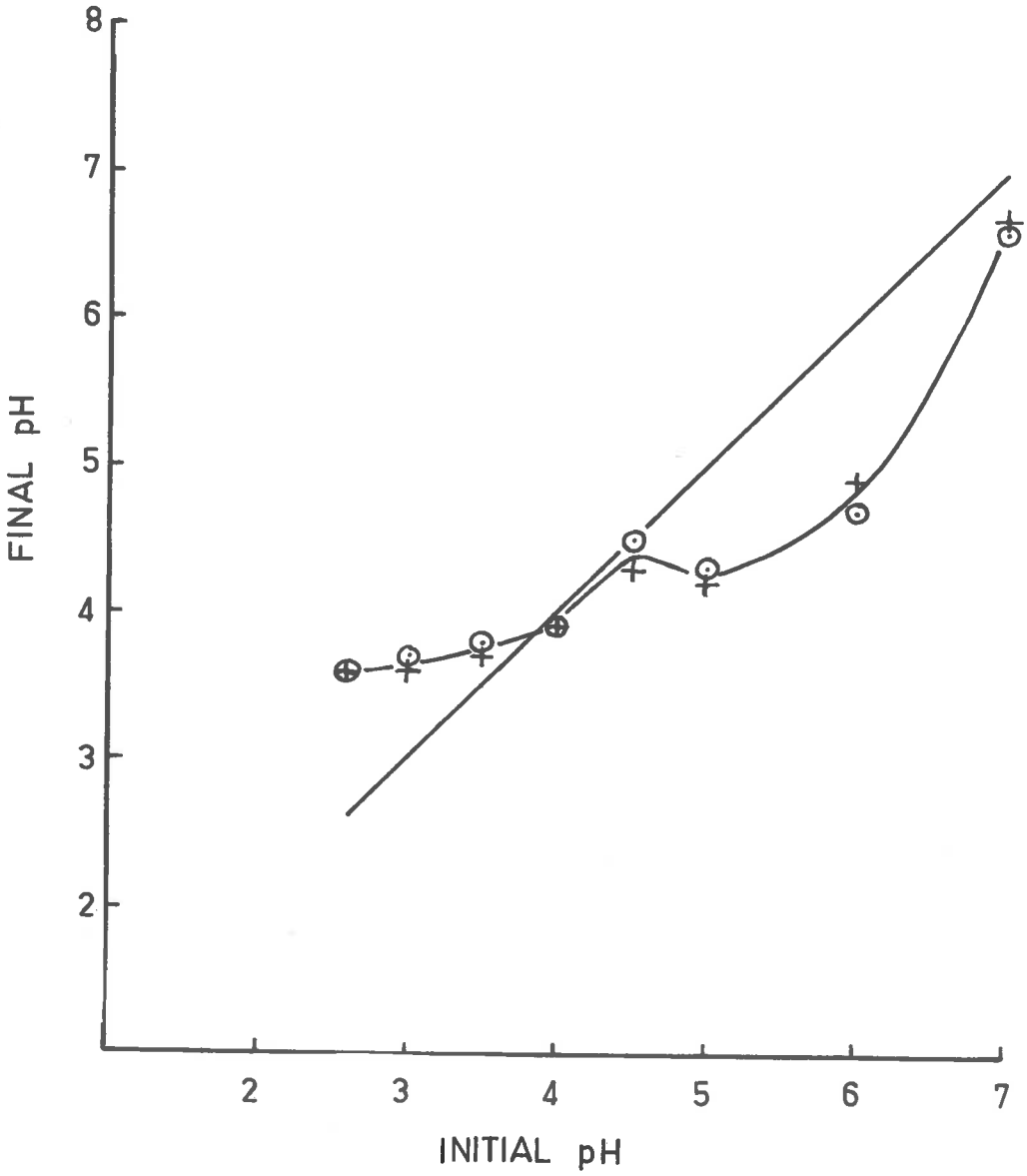
FIGURE 3.231-2

The change in pH during incubation of wheat aleurone tissue. Variety: Olympic. Incubation medium: citrate-phosphate buffer, $2.5 \times 10^{-3}$ M;

⊙ GA (50 µg/ml);

\+ GA (100 µg/ml).

Temperature: 30°C. Time: 24 hours. The straight line represents the situation when no pH shifts occur.


8
7
6
5
4
3
2
FINAL pH
2
3
4
5
6
7
INITIAL pH

and in the absence of GA. The procedure was as in section 3.231. The results are presented in Table 3.232-1 and Figure 3.232-1.

TABLE 3.232-1

Production of α-amylase by wheat aleurone tissue at various concentrations of maleic acid. Variety: Olympic. Incubation medium: maleic acid, 2.5, 5.0 or 10.0 x $10^{-3}$ M; water or GA (100 μg/ml). Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 600 nm per hour per g fresh weight.

| Incubation medium | Maleic acid (x $10^{-3}$ M) | Initial pH | Final pH | α-amylase activity | | |
|---|---|---|---|---|---|---|
| | | | | Ambient | Extract | Total |
| Water | 0 | - | 4.74 | 0 | 76 | 76 |
| " | 2.5 | 2.95 | 4.56 | 0 | 76 | 76 |
| " | 5.0 | 2.71 | 3.84 | 0 | 95 | 95 |
| " | 10.0 | 2.49 | 3.75 | 0 | 95 | 95 |
| GA | 0 | 4.01 | 4.26 | 1,646 | 1,771 | 3,417 |
| " | 2.5 | 2.88 | 4.07 | 5,940 | 2,960 | 8,900 |
| " | 5.0 | 2.65 | 4.07 | 3,085 | 2,680 | 5,765 |
| " | 10.0 | 2.48 | 3.94 | 738 | 3,275 | 4,013 |

There was no effect of maleic acid on α-amylase production in the absence of GA. However, the α-amylase activity was stimulated by maleic acid in the presence of GA. The activity of α-amylase in the extracts was slightly increased (+ 67.8%) at 2.5 x $10^{-3}$ M maleic acid and remained about the same at 5 and 10 x $10^{-3}$ M. The

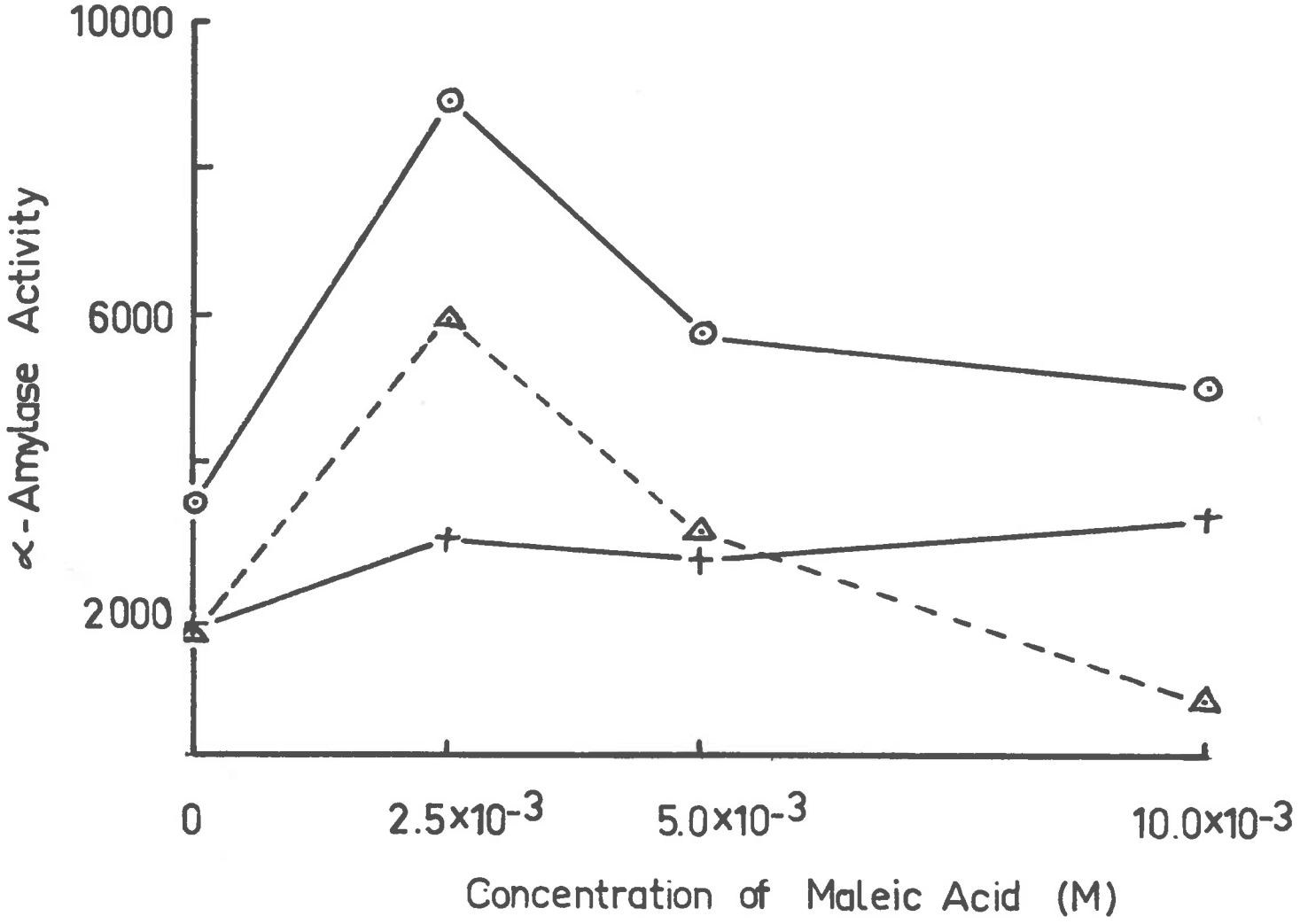
FIGURE 3.232-1

Production of α-amylase by wheat aleurone tissue at various concentrations of maleic acid. Variety: Olympic. Incubation medium: maleic acid 2.5, 5.0 or 10.0 x $10^{-3}$ M; water or GA (100 µg/ml). Temperature 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 600 nm per hour per g fresh weight.

† extracts;

△ ambient solutions;

⊙ total.


10000
6000
2000
∝-Amylase Activity
0
2.5x10-3
5.0x10-3
10.0x10-3
Concentration of Maleic Acid (M)

activity of the enzyme in the ambient solution on the other hand was greatly enhanced at $2.5 \times 10^{-3}$ M (+ 261%), less so at $5 \times 10^{-3}$ M (+ 87.4%) and decreased at $10 \times 10^{-3}$ M (- 55.1%).

The lower amounts of the enzyme measured in the ambient solutions at 5 and $10 \times 10^{-3}$ M maleic acid may represent inactivation during the incubation period since α-amylase is unstable at low pH. At the higher concentrations of maleic acid, the pH of the incubation medium would have remained at a lower level for a longer time. This explanation is made more probable by the fact that there was no change in the amount of enzyme in the extracts at the higher concentrations. Presumably the pH inside the tissue was not affected very much by the acid.

This enhancement of enzyme production at low pH is difficult to assess since the enzyme may be unstable under such conditions. Indeed, the low pH effect may have been entirely obscured if stronger buffer solutions had been used. The results suggest that there may be an initial reaction which has a low pH optimum followed by reactions with higher pH optima.

### 3.24 Calcium

Since calcium is required in the synthesis of

α-amylase as an integral part of the molecule, it is conceivable that addition of calcium may enhance the response. One gram quantities of aleurone tissue were incubated for 24 hours with solutions containing water or GA with $2x10^{-4}$, $2x10^{-3}$ or $2x10^{-2}$ M calcium nitrate. (Calcium nitrate was chosen because it gave results similar to calcium acetate in the α-amylase assay whereas calcium chloride was not as good. Calcium acetate could not be used because it inhibits the GA response at concentrations above $1 \times 10^{-3}$ M.) The α-amylase was measured in both ambient solutions and extracts of the tissue at the end of the incubation period. The results are shown in Table 3.24-1 and Figure 3.24-1.

Calcium nitrate had little effect on α-amylase production in the absence of GA. Similarly, it had little effect on α-amylase production in the presence of GA at the two lowest concentrations. However, at $2 \times 10^{-2}$ M there was a much greater amount of α-amylase in the ambient solution although there was no change in the extracts. This may reflect a greater synthesis and release of the enzyme or, alternatively, a stabilization of the enzyme in the medium.

TABLE 3.24-1

Production of α-amylase by wheat aleurone tissue at various concentrations of calcium nitrate. Variety: Olympic. Incubation medium: calcium nitrate, $2 \times 10^{-4}$, $2 \times 10^{-3}$, or $2 \times 10^{-2}$ M; water or GA (100 μg/ml). Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 600 nm per hour per g fresh weight.

| Incubation medium | Calcium nitrate (M) | α-amylase activity | | |
|---|---|---|---|---|
| | | Ambient | Extract | Total |
| Water | 0 | 0 | 0 | 0 |
| " | $2 \times 10^{-4}$ | 127 | 88 | 215 |
| " | $2 \times 10^{-3}$ | 145 | 150 | 295 |
| " | $2 \times 10^{-2}$ | 66 | 88 | 154 |
| GA | 0 | 1,987 | 2,554 | 4,541 |
| " | $2 \times 10^{-4}$ | 1,603 | 1,855 | 3,458 |
| " | $2 \times 10^{-3}$ | 1,973 | 1,720 | 3,693 |
| " | $2 \times 10^{-2}$ | 6,193 | 2,553 | 8,746 |

## 3.25 Actinomycin D, Sucrose and Glucose

Actinomycin D is known to inhibit the synthesis of DNA-dependent RNA synthesis. However, in some systems this inhibition can be overcome by glucose (Honig and Rabinovitz, 1966). Actinomycin D has no effect on the GA-stimulated release of reducing sugars from barley half-seed (Paleg, 1964) but it does inhibit

FIGURE 3.24-1

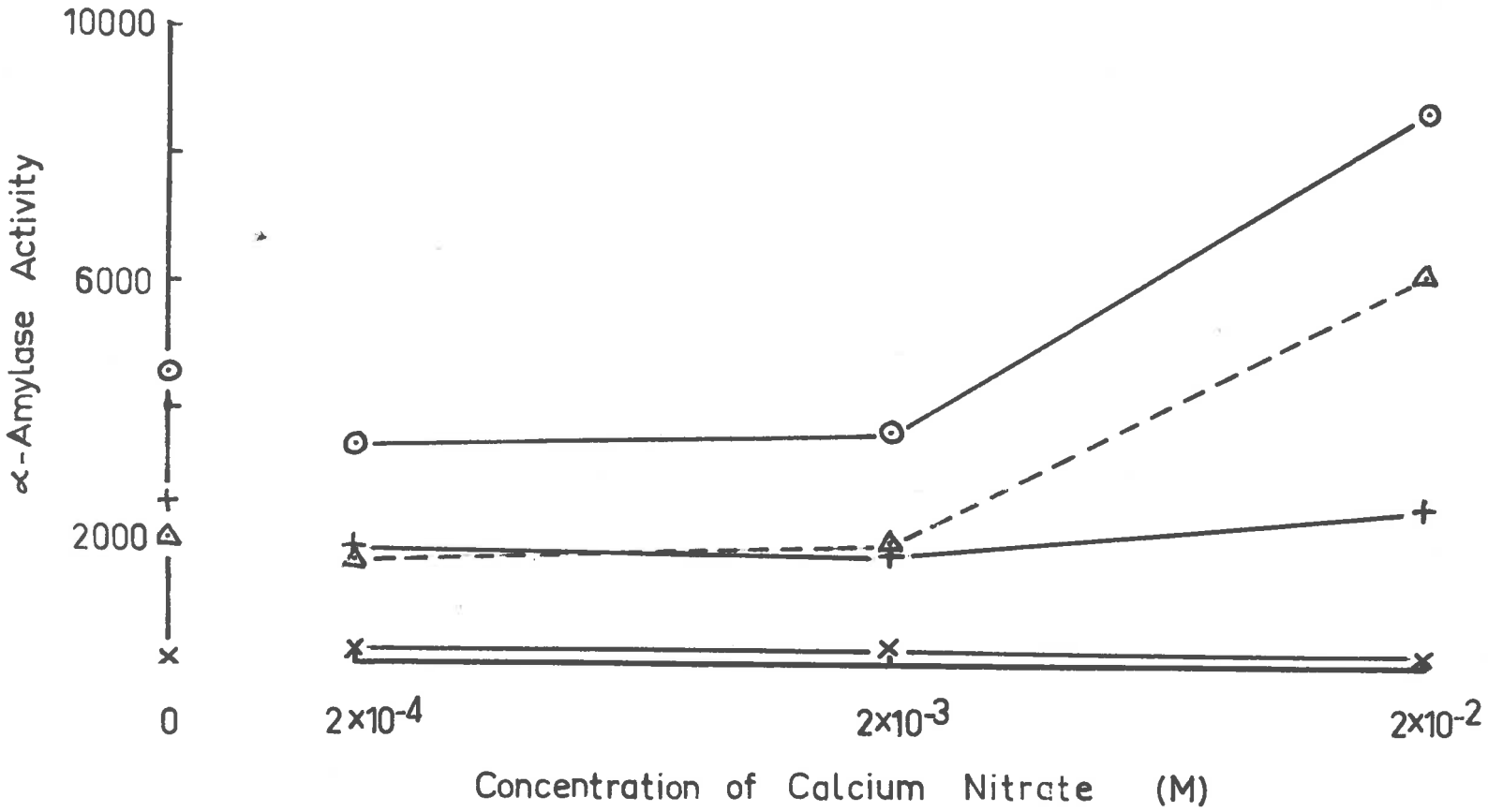
Production of α-amylase by wheat aleurone tissue at various concentrations of calcium nitrate. Variety: Olympic. Incubation medium: calcium nitrate $2 \times 10^{-4}$, $2 \times 10^{-3}$ or $2 \times 10^{-2}$ M; water or GA (100 µg/ml). Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 600 nm per hour per g fresh weight.

× water control;

\+ extracts;

△ ambient solutions;

⊙ total.


10000
6000
2000
0
α-Amylase Activity
2×10-4
2×10-3
2×10-2
Concentration of Calcium Nitrate (M)

the GA-induced production of α-amylase by isolated aleurone layers (Varner and Ram Chandra, 1964). The present experiments were undertaken to ascertain whether the production of α-amylase by wheat aleurone layers is inhibited by actinomycin D and if so, whether glucose has any effect on this inhibition.

3.251 The effect of glucose and sucrose on GA-induced α-amylase production

The effect of glucose itself on the GA-induced α-amylase production was first determined and compared with that of a non-reducing sugar, sucrose.

Samples of aleurone tissue, 0.5 g each, were incubated at 30°C for 24 hours with a total of 2.5 ml of solution containing water or GA with or without $10^{-3}$, $10^{-2}$ or $10^{-1}$ M glucose or sucrose. The α-amylase in the ambient solutions and in the tissue was measured at the end of the incubation period.

A more sensitive method for measuring α-amylase was used in this and in all subsequent experiments. This was modification of the method of Jones and Varner (1967) and is outlined below.

Substrate:

| | |
|---|---|
| Amylose (BDH) | 150 mg |
| $KH_2PO_4$ (16.32%) | 5 ml |
| Calcium acetate ($10^{-2}$ M) | 20 ml |

Water to 100 ml

Boil for 1 minute, cool, centrifuge, adjust pH to 4.6 with acetic acid.

Iodine stock solution:

| | |
|---|---|
| Potassium iodide | 6 g |
| Iodine | 0.6 g |

Water to 100 ml

For assay, dilute 2.5 ml stock solution to 100 ml with water.

Incubation medium:

| | |
|---|---|
| Substrate | 1.0 ml |
| Enzyme | 0.5 ml |

Stop reaction with 0.5 ml diluted IKI solution.

Dilute to 10 ml with water.

Read OD at 620 nm in a Unicam SP350 spectrophotometer.

Results are shown in Table 3.251-1 and Figures 3.251-1 and -2.

The total amount of α-amylase in the tissue increased in the absence of GA at the two highest concentrations of the sugars, but none of this was secreted into the medium. Glucose was more effective than sucrose.

TABLE 3.251-1

Production of α-amylase by wheat aleurone tissue at various concentrations of glucose or sucrose. Variety: Olympic. Incubation medium: glucose or sucrose, $10^{-3}$, $10^{-2}$ or $10^{-1}$ M; water or GA (100 μg/ml). Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 600 nm per hour per g fresh weight.

| Incubation medium | Sugars (M) | α-amylase activity | | |
|---|---|---|---|---|
| | | Ambient | Extract | Total |
| Water | 0 | 14 | 321 | 336 |
| GA | 0 | 36,800 | 55,500 | 92,300 |
| Glucose | $10^{-3}$ | 24 | 857 | 882 |
| " | $10^{-2}$ | 18 | 10,000 | 10,018 |
| " | $10^{-1}$ | 21 | 26,050 | 26,071 |
| GA + glucose | $10^{-3}$ | 22,350 | 78,120 | 100,470 |
| " | $10^{-2}$ | 3,000 | 83,900 | 86,900 |
| " | $10^{-1}$ | 145 | 72,700 | 72,845 |
| Sucrose | $10^{-3}$ | 0 | 348 | 348 |
| " | $10^{-2}$ | 0 | 2,135 | 2,135 |
| " | $10^{-1}$ | 0 | 4,110 | 4,110 |
| GA + sucrose | $10^{-3}$ | 15,280 | 71,500 | 86,780 |
| " | $10^{-2}$ | 225 | 77,700 | 77,925 |
| " | $10^{-1}$ | 0 | 70,500 | 70,500 |

The amount of α-amylase in the extracts increased in the presence of GA at all three concentrations of the sugars. On the other hand, the amount of α-amylase in the ambient solutions decreased, falling nearly to zero at $10^{-2}$ M with both sugars. Thus, the

FIGURE 3.251-1

Production of α-amylase by wheat aleurone tissue at various concentrations of glucose. Variety: Olympic. Incubation medium: glucose, $10^{-3}$, $10^{-2}$ or $10^{-1}$ M; water or GA (100 µg/ml). Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 620 nm per hour per g fresh weight.

× water control;

\+ extracts;

△ ambient solutions;

⊙ total.

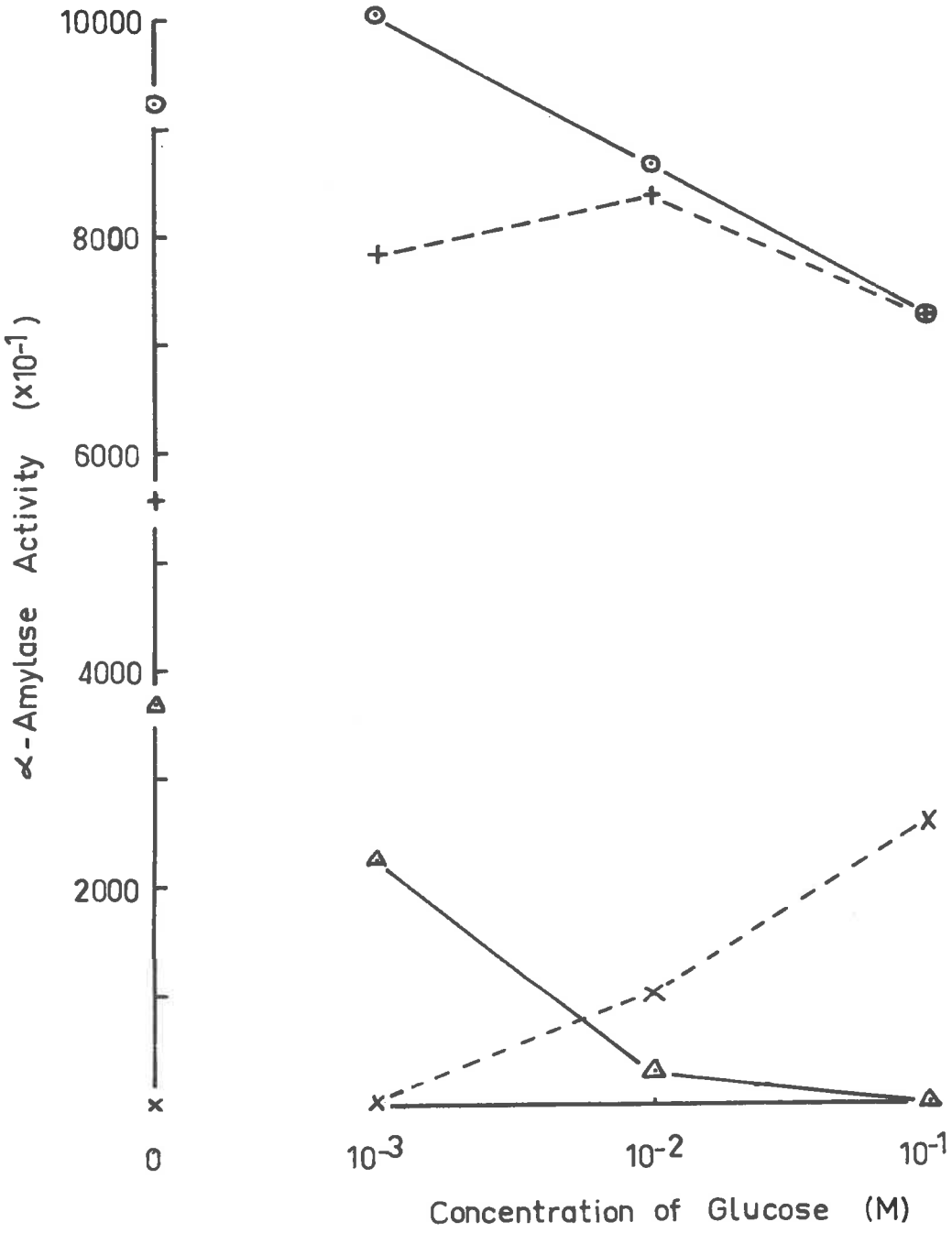

10000
8000
6000
4000
2000
0
α-Amylase Activity (x10⁻¹)
10⁻³
10⁻²
10⁻¹
Concentration of Glucose (M)

FIGURE 3.251-2

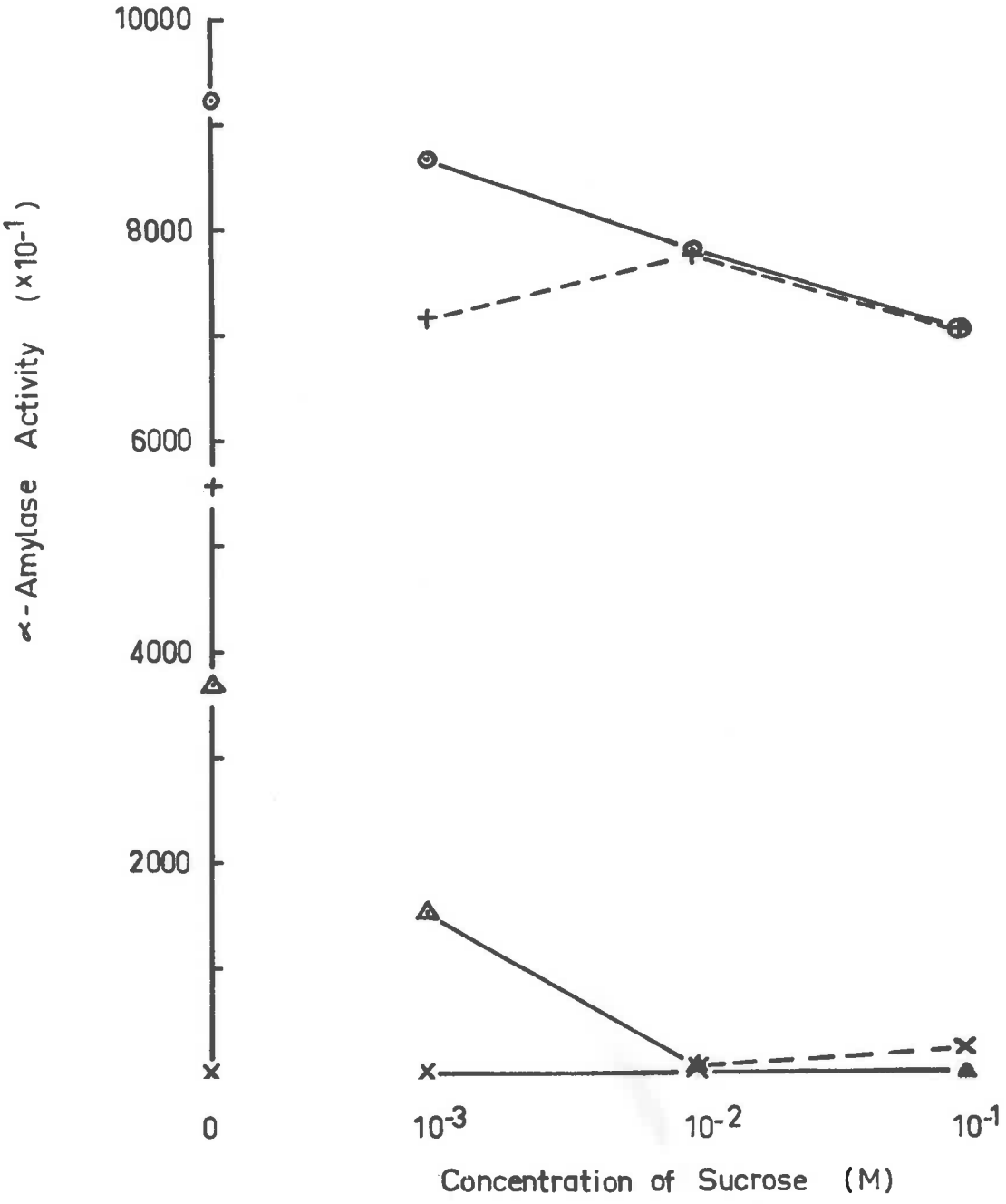
Production of α-amylase by wheat aleurone tissue at various concentrations of sucrose. Variety: Olympic. Incubation medium: sucrose, $10^{-3}$, $10^{-2}$ or $10^{-1}$ M; water or GA (100 µg/ml). Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent OD at 620 nm per hour per g fresh weight.

