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The effects of ethylene on the gibberellic acid-enhanced synthesis and release of amylase by aleurone layers isolated from *Hordeum vulgare* cv Himalaya

by

(C) Kenneth Charles Eastwell

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "The effects of ethylene on the gibberellic acid-enhanced synthesis and release of amylase by aleurone layers isolated from *Hordeum vulgare* cv Himalaya" submitted by Kenneth Charles Eastwell in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Biochemistry.

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ABSTRACT

For studies on effects of ethylene on amylase synthesis and release, methods were developed and extended to enable the incubation of isolated barley aleurone layers under controlled conditions. Particular attention was directed towards creating an atmosphere free of low molecular weight hydrocarbons, common contaminants in most air sources. Other experimental parameters were also investigated and optimized. The characteristics of the response to gibberellic acid were also established.

When isolated barley aleurone layers, in medium containing gibberellic acid, were exposed to ethylene, there was a change in the synthesis and release of amylase, relative to layers maintained in an ethylene-free environment. During the initial 24 h, ethylene accelerated both the appearance of total amylase activity, and the release of this activity from the aleurone layers. However, the isoelectric patterns of amylase and proteins released from control and ethylene-treated aleurone layers were identical. On the other hand, ethylene reduced the total amount of amylase activity that was found after 48 h and 72 h. The nature of the response to ethylene was dependent on the concentration of gibberellic acid in the medium.

Other processes that occur simultaneously with the synthesis and release of amylase were also examined. These included the syntheses of other hydyrolytic enzymes - xylanase, glucanase and haemoglobinase. The development of

xylanase activity was enhanced by ethylene, whereas the rate of glucanase synthesis was unaffected. The role of haemoglobinase activity in the response to ethylene was examined directly, and also through the addition of either potassium bromate or N-ethylmaleimide, inhibitors of sulphydryl enzymes. The sulphydryl group inhibitors prevented the reduction in amylase activity (relative to control samples) otherwise observed in samples that had been exposed to ethylene for longer periods of time (48 h and 72 h). The direct assay of haemoglobinase activity revealed no significant change in proteolytic activity in response to ethylene.

In addition to the biosynthesis of hydrolytic enzymes, the redistribution of ionic species in response to ethylene was explored. Ethylene had no effect on the overall shift in the pH of the medium surrounding the aleurone layers. When the release of individual ionic species was examined, the release of calcium was much more sensitive to ethylene than the release of any of the other ions considered (potassium, magnesium and phosphate). Ethylene induced a 101% increase in the release of calcium from gibberellic acid-treated aleurone layers.

The possible relevance of these events to the action of ethylene on the gibberellic acid-enhanced synthesis and release of amylase by isolated barley aleurone layers is discussed.

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ENZYME NOMENCLATURE

In order to simplify discussion, 1,4-alpha-D-glucan glucanohydrolase (E.C. 3.2.1.1) and 1,4-alpha-D-glucan maltohydrolase (E.C. 3.2.1.2) will be referred to by their recommended trivial names, alpha-amylase and beta-amylase respectively (International Union of Biochemists, 1973). In addition, glucanase will denote 1,4-(1,3:1,4)-beta-D-glucan 3(4)-glucanohydrolase (E.C. 3.2.1.6). Xylanase and endoxylanase refer to 1,4-beta-D-xylan xylanohydrolase (E.C. 3.2.1.8) while exo-xylanase specifies 1,4-beta-D-xylan xylohydrolase (E.C. 3.2.1.37). The term protease will be used to designate the uncharacterized peptide hydrolases (E.C. 3.4) found in barley. Haemoglobinase is used to distinguish the proteases of low pH optima from the proteases with optima at neutral or basic pH (Bhatty, 1968).

I. INTRODUCTION

The effects of ethylene on many plant tissues have been studied. As a consequence of the gaseous nature of ethylene, special procedures were required to contain the gas during the course of the experimentation. In most studies, the techniques have been inadequate, or introduce other variables into the experimental design. It is difficult to define these variables and to assess their influences on parameters measured in the experiment.

The first goal of this study was to develop apparatuses and techniques that would allow careful investigation of ethylene effects. This system was then used to study isolated aleurone layers from *Hordeum vulgare* cv Himalaya. The aleurone layers of cereals offer a unique opportunity to study plant growth regulators. They provide tissue with uniform cell type, and a very limited amount of endogenous plant growth substances. The enhancement, by gibberellic acid, of the synthesis and release of amylase is of particular interest. Amylase is necessary to maintain the germinating embryo by solubilizing storage material in the endosperm. Also, the massive synthesis of amylase in response to gibberellic acid is well documented, providing a solid base for further research with other plant growth substances.

The intent of this investigation is to define the effects of ethylene on this system, and attempt to relate these observations to other metabolic processes that occur

within the aleurone layers of barley during this stage of development.

II. LITERATURE REVIEW

A. Effects of ethylene on seedling growth

Ethylene is the smallest of the plant growth regulators to be recognized. However, the chemical simplicity of ethylene, the fact that it is gaseous at normal environmental conditions and its wide distribution in the environment caused apprehension in its acceptance as a plant growth regulator. These same features have caused ethylene to attract considerable attention in the literature. Despite continued efforts, still very little is known of ethylene's role in the normal development of plants.

The effects of ethylene on development have been particularly well documented in dicotyledonous plants (Abeles, 1973), partially as a result of the more dramatic responses of dicotyledonous plants as compared to the responses of monocotyledonous plants (Abeles, 1973; Smith & Robertson, 1971). Nevertheless, ethylene at low concentrations has been shown to influence growth and development of several monocotyledonous species. The extension of monocotyledonous seedlings is modified by ethylene . This was exemplified by the inhibition of corn top growth by between 10 and 100 nl/ml ethylene (Heck & Pires, 1962), and the severe inhibition of root and shoot extension of wheat in response to 0.2% ethylene (Roberts, 1951). In contrast, as little as 0.5 nl/ml ethylene has promoted the extension of rice seedlings germinated in

darkness (Ku *et al.*, 1970). The response was not saturated until 100 nl/ml to 200 nl/ml of ethylene was applied. Rice seedlings grown in light were also sensitive to ethylene giving a similar elongation response to both endogenous ethylene and exogenously applied ethylene (Suge, Katsura & Inada, 1971). Barley seedlings have also been shown to be influenced by exogenous ethylene. In 1929, Nord & Weichherz observed that ethylene enhanced the rate of shoot growth. This has since been reconfirmed (Cornforth & Stevens, 1973). However, unlike rice, where both shoot and root extension were increased (Abeles, 1973), root development of barley was severely limited in the presence of exogenous ethylene. Smith & Robertson (1971) noted that below 1 nl/ml ethylene, the extension of rice roots was stimulated, but higher concentrations of ethylene inhibited the elongation of roots. Root extension of barley, the most sensitive of the three cereals tested, was inhibited by the lowest ethylene concentration considered, 0.1 nl/ml. Saturation of this response to ethylene was not evident up to 100 nl/ml, a level that resulted in root extension of treated tissue being 80% less than that of the control plants. In addition to the inhibition of root extension, ethylene also induced distinct changes in root form (Cornforth & Stevens, 1973), the ethylene treated roots were thick and tightly coiled. A mat of root hairs was also evident on ethylene treated roots. These morphological changes in root form were similar to those found in wheat seedlings exposed to

ethylene (Roberts, 1951).

The significance of these observations becomes more evident when the levels of ethylene encountered in the environment are considered. It has been demonstrated that several plant species, including the monocotyledonous plants oats (Meheriuk & Spencer, 1964) and rice (Ku *et al.*, 1970), produced significant amounts of ethylene during germination (Wareing & Saunders, 1971). In addition to endogenous ethylene, many plants are exposed to exogenous ethylene from the environment. Smith & Restall (1971) demonstrated that soils produce varying amounts of ethylene and other low molecular weight hydrocarbons, including methane, propane, propylene and ethane. Methane and ethylene were produced in greater quantities than the other hydrocarbons. The rate of ethylene evolution was highly dependent on environmental conditions. Water content, soil type, temperature and the degree of aeration influenced hydrocarbon production (Smith & Restall, 1971). The levels of ethylene measured in water-saturated, anaerobic soils have exceeded 20 nl/ml (Smith & Russel, 1969). Although these high concentrations of ethylene were attained only in artificial environments created within the laboratory, field studies indicated that significant levels of ethylene occurred in natural conditions, particularly with water-logged soils (Smith & Russell, 1969). The levels of ethylene encountered in their field studies, up to 8 nl/ml, would be sufficient to alter the development of many plant seedlings, including those of

the cereals.

B. Choice of experimental system

There are a number of factors that should be considered in the selection of tissue for studies on the effect of ethylene on seedling development of monocotyledonous plants. Tissues used in the past include coleoptiles (in rice, Suge, *et al.*, 1971; in corn, Heck & Pires, 1962; in wheat, Roberts, 1951; in barley, Cornforth & Stevens, 1973), intact root systems (in rice, Abeles, 1973, Smith & Robertson, 1971; in corn, Heck & Pires, 1962; in wheat, Smith & Robertson, 1971, Roberts, 1951; in barley, Cornforth & Stevens, 1973, Smith & Robertson, 1971), and isolated embryos or endosperms (Roberts, 1951). However, in studying the effects of any plant growth regulator, regardless of its origin, special precautions must be observed. The response of most plant tissues to an exogenous plant growth regulator will be modified by a number of endogenous plant growth regulators. Therefore, a plant tissue that is devoid of endogenous plant growth substances, and that has had a limited exposure to these substances in the past, should be sought in order to provide adequate controls and enable unambiguous interpretation of the observed effects. As pointed out by Cline (1976), the aleurone layer of cereals is one of the few plant tissues that approaches these criteria. However, even in this tissue, small amounts of gibberellic acid-like substances have been found (Chrispeels

& Varner, 1967), the concentration being dependent on the age of the seed (Jones & Varner, 1967; Cline, 1976). This variability in endogenous gibberellic acid-like activity may account for the change in sensitivity to exogenous gibberellic acid as seed samples age (MacLeod, 1964).

The use of the aleurone layer for studying hormonal effects offers other advantages. Unlike most other plant tissues, which consist of a composite of cell types, the aleurone layer is of a homogenous cell type, so a more uniform response would be anticipated. The aleurone layer of cereals is only a very few cells in depth, reducing the time interval required for all of the cells to be exposed to an exogenously applied plant growth regulator. Again, this feature should result in a more concerted response to test substances.

The response of the cereal aleurone layer to exogenous plant growth regulators has also been investigated using embryo-less grain. Khan *et al*. (1973) have shown that embryo-less grains of wheat (*Triticum aestivum* cv Yorkstar) could be used in these studies. The use of these atypical grains would reduce any response of the layers to damage by physically cutting away the embryo and scutellum. Several years prior to the proposal of Khan *et al*. (1973), Briggs (1968b) had shown that even relatively minor damage to the aleurone layer of barley would seriously reduce amylase synthesis. However, the use of embryo-less grain would also create difficulties. The plant growth regulators must now

diffuse through the starchy endosperm. The effect of restricted diffusion on the development of the gibberellic acid-response was demonstrated by Briggs (1964). When intact barley was incubated in gibberellic acid solutions, enzyme release from the aleurone layer was initiated at the proximal end, and then gradually extended toward the distal end of the grain. This suggested that gibberellic acid was penetrating the grain near the embryonic region, then diffusing through the endosperm. When slices of the grain were incubated in a gibberellic acid solution, the induction of enzyme secretion in each of the slices occurred simultaneously, indicating that the progressive development of enzyme secreting activity along the aleurone layer of the intact seed was not a consequence of the state of the aleurone cells, but related to the accessibility of the receptor cells to the plant growth regulator.

The use of intact embryo-less grains would be further complicated by the presence of large quantities of both alpha- and beta-amylase. Alpha-amylase is produced in the aleurone layer whereas beta-amylase originates in the endosperm (Bilderback, 1974). It would be possible to circumvent the interference caused by the presence of both enzymes in the sample by using specialized assay procedures that differentiate between the two forms of amylase. These methods include the use of beta-limit dextrin as substrate in the presence of excess added beta-amylase to obscure any contaminating beta-amylase (Bilderback, 1973; Briggs, 1961;

Sandstedt, Kneen & Blish, 1939), the use of heat-treatment to selectively inactivate the beta-amylase (Paleg, 1960b) or the use of low pH to inactivate alpha-amylase (Paleg, 1960b).

The nature of the amylases found in isolated aleurone layers has also been questioned. Frydenberg & Nielsen (1965) used agar gel electrophoresis to separate the amylases found in the kernels of germinating barley. The classical differences betwen alpha- and beta-amylase were used to differentiate the types of amylases comprising each band of activity in the gels. The methods used by Frydenberg & Nielsen included heating the gels to 70 C (which inactivated beta-amylase), the addition of Cu^{+2} or Hg^{+2} to inhibit beta-amylase, or the addition of hexametaphosphate to complex Ca^{+2}, thus inactivating alpha-amylase. By these techniques, five bands with alpha-amylase activity and two bands with beta-amylase activity were identified, as well as two bands of activity displaying hybrid characteristics. The latter were resistant to heat, but they were inhibited by Hg^{+2} and Cu^{+2}, and showed no dependence on the availability of Ca^{+2}. (A number of reports have appeared, summarized in Greenwood & Milne (1968), demonstrating that alpha-amylases from several sources are sensitive to Hg^{+2} and Cu^{+2}.) A very similar approach was used to determine the types of amylase that originated in isolated aleurone layers incubated in solutions of gibberellic acid (Jacobsen, Scandalios &

Varner, 1970). This latter investigation revealed eight bands of enzyme activity towards potato starch. Four of the zones were very minor and exhibited the characteristics of beta-amylase in that they were heat labile, sensitive to Hg^{+2} and insensitive to 5 mM ethylenediaminetetraacetic acid (EDTA). The authors suggested that these minor bands may have originated from small fragments of the endosperm adhering to the aleurone layers. The electrophoretic pattern of the four beta-amylase bands was duplicated by the amylases extracted from isolated endosperm tissue that had been treated with papain, a procedure that activates the beta-amylase zymogens (Rowsell & Goad, 1962). Of the four remaining bands of amylase from aleurone layers, the two most intense bands, containing 75% to 80% of the total amylase activity, had properties typical of alpha-amylase; the remaining two bands demonstrated the hybrid characteristics shown by the preparations of Frydenberg & Neilsen. Paper chromatography of the reaction products of the four major bands indicated that they all had the action pattern typical of alpha-amylase (Jacobsen *et al.*, 1970). Bilderback (1974) on the otherhand, found seven bands of activity after polyacrylamide gel electrophoresis of the extract from isolated aleurone layers. All bands were of alpha-amylase shown by virtue of the fact that they were able to hydrolyse beta-limit dextrins. Two of the bands, however, were insensitive to 50 mM EDTA. When isolated endosperms were imbibed, the resulting amylase preparation

was able to hydrolyze soluble starch, but no activity was present that could digest the beta-limit dextrin. Clutterbuck & Briggs (1973) have demonstrated that some of the enzyme activity from isolated aleurone layers measured by the degradation of soluble starch may have resulted from the presence of alpha-glucosidase. Thus, the exact natures of the "amylases" in barley aleurone layers remain obscure, although alpha-amylases are much more prevalent than any other form of starch hydrolyzing enzyme.

As indicated by the studies cited above, much of the work on isolated aleurone layers has emphasized the production of amylase. The selection of amylase as a marker of processes within the layer arose from several considerations that are presented below.

Amylase was one of the first recognized enzymes, when it was observed than an ethanol precipitate of barley malt yielded a thermolabile component able to liberate oligosaccharides from insoluble starch granules (Payem & Persoz, 1833 sited in Dixon & Webb, 1979). Since the middle of the nineteenth century, the brewing industry has been intensely studying the role that amylase fulfills during the malting process. The consequence of this interest and support has been the knowledge of well defined conditions that yield optimal activity of the barley amylase, and also the development of a number of reagents and conditions that can be used to manipulate the response of aleurone layers to gibberellic acid.

The production of amylase by the aleurone layer is not required for germination, as defined by root protrusion (Chen & Chang, 1972), but it is beneficial for the continued development of the seedling. Amylase is only one of a number of hydrolytic enzymes synthesized and/or secreted at this stage of development (summaries are found in Trewavas, 1976; Yomo & Varner, 1971), but it constitutes the single largest proportion of the enzymes produced. It has been observed that each isolated aleurone layer can produce up to 0.06 mg of alpha-amylase within 24 h of exposure to gibberellic acid (Chrispeels & Varner, 1967). This dramatic rate of amylase production coupled with low background levels of amylase in the absence of gibberellic acid (Chrispeels & Varner, 1967; Paleg, 1960b) facilitates the measurement of amylase production in response to a variety of factors.

C. Effects of growth regulators on amylase synthesis by cereal aleurone tissue

Despite the uncertain character of the amylases produced by aleurone cells, the synthesis and secretion of amylase by isolated barley aleurone layers in response to various plant growth regulators have been studied extensively. The induction by gibberellins of hydrolytic enzyme synthesis and secretion is now one of the best understood responses of a plant tissue to a growth regulator, and offers the closest look at the primary mode

of action of any plant growth regulator. In spite of the advances, some fundamental questions remain unanswered. These are evident from several recent review articles (Chrispeels, 1976; Moore, 1979; Trewavas, 1976; Varner & Ho, 1977). Many features of the response of aleurone tissue to gibberellic acid were typical of control exerted at the transcriptional level. The increase in amylase activity was sensitive to inhibitors of ribonucleic acid (RNA) biosynthesis, but not to inhibitors specific for the synthesis of transfer-RNA or ribosomal-RNA (summarized in Trewavas, 1976). On the other hand, Johnson & Kende (1971) proposed the existence of a preformed messenger-RNA specific for amylase, and that gibberellic acid induced the formation of the protein synthesizing and secretory apparatus within the cell, thus permitting the expression of the latent amylase messenger-RNA. This view was supported by the proliferation of rough endoplasmic reticulum and enzymes of lipid biosynthesis during the lag phase, a period of 8 h to 12 h after the application of gibberellic acid, but before the appearance of amylase (Johnson & Kende, 1971). However, more recent evidence has placed the existence of preformed amylase messenger-RNA in doubt. An *in vitro* translational system isolated from wheat germ did not produce amylase in response to RNA isolated from aleurone layers incubated in the absence of gibberellic acid (Higgins, Zwar & Jacobsen, 1976; Muthukrishnan, Chandra & Maxwell, 1979). The formation of functional amylase messenger-RNA after

gibberellic acid treatment did not appear to be the result of activation of a precursor strand of RNA by deproteinization (Higgins *et al.*, 1976), methylation or guanylylation of RNA (Muthukrishnan *et al.*, 1979). *De novo* synthesis is the apparent origin of functional messenger-RNA. Nevertheless, Muthukrishnan *et al.* (1979) did find that the response of aleurone layers to gibberellic acid was sensitive to inhibitors of protein synthesis, prior to the accumulation of amylase messenger-RNA. Results that contradict this possible mode of action were observed by Jelsema *et al.* (1977). Through their efforts to localize the gibberellic acid receptor in wheat, they found that ^{3}H-gibberellin A1 was preferentially bound to the microsomal fraction rich in aleurone grains. The relevance of this binding was strengthened by the observation that abscisic acid, an inhibitor of gibberellic acid activity, successfully abolished the binding capability of the aleurone grains with respect to labelled gibberellin A1. Therefore it is still uncertain if the primary action of gibberellic acid is directly involved in the enhanced synthesis of amylase, as through gene derepression, or whether the response to gibberellic acid is mediated through its effects on the secretory apparatus of the aleurone cells, or both.