× water control;

\+ extracts;

△ ambient solutions;

⊙ total.


10000
8000
6000
4000
2000
0
α-Amylase Activity (×10⁻¹)
10⁻³
10⁻²
10⁻¹
Concentration of Sucrose (M)

total amount of amylase was only slightly changed at $10^{-3}$ but was decreased at $10^{-2}$ and $10^{-1}$ M. The sugars appeared either to inhibit release of α-amylase or to decrease its stability in solution.

3.252 The effect of actinomycin D on GA-induced α-amylase production in the presence or absence of glucose

The procedure was the same as for the experiment of section 3.251. The results are shown in Tables 3.252-1 and -2, and Figure 3.252-1.

Actinomycin D at both 50 and 100 μg/ml inhibited GA-induced α-amylase production by over 99% in the absence of glucose (Tables 3.252-1 and -2). There was only a slight recovery at $10^{-2}$ and $10^{-1}$ M glucose (Tables 3.252-1 and -2 and Figure 3.252-1). The GA-induced α-amylase production by wheat aleurone tissue, therefore, was inhibited by actinomycin D and there was little reversal of this inhibition by glucose. Indeed, the small amount of recovery observed in the presence of glucose could be accounted for by the stimulation of the GA response by glucose in the absence of actinomycin D (Figure 3.252-1).

## TABLE 3.252-1

Production of α-amylase by wheat aleurone tissue at various concentrations of glucose in the presence or absence of actinomycin D and/or GA. Variety: Olympic. Incubation medium: glucose, $10^{-3}$, $10^{-2}$ or $10^{-1}$ M; water or GA (100 μg/ml); actinomycin D, 100 μg/ml. Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 620 nm per hour per g fresh weight.

| Incubation medium | Glucose (M) | Actinomycin D (μg/ml) | α-amylase activity | | | Inhibition (%) |
|---|---|---|---|---|---|---|
| | | | Ambient | Extract | Total | |
| Water | 0 | 0 | 13 | 90 | 103 | - |
| " | $10^{-3}$ | 0 | 0 | 66 | 66 | - |
| " | $10^{-2}$ | 0 | 0 | 675 | 675 | - |
| " | $10^{-1}$ | 0 | 0 | 1,980 | 1,980 | - |
| GA | 0 | 0 | 2,830 | 5,040 | 9,870 | - |
| " | $10^{-3}$ | 0 | 1,320 | 10,700 | 12,002 | - |
| " | $10^{-2}$ | 0 | 3 | 14,500 | 14,503 | - |
| " | $10^{-2}$ | 0 | 3 | 13,600 | 13,603 | - |
| GA | 0 | 100 | 0 | 45 | 45 | 99.6 |
| " | $10^{-3}$ | " | 0 | 48 | 48 | 99.5 |
| " | $10^{-2}$ | " | 0 | 1,280 | 1,280 | 91.3 |
| " | $10^{-1}$ | " | 0 | 473 | 473 | 93.8 |

## TABLE 3.252-2

Production of α-amylase by wheat aleurone tissue at various concentrations of glucose in the presence or absence of actinomycin D and GA. Variety: Olympic. Incubation medium: glucose, $10^{-3}$, $10^{-2}$ and $10^{-1}$ M; water or GA (100 µg/ml); actinomycin D, 50 or 100 µg/ml. Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 620 nm per hour per g fresh weight.

| Incubation medium | Glucose (M) | Actinomycin D (µg/ml) | α-amylase activity | | | Inhibition (%) |
|---|---|---|---|---|---|---|
| | | | Ambient | Extract | Total | |
| Water | 0 | 0 | 0 | 158 | 158 | |
| GA | 0 | 0 | 2,904 | 1,600 | 4,504 | |
| " | 0 | 50 | 0 | 22 | 22 | 99.5 |
| " | $10^{-3}$ | " | 0 | 198 | 198 | 95.8 |
| " | $10^{-2}$ | " | 0 | 249 | 249 | 94.5 |
| " | $10^{-1}$ | " | 0 | 369 | 369 | 91.5 |
| GA | 0 | 100 | 0 | 18 | 18 | 99.8 |
| " | $10^{-3}$ | " | 0 | 22 | 22 | 99.5 |
| " | $10^{-2}$ | " | 0 | 45 | 45 | 99.1 |
| " | $10^{-1}$ | " | 0 | 19 | 19 | 99.8 |

FIGURE 3.252-1

Production of α-amylase by wheat aleurone tissue at various concentrations of glucose in the presence or absence of actinomycin D and/or GA. Variety: Olympic. Incubation medium: glucose, $10^{-3}$, $10^{-2}$ or $10^{-1}$ M; water or GA (100 µg/ml); actinomycin D, 100 µg/ml. Temperature: 30°C. Time: 24 hours. Enzyme activity: change in percent initial OD at 620 nm per hour per g fresh weight.

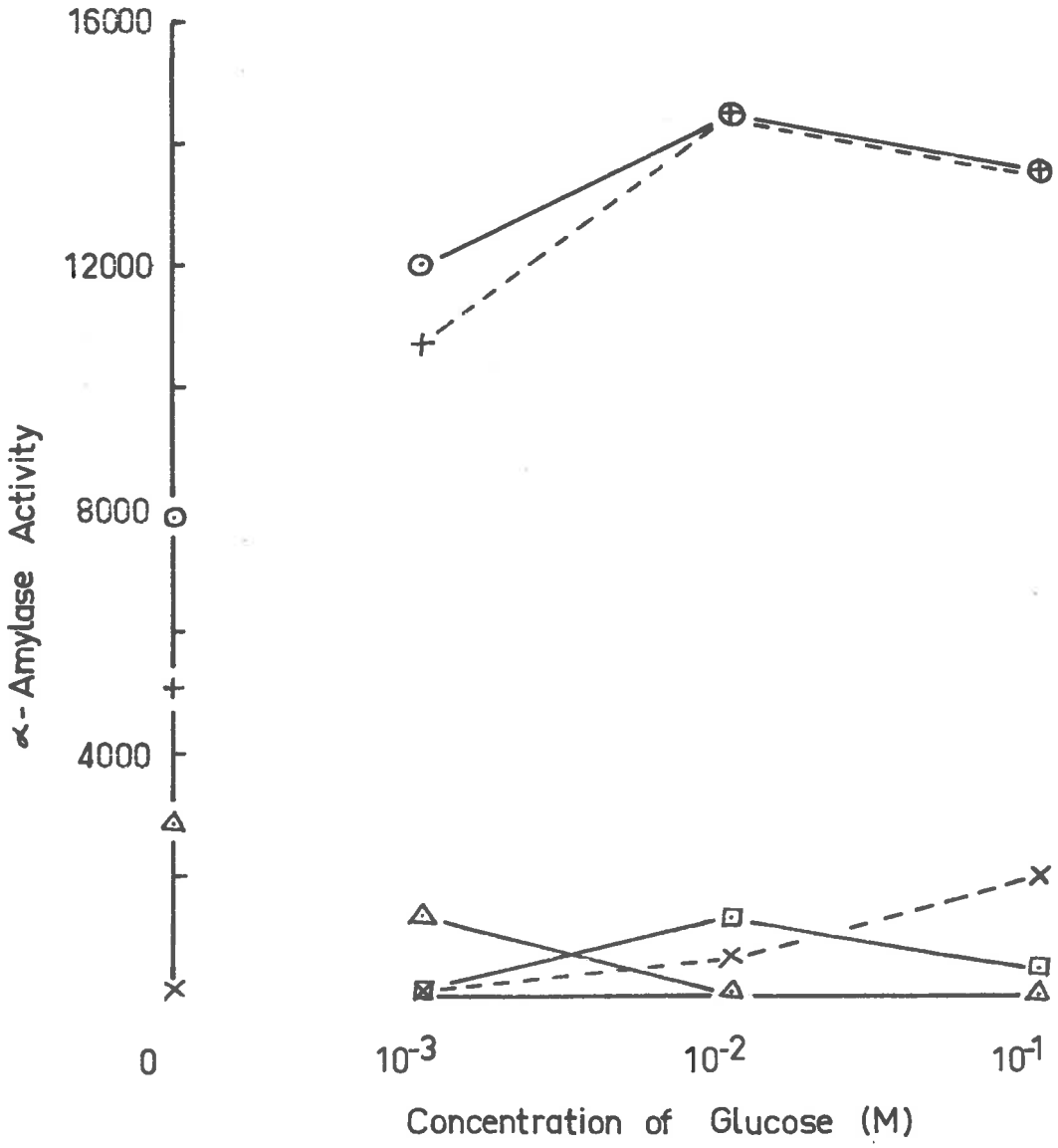
× water control;

+ extracts;

△ ambient solutions;

⊙ total;

⊡ total in presence of actinomycin D and GA.


16000
12000
8000
4000
0
α-Amylase Activity
10-3
10-2
10-1
Concentration of Glucose (M)

## 3.3 Fractionation of the aleurone tissue into sub-cellular components

As mentioned in section 2.9, the ultimate aim of the present investigation was to determine whether any of the GA-induced enzymes is membrane-bound. The mass isolation technique results in wheat aleurone tissue whose response to GA is qualitatively similar to that of barley aleurone tissue. Moreover, this technique permits the isolation of quantities of tissue large enough for the investigation of techniques for the fractionation of subcellular components. The present section deals with the development of such methods of fractionation.

### 3.31 Homogenization

#### 3.311 Choice of an appropriate grinding medium

Both aqueous and non-aqueous media have been used for the fractionation of plant cells. Each kind of medium has its advantages and disadvantages. Some of the considerations that apply to the selection of a grinding medium are listed in Table 3.31-1. Since the aleurone tissue is fully hydrated when it is removed from the seed, and since it is metabolically active only in the hydrated state, an aqueous medium was chosen.

TABLE 3.31-1

Comparison of aqueous and non-aqueous grinding media

| Aqueous | Non-aqueous |
|---|---|
| 1. Tissue must be hydrated. | 1. Tissue must be dry. |
| 2. The medium must try to simulate the internal milieu of the cells. | 2. The medium must be kept dry. |
| 3. The grinding of the tissue and manipulations of homogenates must be carried out at low temperatures (2-4°C) to minimize enzymic changes. | 3. The grinding and manipulations can be carried out at room temperature since enzyme reactions require aqueous conditions. |
| 4. Loss of water-soluble components may occur. | 4. Loss of lipid-soluble components may occur. |
| 5. Solvents are not toxic. | 5. Solvents may be very toxic, e.g. carbon tetrachloride. |

The points which must be taken into account when selecting an aqueous medium are as follows:

1. Osmotic pressure. The medium should be isotonic with the cell contents to minimize structural changes in membrane-bound organelles.
2. pH. The medium should be buffered to minimize changes in pH due to mixing of the compartments, particularly if vacuoles containing acids are present.
3. Salts. Some organelles require specific ions for maintenance of integrity. (e.g. $Mg^{++}$ ($10^{-3}$ M) is required for isolation of ribosomes, $Ca^{++}$ for nuclei.)
4. SH-preservatives. Some enzymes are inactivated if their SH-groups are oxidized. β-Mercaptoethanol, glutathione and cysteine ($10^{-2}$ M) are commonly used as protecting agents (Bonner and Varner, 1965).

The following medium has been shown to be suitable for the isolation of microscopically well preserved plant nuclei, mitochondria, and chloroplasts, and for the maintenance of protoplasmic streaming (Honda et al., 1966).

## BASIC MEDIUM

| Component | Concentration | Function |
|---|---|---|
| Sucrose | 0.25 M | Osmoticum |
| Ficoll (MW 400,000) | 2.5% | Structure preservatives |
| Dextran (MW 40,000) | 5.0% | Adsorption of polyphenols |
| Protein (Bovine serum albumin) | 0.01% | Structure preservative |
| Buffer (Tris-HCl, pH 7.8) | 0.025 M | Maintenance of pH |

Addition of the following components may be necessary to demonstrate protein synthesis:

## ACTIVITY ADDITIVES

| | | |
|---|---|---|
| Glutathione, cysteine or β-mercaptoethanol | $3.9 \times 10^{-3}$ M | SH-group preservative |
| Mg acetate | $1.0 \times 10^{-3}$ M | activity preservatives |
| Mn acetate | $1.0 \times 10^{-3}$ M | activity preservatives |
| EDTA | $2 \times 10^{-3}$ M | Heavy metal chelation |

The basic "Honda" medium was chosen for homogenizing the aleurone cells because cations and EDTA vary in their effects on different organelles (e.g. $Ca^{++}$ ions preserve nuclear integrity but cause mitochondria to swell; EDTA preserves mitochondrial integrity but disrupts ribosomes.)

### 3.312 Choice of a suitable homogenising method

To obtain a cell-free suspension of organelles, the cell walls and protoplasmic membranes must first be ruptured. Since aleurone cells have very thick tough cell walls, fairly harsh methods are required to break them, and many of the organelles may be damaged in the process. It was necessary, therefore, to examine a number of different homogenising methods and to compare the integrity of the cell-free organelles with those of the intact tissue.

#### Procedure

Aleurone tissue was homogenized by various methods in basic Honda medium, the homogenates were centrifuged at 38,000 g for 30 minutes, and the pellets examined in the electron microscope and compared with electron micrographs of intact tissue.

The following procedure was used for preparing pellets for examination in the Electron Microscope.

(1) Fix in 5% glutaraldehyde (Sabatini *et al.*, 1964) in Millonig's buffer (pH 7.1) (Millonig, 1962) for 30 minutes.

(2) Fix in 2% potassium permanganate in Sorenson's buffer (pH 7.1) (Cowdry, Laboratory Techniques, 1952) for 10 minutes.

(3) Dehydrate in acetone according to the following regime:

| Acetone: | 50% | 75% | 95% | 100% | 100% |
|---|---|---|---|---|---|
| Time: | 1 min. | 10 mins. | 30 mins. | 30 mins. | 30 mins. |

Steps (1), (2) and (3) are carried out at 1 or 2°C until the final acetone step.

(4) Embed according to the method of Luft (1961) as follows:

(a) Put tissue through two changes of propylene oxide - 5 minutes each.

(b) Place in fresh propylene oxide diluted 1:1 with araldite - 1 hour.

(c) Add three times the volume of resin to the above mixture - 2-3 hours.

(d) Pour off and embed in fresh araldite overnight.

(e) Embed in fresh araldite and cure at 35°C overnight.

(f) Increase the temperature to 60°C and cure overnight.

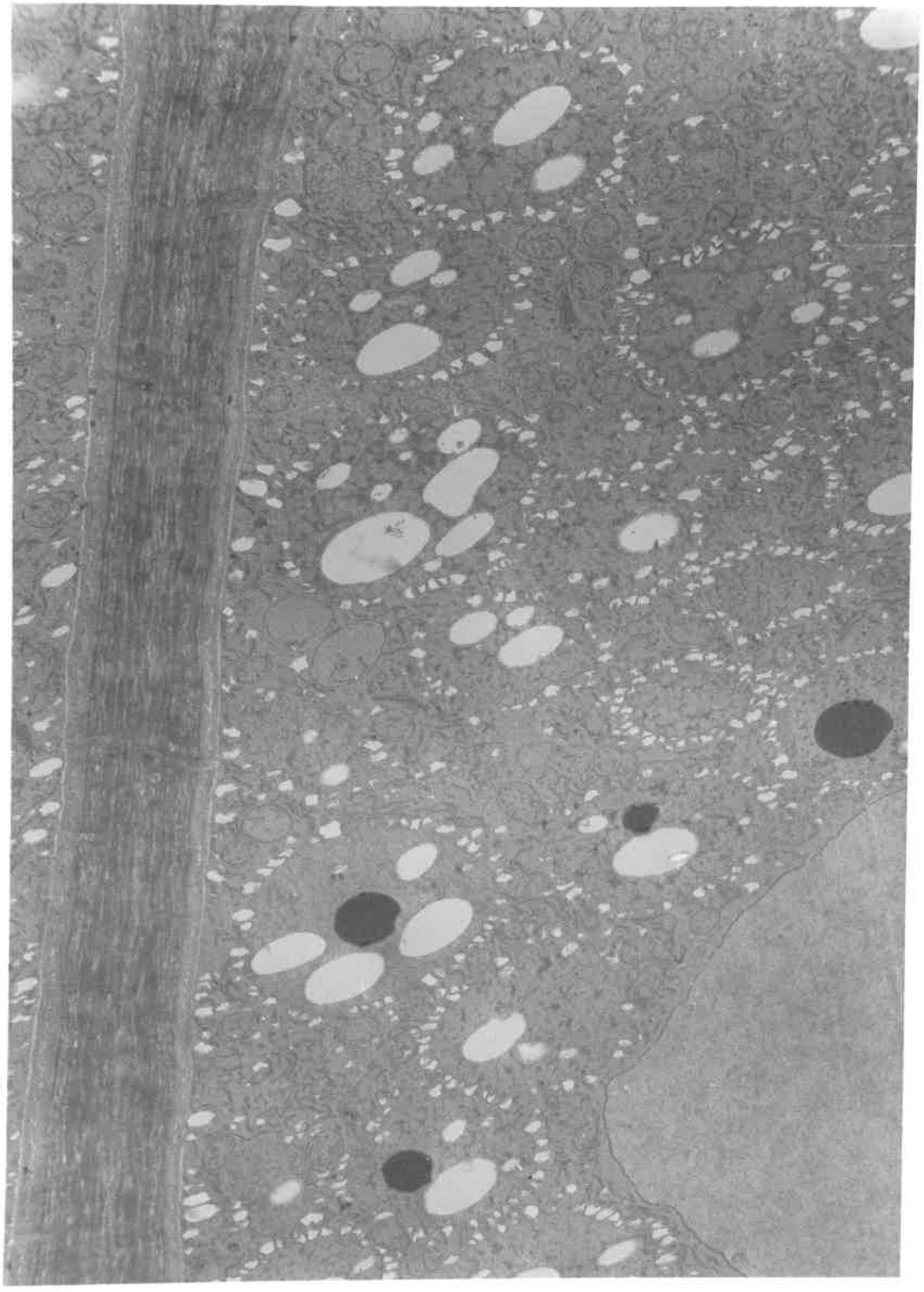
## Results

a) Intact cells

The most obvious features of the aleurone cell are thick cell walls, a prominent nucleus, many large aleurone grains, mitochondria, numerous spherosomes, endoplasmic reticulum, golgi bodies and plastids (Figure 3.312-1).

FIGURE 3.312-1

Electron micrograph of a wheat aleurone cell. Cell wall (left, running top to bottom); nucleus (right, bottom); aleurone grains with electron transparent and/or electron dense inclusions (scattered throughout the cell); spherosomes (small electron transparent bodies lining the cell wall and surrounding the aleurone grains); mitochondria with internal cristae (scattered throughout the cell); endoplasmic reticulum (membranous structures between other organelles); plastids (somewhat larger than mitochondria, centre-left, immediately to the right of the cell wall).
Magnification: 9070x.



b) Mortar and pestle

Only a small proportion of the cells could be broken with a mortar and pestle with added sand or ballotini beads unless the tissue was ground with dry ice or after lyophilization. These latter treatments resulted in complete disruption of cellular structure (Figure 3.312-2).

c) Kontes glass homogenizer

The tissue could be completely disintegrated using a motor-driven Kontes ground-glass homogenizer. If a tight-fitting homogenizer was used, very few organelles were structurally preserved (Figure 3.312-3). If a loose fitting homogenizer was used, some structure was preserved (Figure 3.312-4). Spherosomes and what appear to be aleurone grains in the process of breaking up are recognizable. However, no intact aleurone grains were observed using this method.

d) Omnimixer and Ultra-Turrax

Only a small proportion of the cells could be broken using an Omnimixer. However, the Ultra-Turrax which is a high speed blender with a different kind of cutting blade yielded reasonably intact aleurone grains and spherosomes (Figure 3.312-5).

FIGURE 3.312-2

Electron micrograph of a homogenate of aleurone tissue. Tissue lyophilized, ground in Honda medium with a mortar and pestle, centrifuged at 38,000 g for 30 minutes. Magnification: 20,000x.

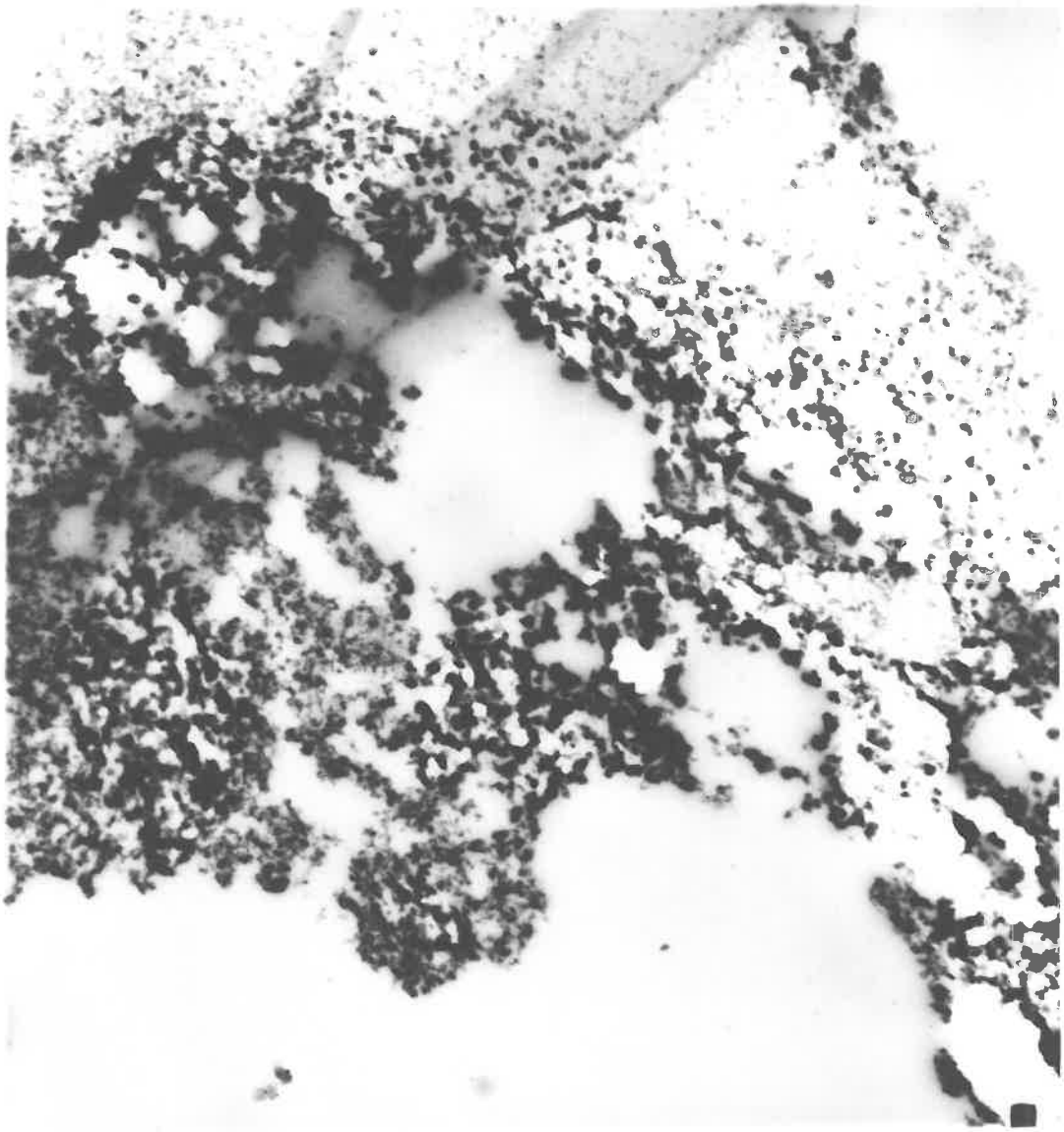


FIGURE 3.312-3

Electron micrograph of a homogenate of aleurone tissue. Tissue ground in Honda medium with a tight-fitting Kontes glass homogenizer, centrifuged at 38,000 g for 30 minutes. Magnification: 20,000x.