The role of ethylene in this well defined system has also been studied, but as a result of often inadequate methodology and conflicting results, its function and mode

of action remain obscure. When intact barley seedlings were exposed to ethylene, an increase in the solubilization of sugars and protein was observed (Nord & Weichherz, 1929). Using barley aleurone layers still attached to the endosperm, Scott & Leopold (1967) found that 10 nl/ml of ethylene caused a 35% decline in the liberation of reducing sugars from the half-seeds treated with gibberellic acid. A decrease in the release of reducing sugars had previously been shown to reflect a decrease in levels of amylase activity (Paleg, 1960b). To re-evaluate the response of amylase synthesis and secretion to ethylene, Jones (1968) used isolated aleurone layers and measured amylase activity directly. After 18 h of exposure to gibberellic acid plus ethylene, the amylase activity secreted by these layers exceeded the activity secreted by layers exposed to gibberellic acid alone. The extent of this enhancement ranged from 6% at 0.04 nl/ml of ethylene, to 38% at 4.0 nl/ml ethylene. By determining the amylase activity retained in the layers as well as the activity secreted into the medium, Jones (1968) found that ethylene had no effect on the total amount of amylase activity induced by gibberellic acid, but ethylene did increase the proportion of amylase that was released into the medium. Jacobsen (1972) also found that ethylene modified the synthesis and secretion of amylase in response to gibberellic acid. Low concentrations of ethylene increased amylase activity in the medium, but unlike the results obtained by Jones (1968),

this was not accompanied by a corresponding reduction of amylase activity within the layers. Thus, ethylene induced an increase in the total amount of amylase produced by the isolated aleurone layers. An investigation of this system by Ho, Abroms & Varner (personal communication) produced results similar to those obtained by Jones (1968). Exposure to 40 nl/ml ethylene during the 24 h incubation period had no effect on the total amylase activity produced, but yielded an 80% increase in the amylase activity in the medium.

Abscisic acid, also, was physiologically active in barley aleurone layers, causing inhibition of the gibberellic acid-induced synthesis of amylase (Chrispeels & Varner, 1966; Ho and Varner, 1976). In addition, Jacobsen (1973) found that exposing isolated aleurone layers to ethylene during the incubation with gibberellic acid would reduce the inhibition of amylase synthesis caused by the addition of abscisic acid to the medium. The magnitude of the increase in gibberellic acid-induced amylase synthesis promoted by ethylene was much greater in the presence of abscisic acid than in the absence of abscisic acid. The concentration of abscisic acid in the medium affected both the extent to which the inhibition of amylase synthesis could be relieved by ethylene, and the concentration of ethylene required to reduce the effect of abscisic acid to a minimum. Ho, Abroms & Varner (personal communication) also found that abscisic acid inhibited the response of aleurone

layers to gibberellic acid, but contrary to the results of Jacobsen (1973), ethylene was found to be ineffectual in reversing the inhibitory effects of abscisic acid.

Jacobsen (1972) conjectured that the anomalous results obtained by different research groups, and the variability in the responses of different seed lots of Himalaya barley may have been the consequence of residual levels of abscisic acid in the seed. Variability in response to plant growth regulators has been observed by other reseachers. Jones & Varner (1967) stated that significant levels of endogenous gibberellins, also, could be found in isolated aleurone layers, and that these levels were governed by the age of the seed. This altered the response of the barley half-seeds to exogenous gibberellic acid. Smith & Robertson (1971) observed that storing seeds under adverse conditions induced appreciable resistance to the inhibitory effects of ethylene on root growth in barley seedlings.

D. Factors that modify gibberellic acid-enhanced amylase synthesis in isolated barley aleurone layers

In selecting isolated barley aleurone layers as an experimental tissue in which to investigate hormonal control of enzyme synthesis and secretion, certain precautions must be exercised in designing the experiment and in the interpretation of the results. It has been demonstrated that many factors other than the naturally occurring plant growth regulators are able to modify the gibberellic

acid-enhanced synthesis of amylase. The nature of the buffering systems employed throughout various phases of the incubation procedure influenced the response of aleurone layers to experimental conditions (Firn & Kende, 1974). Furthermore, under certain temperature conditions, the rate of amylase production was highly dependent on the pH of the medium (Carr & Goodwin, 1972), with the maximum being centered between a pH of 5.00 and 5.05. Buffer concentration also had a direct effect on the amylase activity recovered from the samples. The buffering capacity of the medium had to be sufficient to prevent the pH of the incubation medium from dropping to levels where alpha-amylase would have been unstable (Clutterbuck & Briggs, 1973). Also, it was necessary to surface sterilize the tissue and then to manipulate the isolated layers under antiseptic conditions to prevent microbial contamination. Such an infection generally resulted in suppressed amylase activity (Jones & Varner, 1967). However, two by-products of infection by *Helminthosporium sativum*, helminthosporol and helminthosporic acid, have been shown to induce amylase biosynthesis in barley aleurone layers (Okuda, Kato & Tamura, 1967). The concentration of these compounds required for optimal amylase production was 100 times greater than the molar concentration of gibberellic acid required to elicit a maximal response, and at optimal concentrations of growth regulators, the amount of amylase activity produced in response to helminthosporol and

helminthosporic acid represented only 21% and 25%, respectively, of the amylase activity achieved with gibberellic acid (Okuda *et al.*, 1967). Antibiotics, however, cannot be used indiscriminately to control microbial growth. Clutterbuck & Briggs (1973) found that a number of commonly used antibiotics drastically reduced amylase production by isolated barley aleurone layers. The antibiotics tested included benzyl penicillin, streptomycin, amphotericin B and mystatin; of these, only benzyl penicillin was without effect on amylase production. In the release of amylase by rice endosperm, penicillin was found to mimic gibberellic acid, and also enhanced the response to gibberellic acid when both substances were applied simultaneously (Biswas & Mukherje, 1979). Jones & Varner (1967) found that 0.02 mg/ml chloramphenicol had no effect on amylase synthesis, but 10 mg/ml has been shown to be extremely inhibitory (Varner, 1964). In addition, anaerobic conditions caused more than a 91% inhibition of amylase synthesis in barley half-seeds (Varner, 1964; Yomo & Iinuma, 1964), demonstrating the need for adequate aeration of the sample. The concentration of inorganic salts was also critical. Chrispeels & Varner (1967) found that 20 mM calcium chloride was required for optimal recovery of amylase activity after 24 h. All of these variables were able to modify hormone-induced responses, and must be closely regulated to obtain meaningful and reproducible results.

After aleurone tissue is exposed to gibberellic acid, there is a lag period before the appearance of alpha-amylase. During this delay in amylase synthesis, many structural and metabolic changes occur (Pollard, 1969; reviews by Trewavas, 1976; Varner & Ho, 1976; Yomo & Varner, 1971). The possibility remained that the effect on the induction of alpha-amylase secretion by any perturbance may have been a secondary response. That is, a change in the characteristics of the synthesis and secretion of alpha-amylase may have been a reflection of changes that occurred in other processes. This salient feature of the gibberellic acid-enhanced synthesis of amylase makes explicit interpretation of results difficult.

E. Role of hydrolytic enzymes in the synthesis and release of amylase from aleurone tissue

A number of enzyme systems may be directly involved in the secretion and synthesis of amylase by barley aleurone cells. Research directed towards obtaining an understanding of the secretory process of barley aleurone layer has suggested that the diffusion of secreted enzymes through the cell walls may be a rate limiting step in the release of amylase from the tissue (Varner & Mense, 1972). Therefore, cell wall degrading enzymes may fulfill an important function in permitting the release of enzymes from the aleurone layer. The activities of several cell wall-degrading enzymes have been detected in the malt of a

number of cereals (Preece & MacDougall, 1958). In barley, these were accompanied by the release of a wide range of soluble carbohydrates from the malt (Preece *et al.*, 1958). During the incubation of isolated aleurone layers in buffered solutions of gibberellic acid, soluble beta-1,3-glucans were released within the first 4 to 5 h, whereas amylase did not appear until 8 h of incubation (Pollard, 1969). Direct measurement of glucanase indicated that the activity of this enzyme did appear several hours prior to amylase (MacLeod, Duffus & Johnston, 1964). In the course of germination, the thick cell walls of the aleurone layer became progressively depleted with respect to carbohydrates (Taiz & Jones, 1970) around the cells adjacent to the endosperm. This apparent loss of carbohydrate was paralleled by the declining ability of the cell walls to bind aniline blue, a fluorescence stain that reacts with beta-1,3-linkages. These results implied that the hydrolytic action of glucanase on cell wall material, tentatively identified as a polymer of a beta-1,3-glucan, was essential for enzyme release from the layers. This hypothesis was supported by observations made after histochemical staining of acid phosphatase activity to determine the distribution of this enzyme within the barley aleurone layer (Ashford & Jacobsen, 1974). In the absence of gibberellic acid, acid phosphatase activity accumulated within the cells, but also in a heavily stained narrow band between the plasmalemma and the cell wall. Very little

alteration in this distribution occurred with prolonged incubation. If the layers were incubated in the presence of gibberellic acid, the staining pattern became completely different. The narrow band of enzyme activity was much more diffuse and widely distributed throughout portions of the cell wall. By staining adjacent sections for either acid phosphatase activity or cell wall carbohydrate, Ashford & Jacobsen (1974) were able to relate phosphatase migration to cell wall degradation. Large segments of cell wall became void of carbohydrate, apparently forming channels that permitted the acid phosphatase to readily diffuse away from aleurone cells, into the endosperm or into the surrounding medium. The extensive dissolution of barley aleurone cell walls has also been observed by scanning electron microscopy (Pomeranz, 1972). The enzymes that participated in the depletion of cell wall material could not have been identified until the exact nature of the cell walls was firmly established. Using wheat aleurone tissue, Fulcher, O'Brien & Lee (1972) found that the aniline blue staining procedure was not specific for beta-1,3-linkages, and concluded that the aleurone cell walls were dominated by 1,4-linkages. A much more rigorous analysis of barley aleurone layer cell wall substantiated this conclusion (McNeil *et al.*, 1975). Arabinoxylans constituted 85% of the aleurone cell wall, with beta-1,4-xylosyl linkages forming the structural foundation of this polymer. In light of this discovery, new attempts were made to identify cell wall

degrading enzymes. Taiz & Honigman (1976) found that exo- and endo-xylanase activity increased dramatically in barley aleurone layers in response to applied gibberellic acid. Although the release of endo-xylanase just preceded the release of amylase, suggesting the involvement of endo-xylanase activity in enzyme release, extensive cell wall degradation had occurred several hours prior to the appearance of endo-xylanase activity (Taiz & Honigman, 1976). This accumulated evidence implied that endo-xylanase was central in facilitating enzyme release from layers. However, the irregularity in the time course has yet to be resolved satisfactorily.