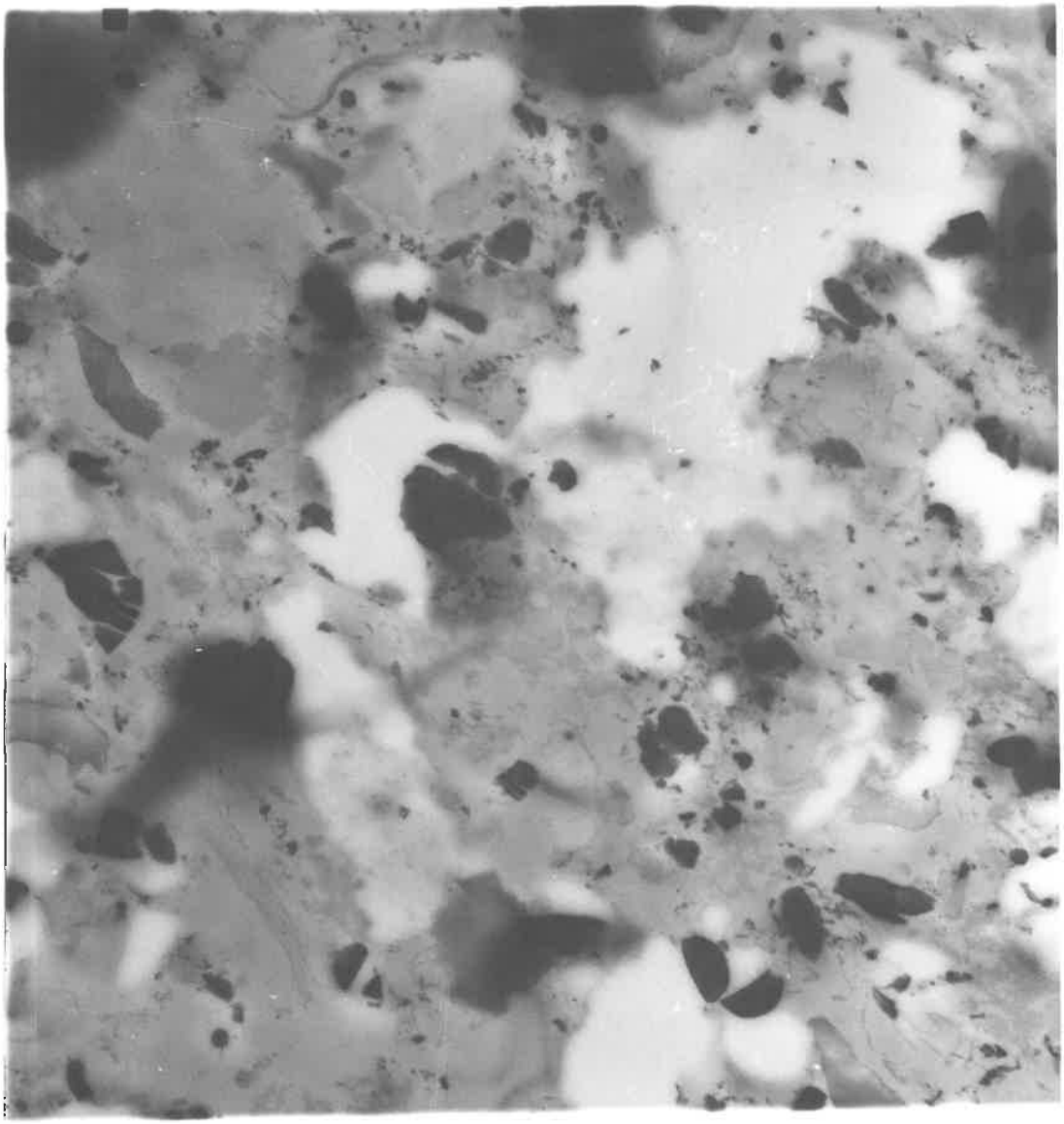


FIGURE 3.312-4

Electron micrograph of a homogenate of aleurone tissue. Tissue ground in Honda medium with a loose-fitting Kontes glass homogenizer, centrifuged at 38,000 g for 30 minutes. Aleurone grain disintegrating (centre) surrounded by spherosomes. Magnification: 18,500x.

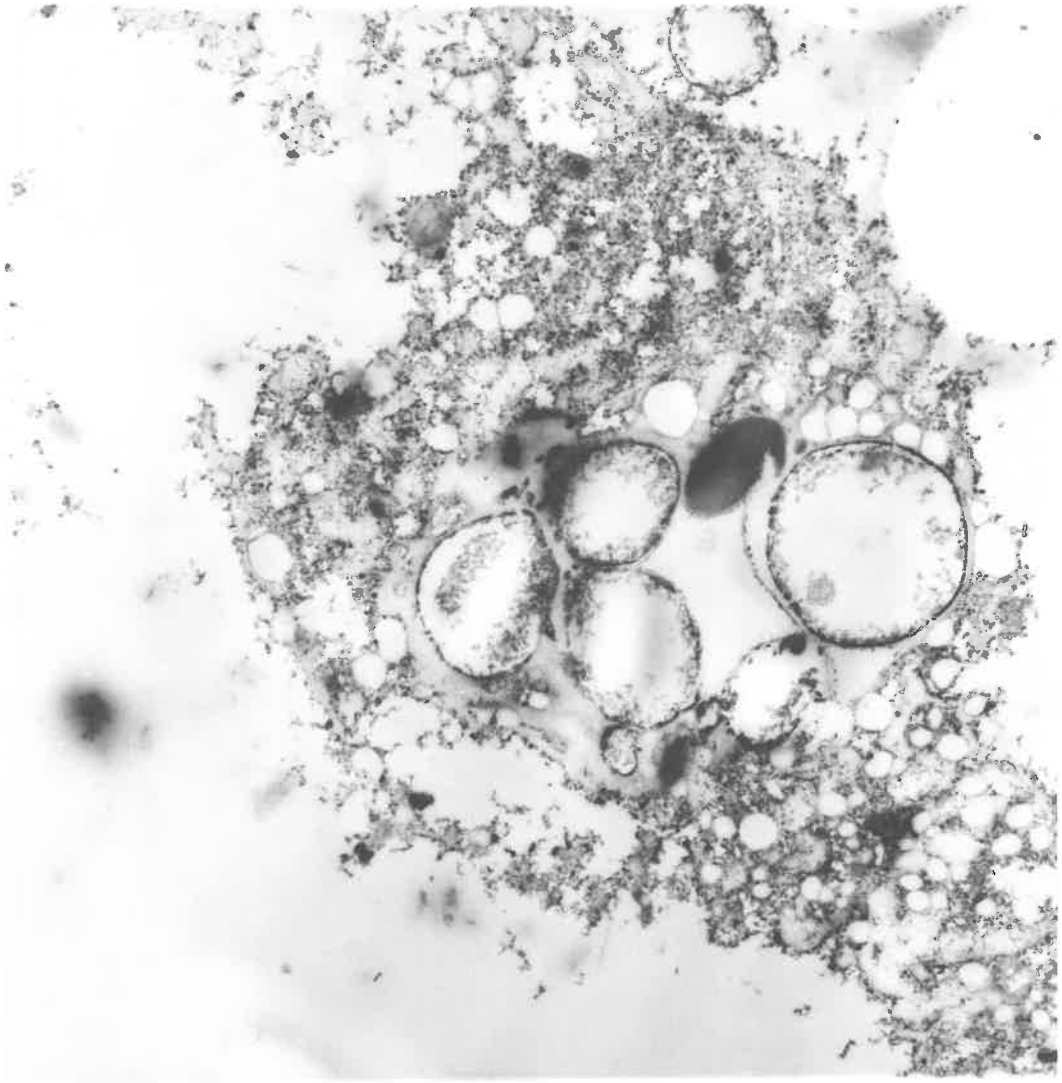


FIGURE 3.312-5

Electron micrograph of a homogenate of aleurone tissue. Tissue homogenized in Honda medium with an Ultra-Turrax, centrifuged at 38,000 g for 30 minutes. Aleurone grains with several electron transparent inclusions and usually one electron dense inclusion; starch grains (large white to greyish particles); aggregates of spherosomes (above centre).
Magnification: 4700x.

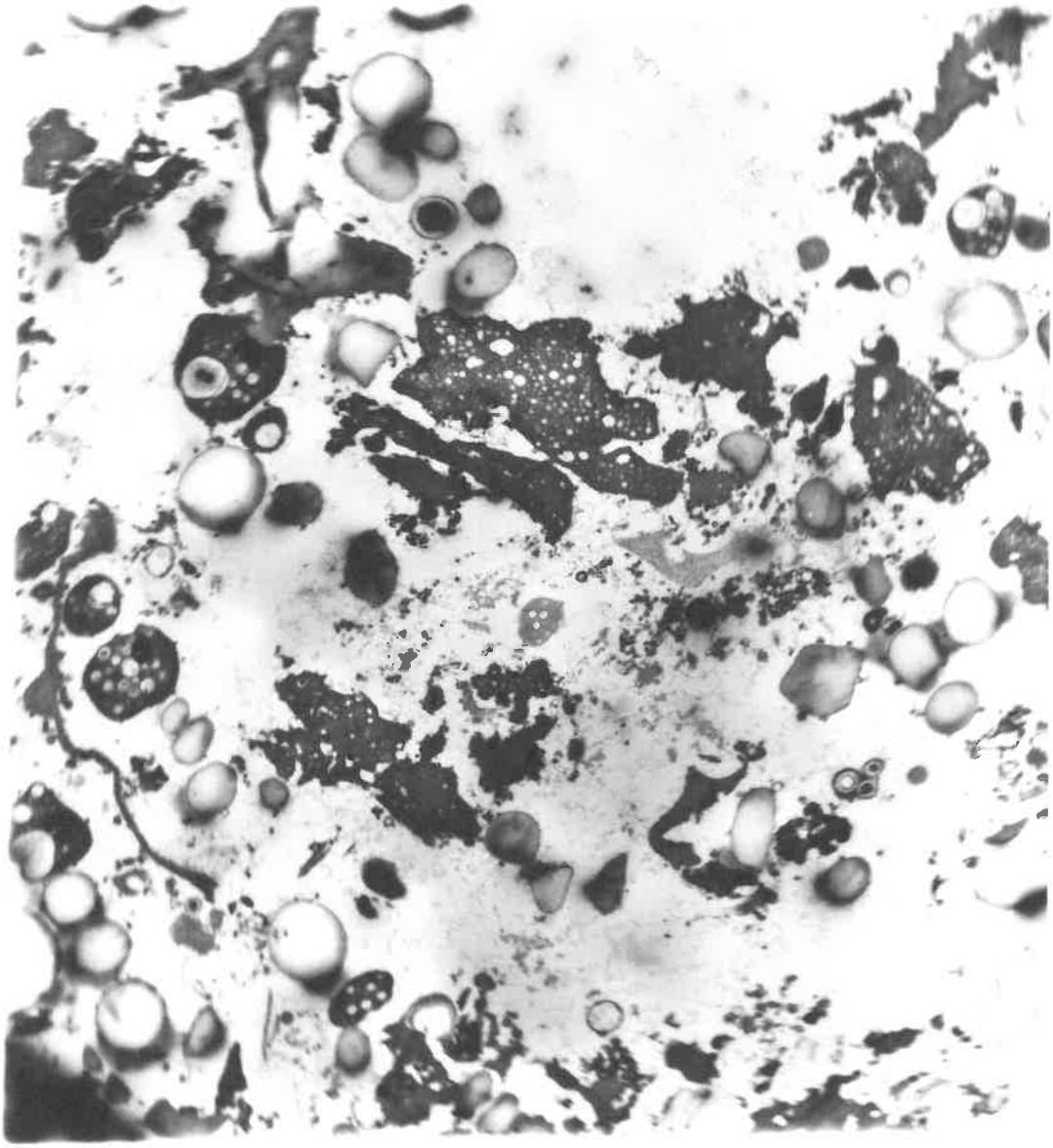
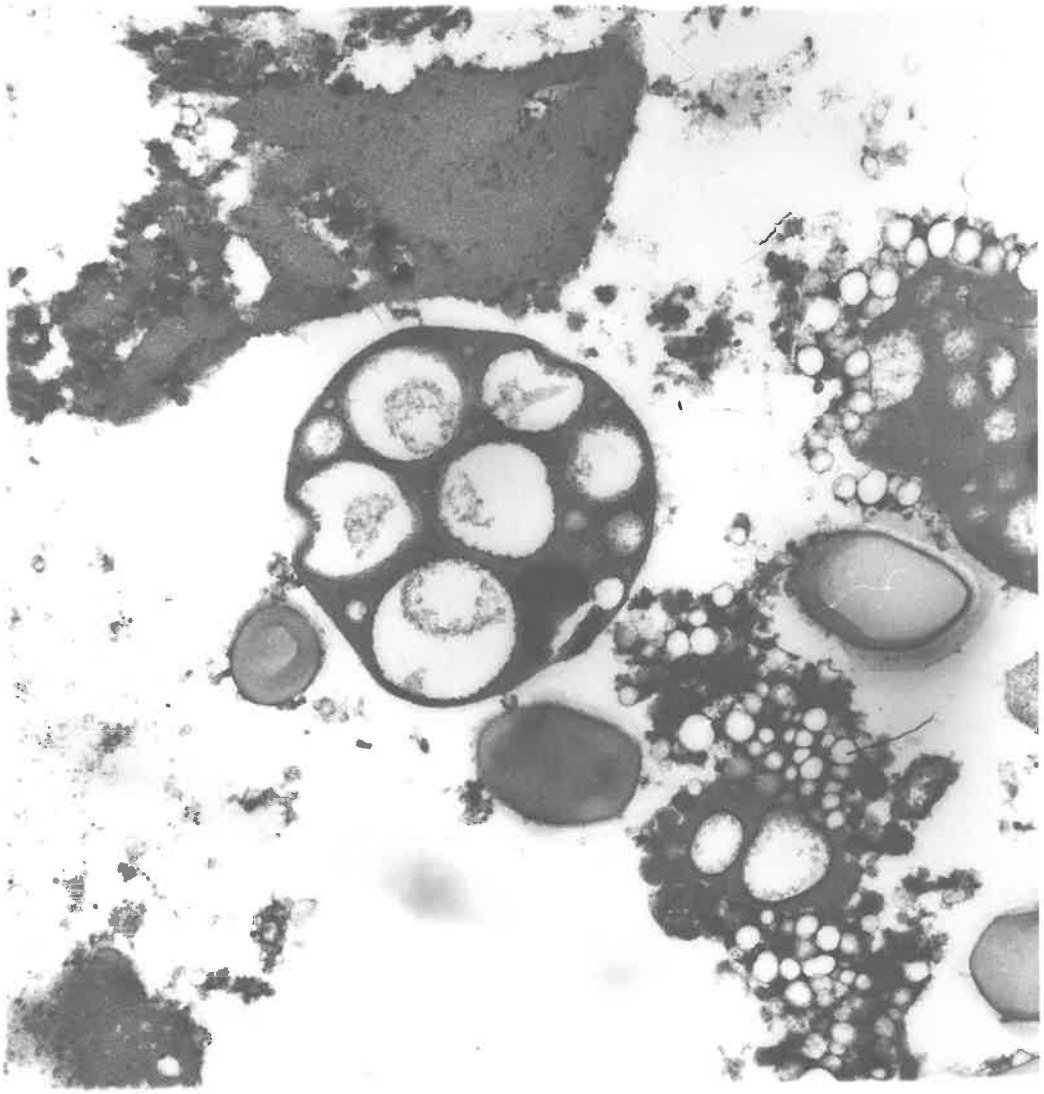


FIGURE 3.312-5a

Electron micrograph of homogenate of aleurone tissue. Tissue homogenized in Honda medium with an Ultra-Turrax, centrifuged at 38,000 g for 30 minutes. Aleurone grain (centre); spherosome aggregates (bottom, right); starch grains (large greyish particles below aleurone grain and centre, right).
Magnification: 21,400x.



e) Razor blades

Honda et al. (1966) used stainless steel razor blades stacked together without spacers to homogenize small amounts of leaf tissue for microscopy. This method worked reasonably well with aleurone tissue if the blades were stacked together with one or two spacers between them, each spacer being the same thickness as one of the razor blades. The tissue had to be chopped several times instead of just once and could not be completely homogenized. However, the aleurone grains and spherosomes appeared to be reasonably well preserved (Figure 3.312-6).

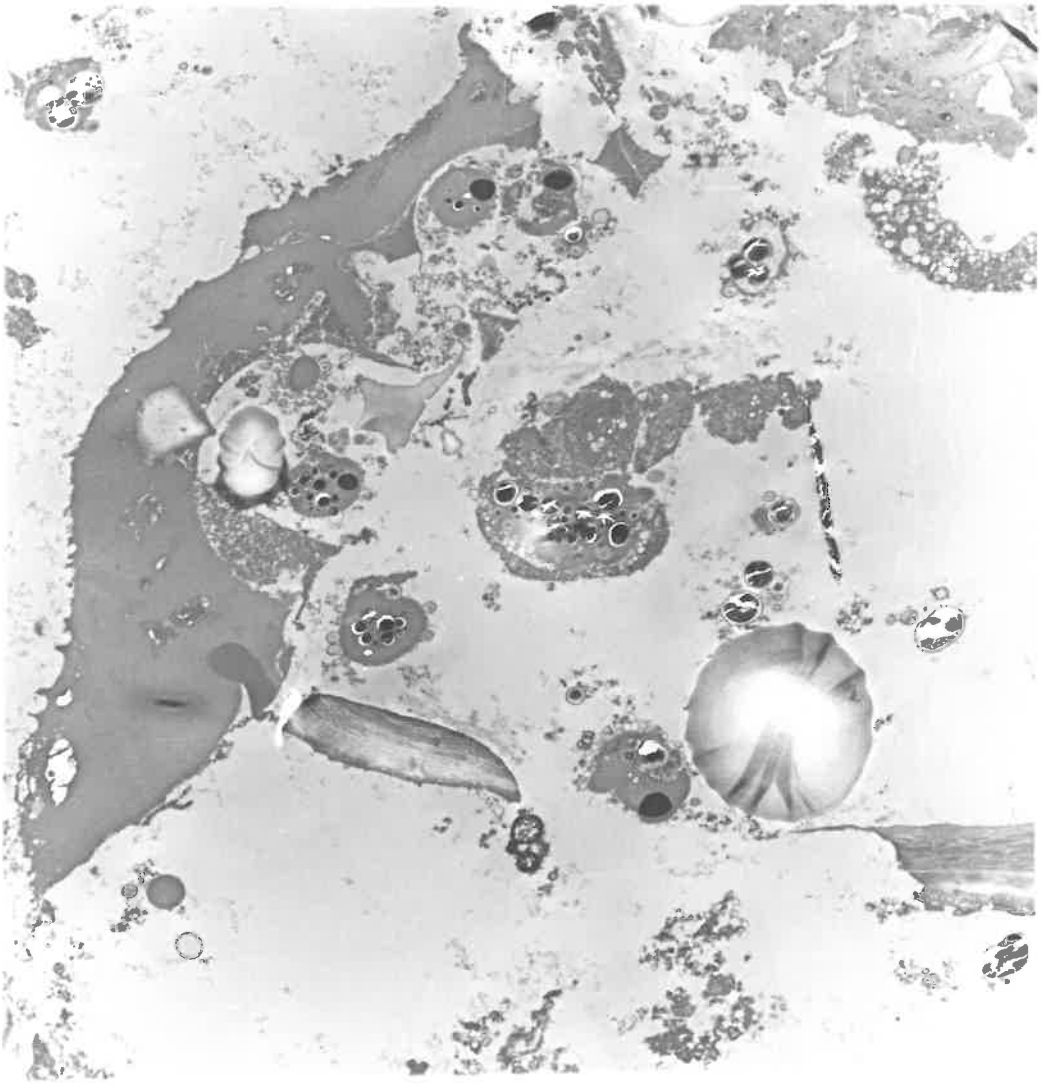
## Conclusions

Both the Ultra-Turrax and the razor blade chopper yielded reasonably well-preserved aleurone grains and spherosomes. The Ultra-Turrax is to be preferred since only a few seconds are required for the homogenization whereas the hand-operated razor blade chopper required one or two hours for the homogenization of 10 g of tissue. Since the Ultra-Turrax was not immediately available for use, the razor blade technique was adopted for the remainder of the experiments.

FIGURE 3.312-6

Electron micrograph of a homogenate of aleurone tissue. Tissue homogenized in Honda medium with stacked razor blades, centrifuged at 38,000 g for 30 minutes. Aleurone grains (centre); starch grains (right of centre and towards bottom); cell wall material (left, diagonally across corner); spherosomal aggregates (top right).

Magnification: 4200x.



## 3.32 Fractionation

### 3.321 Sieving and filtering

All cells in the tissue were not broken by the razor blade technique. In order to achieve some degree of reproducibility, the tissue was chopped in approximately one-gram quantities and forced through a plastic sieve having a pore size of about 0.5 mm. Any tissue not passing through the sieve was re-chopped until it would pass through.

The next step was to remove unbroken cells and larger pieces of tissue as well as most of the cell wall material. This was achieved by passing the sieved homogenate through 12 layers of cheese cloth which had been wetted with Honda medium. (The cheese cloth had been thoroughly washed with boiling distilled water before use.)

### 3.322 Centrifugation

The aim is to achieve a separation of particles with a minimum amount of time and manipulation. Differential centrifugation separates particles on the basis of size and density and yields very heterogeneous fractions. Resuspension of pellets and re-centrifugation is required. If the organelles are at all fragile there may be breakage during these operations. In addition, there is a chance

of forming large aggregates of particles that cannot be resuspended easily.

Density gradient centrifugation involves centrifuging a suspension of particles through a solution of continuously increasing density. When centrifugation is not continued to equilibrium, separation is on the basis of both size and density; as equilibrium is approached, separation tends to complete dependence upon density. The heavier particles will move to the denser regions of the gradient while the lighter ones will remain in the less dense regions of the gradient. If the centrifugation is continued for sufficiently long times, the particles will move to a region of the gradient corresponding to their own density. The main considerations for selecting a density gradient component are that it has a reasonably high molecular weight, forms fairly dense solutions within its solubility range, and that these solutions possess reasonably low viscosities. Sucrose has been used most frequently for this purpose.

### 3.3221 Sucrose density gradient

The initial attempts to fractionate the homogenates combined differential centrifugation with discontinuous sucrose density gradient centrifugation. The procedure is outlined in Scheme 3.3221-1.

SCHEME 3.3221-1

8 g ALEURONE TISSUE

Homogenize with razor blades in Honda medium (40 ml)
Sieve
Filter through 12 layers cheese cloth

FILTRATE (Cell-wall free homogenate) | RESIDUE (Cell walls, unbroken cells)

Centrifuge 350 g/10 min

PELLET | SUPERNATANT

Resuspend in 25 ml Honda medium

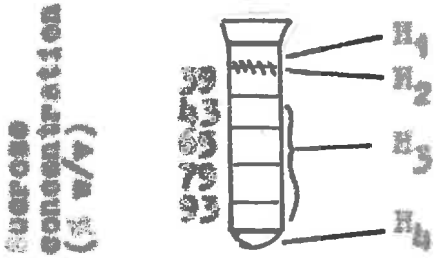
5 ml | 20 ml

Centrifuge 38,000 g/30 min.

$P_1$ | S

Layer onto discontinuous sucrose gradient 39.0-92.5% (w/v) Centrifuge 4,900 g/60 min.

FRACTIONS



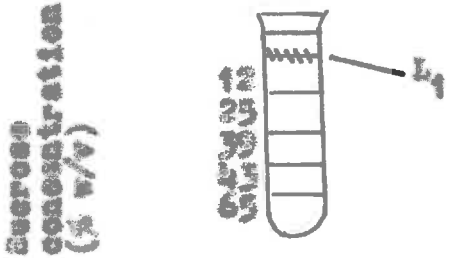

30 ml | 5 ml

Layer onto discontinuous sucrose gradient 12-65% (w/v) Centrifuge 4,900 g/60 min.

Centrifuge 38,000g/30 min

$P_2$ | S

FRACTIONS



Each sucrose solution contained 5% dextran and 2.5% Ficoll in 0.025 M Tris, pH 7.8. Gradients were prepared by layering 5 ml aliquots of different density, one on top of the other, with the heaviest in the bottom. At the end of the centrifugation, fractions were removed from the tubes with a syringe fitted with a 3 inch hypodermic needle bent in a right angle at the tip. The heaviest fractions were diluted with Honda medium to lower their densities (Table 3.3221-1) and then all fractions were centrifuged at 38,000 g for 30 minutes.

TABLE 3.3221-1

Sucrose density gradient fractions

| Fraction | Volume | Volume of Honda added to lower density |
|---|---|---|
| $H_1$ | 10 ml | 0 |
| $H_2$ | 10 ml | 0 |
| $H_3$ | 8 ml | 5 ml |
| $H_4$ | 7 ml | 15 ml |
| $L_1$ | 10 ml | 0 |

Pellets were examined in the electron microscope. The results are shown in Figures 3.312-6 and 3.3221-1, -2, -3, -4 and -5.

$P_1$ was similar to the 38,000 g pellet with well-preserved aleurone grains, spherosomes, and starch

FIGURE 3.3221-1

Electron micrograph of a homogenate of sucrose density gradient fractions, $P_2$ and $L_1$, of Scheme 3.3221-1. Predominantly spherosomal material but with some broken aleurone grains and aleurone grain inclusions.

Magnification: 15,000x.

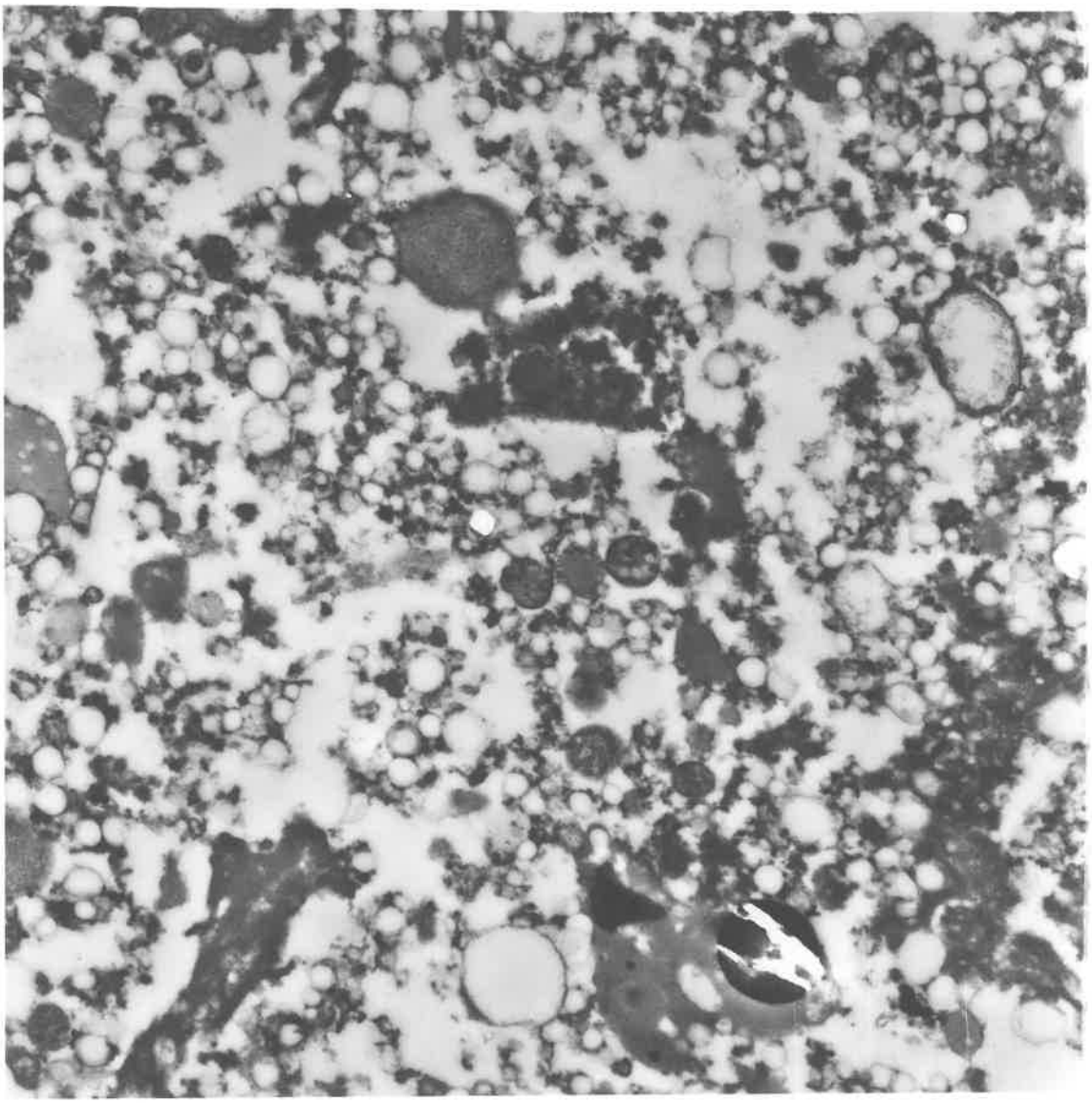


FIGURE 3.3221-2

Electron micrograph of sucrose density gradient fraction $H_4$ of Scheme 3.32221-1. Predominantly spherosomal aggregates; an occasional mitochondrian (bottom left); debris from ruptured aleurone grains.

Magnification: 11,700x.

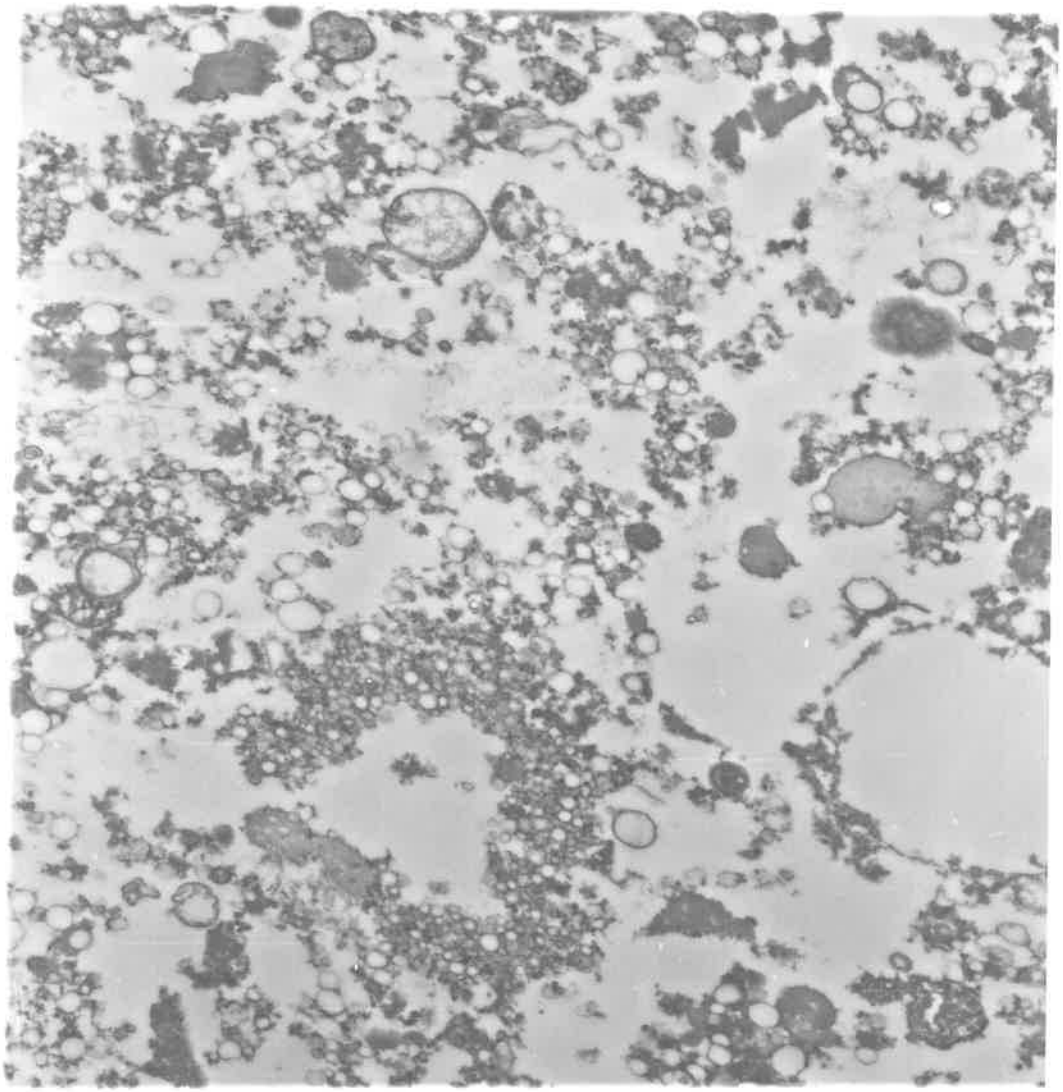


FIGURE 3.3221-3

Electron micrograph of sucrose density gradient fraction $H_2$ of Scheme 3.3221-1. Predominantly distorted and shattered aleurone and starch grains; cell wall debris.

Magnification: 4600x.

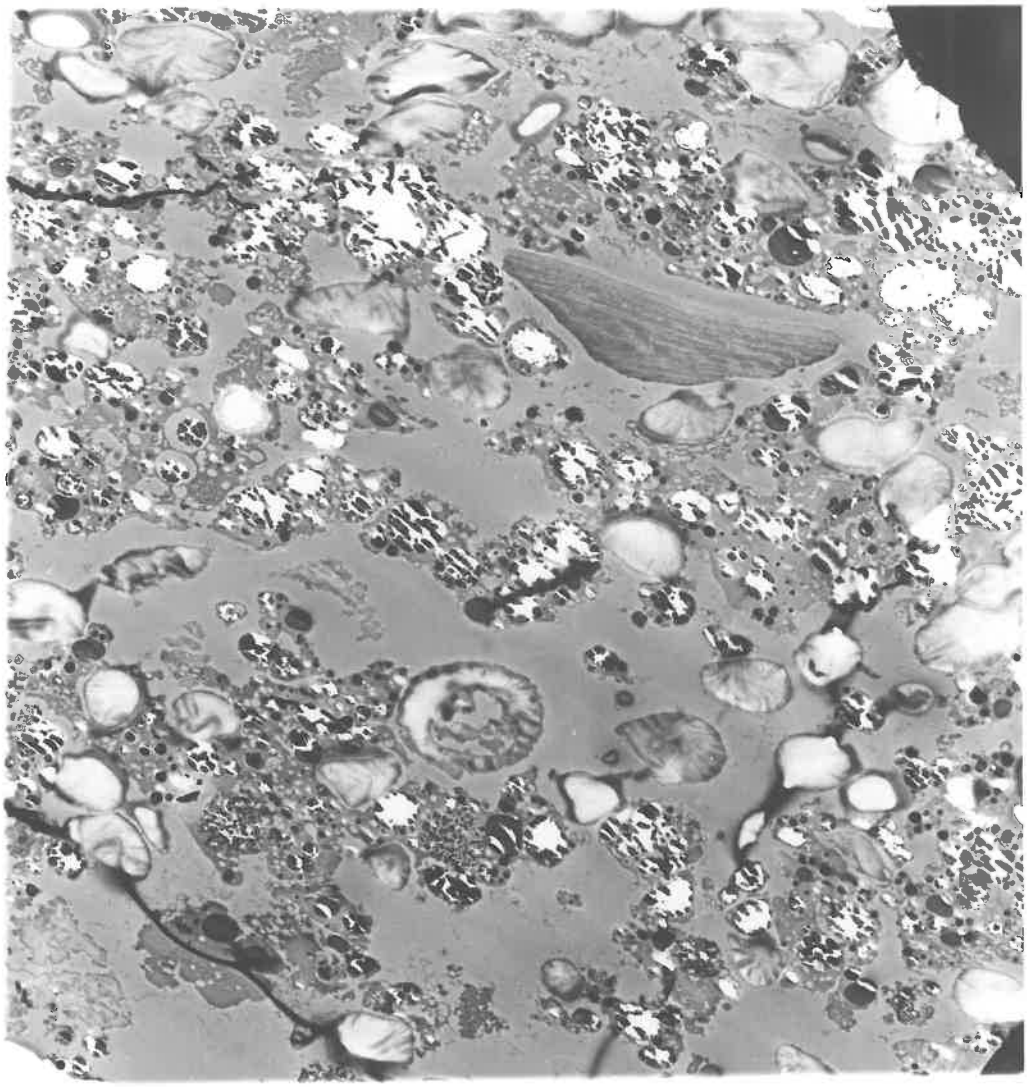


FIGURE 3.3221-4

Electron micrograph of sucrose density gradient fraction $H_3$ of Scheme 3.3221-1. Distorted aleurone grains. Magnification: 24,000x.

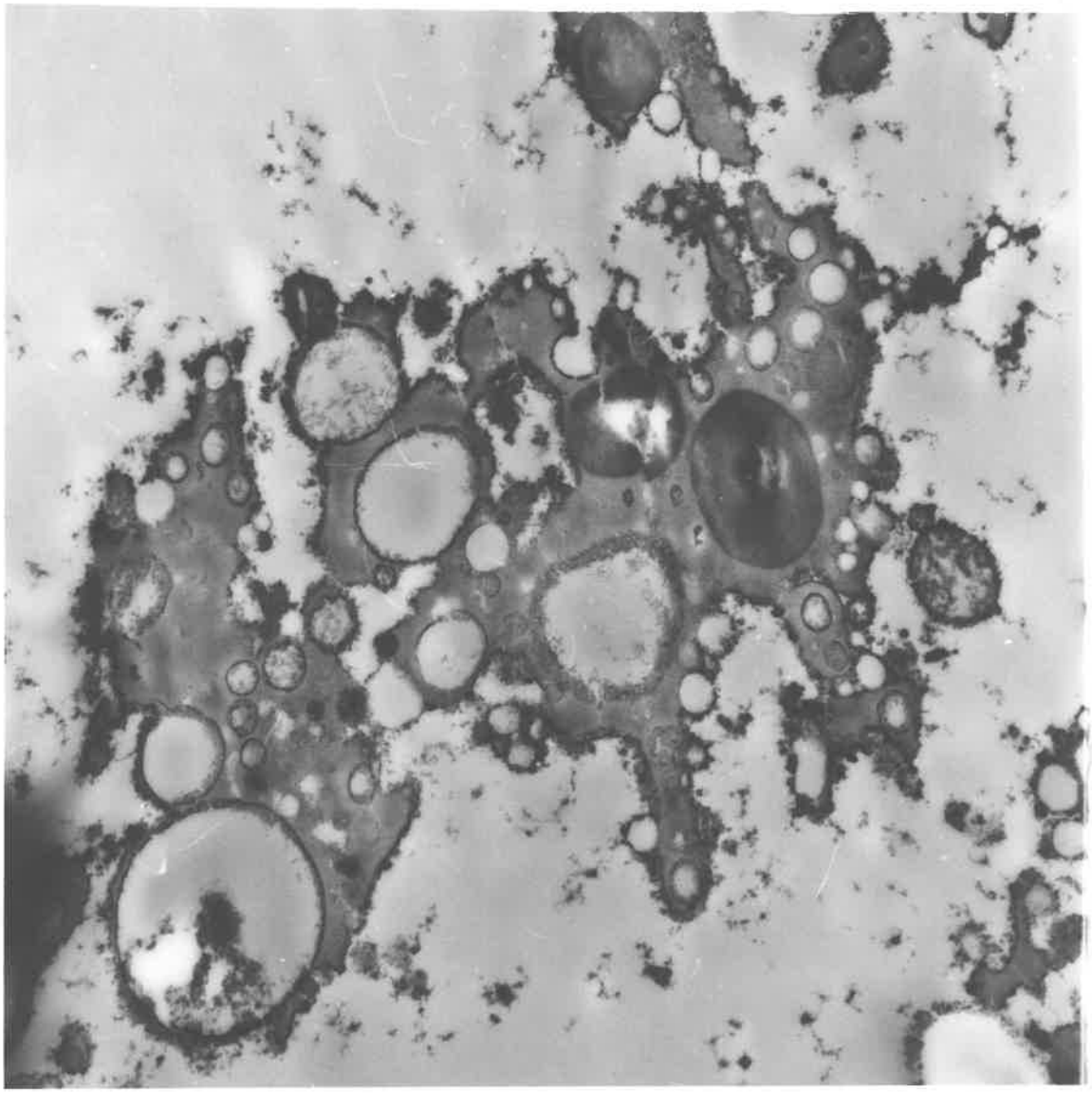
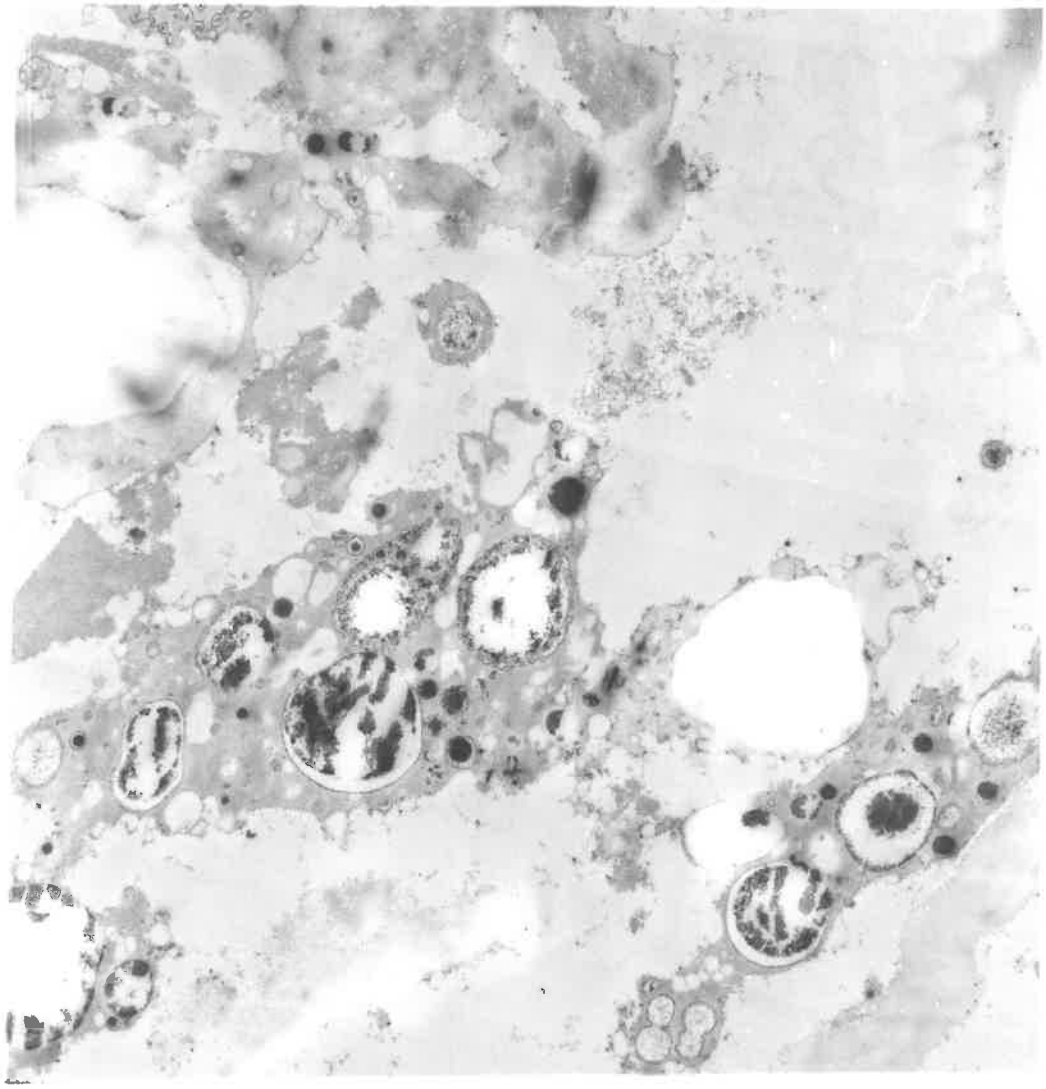


FIGURE 3.3221-5

Electron micrograph of sucrose density gradient fraction $H_4$ of Scheme 3.3221-1. Distorted aleurone grains; starch grains.

Magnification: 12,500x.



grains, but with less cell wall debris (Figure 3.312-6). $P_2$ and $L_1$ were very similar and very heterogeneous. Spherosomes were the most predominant component in the fraction but there were also fragments of aleurone grains, probably mitochondria and other debris (Figure 3.3221-1). $H_1$ was very similar to $P_2$ and $L_1$ but perhaps with more aggregates of the spherosomal material (Figure 3.3221-2). $H_2$ consisted of starch grains and shattered aleurone grains (Figure 3.3221-3). $H_3$ contained mainly aleurone grains which appeared to be very much distorted (Figure 3.3221-4). $H_4$ was similar to $H_3$ with starch granules and distorted aleurone grains (Figure 3.3221-5).

The aleurone grains were well preserved in the 350 g pellet in Honda medium but became distorted and disintegrated when centrifuged through dense sucrose solutions. On the other hand, the spherosomes in the supernatant fractions - whether layered onto sucrose gradients or not, seemed to resemble those of the whole cells. Therefore, it was concluded that:

a) the osmotic pressure of the dense sucrose solutions was too high to maintain the integrity of the aleurone grains, and

b) there was little advantage to the initial low speed centrifugation since fractions $P_2$, $L_1$ and $H_1$ were all very similar.

### 3.3222 Ficoll and Dextran density gradients

The deleterious effect of the high sucrose concentrations might be overcome if a compound with a high molecular weight were used. For a given density, the osmotic pressure would be lower. Ficoll, a polymer of sucrose, and a component of the isolating medium, has a molecular weight of 400,000 and is soluble at least to a concentration of 30% (corresponding to a specific gravity of about 1.172). If 0.25 M sucrose and 5% dextran are added to this, the specific gravity becomes about 1.21.

Scheme 3.3222-1 outlines an experiment designed to test Ficoll as a density gradient component.

Fractions were removed with a bent hypodermic needle. Fraction 4 was diluted with an equal volume of Honda medium and fraction 5 was resuspended in Honda. Each fraction was centrifuged at 38,000 g for 30 minutes. Pellets of fractions 2, 4 and 5 were examined in the electron microscope. Fractions 1 and 3 did not yield sufficient material for electron microscopy. Electron micrographs are shown in Figures 3.3222-1, -2 and -3.

Fraction 1 contained mainly broken aleurone grains and spherosomal aggregates (Figure 3.3222-1). Fraction 4 contained mainly aleurone grains contaminated with

SCHEME 3.3222-1

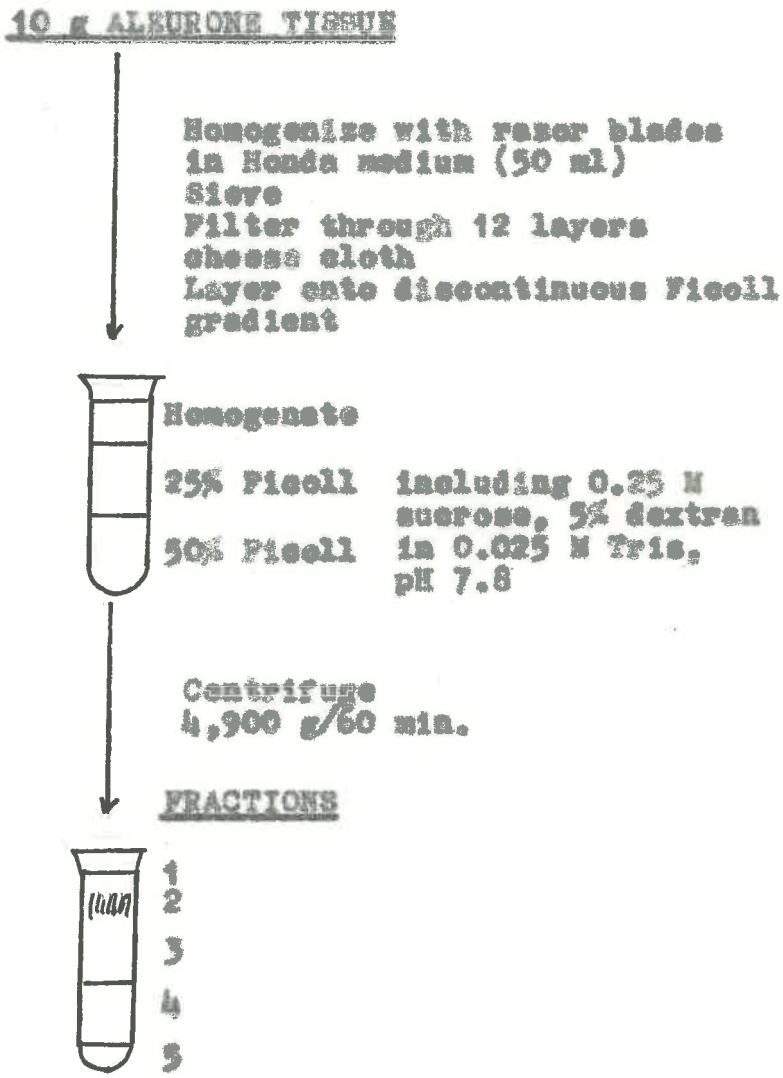


FIGURE 3.3222-1

Electron micrograph of Ficoll density gradient fraction 2 of Scheme 3.3222-1. Mainly spherosomal aggregates and aleurone grain fragments, some cell wall debris.

Magnification: 5800x.

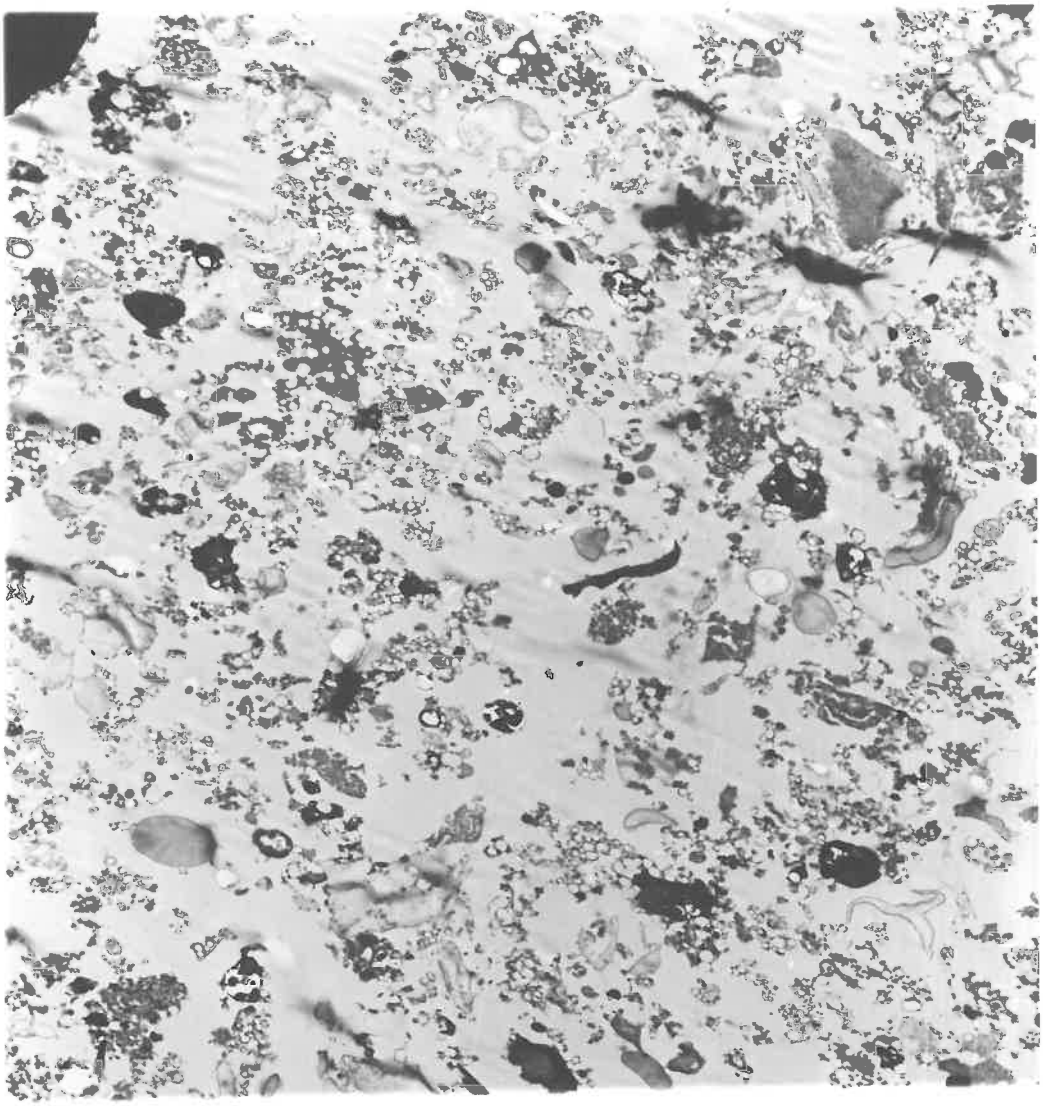


FIGURE 3.3222-2

Electron micrograph of Ficoll density gradient fraction 4 of Scheme 3.3222-1. Mainly damaged aleurone grains and some spherosomal aggregates.

Magnification: 20,700x.

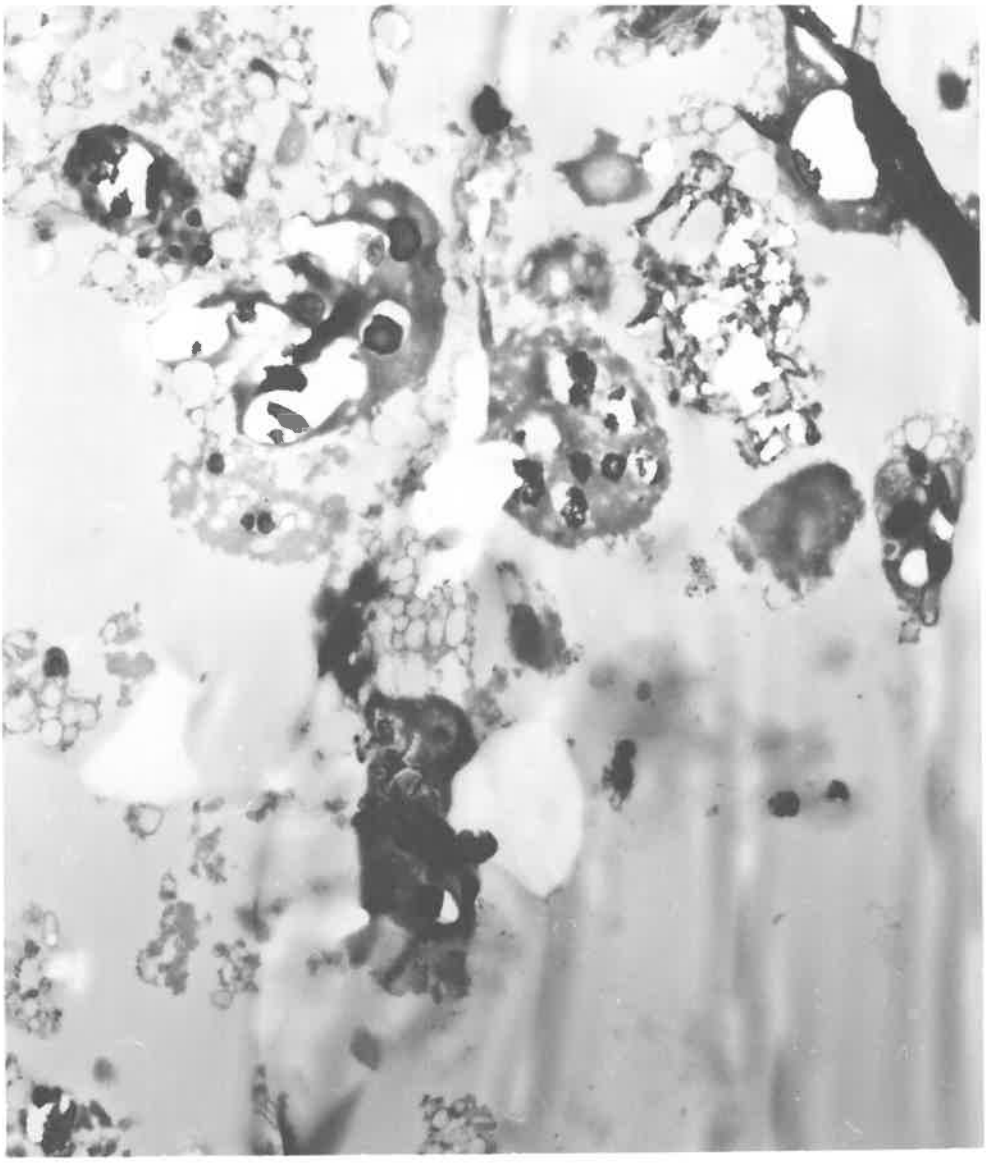


FIGURE 3.3222-2a

Electron micrograph of Ficoll density gradient fraction 4 of Scheme 3.3222-1. Occasionally a whole protoplast was observed in this fraction. Magnification: 11,000x.