The effect of ethylene on the release of amylase from aleurone tissue may have also been dependent on the activity of cell-wall degrading enzymes. Examination of the role of ethylene in amylase production was conducted by Ho, Abroms & Varner (1977) to determine if ethylene affected the secretion of the amylase protein from the cell, or the diffusion of amylase through the thick cell walls of barley aleurone cells. The latter process would be sensitive to the activity of cell wall degrading enzymes. Amylase is very sensitive to low pH, and it has been found that aleurone tissue can be exposed to 1 mM hydrochloric acid without disrupting the integrity of the cells (Varner & Mense, 1972). This treatment inactivated extracellular amylase (Varner & Mense, 1972), but membrane-bound amylase was protected (Locy & Kende, 1978). Using this methodology,

Ho, Abroms & Varner (personal communication) determined that ethylene reduced the amount of amylase activity associated with the cell wall to less than 12% of the quantity of bound amylase in the absence of ethylene. Therefore, ethylene facilitated the passage of amylase out of the aleurone tissue, but not out of the individual cells. They speculated that this response could be mediated through the action of ethylene on the synthesis of cell wall degrading enzymes by the aleurone cells. However, contrary to this hypothesis, ethylene did not stimulate the release of acid phosphatase from aleurone layers

The proteolytic enzymes of aleurone tissue may also influence the amylase activity found after exposure to gibberellic acid. The *de novo* synthesis of protease paralleled the synthesis of amylase, with respect to both time of release (MacLeod *et al.*, 1964; Jacobsen & Varner, 1967) and dependence on the concentration of available gibberellic acid (Jacobsen & Varner, 1967). However, it was not until several years later that the interdependence of these two enzyme systems was realized. Bromate is an effective inhibitor of barley protease (Bhatty, 1968; Enari, Puputti & Mikola, 1964) and has been used extensively to reduce the solubilization of protein during the malting process (Macey & Stowell, 1961). The ability of bromate to inhibit the solubilization of protein and release of amino acids from isolated barley aleurone layers has also been demonstrated (Melcher & Varner, 1971). The presence of 5 mM

potassium bromate in the incubation medium reduced total amylase production in the first 24 h period by 75% (Ho & Varner, 1978; Melcher & Varner, 1971). To demonstrate that the suppression of amylase synthesis was a result of the reduced availability of amino acids for protein synthesis, a consequence of inhibited protease activity, free amino acids were added to the medium in addition to the bromate. Supplementing the medium with 10 mg/ml casein hydrolyzate reduced the inhibition by bromate to 43% (Melcher & Varner, 1971), while the addition of 10 mg/ml casein hydrolyzate plus 0.5 mg/ml tryptophan to the bromate medium produced a total restoration of amylase synthesis to control levels (Ho & Varner, 1978).

In addition to the importance of the proteolytic enzymes to amylase synthesis during the early stages of germination, these enzymes may have an equally significant role in the degradation of amylase at later stages of development. Many papers have shown that amylase activity increased to a maximum, then plateaued or declined with extended incubation periods. This effect has been observed with intact seedlings (Briggs, 1968a), half-seeds exposed to gibberellic acid (Briggs, 1968a; Varner & Chandra, 1964; Varner, Chandra & Chrispeels, 1965) and isolated aleurone layers, also exposed to gibberellic acid (Clutterbuck & Briggs, 1973; Jacobsen & Varner, 1967; Taiz & Honigman, 1976). Briggs (1968a) demonstrated that the decline in amylase activity in the intact seedlings was accompanied by

a decline in the pH of the endosperm. The lower pH values not only destablized amylase (Greenwood & MacGregor, 1965), but also approached the optimal pH for protease activity (Bhatty, 1968; Enari & Mikola, 1968). A similar drop in pH was also observed in an inadequately buffered culture medium containing either half-seeds (Briggs, 1968a) or isolated aleurone layers (Clutterbuck & Briggs, 1973). These observations suggested that decreasing pH and increasing proteolytic activity may have been instrumental in causing the decline in amylase activity during prolonged exposure to gibberellic acid.

F. The effects of inorganic ions on enzyme release from aleurone tissue

The availability of inorganic ions also served a regulatory function during the gibberellic acid induction of enzyme release from aleurone layers. Studies of the relationships between synthesis and release of alpha-amylase and inorganic ions were hampered by the dependence of barley alpha-amylase on Ca^{+2} for activity and stability (Greenwood & MacGregor, 1965). Calcium ions had to be added to the medium suspending the isolated aleurone layers, otherwise, very little amylase activity would be observed (Chrispeels & Varner, 1967; Clutterbuck & Briggs, 1973) after the incubation period. Calcium ions could not be replaced by salts of magnesium, barium or cadmium, although strontium salts were almost as effective as calcium salts in

permitting the recovery of amylase activity (Chrispeels & Varner, 1967). It was impossible to ascertain from these experiments whether the other cations were preventing the synthesis of amylase, or if the amylase was being deactivated in the absence of the stabilizing effect of calcium, although the concentration of calcium required for maximum amylase synthesis exceeded the calcium concentration required to satisfy the calcium-dependence of amylase activity (summarized in Carr & Goodwin, 1972). To resolve this ambiguity, a flow-through cell was employed that permitted the aleurone tissue to be incubated in a medium lacking calcium, but as the medium passed around the layers, it was immediately added to a buffered calcium solution. This would aid in the preservation of any amylase that had been released into the medium (Varner & Mense, 1972). Using this approach, it was found that calcium, magnesium and potassium salts facilitated the release of amylase from isolated layers. The aleurone layer was the major source of inorganic ions in cereals (Hinton, 1959; Liu & Pomeranz, 1975) and the release of large quantitites of these inorganic ions from isolated aleurone layers was dependent on the application of gibberellic acid (Jones, 1973). The inorganic ions released from the aleurone of germinating intact seedlings were able to, in turn, stimulate the release of amylase from isolated aleurone layers that had been exposed to gibberellic acid (Varner & Mense, 1972). Although the prescribed liberation of these ions may be one

means of regulating amylase release from aleurone layers, the release of amylase directly from the lysosomal fraction of aleurone cells was not significantly enhanced by the addition of calcium ions. It was conceded, however, that sufficient calcium may have been bound to various cell fractions to saturate the dependence on calcium (Gibson & Paleg, 1977). The distribution of inorganic ions could play a significant role in modifying the gibberellic acid-induced responses of aleurone cells through other mechanisms. As previously mentioned, gibberellin A1 was preferentially bound to the aleurone grain fraction from the aleurone cells of wheat (Jelsema *et al.*, 1977). This binding was dependent on the availability of free calcium ions. The omission of calcium from the incubation medium or the addition of the calcium-chelating agent ethyleneglycol-bis (beta-amino ethyl ether) N,N'-tetraacetic acid (EGTA) eliminated the association of tritiated gibberellin A1 with aleurone bodies.

Another aspect of the possible involvement of calcium ions was investigated using intact barley seedlings germinated on filter paper (Briggs, 1968a). The levels of free calcium in the endosperm were rapidly depleted as seedling growth proceded. This decline in available calcium was accompanied by a sharp loss of amylase activity in the grain. The loss of calcium from the endosperm may tend to destabilize the amylase, rendering it more sensitive to changes in pH and more susceptible to proteolytic attack

(Briggs, 1968a).

The inorganic ions of aleurone layers are predominantly associated with the aleurone grains (Pomeranz, 1973; Liu & Pomeranz, 1975), and phosphate, potassium, magnesium and calcium are the dominant ions. The very large amount of phosphate was indicative of the presence of phytin, a major component of aleurone grains (Jacobsen, Knox & Pyliotis, 1971). During germination, phytin was hydrolyzed to phosphate and myo-inositol by phytase, another enzyme produced in response to gibberellic acid during germination (Srivastava, 1964). This could account for the large efflux of phosphate, potassium and magnesium from the aleurone layers (Jones, 1973). Presumably, calcium would also be released, as indicated by the loss of calcium from the caryopsis of germinated barley (Briggs, 1968a). On the basis of these observed responses involving inorganic ions, it is possible that any factor that can modify the normal distribution of ions in the tissue may also affect normal enzyme production and secretion. Ethylene has been shown to cause a stimulation of ion release from some tissues as exemplified by the release of chloride and rubidium from flower petals (Hanson & Kende, 1975). The nature of this induced ion transport was not known, but a general increase in membrane permeability appeared unlikely (Mehard, 1969).

G. Ethylene methodology

Ethylene, a gas at laboratory temperature and pressure, is capable of modifying many phases of plant growth and development (Abeles, 1973). Many of these effects have been detected in response to very low concentrations of ethylene. In order to obtain meaningful results, experiments and apparatuses must be carefully designed to accomodate the volatile nature of ethylene and the sensitivity of many tissues to trace quantities of ethylene. Similarily, potential environmental sources of ethylene must be eliminated. As previously mentioned, soils release ethylene under some circumstances, but urban air (Abeles, Forrence & Leather, 1971; Jacobsen, 1973), laboratory air (Jacobsen, 1973) and compressed air cylinders (Eastwell, Bassi & Spencer, 1978) also contain significant amounts of ethylene. In addition, components of experimental equipment may contribute ethylene to the test environment (Bassi & Spencer, 1979; Smith and Restall, 1971). The need for adequate controls was emphasized by a study on the ethylene induction of increased glucanase activity in bean leaves (Abeles *et al.*, 1971). Ambient levels of ethylene were sufficient to saturate the response to ethylene, therefore, the introduction of additional ethylene produced no detectable effect and a potential ethylene response had been masked. However, when an adsorbent was employed to reduce the ethylene concentration in the control environment, a response to ethylene was readily observed. Jacobsen (1972)

suggested that a similar situation existed in the studies of barley aleurone tissue. Ambient ethylene may have partially masked the response to any increment in ethylene concentration over the control levels. Therefore, it is essential, for quantitative results, to eliminate ethylene from the control environment.

For ease of handling and analysis, experiments on ethylene effects and production typically have been conducted in closed systems (Ward *et al.*, 1978). As a consequence of enclosure, tissues were exposed to accumulating volatiles including ethylene (Ku *et al.*, 1970; Jacobsen, 1973) and carbon dioxide (Ku *et al.*, 1970). Carbon dioxide interacts wih ethylene in several systems (for example Abeles, 1973; Burg & Burg, 1967; Toole, Bailey & Toole, 1964). The concentration of oxygen also varies, through respiratory or photosynthetic activity. The modification of the atmosphere surrounding the tissue could be reduced by directing a stream of air continuously through the sample (Ward *et al.*, 1978). The effects of ventilation were exemplified by Ku *et al.* (1970). Their results showed that the addition of ethylene to the air of sealed flasks (final concentration, 10 nl/ml) resulted in rice coleoptile extension 53% greater than controls also maintained in sealed flasks , but 240% greater than controls in a continuous flow system. In the sealed flasks containing the rice seedlings, carbon dioxide had accumulated to concentrations exceeding 12%. This illustrated the need for

adequate gas exchange during plant physiological studies.