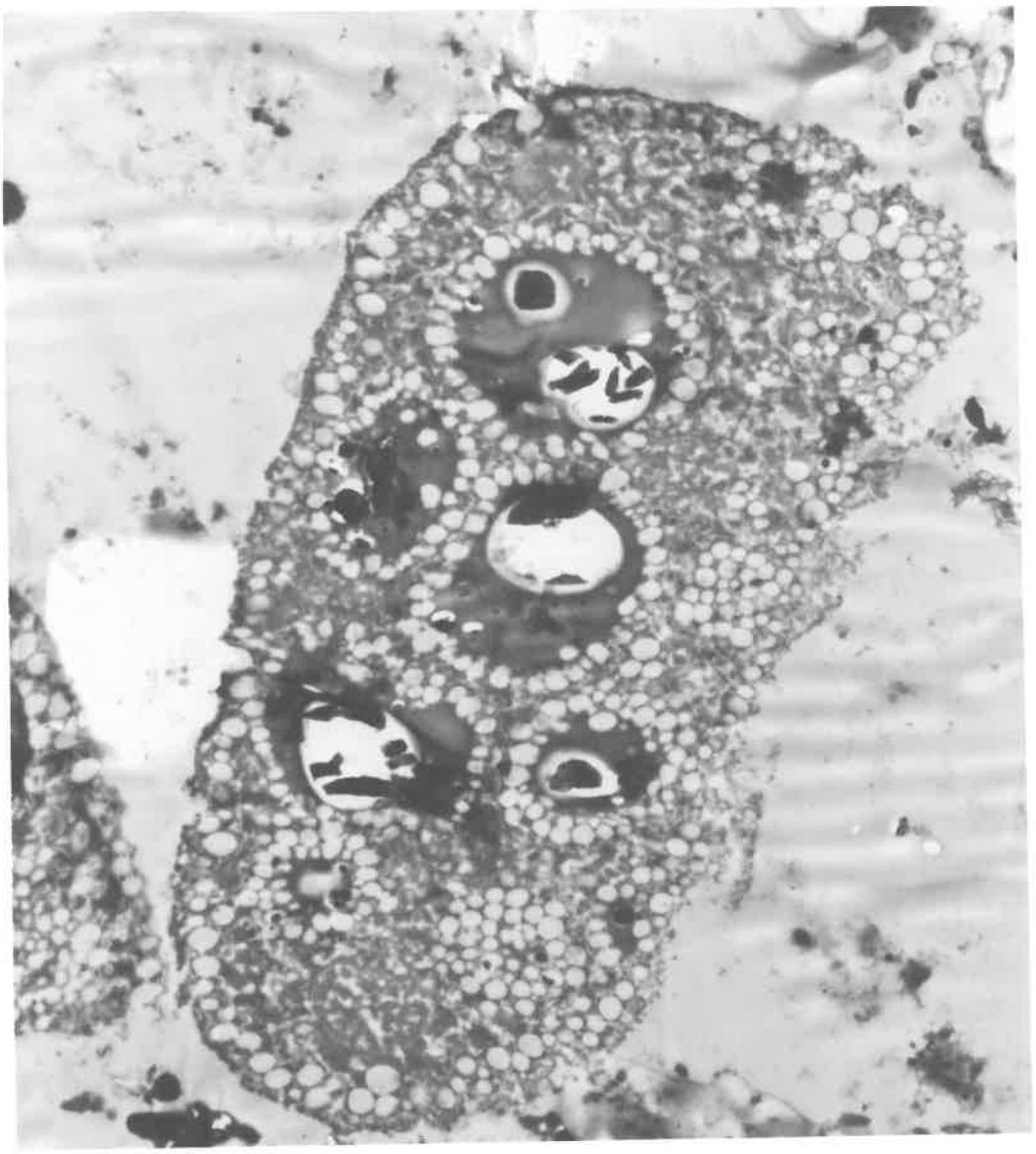
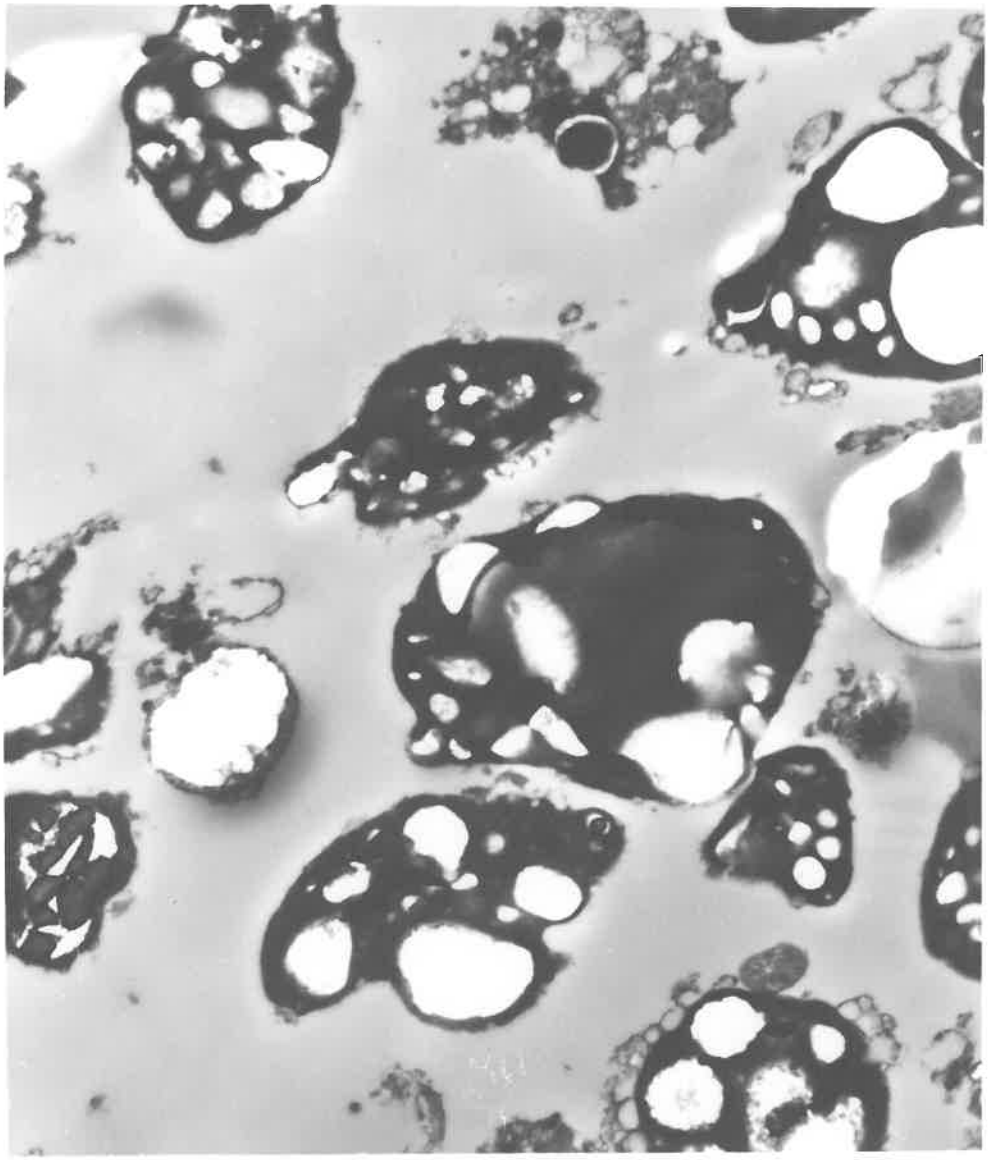


FIGURE 3.3222-3

Electron micrograph of Ficoll density gradient fraction 5 of Scheme 3.3222-1. Predominantly aleurone grains and starch grains.

Magnification: 3,000x.



spherosomes and a few whole protoplasts (Figure 3.3222-2). Fraction 5 contained mainly whole aleurone grains, some with spherosomes adhering to them, and starch grains (Figure 3.3222-3).

The aleurone grains, although somewhat distorted remained intact after centrifuging into the heavy Ficoll solution. Therefore, this would appear to be a more suitable density medium than sucrose for separation of the heavy aleurone and starch grains from the lighter fractions.

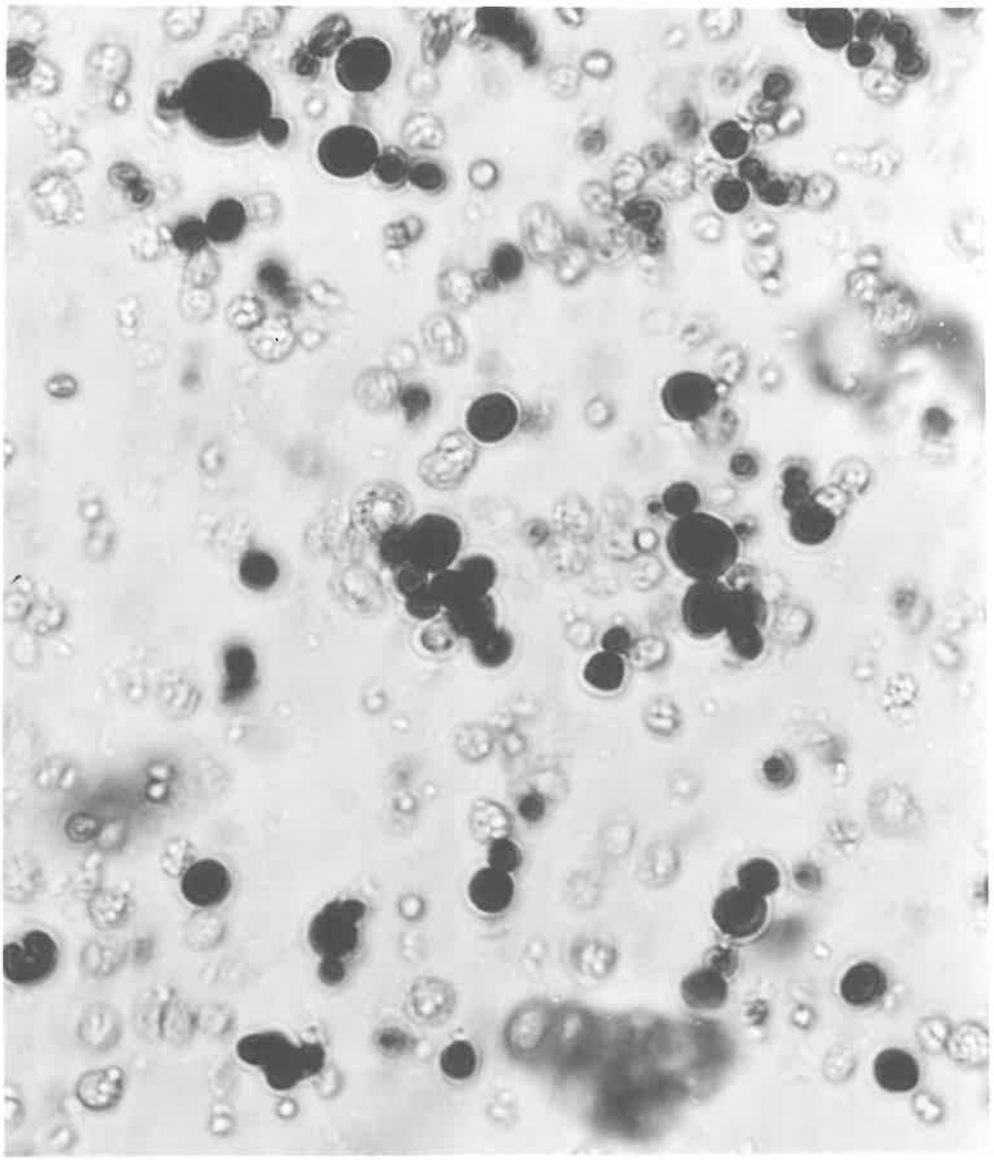
The heavy Ficoll solutions, however, were very viscous and therefore difficult to handle. According to Mack and Lasko (1968), dextran (MW 40,000) which is less viscous than Ficoll at the same density can be used as a density gradient component. When dextran was substituted for Ficoll and the previous experiment repeated, very similar results were obtained. The dextran was less viscous, easier to handle, and yielded intact aleurone grains as observed in the light microscope (Figure 3.3222-4). Although the molecular weight of dextran was $^{1}/_{10}$ that of Ficoll, its osmotic pressure in solution is still considerably less than that of sucrose in solution at the same concentration.

### 3.3223 Conclusions

Most of the heavy aleurone and starch grains were effectively separated from the lighter fractions

FIGURE 3.3222-4

Light micrograph of dextran density gradient fraction. Stained with IKI. Black particles are starch grains; other particles are aleurone grains or aleurone grain inclusions. Magnification: 1200x.



using Ficoll or dextran density gradients. The spherosomes and mitochondria were pelleted from Honda medium by centrifuging at 38,000 g for 30 minutes. However, no further fractionation of the lighter components was achieved.

## 3.4 Distribution of hydrolytic enzymes among sub-cellular fractions of aleurone tissue

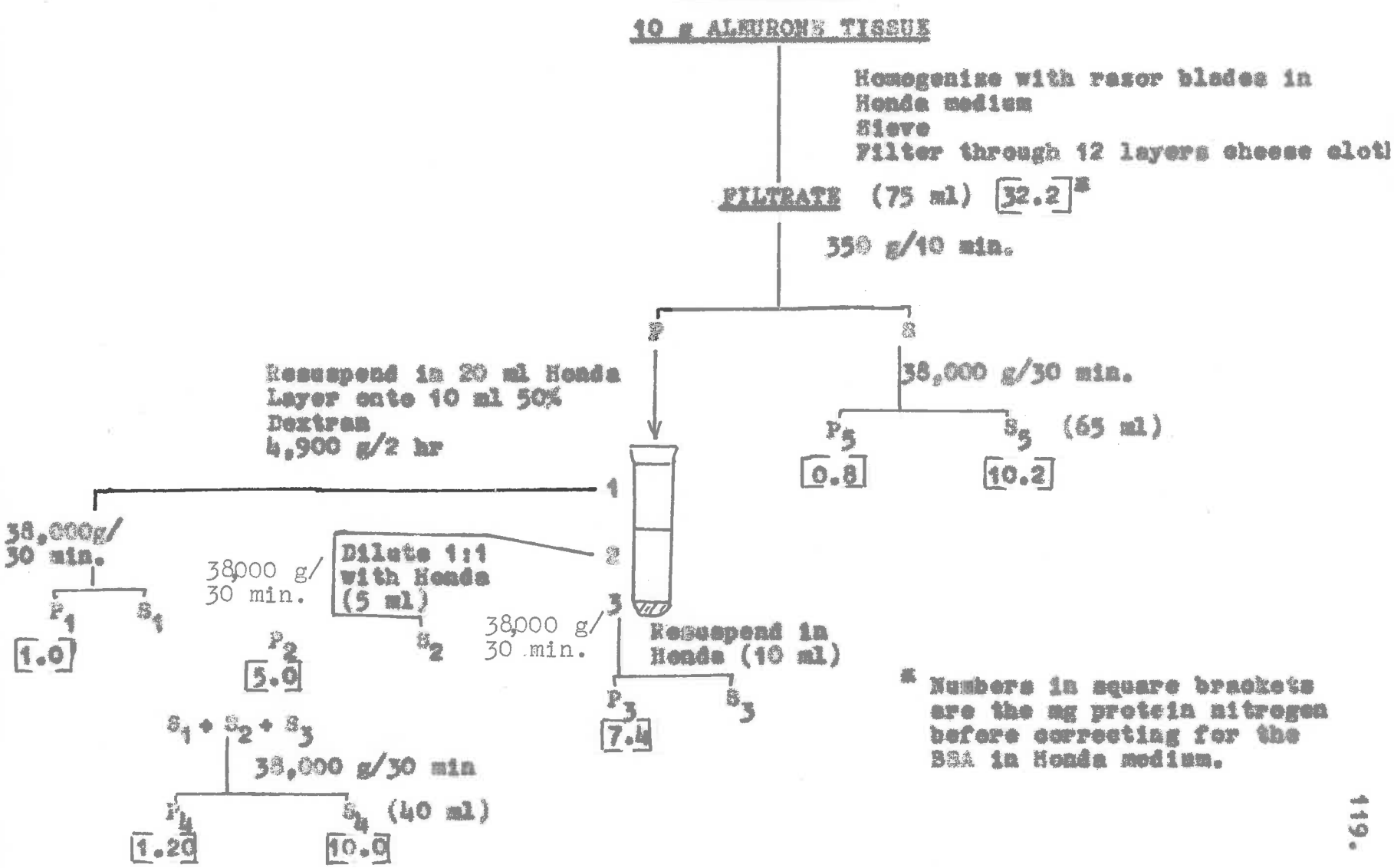
Phosphatase, proteinase and RN-ase, among other hydrolytic enzymes, increase in activity during GA treatment (see section 2). The development of methods for measuring the distribution of these enzymes among the various fractions of the untreated aleurone tissue is dealt with in the present section.

### 3.41 Distribution of total protein

Aleurone tissue was homogenized as described in section 3.312, and the homogenate fractionated as outlined in Scheme 3.41-1.

Protein in each fraction of Scheme 3.41-1 was isolated by precipitating with trichloroacetic acid (TCA) (enough TCA added to give a final concentration of 5%), washing the precipitates once with 10% TCA, and redissolving in 1 N NaOH. Protein was measured by the Biuret method. Since the Honda medium contained

SCHEME 3.41-1




* Numbers in square brackets are the mg protein nitrogen before correcting for the BSA in Honda medium.

0.1 mg/ml of bovine serum albumin (BSA), the supernatant values were corrected by subtracting the amount of BSA added, assuming that it was all in the supernatants.

TABLE 3.41-1

Distribution of protein nitrogen between particulate and soluble fractions of wheat aleurone tissue fractionated as outlined in Scheme 3.41-1.

| Fraction | Protein Nitrogen (mg) | Percent Distribution |
|---|---|---|
| Filtrate | 24.7 | - |
| Supernatant (combined) | 9.7 | 38.6 |
| Pellets (combined) | 15.4 | 61.4 |
| Recovery | 25.1 | 101.0 |

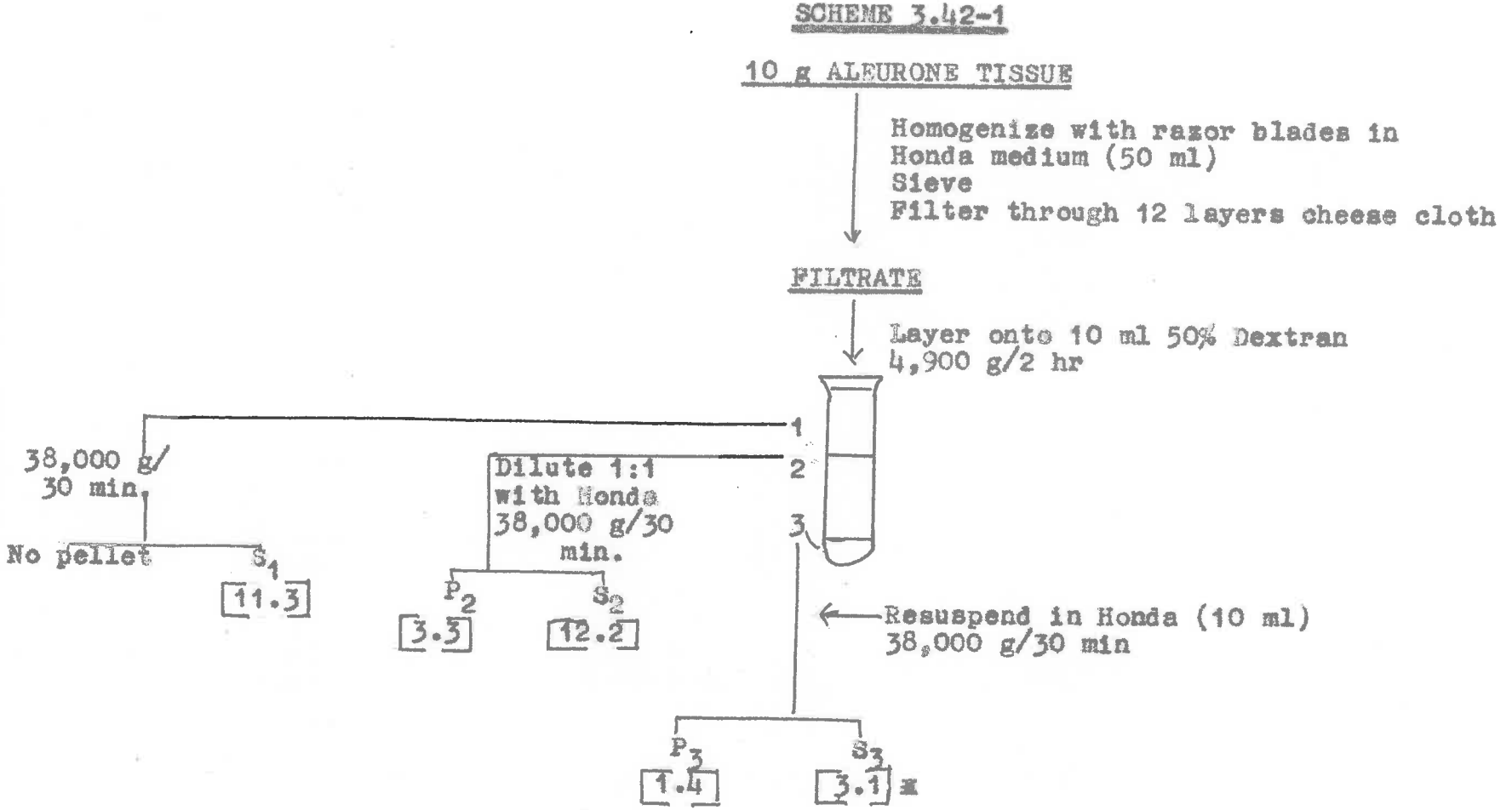
Table 3.41-1 shows that about 60% of the protein is present in the pellets and about 40% in the supernatant.

## 3.42 Distribution of acid phosphatase

The procedure was similar to that of section 3.41 with the exception that the initial 500 g centrifugation was omitted. The procedure is outlined in Scheme 3.42-1.

Acid phosphatase activity was measured by incubating aliquots of the various fractions with sodium β-glycerophosphate at 30°C as indicated below:

SCHEME 3.42-1




* The numbers in square brackets represent the total activity of acid phosphatase in each fraction. Pellets were resuspended in Honda medium for acid phosphatase determination.

Acid phosphatase assay:

| | |
|---|---|
| Sodium β-glycerophosphate (0.05 M) in Tris-acetate buffer (0.2 M, pH 5.0) | 0.5 ml |
| Enzyme preparation | 0.5 ml |
| Stop reaction by adding 10% TCA | 1.0 ml |

Centrifuge and measure inorganic phosphate in supernatants by the method of Taussky and Shorr (1953) using 1 mM sodium monohydrogen phosphate as a standard. Enzyme activity is expressed as the μmoles of inorganic phosphate liberated from the substrate per hour per total volume of the fraction.

Table 3.42-1 shows the distribution of the enzyme between particulate and soluble fractions. Most of the enzyme activity is present in the supernatant. However, there is an appreciable amount in the pellet.

TABLE 3.42-1

Distribution of acid phosphatase between particulate and soluble fractions of wheat aleurone tissue.

| Fraction | Acid Phosphatase (μmoles Pi released/ hour) | Percent Distribution |
|---|---|---|
| Pellets | 4.7 | 15.0 |
| Supernatants | 26.6 | 85.0 |
| Total | 31.3 | 100.0 |

## 3.43 Distribution of ribonuclease (RN-ase), acid proteinase and acid phosphatase

In order to be able to assay fractions for several enzymes at the same time, it was necessary to shorten the fractionation procedure. Scheme 3.43-1 represents the procedure finally adopted. Twenty-five percent dextran was substituted for the 30% used previously so that a shorter time could be used for the initial centrifugation. This means that the fractions obtained may be slightly different than previously. However, light and electron microscopic examination of the aleurone grain fraction showed that it was similar to that obtained with either 30% dextran or 30% Ficoll.

Fractions $P_1$ and $P_2$ were combined and made to 25 ml with Honda medium. Fraction S was made to 50 ml with Honda medium. Each fraction was assayed for acid phosphatase, acid ribonuclease and acid proteinase.

Enzyme assays:

a) Acid phosphatase

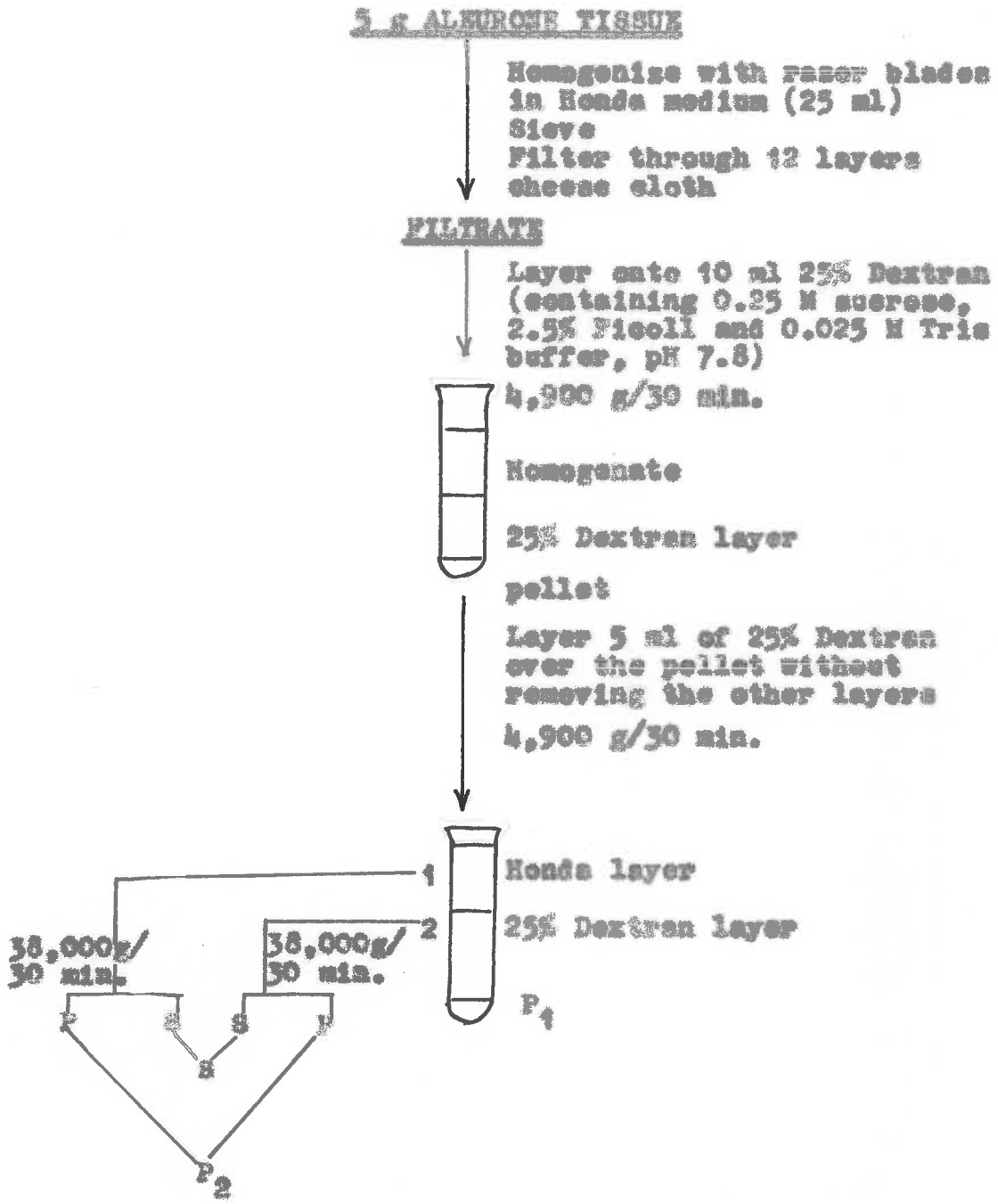
Procedure as described in section 3.42.

b) Acid ribonuclease

Incubation medium:

| | |
|---|---|
| * RNA (1% in 0.2 M tris-acetate, pH 5.0) | 0.4 ml |
| KCl (0.1 M) | 0.1 ml |
| Enzyme | 0.5 ml |
| Stop reaction with a mixture of 2.5% TCA and 0.25% uranyl acetate | 1.0 ml |

SCHEME 3.43-1


5 g ALEURONE TISSUE
Homogenise with razor blades in Honda medium (25 ml)
Sieve
Filter through 12 layers cheese cloth
FILTRATE
Layer onto 10 ml 25% Dextran (containing 0.25 M sucrose, 2.5% Ficoll and 0.025 M Tris buffer, pH 7.8)
4,900 g/30 min.
Homogenate
25% Dextran layer
pellet
Layer 5 ml of 25% Dextran over the pellet without removing the other layers
4,900 g/30 min.
1
Honda layer
2
25% Dextran layer
38,000g/ 30 min.
38,000g/ 30 min.
P1
P2

Let stand 5 minutes.
Centrifuge and dilute supernatants to 10 ml.
Measure optical density at 260 nm.
Enzyme activity expressed as the change in OD at 260 nm per hour per fraction.

x Yeast RNA (BDH) purified by precipitating from a 1% solution of RNA in 0.1 M acetate buffer, pH5 with three volumes of ethanol and one-fifth volume of glacial acetic acid (Cantoni and Davies, 1966).

e) Acid proteinase

Substrate:

Hemoglobin (BDH) 2.5% was dialysed at 2°C against 2 l of 1 mM EDTA for 16 hours, followed by 2 l distilled sterilised water for 8 hours. To 80 ml of this dialysed solution were added 20 ml of 0.3 M HCl, pH 2.0.

Incubation medium:

| | | |
|---|---|---|
| Hemoglobin | (2%, pH 2.0) | 0.5 ml |
| NaCl | (1 M, pH 2.0) | 0.5 ml |
| Enzyme | | 0.5 ml |
| Stop with 10% TCA | | 1.5 ml |

Centrifuge, dilute with water, and read optical density at 280 nm.
Enzyme activity expressed as the change in OD at 280 nm per hour per fraction.

TABLE 3.43-1

Distribution of acid phosphatase, ribonuclease and acid proteinase between particulate and soluble fractions of wheat aleurone tissue.

| Fraction | Acid Phosphatase | | Ribonuclease | | Acid Proteinase* | |
|---|---|---|---|---|---|---|
| | Activity | Percent | Activity | Percent | Activity | Percent |
| Pellet | 0.26 | 0.8 | 0 | 0 | 6.0 | 73.2 |
| Supernatant | 34.5 | 99.2 | 31.0 | 100 | 2.2 | 26.8 |

* This is the rate determined after 30 minutes incubation. There was no change in the OD from 0 to 30 minutes. From 30 to 90 minutes the rate was constant.

It appears that virtually all of the acid phosphatase, and ribonuclease are present in the supernatants. However, the acid proteinase was mainly in the pellet with an appreciable amount in the supernatants as well.

### 3.5 Distribution of hydrolytic enzymes among particulate and soluble fractions of wheat aleurone tissue incubated for various times in the presence or absence of GA

The distribution of three hydrolytic enzymes was determined in section 3.43 for untreated tissue. The present section deals with the distribution of these three enzymes and amylase between the same fractions after various times of incubation either in GA or in water.

Five gram samples of aleurone tissue were homogenised immediately or incubated with either GA (10 µg/ml) or water. For incubation each sample was divided into 1 g lots and each of these placed in a 9 cm petri dish with 8 ml of solution. The samples were incubated at 30°C for 6, 10 and 16 hours. At the end of these times the samples were transferred to a cold room (at 2°C), the ambient solutions decanted and the tissues rinsed once with 10 ml of ice cold water. Excess moisture was squeezed out of the tissue samples and they were homogenised as described in section 3.312.

The homogenates were fractionated according to

Scheme 3.43-1 and fractions $P_1$ and $P_2$ were each resuspended in 25 ml of Honda medium while fraction S was made to 50 ml. Each fraction was then assayed for acid phosphatase, ribonuclease, and acid proteinase as described in sections 3.42 and 3.43 and for total amylase as described in section 3.251.

The distribution of acid phosphatase and ribonuclease among the three fractions after 0, 6, 10 and 16 hours in GA or water is shown in Tables 3.5-1 and -2. Both enzymes were predominantly in the supernatant fractions at all times and there was no change in the distribution as a result of GA treatment. Neither enzyme increased after GA treatment at any of the incubation times.

Acid proteinase was measured as described previously but no activity was found in any fraction. The reason for this lack of activity is not clear. Either something interfered with the enzyme assay (such as irreversible binding of the enzyme to phytic acid or inactivation by the high pH) or there was no acid proteinase present.

Total amylase activity is shown in Table 3.5-3. Only the 6 and 16 hour samples were measured.

TABLE 3.5-1

Distribution of acid phosphatase among fractions of wheat aleurone tissue incubated for various times with or without GA (10 µg/ml).

| Time (hours) | 0 | 6 | 6 | 10 | 10 | 16 | 16 |
|---|---|---|---|---|---|---|---|
| Treatment | No treatmt. | Water | GA | Water | GA | Water | GA |
| S | 36.0(97.0)* | 20.0(91.1) | 28.3(91.5) | 33.3(95.2) | 33.0(96.5) | 22.2(88.3) | 32.2(95.5) |
| $P_2$ | 1.15(3.0) | 0.67(3.1) | 1.11(3.6) | 0.5(1.4) | 0 (0) | 1.50(6.0) | 0.67(1.9) |
| $P_1$ | 0 (0) | 1.28(5.8) | 1.50(4.9) | 1.15(3.3) | 1.15(3.5) | 1.44(5.7) | 0.89(2.6) |
| Total | 37.2(100) | 22.0(100) | 30.9(100) | 35.0(100) | 34.2(100) | 25.1(100) | 33.8(100) |

* Numbers in brackets represent percent of total enzyme

TABLE 3.5-2

The distribution of ribonuclease among fractions of wheat aleurone tissue incubated for various times with or without GA (10 μg/ml)

| Time (hours) | 0 | 6 | 6 | 10 | 10 | 16 | 16 |
|---|---|---|---|---|---|---|---|
| Treatment | No treatmt. | Water | GA | Water | GA | Water | GA |
| S | 10.7(100)[a] | 17.5(86.4) | 19.7(91.1) | 12.5(100) | 15.7(100) | 15.2(92.6) | 23.0(88.4) |
| $P_2$ | 0 (0) | 0.33(1.6) | 0.83(3.8) | 0 (0) | 0 (0) | 1.11 (6.8) | 0 (0) |
| $P_1$ | 0 (0) | 2.44(12.0) | 1.11(5.1) | 0 (0) | 0 (0) | 0.11(0.6) | 3.11(11.9) |
| Total | 10.7(100) | 20.3(100) | 21.6(100) | 12.5(100) | 15.7(100) | 16.4(100) | 26.1(100) |

[a] Numbers in brackets represent percent of total enzyme

TABLE 3.4-3

The distribution of total amylase among fractions of wheat aleurone tissue incubated for various times with water or GA (10 µg/ml). Fractionation as outlined in Scheme 3.43-1.

| Time (hours) | 6 | 6 | 16 | 16 |
|---|---|---|---|---|
| Treatment | Water | GA | Water | GA |
| S | 6,970(90.0)[a] | 6,390(86.1) | 9,050(93.3) | 12,780(54.6) |
| $P_2$ | 360(4.7) | 510(6.9) | 476(4.9) | 4,740(20.2) |
| $P_1$ | 407(5.3) | 521(7.0) | 175(1.8) | 5,900(25.2) |
| Total | 7,737(100) | 7,420(100) | 9,701(100) | 23,420(100) |

[a] Numbers in brackets represent percent of total enzyme

There was very little effect of GA on the amount or distribution of amylase at 6 hours. However, GA caused both an increase and a change in the distribution of amylase at 16 hours. Whereas over 90% of the enzyme was in the supernatant fraction of the water controls, only 55% was in this fraction of the GA-treated sample. The remainder was distributed between the pellets.

α-Amylase is synthesized de novo in response to GA whereas β-amylase, already present in the starchy endosperm, is activated. It was of interest to determine whether any of the enzyme in the pellets after 16 hours in GA was α-amylase.

The samples were stored at 0°C overnight and the total amylase of all three of the 16 hour GA fractions measured again. These three fractions along with the supernatant fractions of the other three samples were then made 5 mM with calcium acetate and heated at 70°C for 20 minutes to destroy β-amylase. The α-amylase activity was then measured. No activity was found in either the 6 hour supernatants or the 16 hour water control supernatant. The activity of the 16 hour GA fractions is shown in Table 3.3-4.

TABLE 3.5-4

Distribution of total amylase and α-amylase after storage of the 16 hour GA fractions for 24 hours at 0°C.

| Fraction | Total amylase (not stored) | Total amylase (stored 24 hours) | α-amylase (stored 24 hours) |
|---|---|---|---|
| S | 12,780 (54.6) | 13,820 (93.0) | 2,620 (89.8) |
| $P_2$ | 4,740 (20.2) | 184 (1.2) | 0 (0) |
| $P_1$ | 5,900 (25.2) | 880 (5.8) | 298 (10.2) |
| Total | 23,420 (100) | 14,884 (100) | 2,918 (100) |

There was no effect of storage on the amylase activity of the supernatant. However, the activity of both pellets was greatly decreased after 24 hours storage. α-Amylase accounted for at least part of the activity in the supernatant and in $P_1$. A proportion of the activity in the pellets (Table 3.5-3) was probably due to β-amylase which was possibly associated with the starch and which was activated by GA treatment. However, as noted above at least some of the particulate amylase was α-amylase.

Most of the GA-induced enzymes appeared to be localised in the 38,000 g supernatant. To test the possibility that these enzymes might be present in a very light particulate fraction, the experiment was repeated

and the 38,000 g supernatants further centrifuged at 150,000 g for 60 minutes.

Procedure

Five gram lots of aleurone tissue were incubated with water or GA (10 µg/ml) for 0, 6, 18 or 24 hours. At the end of these times the ambient solutions were decanted, each 1 g lot of tissues was rinsed once with 10 ml of water and the rinses combined with the ambient solutions which were then made to 100 ml with water and stored at 2°C until required. The tissue was then homogenized and fractionated as outlined in Scheme 3.5-1. The fractions were dealt with as follows:

$P_1$ - resuspended in 25 ml Honda

$P_2$ and $P_3$ - combined and resuspended in 50 ml Honda

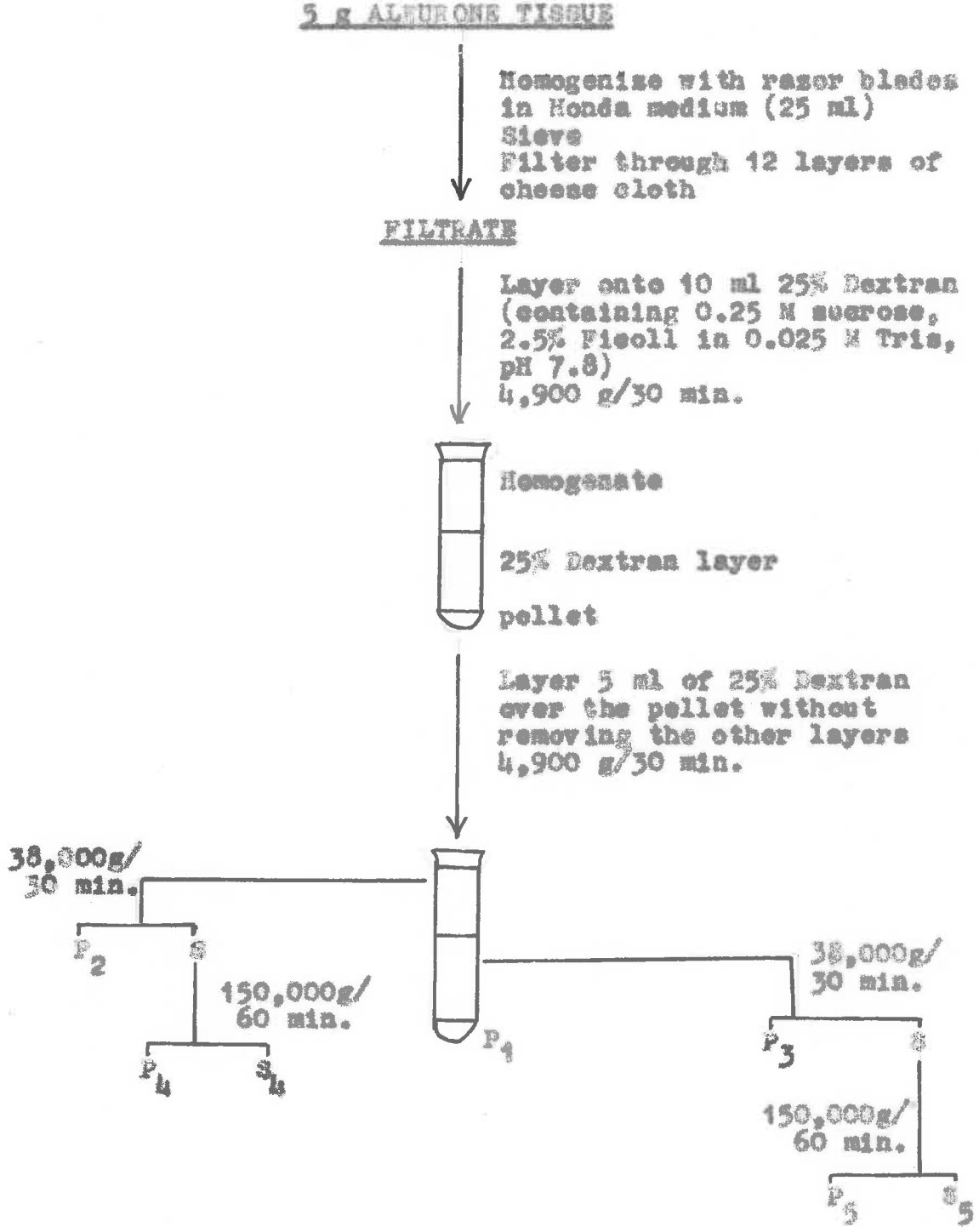
$P_4$ and $P_5$ - combined and resuspended in 10 ml Honda

$S_4$ and $S_5$ - combined and made to 50 ml with Honda.

Acid phosphatase, RN-ase and amylase were measured in each fraction as described for the previous experiment. The results are shown in Tables 3.5-5, -6, -7 and -8.

Once again, most of the acid phosphatase was present in the supernatant fraction. Only small amounts were pelleted at 150,000 g and there was no effect of GA on this fraction.

SCHEME 3.5-1


5 g ALEURONE TISSUE
Homogenize with razor blades in Honda medium (25 ml)
Sieve
Filter through 12 layers of cheese cloth
FILTRATE
Layer onto 10 ml 25% Dextran (containing 0.25 M sucrose, 2.5% Ficoll in 0.025 M Tris, pH 7.8)
4,900 g/30 min.
Homogenate
25% Dextran layer
pellet
Layer 5 ml of 25% Dextran over the pellet without removing the other layers
4,900 g/30 min.
38,000g/ 30 min.
P2
S
150,000g/ 60 min.
P4
S4
P1
38,000g/ 30 min.
P3
S
150,000g/ 60 min.
P5
S5

## TABLE 3.5-5

Distribution of acid phosphatase among fractions of wheat aleurone tissue incubated for various times with water or GA (10 μg/ml). Fractionation as outlined in Scheme 3.5-1.

| Time (hours) | 0 | 6 | 6 | 18 | 18 | 24 | 24 |
|---|---|---|---|---|---|---|---|
| Treatment | None | Water | GA | Water | GA | Water | GA |
| $S_4 + S_5$ | 16.6(87.6)[a] | 33.4(92.0) | 33.9(95.0) | 19.9(86.2) | 25.4(88.6) | 25.5(93.8) | 39.9(95.4) |
| $P_4 + P_5$ | 0.3(1.6) | 0.4(1.1) | 0.3(0.8) | 0.5(2.1) | 0.7(2.4) | 0 (0) | 0 (0) |
| $P_2 + P_3$ | 1.1(5.5) | 2.1(5.8) | 1.1(3.1) | 1.5(6.5) | 1.5(5.2) | 0.9(3.3) | 1.0(2.4) |
| $P_1$ | 1.0(5.3) | 0.4(1.1) | 0.4(1.1) | 1.2(5.2) | 1.1(3.8) | 0.8(2.9) | 0.9(2.2) |
| Total | 19.0(100) | 36.3(100) | 35.7(100) | 23.1(100) | 28.7(100) | 27.2(100) | 41.8(100) |

[a] Numbers in brackets represent percent of total enzyme

## TABLE 3.5-6

Distribution of ribonuclease among fractions of wheat aleurone tissue incubated for various times with water or GA (10 µg/ml). Fractionation as outlined in Scheme 3.5-1.

| Time (hours) | 0 | 6 | 6 | 18 | 18 | 24 | 24 |
|---|---|---|---|---|---|---|---|
| Treatment | None | Water | GA | Water | GA | Water | GA |
| $S_4 + S_5$ | 20.8(82.8)[a] | 33.3(74.7) | 36.5(76.2) | 20.2(94.8) | 34.5(91.3) | 26.8(100.0) | 45.3(92.6) |
| $P_4 + P_5$ | 0.6(2.4) | 1.0(2.2) | 0.9(1.9) | 0 (0) | 0 (0) | 0 (0) | 0.8(1.6) |
| $P_2 + P_3$ | 2.0(8.0) | 7.3(16.4) | 7.3(15.2) | 1.1(5.2) | 3.3(8.7) | 0 (0) | 1.8(3.7) |
| $P_1$ | 1.7(6.8) | 3.0(6.7) | 3.2(6.7) | 0 (0) | 0 (0) | 0 (0) | 1.0 (2.1) |
| Total | 25.1(100) | 44.6(100) | 47.9(100) | 21.3(100) | 37.8(100) | 26.8(100) | 48.9(100) |

[a] Numbers in brackets represent percent of total enzyme.

TABLE 3.5-7

Distribution of total amylase among fractions of wheat aleurone tissue incubated for various times with water or GA (10 µg/ml). Fractionated as outlined in Scheme 3.5-1.

| Time (hours) | 0 | 6 | 6 | 18 | 18 | 24 | 24 |
|---|---|---|---|---|---|---|---|
| Treatment | None | Water | GA | Water | GA | Water | GA |
| $S_4 + S_5$ | - | 6,768(88.7)[a] | 7,520(88.2) | 10,655(90.6) | 51,760(89.1) | 9,450(62.1) | 34,780(62.4 |
| $P_4 + P_5$ | - | 0 (0) | 0 (0) | 348(2.9) | 1,721(3.0) | 1,165(7.7) | 6,450(11.6 |
| $P_2 + P_3$ | - | 694(9.6) | 721(8.4) | 0 (0) | 1,758(3.0) | 598(3.9) | 10,450(18.7 |
| $P_1$ | - | 208(2.7) | 288(3.4) | 775(6.5) | 2,840(4.9) | 3,990(26.3) | 4,050(7.3) |
| Total | - | 7,630(100) | 8,529(100) | 11,778(100) | 58,079(100) | 15,203(100) | 55,730(100) |

[a] Numbers in brackets represent percent of total enzyme.

TABLE 3.5-8

Distribution of α-amylase among fractions of wheat aleurone tissue incubated for various times with water or GA (10 μg/ml). Fractionated as outlined in Scheme 3.5-1.

| Time (hours) | 18 | 18 | 24 | 24 |
|---|---|---|---|---|
| Treatment | Water | GA | Water | GA |
| $S_4 + S_5$ | - | 7,785(95.9)* | 2,993(56.8) | 41,035(85.7) |
| $P_4 + P_5$ | - | 0 (0) | 322(6.1) | 861(1.8) |
| $P_2 + P_3$ | - | 333(4.1) | 0 (0) | 2,335(4.9) |
| $P_1$ | - | 0 (0) | 1,955(37.1) | 3,650(7.6) |
| Total | | 8,118(100) | 5,270(100) | 47,881(100) |

* Numbers in brackets represent percent of total enzyme.

RN-ase also was predominantly in the supernatant particularly at 18 and 24 hours. Its distribution among the fractions did not appear to be affected by GA although GA caused an increase in the total amount of enzyme after 24 hours.

Total amylase was located mainly in the supernatant at both 6 and 18 hours. However, there was an increase in amylase in all fractions after 18 hours in GA. At 24 hours almost 40% of the total amylase was present in the pellets in both water controls and GA samples. GA caused a considerable increase in amylase particularly in the supernatant, 38,000 g pellet and 150,000 g pellet. Table 3.5-8 shows that α-amylase makes up a large percentage of this enzyme activity particularly after 24 hours in GA and especially in the supernatant and in the 4,900 g pellet ($P_4$).

Since the GA-induced enzymes are largely secreted by the aleurone layers into the surrounding medium, the amount of these enzymes was also measured in the ambient solutions. The distribution of the enzymes between the ambient solutions and the tissue is shown in Table 3.5-9.

GA had no effect on the distribution of acid phosphatase until 24 hours when a greater proportion was found in the ambient solutions. Similarly there was no effect of GA on the distribution of RN-ase even at

TABLE 3.5-9

Distribution of acid phosphatase, ribonuclease, total amylase and α-amylase of wheat aleurone tissue incubated for various times with water or GA (10 μg/ml) between the tissue and the ambient solutions.

ACID PHOSPHATASE

| Time (hours) | 0 | 6 | 6 | 18 | 18 | 24 | 24 |
|---|---|---|---|---|---|---|---|
| Treatment | None | Water | GA | Water | GA | Water | GA |
| Tissue | 19.0 | 36.3(59.6) | 35.7(60.5) | 23.1(49.3) | 28.7(57.4) | 27.0(100) | 41.8(28.7) |
| Ambient | - | 24.6(40.4) | 23.3(39.5) | 23.7(50.7) | 21.3(42.6) | 0 (0) | 104.0(71.3) |
| Total | 19.0 | 60.9(100) | 59.0(100) | 46.8(100) | 50.0(100) | 27.0(100) | 146 (100) |

RIBONUCLEASE

| Time (hours) | 0 | 6 | 6 | 18 | 18 | 24 | 24 |
|---|---|---|---|---|---|---|---|
| Treatment | None | Water | GA | Water | GA | Water | GA |
| Tissue | 25.1 | 44.6(52.7) | 47.0(51.4) | 21.3(49.8) | 37.8(53.2) | 26.8(100) | 48.9(84) |
| Ambient | - | 40.1(47.3) | 44.5(48.6) | 21.5(50.2) | 33.2(46.8) | 0 (0) | 9.4(16) |
| Total | 25.1 | 84.7(100) | 91.5(100) | 42.8(100) | 71.0(100) | 26.8(100) | 58.3(100) |

(continued)

TABLE 3.5-9 (continued)

TOTAL AMYLASE

| Time (hours) | 0 | 6 | 6 | 18 | 18 | 24 | 24 |
|---|---|---|---|---|---|---|---|
| Treatment | None | Water | GA | Water | GA | Water | GA |
| Tissue | - | 7,630(79.5) | 8,529(81.3) | 11,778(29.2) | 58,079(25.4) | 15,203(24.0) | 55,730(8.8) |
| Ambient | - | 1,970(20.5) | 1,970(18.7) | 28,630(70.8) | 171,200(74.6) | 48,210(76.0) | 577,000(91.2 |
| Total | - | 9,600(100) | 10,499(100) | 40,408(100) | 229,279(100) | 63,413(100) | 632,730(100) |

α-AMYLASE

| Time (hours) | 0 | 6 | 6 | 18 | 18 | 24 | 24 |
|---|---|---|---|---|---|---|---|
| Treatment | None | Water | GA | Water | GA | Water | GA |
| Tissue | - | - | - | - | 8,118(37.4) | 5,270(44.1) | 47,881(20) |
| Ambient | - | - | - | - | 13,550(62.6) | 6,670(55.9) | 191,800(80) |
| Total | - | - | - | - | 21,668(100) | 11,940(100) | 239,681(100) |

* Numbers in brackets represent percent of total enzyme.

24 hours. At 6 and 18 hours RN-ase was more or less evenly distributed between the tissues and the ambient solutions, while at 24 hours most of it was found inside the tissue. Amylase, on the other hand, was predominantly inside the tissue at 6 hours but 70 to 75% was in the ambient solution at 18 hours in both water control and GA-treated samples. At 24 hours there was no further change in the distribution in the water controls but over 90% of the amylase of the GA samples was in the ambient solutions. The distribution of α-amylase followed a similar pattern at 18 and 24 hours but with a somewhat larger proportion of the enzyme inside the tissue.

Conclusions:

Small amounts of the enzymes are always found in particulate fractions but these generally represent only a small proportion of the total. There does not appear to be any pronounced increase in any of the enzymes in the 150,000 g pellet. Large amounts of the enzymes, acid phosphatase and amylase, are secreted into the ambient solutions particularly at 24 hours, whereas RN-ase is retained inside the tissue.

# 4. DISCUSSION

## 4.1 The mass isolation technique

In answer to a question about isolating a cell-free protein-synthesizing system from barley aleurone cells, Varner replied: "These cells have very heavy cell walls, and there is some problem of logistics in getting hundreds of grams or even tens of grams of aleurone layers" (in discussion of paper by Varner et al., 1965). Duffus (1967) also stated that " ... the isolation of aleurone in quantity, free from starchy endosperm, is a very time-consuming task and changes are taking place in the aleurone throughout this period". The method for isolating wheat aleurone tissue, developed in the present investigation, has overcome these difficulties.