In spite of these general considerations favouring free air exchange for experimental tissues, previous studies on the effects of ethylene on barley aleurone layers have been conducted exclusively in closed systems (Jacobsen, 1973; Jones, 1968; Scott & Leopold, 1967). Jacobsen (1973) claimed that enclosure of barley aleurone layers had no effect on amylase synthesis and surmised that changes in oxygen and carbon dioxide required no further considerations. Nevertheless, the consequences of varying carbon dioxide and oxygen concentrations on ethylene responses and production, respectively, have been well documented in a number of plant tissues (Abeles, 1973; Abeles & Forrence, 1970; Kang *et al.*, 1967; Keys *et al.*,1975). During a study on the synthesis of amylase by barley aleurone layers, it was observed that the concentration of ethylene in sealed flasks increased as a result of ethylene production by the tissue (Jacobsen, 1973). This inadequate control of gas composition could lead to erroneous conclusions. Therefore, for the present study, it was necessary to devise a continuous flow system to prevent the accumulation of abnormal gas concentrat is during the incubation of isolated aleurone layers.

A continuous flow of air through the sample was also required during the initial incubation period of barley half-seeds. It has been shown that the exposure of aleurone tissue to ethylene before the addition of gibberellic acid

suppressed amylase release (Jones, 1968). An open system would alleviate the possible accumulation of ethylene around the half-seed.

A continuous flow system is only a partial solution. Most sources of air contain ethylene as well as other hydrocarbons. Some methods were available to remove ethylene from an air system (Abeles, 1973) but little attention has been directed to other light hydrocarbons. Research has shown that ethane and other hydrocarbons were capable of accelerating mitochondrial swelling (Mehard, 1969; Mehard & Lyons, 1970) and altering the physical properties of model membrane systems (Mehard, 1969; Mehard, Lyons & Kumamoto, 1970). In spite of these effects, however, several studies have failed to detect any physiological response of plants to methane or ethane (Burg & Burg, 1967; Heck & Pires, 1962; Jacobsen, 1973). The environment of the plant should be free from any hydrocarbons that may influence the normal biochemical processes within the sample. Most methods currently in use for plant physiology studies were inadequate, and new methodology had to be developed.

H. Summary

From the discussion, it is apparent that isolated aleurone layers from cereals offer a unique opportunity to study hormonal control. Three plant growth regulators (gibberellins, ethylene and abscisic acid) have been shown

to be active in this biological system, but large voids remain in our understanding of their actions and their interactions. Despite the apparent simplicity of the tissue, the interdependence of several metabolic processes is evident. These relationships make it hazardous to interpret effects of any one segment of the hormonal response of aleurone layers, without considering possible interferences from other responses within the same tissue. Aleurone cells, like most other plant and animal cells, are sensitive to their environment. In the case of isolated aleurone layers, factors relevant to the hormone-induced production of hydrolytic enzymes include: inorganic ion concentration, pH, extent of aeration, microbial attacks, the presence of antibiotics and ambient ethylene concentrations. All of these parameters must be carefully controlled in order to permit the acquisition of explicit results. Many existing practices are deficient in environmental conditions, and it is considered necessary to expand or develop certain new techniques.

III. METHODS AND MATERIALS

A. Analysis of gases

Gaseous samples

The hydrocarbon and carbon dioxide contents of sample gases were determined by gas chromatography with a Hewlett-Packard (Model 5830A) gas chromatograph equipped with four-way sideport valves. Typically, 1 ml to 5 ml gas samples were injected onto the analytical column, a stainless steel column (300 cm x 0.3175 cm OD) packed with PorapaK Q (80 to 100 mesh, Waters Associates Inc., Milford, Mass.). When analyzing the very low concentrations of hydrocarbons encountered in many of the air samples, a fine needle (26 guage x 1.27 cm long) had to be used with the gas tight syringe. The penetration of the septum by a coarser needle consistently produced a sizeable peak that coincided with the methane peak on the chromatogram. This effect was observed regardless of the brand or style of septum used. The flow rate of the carrier gas, helium, was maintained at 40 ml/min. The analyses were performed isothermally at 40 C. A 1:1 effluent splitter was attached to the end of the column; one-half of the flow was directed through a hydrogen flame ionization detector (FID) for the resolution of hydrocarbon peaks, while the remaining portion of the gas flowed through a thermal conductivity detector (TCD) for the determination of carbon dioxide. An auxiliary flow of 30 ml/min of carrier gas was introduced into the jet of the

hydrogen flame to improve the stability of the signal from the flame ionization detector. The limit of detection, corresponding to a signal-to-noise ratio of approximately 2, was 0.05 ng of each hydrocarbon. The peak areas on the chromatograms were quantified by a built-in electronic integrator (sensitivity limit 0.5 ng). The areas of smaller peaks were determined manually by height-width measurements. The areas of peaks were converted to units of nl/ml of gas by comparison to chromatograms of external standards. These standards were analyzed hydrocarbon/helium mixtures (Union Carbide of Canada Ltd., Oakville, Ont.) and analyzed carbon dioxide/air mixtures (Matheson of Canada Ltd., Whitby, Ont.). The hydrocarbon mixtures were further checked against analyzed standards (Union Carbide of Canada Ltd., Oakville, Ont.) that had been tested by an independent laboratory (Chemical and Geological Laboratories Ltd., Edmonton, Alberta).

When a more sensitive method of detecting ethylene was required, the effective sample size was increased by the use of a collection trap (De Greef, De Proft & De Winter, 1976; Stinson & Spencer, 1969). Ethylene was trapped on 1.5 g silica gel (30 to 60 mesh; Matheson, Coleman and Bell, Cincinnati, Ohio) packed in a copper U-tube (16 cm x 0.635 cm OD). Preliminary experiments indicated that during manipulation of the trap, sufficient room air was retained in the U-tube to contribute detectable amounts of hydrocarbons to the sample. A four-way ball valve (Whitey

Co., Oakland, Calif.) was attached to the U-tube to exclude room air from the trap. Residual hydrocarbons from previous runs or from room air were exhausted by immersing the trap in boiling water and purging with sample gas for 10 min. At this elevated temperature, the retention of ethylene was prevented. After purging the trap, the ball valve was rotated to bypass the U-tube, and the trap was immersed in a dry ice/acetone slurry. It reached temperature equilibirium in 2 to 3 min. The flow of sample gas through the U-tube was then re-established by turning the ball valve. This procedure trapped any ethylene present in the gas sample. After an appropriate length of time, the valve was again turned so that the gas flow bypassed the trap. The collection system, while still immersed in dry ice/acetone, was attached to the sideport valve on the gas chromatograph. Carrier gas was flushed through the sideport connections, while the U-tube containing the trapped ethylene was heated in a boiling water bath for 10 to 15 min to dissociate the ethylene from the adsorbent. The ball valve of the trap was then turned to direct the carrier gas through the U-tube and sweep the liberated ethylene to the gas chromatography column. Operated in this manner, the trap functioned at 100% efficiency with respect to the collection and subsequent release of ethylene from an air stream. It has also been noted that smaller quantities of silica gel could be used in the U-tube if it was necessary to reduce the back pressure created by the collection system (Bassi & Spencer,

1979).

The concentration of oxygen in gases was determined by drawing the sample into a portable oxygen analyzer (Model D2; Beckman Instruments, Inc., Fullerton, Calif.)

The concentration of the oxides of nitrogen in an air stream was determined by absorbing nitrogen dioxide in an aqueous triethanolamine solution and measuring the final nitrogen dioxide concentration in the absorbent by the Griess-Saltzman reaction (American Public Health Association, 1977b). Although nitric oxide should have been spontaneously oxidized to nitrogen dioxide (Nash, 1950), a constant humidity regulator and a nitric oxide oxidizer (American Public Health Association, 1977a) were placed in series upstream from the absorbing reagent to ensure complete conversion of nitric oxide to nitrogen dioxide for the analysis. The technique was calibrated using standard solutions of sodium nitrite.

Dissolved gases

The gases dissolved in liquids were determined by two methods: (1) vacuum extraction of gases from solution, and (2) stripping of gases from solution with an inert carrier gas. In both cases, the dissolved hydrocarbons were subsequently analyzed by gas chromatography. The gas chromatograph was as described above, but to accommodate the larger volume of gas sample entering the column, a stainless steel column of a greater diameter was used (245 cm x 0.635 cm OD). The column was packed with either Porapak Q

(80 to 100 mesh; Waters Associates Inc., Milford, Mass.) or activated alumina (80 to 200 mesh; Matheson, Coleman and Bell, Cincinnati, Ohio). In accordance with the larger column diameter, the flow rate of the helium carrier gas was increased to 60 ml/min unless otherwise stated. The carrier gas passed directly from the column to the hydrogen flame ionization detector. The analysis was performed isothermally at 40 C, but after each run, the column was heated to 120 C for 5 min with the carrier gas flowing to expel excess moisture from the column. The absolute amount of ethylene in each sample was calculated from the chromatograms as described above.

The theoretical values for the amounts of ethylene dissolved in water were estimated through Henry's law:

$$p = (X)(k)$$

where p was the partial pressure of gaseous ethylene in equilibrium with the solution and X was the mole fraction of dissolved ethylene. Henry's law constant was represented by k, and was equal to 8.67×10^6 mm mercury/mole fraction for a solution of ethylene in water at 25 C. For dilute aqueous solutions, this estimate was valid to within 3% of the actual values (Daniels & Alberty, 1966).