The isolation of barley aleurone tissue required pre-incubation of the half-seed in water for two to three days, whereas the wheat system requires only 26 hours (including the 2 hour sterilisation period in calcium hypochlorite). Hand-peeling the barley half-seed was time-consuming, the tissue obtained was heterogeneous because of variations in time and handling of individual layers, and only relatively small quantities could be obtained. The wheat system, on the other hand, requires only 45 minutes for the isolation of ten to twenty grams

of tissue. Larger or smaller amounts can be obtained with slight modifications to the procedure. The nature of the method allows for very strict control over treatment of the tissue from one experiment to another and results in a reproducible population of uniformly treated aleurone layers. Although there is still some contamination from the starchy endosperm, this is reproducible (section 3.133) and is probably not appreciably different from that of the hand-peeled barley aleurone tissue.

## 4.2 The response of the wheat tissue to GA

The response of the wheat aleurone tissue to GA is qualitatively similar to that of barley aleurone. α-Amylase production increases with increasing GA concentration and there is no real optimum for the response (section 3.21). Similar results had been found previously for the production of reducing sugars by barley half-seed (MacLeod and Millar, 1962) and for the production of α-amylase by barley aleurone layers (Chrispeels and Varner, 1967a).

α-Amylase is produced after a short lag period and is secreted into the medium. The time-course of development of the enzyme is comparable with that of

barley tissue isolated by hand-peeling, enzyme digestion or grinding soaked half-seed with a mortar and pestle (Varner and Ram Chandra, 1964; Yomo and Iinuma, 1964; MacLeod *et al.*, 1964). In the experiment reported in section 3.22, an increase in α-amylase production was detected after 16 hours incubation with GA. However, a relatively insensitive method for assaying the enzyme was used in this experiment. When the more sensitive method described in section 3.25 was used, increased α-amylase production was evident after only 9 hours incubation. This agrees well with the time course of development of α-amylase by hand-peeled barley aleurone layers reported by Chrispeels and Varner (1967a).

The pH studies of section 3.23 indicated that α-amylase production was enhanced at low pH. However, there was always a shift during the incubation period to between pH 4.0 and 4.5. A similar enhancement at low pH was found for the GA-induced sugar release by barley half-seed and a pH optimum of about 4.3 was established for this response using higher concentrations of the buffers (Paleg, unpublished). Briggs (1968) also found a drift in pH to 3.01, 3.58 and 4.00 when barley half-seed were incubated with unbuffered GA (50 µg/ml). This shift in pH is probably due to accumulation of fatty and amino acids in the ambient solutions following

hydrolysis of lipids and proteins by the GA-induced enzymes. Thus, the wheat tissue is similar to the barley half-seed in respect to both the low pH enhancement and the pH shift during incubation.

The activity of α-amylase in the ambient solutions was enhanced by $2 \times 10^{-2}$ M calcium ions (section 3.24) as was also found for barley α-amylase (Chrispeels and Varner, 1967a). Chrispeels and Varner showed that the calcium effect was on the stability of the enzyme in solution rather than on its synthesis or secretion. This is probably also true for the wheat system since there was no effect of calcium on the amount of enzyme remaining in the tissue.

Actinomycin D (50 and 100 µg/ml) inhibited the production of α-amylase by the wheat aleurone tissue by 99% whereas it inhibited the barley system by only 60% (Chrispeels and Varner, 1967a). This difference in sensitivity to the antibiotic could be due to a number of factors. Since the wheat aleurone layers are only one cell thick, it is conceivable that the antibiotic penetrated more rapidly and perhaps in a higher concentration than into the barley tissue which is three cells thick. The effect of the antibiotic on glucose metabolism reported by Honig and Rabinovitz (1965)

was not responsible for the inhibition in wheat tissue since addition of glucose did not cause a significant reversal of the inhibition. Indeed, the small amount of recovery observed with glucose could be entirely accounted for by the enhancement of α-amylase production caused by glucose in the absence of actinomycin D (section 3.251). Another possible explanation for the greater inhibition by actinomycin D in the wheat tissue may be that the method of isolation, although relatively gentle, is harsher than hand-peeling. This may result in a wounding effect which in other tissue causes an increase in RN-ase activity (Bagi et al., 1967). The increased RN-ase may, in turn, result in destruction of RNA which must be replenished before protein synthesis can proceed. Actinomycin D would prevent this replenishment.

In general, then, the wheat aleurone tissue responds to GA in a way which is qualitatively similar to barley aleurone tissue. The ease with which it is obtained, the uniformity of handling which is possible and the large quantities which can be secured make it a very attractive experimental material with which to study the GA response.

## 4.3 Evidence for particulate enzymes

The electron microscope studies of the aleurone tissue of germinating barley seed (section 2.82) suggested that vesicles were pinched off from endoplasmic reticulum, transported to the plasmalemma and discharged outside the cell (Van der Eb and Nieuwdorp, 1967). Similar studies of wheat aleurone tissue (isolated by the method in section 3.13 and incubated for various times with GA) indicated that different kinds of sub-cellular structures appeared as a result of GA treatment (compare Figures 4.3-1 and -2 with Figure 3.312-1). These structures, which were irregular in outline and electron dense after $KMnO_4$ fixation, were observed fairly frequently after 24 hours treatment with GA. Occasionally, as shown in the figures, these particles appeared to be discharging their contents at the cell membrane. However, this latter phenomenon was observed infrequently and only at specific regions of the cell wall. For this and other reasons the electron micrograph studies are as yet inconclusive. Nevertheless, they do suggest the possibility that the GA induced enzymes of the wheat aleurone tissue may be present in particulate fractions. The large amounts of reactive tissue made available by the mass-isolation technique permitted the investigation of methods for homogenizing and fractionating the

FIGURE 4.3-1

Electron micrograph of part of an aleurone cell from tissue that has been incubated with GA (100 μg/ml) for 24 hours. Cell wall extends across the bottom; dense irregular particles scattered throughout the cell and appear to be discharging their contents at the cell wall. Magnification: 22,500x.

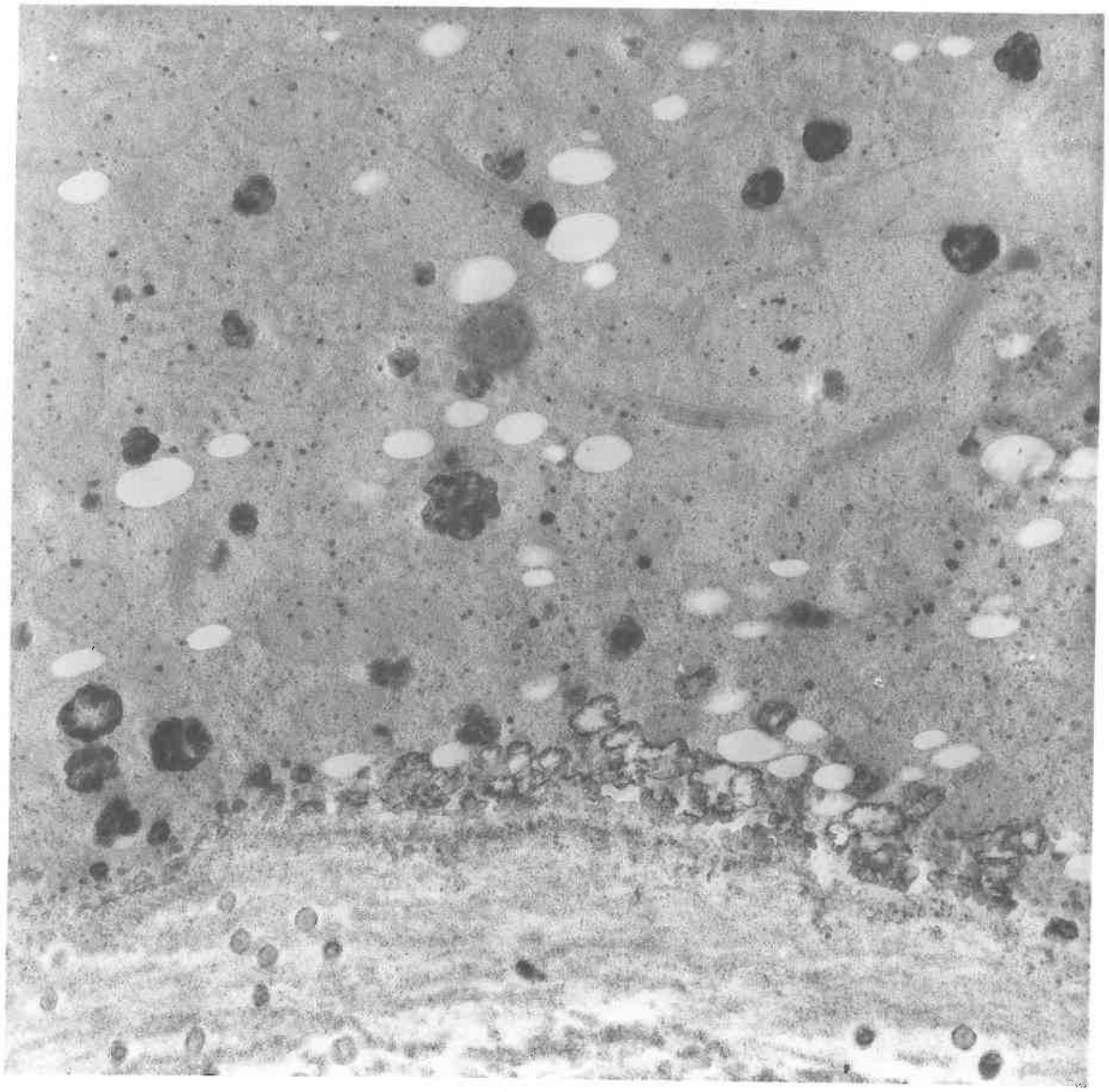
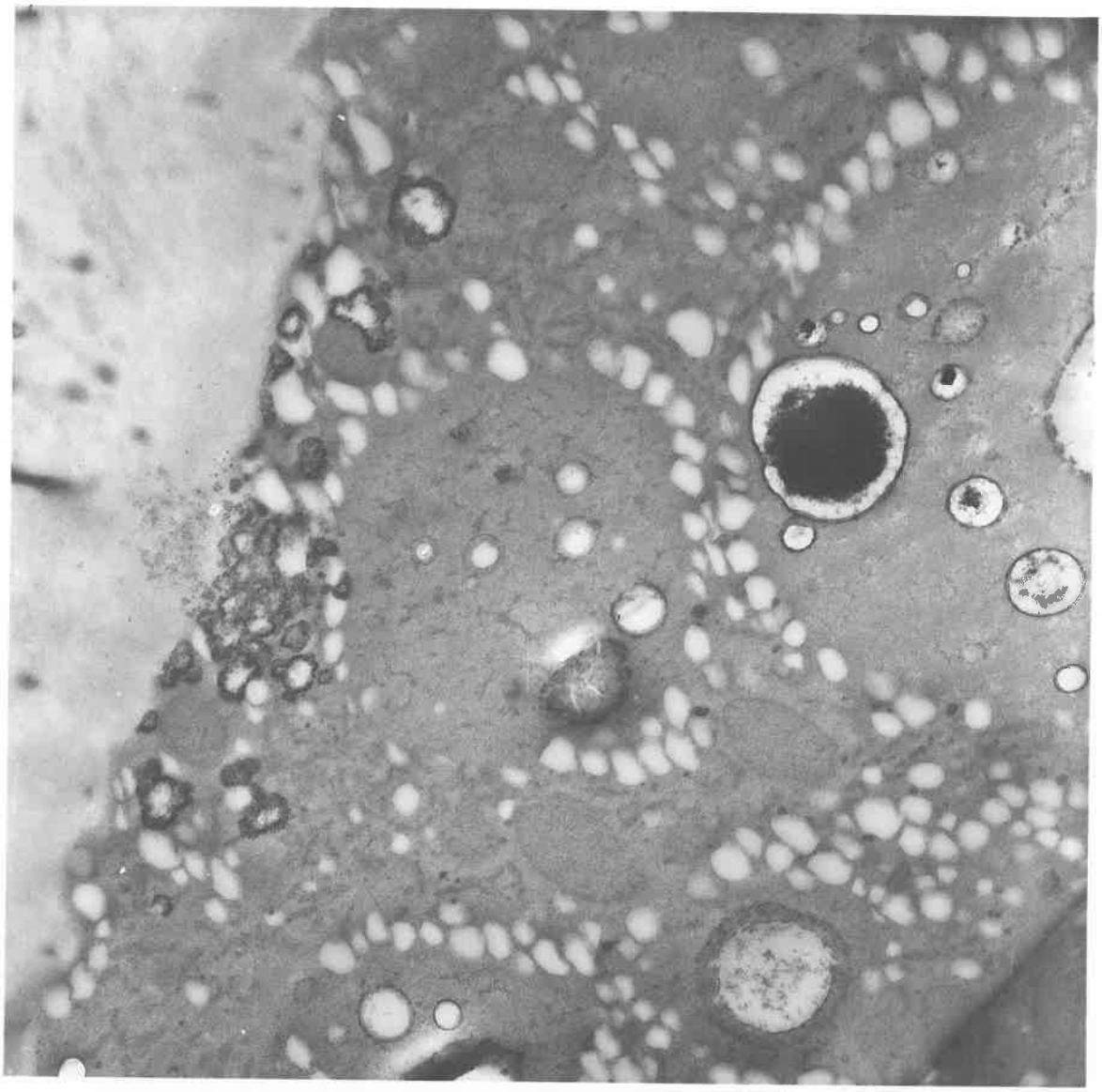


FIGURE 4.3-2

Electron micrograph of part of an aleurone cell from tissue that has been incubated with GA for 24 hours. Cell wall extends from top to bottom along the left-hand side; particles appear to be discharging their contents into the cell wall. Magnification: 22,500x.



aleurone tissue in order to examine the above suggestion.

## 4.31 Fractionation of wheat aleurone tissue

Homogenization of the aleurone tissue did not prove to be a simple task. As Duffus (1967) stated " ... the aleurone cells are protected by thick walls and, though this facilitates their isolation, it hinders their homogenization." Only methods which sliced the tissue cleanly without shearing or crushing were suitable for isolating morphologically intact aleurone grains (section 3.312). The razor-blade technique finally adopted, although perhaps the gentlest method of homogenization, is time-consuming. The major disadvantage of the method is that the crude homogenates have to stand for one to two hours, albeit in the cold, before fractionation can be achieved. This increases the chances of damage to membranes and of leakage of enzymes.

Fractionation of the homogenates into subcellular constituents again posed difficulties. Sucrose, which is normally used as a density gradient component and which has been used to isolate protein bodies from both immature wheat endosperm (Graham, Morton and Nelson, 1963) and soybean cotyledons (Tombs, 1967),

caused the aleurone grains to disintegrate at high concentrations. Ficoll or dextran had to be substituted as a density gradient component. Using these high molecular weight compounds, it was possible to obtain morphologically intact aleurone grains and to separate them, at least partially, from the lighter components. Although the fractions isolated consisted of more than one component, each fraction was generally enriched with one or two specific subcellular organelles. For example, the 4900 g pellet consisted mainly of starch and aleurone grains, while the 38,000 g pellet was comprised mainly of spherosomes and mitochondria.

## 4.32 Distribution of hydrolytic enzymes between particulate and soluble fractions

The results of attempts to localize the amylase, acid phosphatase and RN-ase in either soluble or particulate fractions were inconclusive.

From 4.6 to 13.8% of the acid phosphatase, 0 to 25.3% of the RN-ase, 9.4 to 57.9% of the total amylase, and 4.1 to 43.2% of the α-amylase were present in particulate fractions after various treatments. For acid phosphatase and RN-ase the percentages of enzyme in the particulate fractions represented a relatively small amount of total enzyme. Furthermore,

GA did not induce any substantial changes in the distribution of either of these enzymes. For the amylases on the other hand, even the smallest percentage of enzyme found in particulate fractions represented a substantial amount of activity. Moreover, the distribution of the enzymes between particulate and supernatant fractions was changed after incubation of the tissue with GA. For example, after 24 hours incubation with water 37.9% of the total amylase was in the particulate fraction and the greatest proportion of this was in the 4900 g pellet. The same total percentage of enzyme was in the particulate fraction after 24 hours in GA but it was more evenly distributed between the fractions with the largest amount in the 38,000 g pellet and the least in the 4900 g pellet. On the other hand, 43.2% of the a-amylase was in particulate fractions after 24 hours incubation with water and again most of this was in the heaviest fraction. Only 14.3% of the enzyme was present in the particulate fraction after 24 hours in GA with substantial amounts in both the 4900 g and 38,000 g pellets as well as a small amount in the lighter fraction.

The question is whether the enzymes present in the pellets represent particulate enzymes.

The following considerations may bear upon this question.

4.321 Changes in enzyme distribution during incubation and fractionation

If the GA-induced enzymes are synthesized or packaged in particles, then the nature of the homogenisation and fractionation technique, and the intrinsic qualities of the particles themselves, will determine whether the enzymes can be detected in particulate fractions.

The ease with which particles can be separated and enzymes measured, depends on several factors:

i) The nature of the tissue;

ii) The amount of enzyme present within the tissue initially, and in the control at subsequent times;

iii) The amount of enzyme induced in the tissue by hormone treatment;

iv) The sensitivity of the assays;

v) The free access of enzymes to substrate during the assay;

vi) Rupture of particles during fractionation.

Because of the unique qualities of the aleurone layer and the response induced within it by GA, all of the above points are relevant, as shown in the following discussion.

i) The nature of the tissue

As indicated earlier, the aleurone cells possess very thick tough cell walls necessitating relatively severe methods of homogenisation. This probably resulted in an unknown proportion of the subcellular particles being damaged mechanically. In addition it is likely that a certain amount of osmotic or enzymatic damage also occurred because of possible differences in osmotic pressure between individual particles and the medium, and the long times (one or two hours) required for homogenization.

These factors would tend to cause an increase in the enzymes in the supernatants and a decrease in the particulate fractions.

ii) Enzyme activities in tissues and ambient solutions

Examination of Table 3.5-9 reveals that the total activities of acid phosphatase and RN-ase were extremely low. In addition, 40% or more of the enzymes were secreted into the medium (except for RN-ase at 24 hours) leaving only very small amounts detectable inside the tissue. Amylase activities, on the other hand, were considerably higher, and although 50% was secreted into the medium, the amount remaining in the tissue was substantial, especially at 24 hours. Even allowing for

the disruption of a proportion of the particles during fractionation, the high levels of amylase activity within the tissue increases the possibility of detecting these enzymes if they are in particulate fractions.

iii) Magnitude of the GA-induced increase in the enzymes within the tissues

Table 4.32-1 shows that, in addition to the activities of acid phosphatase and RN-ase being very low, GA induced only a small (less than a 2-fold) increase in their levels in the tissue, even after 24 hours. On the other hand, total amylase and α-amylase activities were much higher initially, and there was a 4- and 9-fold increase, respectively, in the tissue after 24 hours in GA.

Once again there is a greater chance of finding the amylases in particulate fractions than the other two enzymes.

TABLE 4.32-1

Ratio of the enzyme activities in the GA samples to those in the water controls. Data derived from values presented in Table 3.5-9.

Acid Phosphatase

| Time (hours) | 6 | 18 | 24 |
|---|---|---|---|
| Total | 1.0 | 1.1 | 5.4 |
| Tissue | 1.0 | 1.2 | 1.6 |
| Ambient | 1.0 | 0.9 | - |

RN-ase

| Time (hours) | 6 | 18 | 24 |
|---|---|---|---|
| Total | 1.1 | 1.7 | 2.2 |
| Tissue | 1.1 | 1.8 | 1.8 |
| Ambient | 1.1 | 1.6 | - |

Total Amylase

| Time (hours) | 6 | 18 | 24 |
|---|---|---|---|
| Total | 1.1 | 5.7 | 10.0 |
| Tissue | 1.1 | 4.9 | 3.7 |
| Ambient | 1.0 | 6.0 | 12.0 |

α-Amylase

| Time (hours) | 6 | 18 | 24 |
|---|---|---|---|
| Total | - | - | 20.0 |
| Tissue | - | - | 9.1 |
| Ambient | - | - | 29.0 |

iv) Sensitivity of the enzyme assays

β-Glycerophosphate, the substrate used for acid phosphatase, is non-specific and may be hydrolysed by a number of enzymes having different degrees of affinity for it. Yeast RNA, the substrate for RN-ase, may or may not be the best substrate for the aleurone enzyme.

In addition, the physical parameters which might be expected to influence enzyme activity (i.e., pH, buffer molarity, inhibitory effects of components of the fractionating medium, etc.) were not thoroughly investigated.

On the other hand, amylose is highly specific for the amylase enzymes and the requirements for the assay of these enzymes are well known.

v) Rupture of particles containing enzymes

Furthermore, it was assumed that the conditions of assay would be sufficient to lyse any particles that might be present and thus release the enzymes into solution. Therefore, no attempt was made to ensure that particles were ruptured before assaying. However, if only a small proportion of the particles were ruptured during the assay, substantial numbers of particles would have to be present to detect very much enzyme activity. This again would favour the

detection of amylases in particles rather than the other two enzymes.

vi) Rupture of particles during fractionation

In Table 3.5-10 the enzyme activities of the crude homogenates are compared with that obtained by summation of the enzyme activities of each of the fractions. The values for the crude homogenates were obtained by measuring enzyme activities after the filtration step but before fractionation (see Scheme 3.5-1). Theoretically, the enzyme activity of the crude homogenates should be the same as that of the combined fractions when corrected for volume differences. This was true for the amylases, but the values for acid phosphatase and RN-ase were generally lower in the crude homogenates.

Two explanations may be put forward to explain the lower activities of acid phosphatase and RN-ase in the crude homogenates:

i) There is an inhibitor in the crude homogenate which is seperated from the enzymes during fractionation.

ii) The enzymes are present in membrane-bound particles, a proportion of which is disrupted when the tissue is fractionated.

TABLE 3.5-10

Comparison of the enzyme activities of the crude homogenates with the sum of the activities of the various fractions.

ACID PHOSPHATASE

| Time (hours) | 0 | 6 | 6 | 18 | 18 | 24 | 24 |
|---|---|---|---|---|---|---|---|
| Treatment | None | Water | GA | Water | GA | Water | GA |
| Crude homogenate | 4.3 | 27.3 | 31.2 | 15.6 | 15.6 | 10.7 | 16.9 |
| Combined fractions | 19.0 | 36.3 | 35.7 | 23.1 | 28.7 | 27.0 | 41.8 |

RIBONUCLEASE

| Time (hours) | 0 | 6 | 6 | 18 | 18 | 24 | 24 |
|---|---|---|---|---|---|---|---|
| Treatment | None | Water | GA | Water | GA | Water | GA |
| Crude homogenate | 9.3 | 39.1 | 39.1 | 19.4 | 19.3 | 25.0 | 79.2 |
| Combined fractions | 25.1 | 44.6 | 47.0 | 21.3 | 37.8 | 26.8 | 48.9 |

TOTAL AMYLASE

| Time (hours) | 0 | 6 | 6 | 18 | 18 | 24 | 24 |
|---|---|---|---|---|---|---|---|
| Treatment | None | Water | GA | Water | GA | Water | GA |
| Crude homogenate | - | 6,960 | 6,960 | 12,712 | 35,360 | 19,840 | 70,400 |
| Combined fractions | - | 7,630 | 8,529 | 11,778 | 58,079 | 15,203 | 55,730 |

α-AMYLASE

| Time (hours) | 0 | 6 | 6 | 18 | 18 | 24 | 24 |
|---|---|---|---|---|---|---|---|
| Treatment | None | Water | GA | Water | GA | Water | GA |
| Crude homogenate | - | - | - | 800 | 11,580 | - | 101,200 |
| Combined fractions | - | - | - | - | 8,118 | 5,270 | 47,881 |

4.322 Evidence for particulate α-amylase

Tables 3.5-5, -6, -7 and -8 show that only small amounts of acid phosphatase and RN-ase are present in the particulate fractions whereas substantial amounts of the amylases are found in the pellets. This evidence suggests that at least the GA-induced α-amylase may be present in a particulate fraction. In view of the considerations mentioned in the preceding section, the differences between RN-ase and acid phosphatase on the one hand, and amylase on the other, may not necessarily represent differences in hormonal control mechanisms.

However, the data are by no means conclusive. The small proportions of enzymes present in the pellets may simply represent trapping of soluble enzymes between particles or adsorption onto particles. Improvements in the methods of homogenisation, fractionation and purification of particulate fractions are necessary before the particulate or soluble nature of the enzymes can finally be determined.

It is interesting to note that in at least one other investigation of cereal seed, α-amylase has been localised in a heavy particulate fraction (Novellie, 1960).

## 4.4 Hormonal control: the lysosome hypothesis

As suggested in section 2.9, the GA-induced enzymes of the cereal aleurone layer are analagous in many ways to the lysosomal enzymes of animal cells. The electron micrographs of aleurone tissue indicated that different kinds of particulate structures appeared as a result of GA treatment. It is still attractive to speculate that these particles may contain the synthesized enzymes. However, the results of the attempts made during the present investigation to localize the enzymes in such particulate fractions were inconclusive.

Thus it is not yet possible to determine whether the mechanism involved in the initiation and production of the GA-induced enzymes of the aleurone layer is similar to that postulated for the control of animal lysosomes.

# 5. CONCLUSIONS

1) The technique developed for the isolation of large quantities of wheat aleurone tissue is quite satisfactory in that it is rapid, reproducible, and produces a viable tissue.

ii) The response of this wheat aleurone tissue is qualitatively similar to that of hand-peeled barley aleurone.

iii) The centrifugation technique used for fractionation of the subcellular particles provides only heterogeneous fractions.

iv) α-Amylase occurs in substantial amounts in particulate fractions.

v) Little evidence of RN-ase and acid phosphatase was found in fractions other than the supernatant.

# APPENDIX

## ELECTROPHORESIS:

The wheat mill bran of section 3.11 did not respond to GA by producing α-amylase. However, other proteins may be affected by GA as is the case for barley aleurone. Therefore, using electrophoresis, the effect of GA on wheat mill bran was compared with its effect on barley aleurone tissue with respect to changes in protein and enzyme spectrum. In addition, protein secretion and α-amylase production were measured to check whether the tissue was responding to GA.

### A1. Preparation of the tissue

Wheat mill bran from the variety, Wollongong, was isolated as described in section 3.11. Barley aleurone tissue from the varieties, Naked Washington Trail (NWT) and Naked Blanco Mariout (NBM), was isolated by hand-peeling as described for wheat in section 3.12 except that the imbibition period was 72 instead of 16 hours.

### A2. α-Amylase response

As noted above, the wheat mill bran did not produce α-amylase in response to GA (see also section 3.11).

Barley aleurone, on the other hand, did respond to GA in this way.

Six lots of 20 aleurone layers were isolated from each of the two varieties (NBM and NWT). For each variety, three lots were incubated with 5 ml of water and three with 5 ml of GA (100 μg/ml) in 100 ml erlenmeyer flasks in a shaking water bath at 30°C. After 24 hours the ambient solutions were decanted, diluted with an equal volume of $10^{-2}$M calcium acetate and heated to 70°C for 20 minutes. The α-amylase activity of the heated solutions was measured as described in section 3.11. The percent initial OD at 600 nm of the starch-iodine complex is plotted against time in Figures A2-1 and -2 for the two varieties. In the presence of GA the optical density of the starch-iodine solutions decreased to about 2% of the initial value within 18 hours. In the absence of GA the optical density also decreased but only to about 85% of the initial value after 18 hours. Thus, the aleurone tissue of both varieties responded to GA by producing α-amylase, and very little of the enzyme was produced by the water controls.