1. Vacuum extraction method.

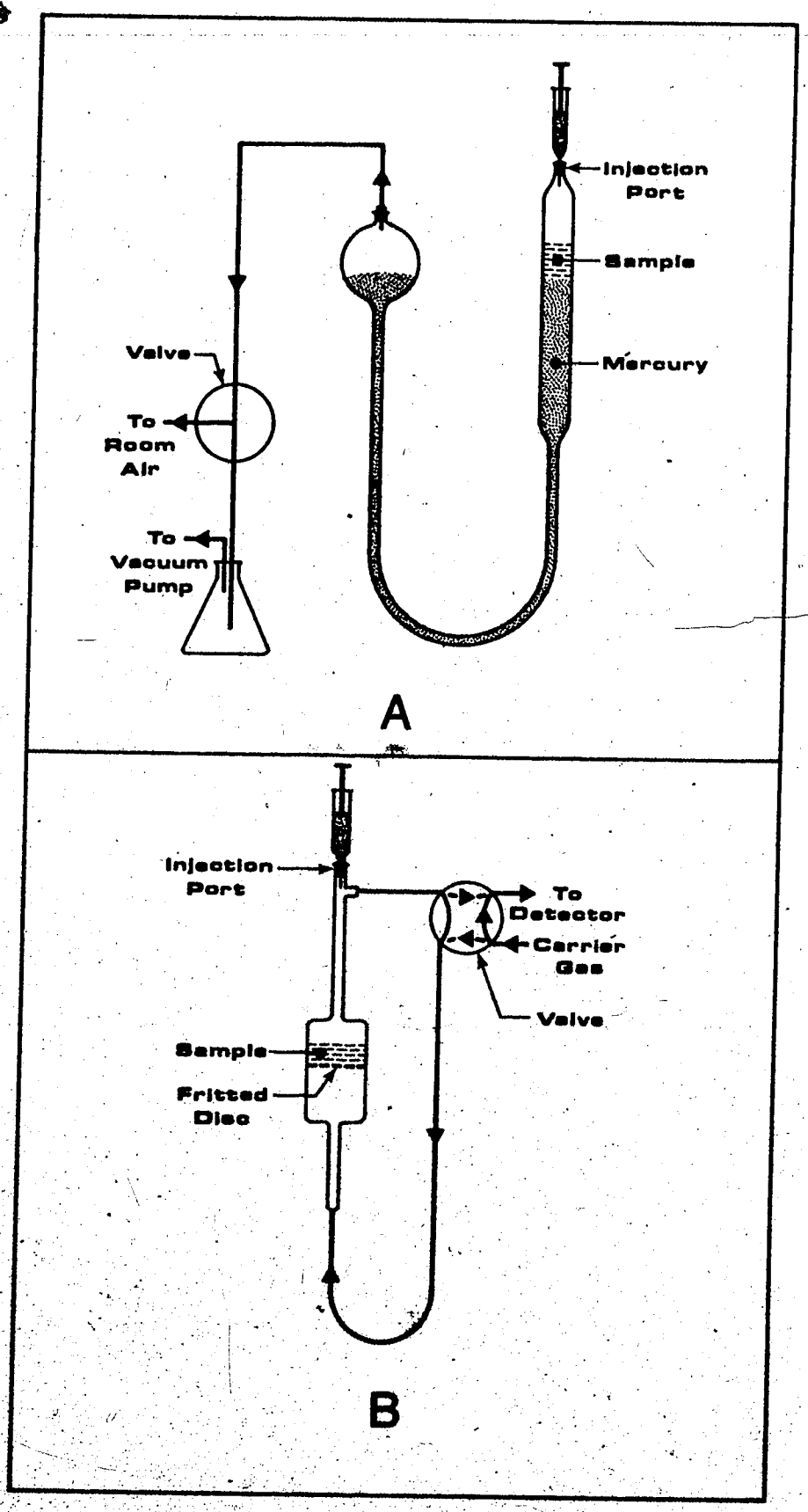
The vacuum extraction technique depended on reduced pressure in the sample chamber to reduce the solubility of gases in liquid. The vacuum was generated with a vacuum pump, but the vacuum was transmitted through a mercury

column to permit containment of the sample. The apparatus (Figure 1A) consisted of a cylindrical separatory funnel (12 cm x 3.3 cm OD) filled with mercury, with the outlet connected to a mercury reservoir with Tygon tubing. The top of the funnel was sealed with a hollow ground glass stopper that had been modified to accomodate a silicon rubber septum. To measure the amount of dissolved ethylene, hydrocarbon-free air (Eastwell *et al.*, 1978) was injected into the funnel, followed by the appropriate volume of liquid sample. Then a vacuum (508 mm of mercury in our system) was applied to the reservoir by the vacuum pump, and the height of the funnel was adjusted to equalize the level of the mercury column in the reservoir and in the funnel. While the vacuum was applied, the funnel was gently tapped to dislodge gas bubbles that formed on the surface of the glass and mercury. After the desired extraction time, the vacuum was released and the height of the funnel was again adjusted to equalize the mercury levels. The entire gas mixture was quickly withdrawn through the septum into a gas tight syringe and injected into the gas chromatograph for analysis. The vacuum pump was vented into a fume hood to prevent the possible contamination of the laboratory with mercury vapours.

2. Gas stripping method.

This technique used the carrier gas of the gas chromatograph to displace dissolved gases from a liquid sample, and carry the displaced gases directly onto an

Figure 1. Schematic diagrams of the apparatuses for extraction of ethylene from aqueous solutions. A. Vacuum extraction technique. B. Gas stripping technique.


Injection Port
Sample
Mercury
Valve
To Room Air
To Vacuum Pump
A
Injection Port
To Detector
Carrier Gas
Valve
Sample
Fritted Disc
B

analytical column (Swinnerton, Linnenbom & Cheek, 1962). The sample chamber (Figure 1B) consisted of a glass cuvette attached to the sideport valve of the gas chromatograph. The top of the cuvette was sealed with a septum contained within an injection port. A fritted glass disc (20 to 100 micron pore size) bisected the glass bulb of the cuvette. Prior to each analysis, the cuvette was flushed with carrier gas for 5 min. Then the sideport valve was turned so that the carrier gas bypassed the cuvette. Any residual back pressure in the cuvette was released by briefly perforating the septum with a fine hypodermic needle. The liquid sample was injected into the cuvette, and the flow of carrier gas through the chamber was quickly restored to the specified flow rate by turning the sideport valve. The stripped gases were carried directly onto the column for analysis.

3. Comparison of methods for dissolved gases.

The performances of the methods described above were evaluated by comparing the results obtained from analysis of standard solutions. Sample gases were prepared by mixing hydrocarbon-free air (Eastwell *et al.*, 1978) and a commercial mixture of 100 nl/ml ethylene in air, at different relative flow rates. The concentration of ethylene in the resultant mixture was determined by gas chromatography. The ethylene/air mixture was then bubbled overnight through deionized glass distilled water in a 1 l gas washing bottle. Since the solubility of gases is temperature dependent, the bottle was maintained at 25 C in

a water bath. The bottle outlet allowed samples of the solution to be drawn into a syringe through a long hypodermic needle. The syringe was flushed with the liquid phase three times before samples were taken. The liquid was withdrawn slowly to avoid premature release of dissolved gases.

B. Methods for removing hydrocarbons from air

Preliminary analysis indicated that laboratory air, air from compressed air cylinders and from on site compressed air lines, contained appreciable amounts of low molecular weight hydrocarbons. In an effort to eliminate these contaminants from air supplies, a number of methods were evaluated for their ability to remove hydrocarbons, including ethylene, from an air stream.

In order to compare the efficiencies of each of the methods tested, a single cylinder of compressed air was used throughout the evaluation procedure. Analysis by gas chromatography indicated that this cylinder contained 9.16 nl/ml methane, 0.020 nl/ml ethylene and 0.420 nl/ml ethane. Analysis of other air cylinders indicated that they contained similar amounts of these low molecular weight hydrocarbons.

The methods that were compared for their suitability for biological studies are summarized in Table I, and included the use of adsorbents (methods 1 to 9), liquid traps (methods 10 and 11) and solid reactive materials

Table I. Relative effectiveness of methods compared for their ability to oxidize hydrocarbons in an air stream

The preparation of the reagents and the assembly of the apparatus are described in the text. The method numbers correspond to the numbers given in the text. Time 0 refers to the time when the first samples were taken after the apparatus had been purged with compressed air for 10 minutes.

Method Number	Description	Temp. (C)	Hydrocarbon Concentration (Per Cent of Control[a])								
			Time of Sampling (min)								
			0			30			60		
			Methane	Ethane	Ethylene	Methane	Ethane	Ethylene	Methane	Ethane	Ethylene
1	Coconut charcoal (50 - 200 mesh)	22	92.6	0	0	91.2	0	0	100.8	0	0
2	Coconut charcoal (50 - 200 mesh)	-86	8.8	0	0	7.3	0	0	15.1	0	0
3	Coconut charcoal (50 - 200 mesh)	-196	0.6	0	0	0.6	0	0	0.5	0	0
4	Silica gel (30 - 60 mesh)	-86	3.2	0	0	100.6	0	0	99.1	0	0
5	Matrasorb T (4 - 12 mesh)	-86	89.8	0	0	100.0	0	0	100.5	0	0
6	Molecular sieve 13X (60 - 80 mesh)	-86	40.1	0	0	98.6	0	0	101.3	0	0
7	Molecular sieve 13X (60 - 80 mesh)	-196	2.1	0	0	2.7	0	0	30.1	0	0
8	Davison molecular sieve, 0.3 nm pore size (4 - 8 mesh)	-86	97.8	13.3	0	100.0	97.1	0	100.4	104.3	0
9	Davison molecular sieve, 0.3 nm pore size (4 - 8 mesh)	-196	74.4	0	0	56.6	0	0	89.1	0	0
10	40% aqueous formaldehyde in concentrated sulphuric acid (3.3 : 100)	22	96.4	94.8	75.0	98.7	99.8	80.0	98.1	94.8	80.0
11	40% aqueous formaldehyde in fuming sulphuric acid (3.3 : 100)	22	96.1	89.8	0	97.8	102.4	b	97.2	97.6	75.0
12	Brominated charcoal (20% w/w) followed by activated coconut charcoal	22	93.9	0	0	96.3	0	0	97.6	b	0
13	20% (w/w) potassium dichromate on silica gel with 9% (v/w) fuming sulphuric acid	22	96.2	92.1	0	94.2	96.7	0	93.6	96.7	0
14	20% (w/w) potassium permanganate on silica gel with 9% (v/w) fuming sulphuric acid	22	97.4	96.7	0	99.1	101.7	0	99.0	102.1	0
15	20% (w/w) potassium permanganate on silica gel with 9% (v/v) fuming sulphuric acid followed by 20% (w/w) chromotropic acid on silica gel	22	98.9	71.7	0	99.9	79.0	0	100.4	73.6	0
16	20% (w/w) iodine pentoxide on silica gel with 9% (v/v) fuming sulphuric acid	200	96.5	98.6	0	93.8	b	0	89.5	0	0
17	20% (w/w) selenium oxide on silica gel with 9% (v/v) fuming sulphuric acid	22	97.3	98.6	0	96.8	102.6	0	96.4	96.2	0
18	9% (v/v) fuming sulphuric acid on silica gel	22	100.1	99.8	60.0	101.0	106.2	66.0	99.3	103.1	70.0
19	2% (w/w) silver nitrate on silica gel	22	100.1	102.1	0	104.0	100.0	0	97.9	103.8	0
20	30% (w/w) cupric oxide, 0.3% (w/w) ferric oxide in Kieselguhr pellets	800	0	0	0	0	0	0	0	0	0
21	90% (w/w) cupric oxide powder dispersed in Chromosorb P (42 - 60 mesh)	800	0	0	0	0	0	0	0	0	0
22	5% (w/w) platinum on asbestos fibres	650	0	0	0	0	0	0	0	0	0

a The concentration of methane, ethane and ethylene in the control air supply was 9.16 ml/ml, 0.420 ml/ml and 0.020 ml/ml respectively. These concentrations were regarded as 100.0% for ease of comparison.

b The hydrocarbon peak on the gas chromatogram was detectable, but below the limit of integration.

(methods 12 to 22). The solid adsorbents (Stinson & Spencer, 1969; Szepesy, 1970) were activated at 200 C for at least 16 hours before being packed into a copper U-tube (45 cm x 1.27 cm OD). Cocoanut charcoal, Molecular Sieve 13X and Davison Molecular Sieve were obtained from Fisher Scientific Co. (Fair Lawn, N.J.) while silica gel was from Matheson, Coleman and Bell (Cincinnati, Ohio). Natrasorb T, an amorphous form of silica gel, was distributed by Cullen Industries Inc. (Buffalo, N.Y.). When the solid adsorbents were used at -86 C, the U-tube was immersed in a dry ice/acetone slurry. A temperature of -196 C was attained by placing the U-tube in a liquid nitrogen bath. The liquid traps (methods 10 and 11) were constructed from gas washing tubes. Ten ml of the absorbing reagent, 40% aqueous formaldehyde in either concentrated sulphuric acid (Weber, 1953) or fuming sulphuric acid, were pipetted into the tube and the air stream was dispersed in the liquid through a fritted glass aerator of medium porosity. The solid reactive materials (methods 12 to 22) were packed in a stainless steel tube (45 cm x 1.27 cm OD). When necessary, higher temperatures were attained by enclosing the steel tube in the 18 cm long heating chamber of a semi micro-combustion furnace (Sargent-Welch Scientific Co.). Cocoanut charcoal (method 12) was brominated by slowly adding bromine to the charcoal with constant stirring (Southwick & Smock, 1943). The charcoal and bromine were allowed to equilibrate overnight in a stoppered flask. The

leading half of the trap was filled wih brominated charcoal and followed by activated charcoal in the second half to trap any residual bromine that had not been completely adsorbed. When potassium dichromate (method 13; Kitagawa & Kobayashi, 1955) and potassium permanganate (methods 14 and 15; Forsyth, Eaves & Lockhart, 1967 and Drager, 1957, respectively) were used as active reagents, they were added to the silica gel as a 40% (w/v) suspension in water, then dried at 200 C overnight. Chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid) was deposited on silica gel (Drager, 1957) in the same way, and then dried at 60 C *In vacuo* for 3 hours (method 15). Crystalline iodine pentoxide (method 16) was triturated and added directly to the silica gel to obtain a uniform suspension (Anonymous, 1957; Vogel, 1968). Selenium oxide reagent (Grosskopf, 1957) was prepared by adding selenious acid to silica gel and heating the mixture to 200 C overnight (method 17). Sublimation to yellow crystals in the stoppered flask indicated the formation of selenium oxide. Silver nitrate (method 19) was deposited on silica gel as a 4% (w/v) solution and dried at 90 C for 3 hours (Nelson & Mulun, 1957). Fuming (30% sulphur trioxide) or concentrated sulphuric acid, when used in any of the above methods, was added immediately before packing the column. Cupric oxide (copper (II) oxide) pellets (method 20; Nash, 1950) were prepared essentially as described by Schenk (1963) with the following modifications. Ferric oxide (iron (III) oxide)

powder was added to the cupric oxide/Kieselguhr paste to improve the efficiency of the catalyst (Vogel, 1968). The mixture was extruded through a syringe and dried at 200 C to form pellets (0.8 cm x 0.2 cm OD). The reduction of cupric oxide with hydrogen [illegible] 1963) was omitted. Method 21 also used cupric oxi[illegible]talyst (Gane, 1936; Norem, 1958). To reduce [illegible] pressure created by the fine powder, the cupric oxide was dispersed in Chromosorb P (Norem, 1958). Platinum catalysts (method 22: Kobe & MacDonald, 1941; Nash, 1950) were commercially available (K and K Laboratories Inc., Plainsview, N.Y.).

All connections and tubing were metal or glass to prevent contamination of the air stream with hydrocarbons released by the apparatus. In each case, the apparatus was allowed to achieve temperature equilibrium before the flow rate of 100 ml/min of air was established through each purification system. The apparatus was then flushed for 10 min before the first samples were taken from the effluent air stream through a minimum bleed septum mounted in a metal tee. Succeeding samples were then taken at 30 min intervals. At each designated time, three 5.0 ml samples were drawn into 5 ml syringes. Each syringe was flushed with effluent air five times before taking the sample, and the delay between sampling and analysis was kept to a minimum. Analysis of the air samples was performed as described in a previous section. Methods 20 to 22 were subjected to closer scrutiny by making 30 min collections to

detect lower concentrations of ethylene.

C. Gas mixing and delivery systems

Two separate systems were constructed to allow samples to be exposed to the desired experimental conditions. One system was used predominantly for the preliminary incubation of barley half-seeds, while the other was used for the ethylene studies on isolated barley aleurone layers. (The preparation of these tissue-types will be described in detail in a later section.)

A relatively simple system was used to administer air during the incubation of barley half-seeds. The air source for this system was the air compressed on site. A two-stage regulator reduced the line pressure to 350 g/cm^2 and the flow rate of air through the system was regulated and maintained at 100 ml/min by a needle valve. The air passed through a heated column of platinized asbestos catalyst to oxidize hydrocarbons. The effluent air line entered a growth cabinet (Model Cel-8, Sherer-Gillett Co., Marshall, Mich.) maintained at 25 C. Inside the chamber, the air bubbled through a 125 ml gas washing bottle filled with deionized, glass distilled water. This humidified the air to prevent desiccation of the tissue. The outlet was connected to a cross union. One branch was sealed with a minimum bleed septum to permit sampling at regular intervals for air analysis, while the two remaining branches led to the samples. For the routine preliminary incubation of

barley half-seeds, the air purged flasks containing sterile half-seeds (Figure 2). Each air line passed through a 2 micron sintered steel filter (Nupro Co., Willoughby, Ohio) to trap particulates and water mist that may have escaped from the gas washing bottle. The effluent air passed through a bulkhead union in the metal culture tube closure of a longnecked 250 l erlenmeyer flask. The opposite end of the union was attached to a segment of stainless steel tubing (~11 cm x 0.3175 cm OD). Thus, the gas inlet was at the bottom of the flask, and the excess air was allowed to vent from underneath the metal closure.

The second system allowed the introduction of various amounts of ethylene into the air stream. In this case, however, the air supply was a commercial cylinder of compressed air. The pressure was reduced to 700 g/cm² through a two-stage regulator. The air passed through a heated column of platinized asbestos catalyst and to a tee union. A needle valve on either side of the tee was used to adjust the flow rates. The air on each side of the tee was then dispersed in a 5% (w/v) solution of sodium hydroxide in gas washing tubes. The effluent from each tube was split at another tee, and each branch was again connected to a needle valve. These valves permitted balancing the flow rate through both branches of the latter tee. As the air emerged from each valve, it again entered a tee. One branch was sealed with a minimum bleed septum and served as a sampling port for analysis of the air. The remaining branch was

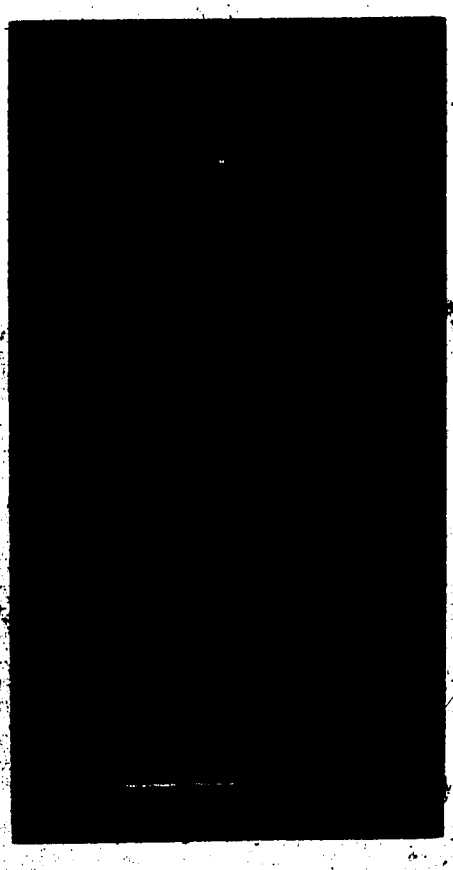


Figure 2. Flask assembly for the incubation of barley half-seeds in hydrocarbon-free air.

typically connected to a culture tube containing isolated aleurone layers.

When desired, ethylene was introduced into the air stream through a tee in one of the two air lines. (The ethylene inlet was situated beyond the needle valve after the air flow was split into two components.) Therefore, when ethylene was supplied, one half of the air flow remained hydrocarbon-free, while the other contained metered amounts of ethylene. Ethylene was supplied from one of two cylinders of specialized mixture, either 113 nl/ml or 219 nl/ml of research grade ethylene in ultra-zero air (Matheson of Canada Ltd., Whitby, Ont.). The pressure was reduced to 700 g/cm² through a standard two-stage regulator, and then further reduced to between 0 and 500 g/cm² through a low-pressure regulator (Model 70, Matheson of Canada Ltd., Whitby, Ont.). The ethylene mixture then passed through a capillary tube and into the tee connected to the air supply. The relative amount of the ethylene-mixture introduced into the air supply was governed by the pressure established with the low-pressure regulator, and by the length of the capillary tubing. The segments of tubing ranged in length from 29 cm to 58 cm.