FIGURE A2-1

Production of α-amylase by hand-peeled barley aleurone tissue. Variety: Naked Washington Trail. Incubation medium: water or GA (100 µg/ml). Temperature: 30°C. Time: 24 hours. Results: percent IKI - starch absorbance at zero time plotted against time of sampling during assay.

△, □, ▽ water controls;

X, ○ + GA treatments.

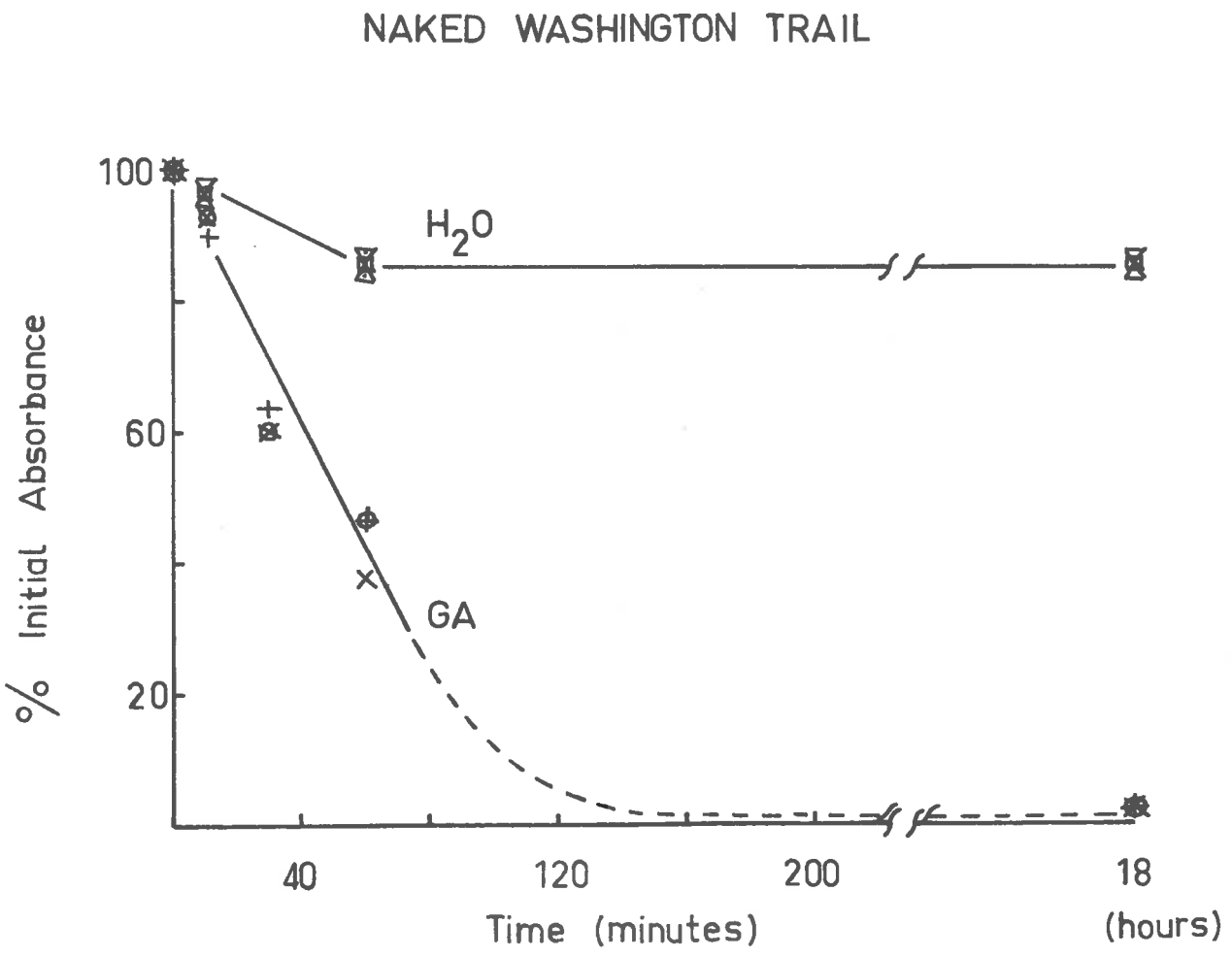
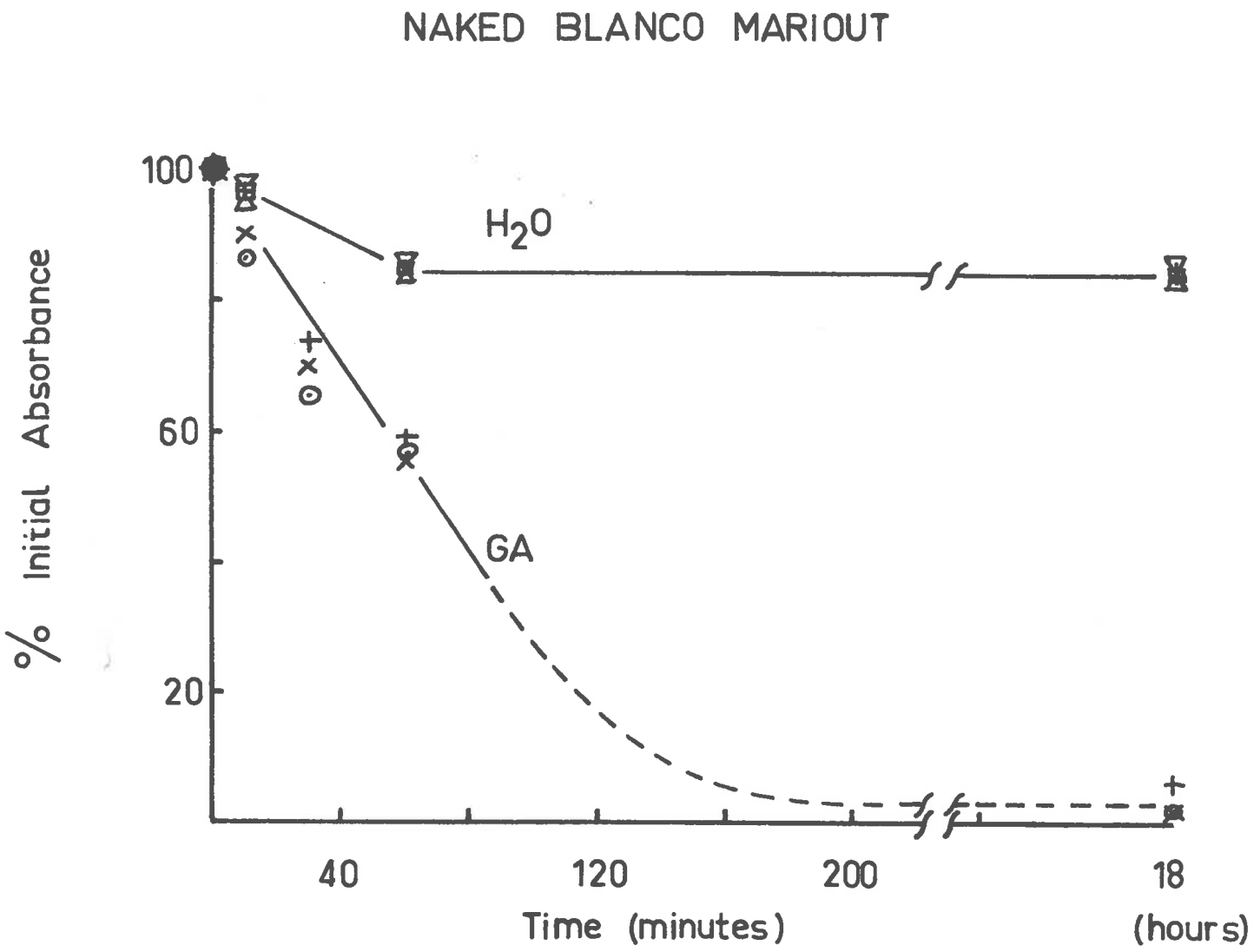

NAKED WASHINGTON TRAIL
100
60
20
% Initial Absorbance
$H_2O$
GA
40
120
200
18
Time (minutes)
(hours)

FIGURE A2-2

Production of α-amylase by hand-pealed barley aleurone tissue. Variety: Naked Blanco Mariout. Incubation medium: water or GA (100 μg/ml). Temperature: 30°C. Time: 24 hours. Results: percent IKI - starch absorbance at zero time plotted against time of sampling during assay.

△, □, ▽ water controls;

×, ○, + GA treatments.


NAKED BLANCO MARIOUT
100
60
20
% Initial Absorbance
$H_2O$
GA
40
120
200
18
Time (minutes)
(hours)

## A3. Protein secretion

The protein secreted by wheat mill bran or barley aleurone tissue after incubation for various times with GA or water was measured by the method of Lowry *et al.* (1951) using Bovine Serum Albumin as a standard.

Wheat mill bran was incubated as described in section 3.11. Barley aleurone was incubated as described above for α-amylase determinations. The results are shown in Table A3-1.

### TABLE A3-1

Secretion of protein by wheat mill bran and barley aleurone tissue. Varieties: wheat, Wollongong; barley, Naked Washington Trail and Naked Blanco Mariout. Incubation medium: water or GA (100 µg/ml). Temperature: 30°C. Protein: µg protein nitrogen.

| Time of incubation (hours) | Wheat Mill Bran | | Barley Aleurone | | | |
|---|---|---|---|---|---|---|
| | | | NWT | | NBM | |
| | Water | GA | Water | GA | Water | GA |
| 18 | 634 | 861 | - | - | - | - |
| 21 | 767 | 722 | - | - | - | - |
| 22.5 | 101 | 104 | - | - | - | - |
| 24 | 955 | 863 | 78 | 203 | 42 | 225 |
| 24 | 900 | 725 | 96 | 215 | 189 | 244 |
| 43 | 572 | 828 | 103 | 401 | 83 | 477 |
| 48 | - | - | - | - | 515 | 355 |

There was little consistency in the effect of GA on the proteins secreted by the wheat mill bran. However, barley aleurone tissue always secreted more protein in the presence of GA than in its absence.

## A4. Starch gel electrophoresis

Electrophoresis was carried out in horizontal starch gels according to the method of Smithies (1955). The starch (Hydrolysed Starch from Connaught Laboratories, Toronto) was made up at a concentration of 11% in Tris-citrate buffer (0.076 M, pH 9.0) containing 5 mM EDTA. The moving phase was sodium borate buffer (0.3M), pH 9.0. Protein solutions were applied to the gels by absorbing aliquots onto pieces of filter paper and then inserting these into slots previously made in the gels. If the solution was dilute, more than one piece of filter paper could be used to apply a larger volume of protein solution. The borate buffer was placed in electrode vessels at each end of the gel and connected to the gel by means of Whatman No. 1 filter paper wicks. A constant current of 35 ma was applied for two to three hours until the moving front reached a predetermined end point. The gel was cooled before application of the sample and, during electrophoresis, was placed in a refrigerator at about 5°C with a container of ice resting on top of it.

At the end of a run the gel was sliced in half as described by Smithies (1955) and stained for protein and peroxidase. Amylase was located by observing hydrolysis of the starch gel *in situ*.

Samples of wheat mill bran or barley aleurone tissue were incubated with GA (100 µg/ml) or water for 24 or 48 hours as described for α-amylase determinations. Ambient solutions were concentrated to small volumes by (a) freeze drying and resuspending in a small volume, or (b) by dialysis against carbowax 20M (polyethylene glycol, MW $20 \times 10^6$). Tissues were ground with sand and dry ice with a mortar and pestle and extracted with Tris-citrate buffer, pH 8.5 (0.076M). The results are shown in Figures 44-1, -2, -3 and -4.

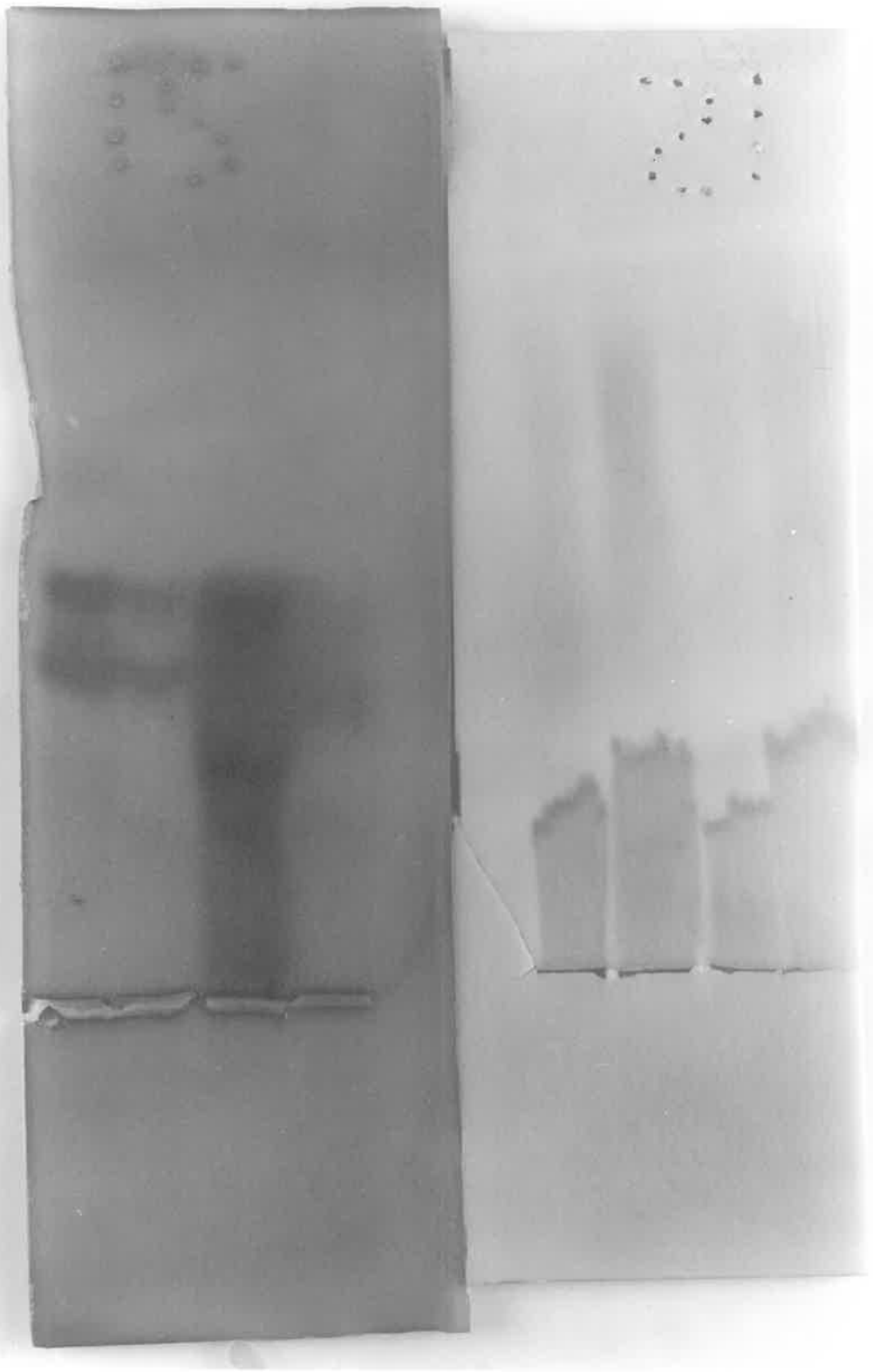
Figures 44-1 and -2 are representative of several attempts to separate proteins and enzymes of the ambient solutions of wheat mill bran by electrophoresis. The only consistent effect of GA was what appeared to be either a reduced amount or a lowered mobility of β-amylase. The peroxidase activity was lower in the GA-treated sample but this effect of GA was variable from one experiment to another. Extracts of tissue were similar and yielded very few protein bands other than β-amylase. This β-amylase is probably a contaminant from sub-aleurone tissue.

FIGURE A4-1

Starch gel electrophoresis of ambient solutions from wheat mill bran. Variety: Wollongong. Incubation medium: water or GA (100 µg/ml). Temperature: 30°C. Time: 24 hours. Left: stained for peroxidase activity with hydrogen peroxide and pyrogallol; left-hand sample: water control, right-hand sample, GA-treatment. Right: stained with amido black; left-hand sample: GA treatment, right hand sample: water control.

FIGURE A4-2

Starch gel electrophoresis of ambient solutions from wheat mill bran. Variety: Wollongong. Incubation medium: water or GA (100 µg/ml). Temperature: 30°C. Time: 48 hours. Left: stained for peroxidase; from left to right: water control, GA treatment, water control, GA treatment. Right: stained with amido black; from left to right: GA treatment, water control, GA treatment, water control.



Figures A4-3 and -4 summarize the effects of GA on proteins of both ambient solutions and extracts of barley aleurone tissue.

## Ambient solutions

At 24 hours there was only one fast-moving protein band in the ambient solutions from the water control tissue. On the other hand, there were at least three protein bands in the ambient solutions from GA-treated tissue; two of these were amylases and ran very close to the origin toward the anode.

At 48 hours there were several protein bands in ambient solutions from both control and treated tissue. These bands were the same in both samples except for two relatively fast moving ones which were missing from the GA-treated samples. There were three areas of peroxidase activity in both samples: two with the same mobility, and one with different mobility.

## Extracts

Extracts of untreated tissue contained four fast-moving and two slow-moving protein bands. These latter two bands exhibited amylase activity. Extracts of water control tissue were almost identical with those of the untreated tissue except that the faster moving of the two

FIGURE A4-3

Starch gel electrophoresis of ambient solutions and extracts from hand-peeled barley aleurone tissue. Variety: Naked Blanco Mariout. Incubation medium: water or GA (100 µg/ml). Temperature: 30°C. Time: 24 hours. Stain: amido black or hydrogen peroxide and pyrogallol. Solid black bands represent either amido black staining bands or peroxidase bands; cross-hatched bands represent areas of the starch-gel which have been degraded by amylase activity.

INCUBATION 24 HOURS

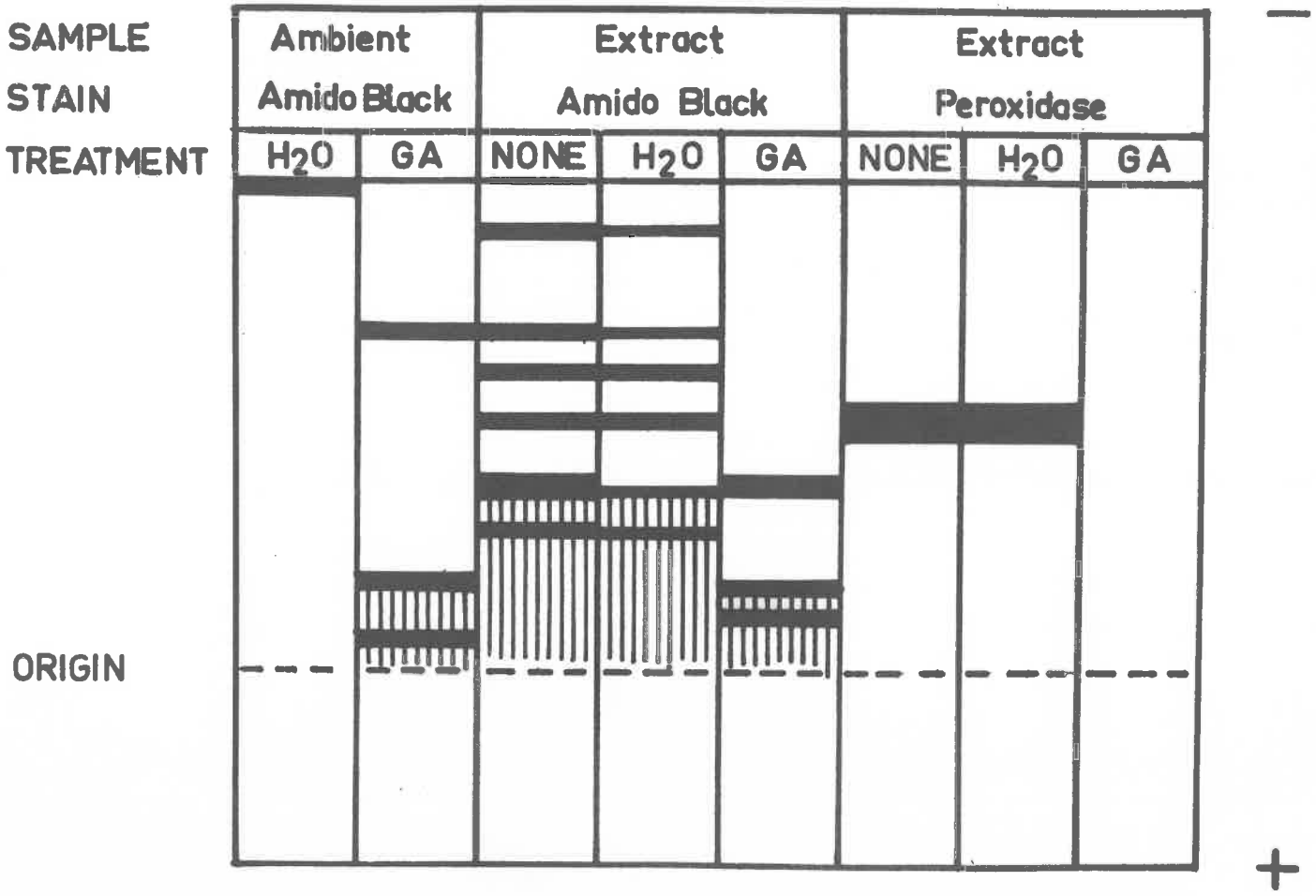
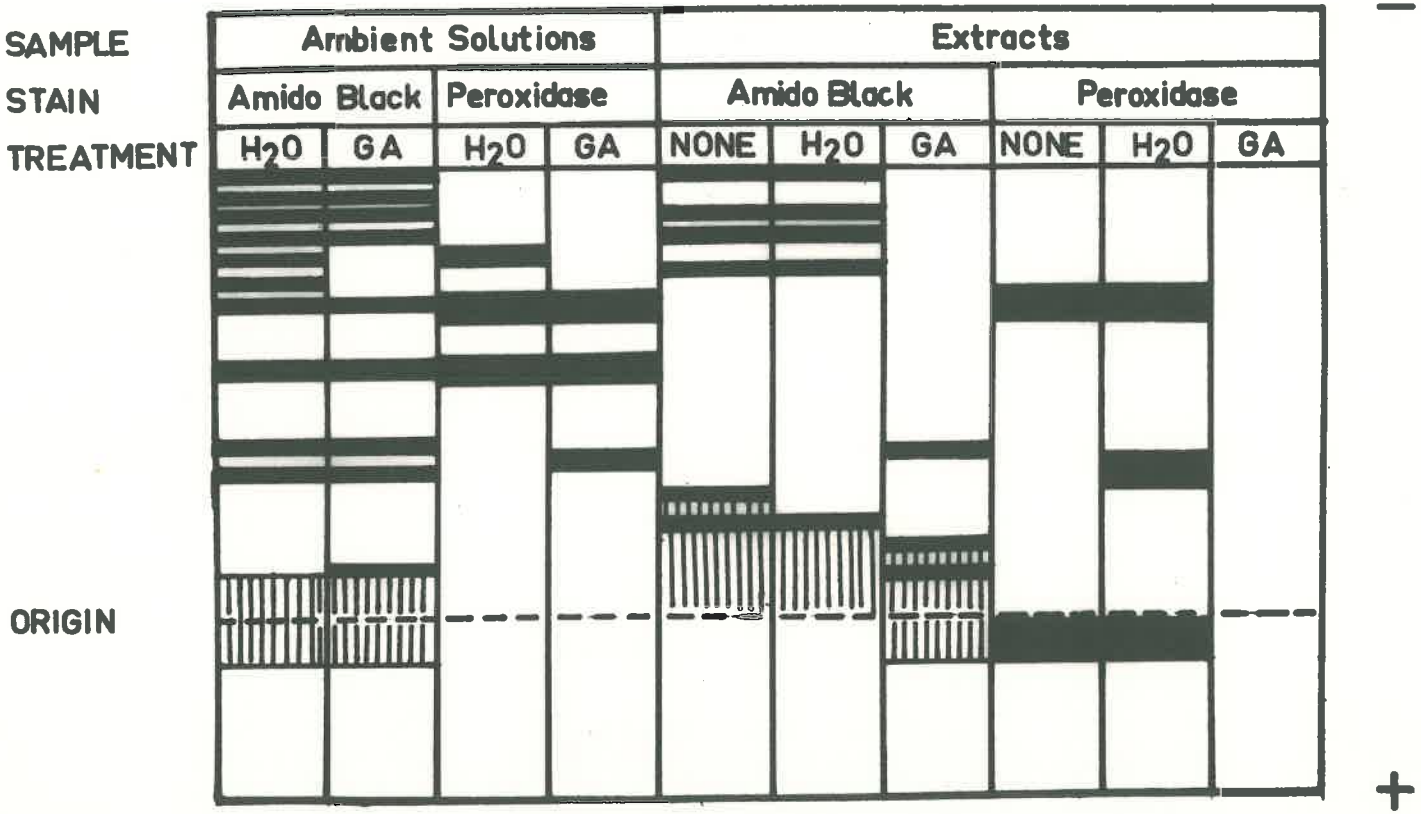


FIGURE A4-4

Starch gel electrophoresis of ambient solutions and extracts from hand-peeled barley aleurone tissue. Variety: Naked Blanco Mariout. Incubation medium: water or GA (100 µg/ml). Temperature: 30°C. Time: 48 hours. Stain: amido black or hydrogen peroxide and pyrogallol. Solid black bands represent either amido black staining bands or peroxidase bands; cross-hatched bands represent areas of the starch-gel which have been degraded by amylase activity.

INCUBATION 48 HOURS



amylase bands was missing at 48 hours. Extracts of GA-treated tissue lacked the four fast-moving and contained only three slow-moving bands, two of which were amylases, at both 24 and 48 hours.

The untreated tissue contained one peroxidase band at 24 hours and two at 48 hours. The water controls contained these same bands with an additional one of intermediate mobility in the 48 hour experiment. The GA-treated tissue did not contain any peroxidase activity at either 24 or 48 hours.

## A5. Discussion

GA affected many proteins both in ambient solutions and in extracts of barley aleurone tissue. It had virtually no effect on the proteins of wheat mill bran. It seems likely that the wheat mill bran was too severely damaged to respond to the hormone.

Briggs (1968) observed that damaging the aleurone layers of barley half-seed by drilling small holes or burning small areas resulted in a much greater reduction in the amount of GA-induced α-amylase than could be accounted for by the number of cells damaged. He concluded that the aleurone layer functions as a single unit. Although this conclusion may not be valid, it

does appear that even slight damage to the barley aleurone tissue reduces its response to GA. The same seems to be true for the wheat aleurone layer.

## ABBREVIATIONS

| | | |
|---|---|---|
| BSA | - | bovine serum albumin |
| DW | - | dry weight |
| EDTA | - | ethylene diamine tetracetic acid |
| ER | - | endoplasmic reticulum |
| FW | - | fresh weight |
| GA | - | gibberellic acid |
| IKI | - | iodine potassium iodide |
| M | - | molar |
| MW | - | molecular weight |
| NBM | - | Naked Blanco Mariout |
| NWT | - | Naked Washington Trail |
| OD | - | optical density |
| RNA | - | ribonucleic acid |
| mRNA | - | messenger ribonucleic acid |
| rRNA | - | ribosomal ribonucleic acid |
| tRNA | - | transfer ribonucleic acid |
| RN-ase | - | ribonuclease |
| SH | - | sulphydryl |
| TCA | - | trichloroacetic acid |

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