This arrangement thus provided gas outlets for four samples. When ethylene studies were conducted, two of the samples were exposed to ethylene, and two samples served as controls. Unless otherwise stated, the flow rates were adjusted to 15 ml/min of gas through each sample. The

culture tubes and the gas washing tubes were partially immersed in a temperature controlled water bath. The temperature was constant at 25 C.

The culture tubes (Figure 3) were designed to offer aseptic conditions, and to avoid contact of the air with plastics or rubber. The female portion of a 12/30 ground glass joint was sealed to form a tube (7 cm x 1.6 cm OD), and a sidearm was attached to the tube. The sidearm was loosely blocked with a plug of glass wool, and functioned as a vent to allow air to escape from the culture tube. The top of the male portion of the joint was fused to a glass tube (0.625 cm OD) which extended approximately 3 cm above the joint, and the bottom was sealed to a tube (0.625 cm OD) that extended to within 3 mm of the bottom of the culture tube. The tip of the lower tube, which served as the gas inlet, was drawn to a taper to provide greater dispersion of the gases. These culture tubes were produced in the University of Alberta glass-shop.

D. Preparation of samples

Seeds

Seeds of barley (*Hordeum vulgare* cv Himalaya, 1974 harvest) were used throughout. The seeds, obtained from Dr. J. D. Maguire, Washington State University, Pullman, Washington in 1975, were stored at 4 C until used.

The viability of the seeds was tested occasionally during the course of experimentation. Seeds were sterilized

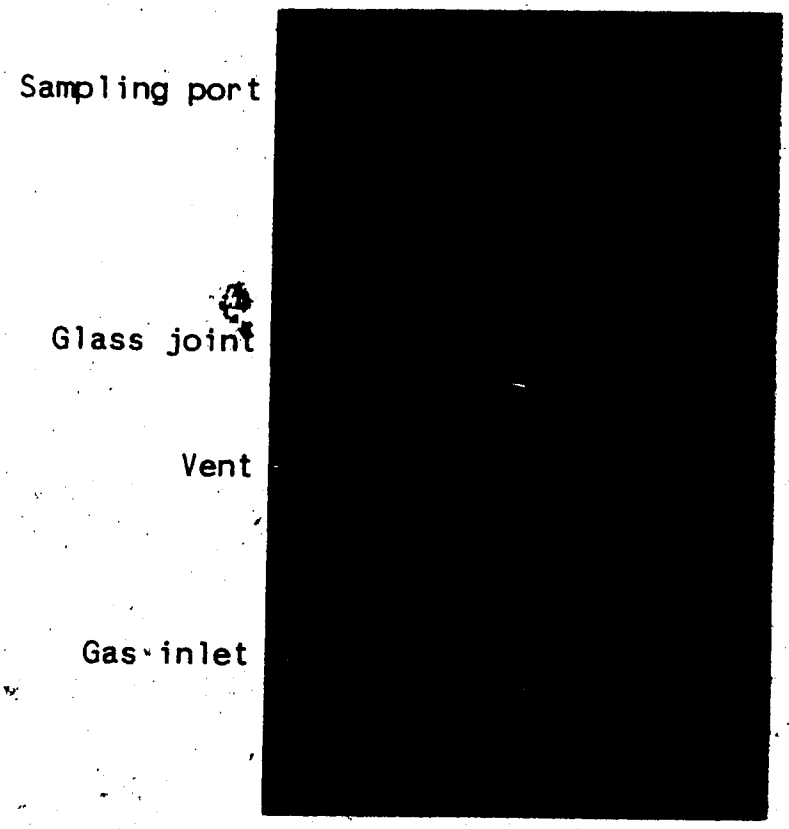


Figure 3. Culture tube for incubating isolated barley aleurone layers in either hydrocarbon-free air or air containing carefully regulated concentrations of ethylene.

as described below for half-seeds, then placed on sterile filter paper in trays, and soaked with distilled water. The trays were wrapped in foil and incubated for 96 h at 25 C before the number of germinated seeds was counted. Over the course of the experimental period, the average germination rate was 97%.

Preparation of half-seeds.

Half-seeds of barley were prepared essentially as described by Chrispeels and Varner (1967). Approximately 0.75 mm of the distal end was removed from each seed and discarded, then a segment 3 mm in length was removed from the adjacent tissue. This was considered a half-seed. If any shrivelling or discolouration of the seed was observed, or if any embryonic tissue remained attached to the half-seed, that sample was discarded.

The half-seeds were sterilized by rinsing the half-seeds for 2 min in 75% ethanol in a sterile 250 ml erlenmeyer. All subsequent steps in the preparation of half-seeds and isolated aleurone layers were performed on a laminar flow bench (Edgegard Hood No. EG4252, Baker Co., Inc., Sanford, Maine) to help maintain sterile conditions. The ethanol was decanted off and the half-seeds were rinsed in 25 ml sterile distilled water for an additional 2 min, then thoroughly drained. Approximately 50 ml 4% sodium hypochlorite was added to the flask, and the flask was gently agitated for 20 min. The hypochlorite solution was carefully and thoroughly decanted away from the half-seeds.

The half-seeds were rinsed five times, each for 2 min, with 30 ml sterile distilled water. The rinse water was periodically collected and the concentration of hypochlorite was determined by the chlorpromazine method (Collier, 1974). This offered a means of evaluating the efficiency of the rinsing procedure. Sixty half-seeds were transferred to each 250 ml flask (Figure 2) containing 3 layers of Whatman #1 filter paper (7 cm dia.) and 6 ml distilled water. (The flask had been fitted with the filter assembly and metal closure previously described and autoclaved before use.) After the half-seeds were transferred to the flasks, the flasks were wrapped in foil and connected to the air supply described for incubation at 25 C.

Incubation of isolated aleurone layers

Aleurone layers were prepared by carefully removing the softened endosperm from half-seeds that had been imbibed for 72 h. If any of the half-seeds showed visible signs of microbial contamination, the contents of the flask were discarded.

The layers were transferred to the appropriate culture medium after being rinsed with sterile distilled water. For determining the dose response of gibberellic acid, incubation was continued in 25 ml culture flasks with metal closures. The flasks were agitated on a gyrotary shaker in a growth cabinet at 25 C. The experiments for ethylene control were conducted in the culture tubes previously described. Each tube or flask contained 10 aleurone layers

suspended in 2 ml of test medium.

In all cases, the solution of gibberellic acid (Grade III, Sigma Chemical Co., St. Louis, Mo.) in buffer was autoclaved in the culture tube or flask. The incubation buffer typically consisted of 20 mM succinic acid, 20 mM calcium chloride, adjusted with sodium hydroxide to pH 5.5 at 25 C. The given concentration of gibberellic acid is representative of the initial value only. Some of the activity may have been destroyed during the autoclaving process, but the consequences of this were minimized by autoclaving all gibberellic acid solutions under consistent and reproducible conditions. When the addition of inhibitors or supplements to the medium was required, a two-fold concentrated solution of gibberellic acid was autoclaved in the culture vessel, and a two-fold concentrated solution of additive was filter sterilized (0.22 micron membrane Millipore filter) and introduced into the culture vessel through a sterile hypodermic needle. As a result of the limited solubility in water of N-ethylmaleimide (CalBiochem, San Diego, Calif.), a concentrated stock solution was prepared in 95% ethanol, and a maximum of 0.005 ml of stock solution was added to each 2 ml of incubation medium. The solutions of casein hydrolyzate (acid hydrolyzed, vitamin free, Difco Laboratories Inc., Detroit, Mich.) and L-tryptophan (Sigma Chemical Co., St. Louis, Mo.) were prepared immediately before use. When required, the final concentrations of

casamino acids and tryptophan in the culture medium were 5 mg/ml and 0.25 mg/ml respectively. These concentrations were lower than those previously used (Ho & Varner, 1978). It was necessary to reduce the concentration of the amino acid supplement by one-half to eliminate foaming as the air bubbled through the incubation medium.

Sample preparation

Samples of media were prepared for enzyme assays after the isolated aleurone layers had been incubated for an appropriate period of time. The original culture medium was decanted into conical centrifuge tubes. The layers and culture tubes were rinsed with 2 ml of buffer that contained 20 mM potassium acetate, 20 mM calcium chloride and had been adjusted with hydrochloric acid to pH 5.5 at 25 C. This rinse buffer was combined with the medium and centrifuged at 980 x g for 20 min (centrifuge model C1 with rotor 221, International Equipment Co., Needham, Mass.). The centrifuge was operated in a cooler maintained at 4 C. The supernatant layer was decanted, diluted to 5 ml with buffer and stored on ice.

Extracts from the layers were also prepared. The layers were rinsed again with 2 ml buffer, which was discarded. The layers were gently blotted dry between filter papers then transferred to test tubes (15 cm x 2.5 cm OD) containing 4.5 ml of the potassium acetate-calcium chloride buffer. The layers were homogenized with a Polytron (Kinematica, Lucerne, Switzerland) operated at 70%

of full power for 80 seconds. During the homogenization procedure, the samples were cooled in an ice-water slurry. The resulting suspension was centrifuged at 980 x g for 20 min as described above. The supernatant layer was decanted and diluted to 5 ml with buffer.

For routine enzyme determinations, between 0.01 ml and 0.10 ml of sample was removed and diluted to 0.5 ml for the amylase assay. The remainder of the sample was precipitated (Taiz & Honigman, 1976) with 3 volumes of cold 3.9 M ammonium sulphate (special enzyme grade, Serva Feinbiochemica, Heidelberg, W. Germany) and collected by centrifugation in a refrigerated centrifuge at 2 C (model J-21B, Beckman Instruments Inc., Palo Alto, Calif.). The samples were spun for 30 min at 15300 x g (14000 rpm, model JA-20 rotor). The precipitate, after resuspension in 1 ml of the potassium acetate-calcium chloride buffer and dialysis, was used for determination of glucanase and xylanase. For the xylanase assay, a sample volume of 0.30 ml was normally required, whereas for the glucanase assay, 0.010 ml was diluted to 0.30 ml with buffer.

Separate samples were required for the determination of haemoglobinase activity. The protein fraction that precipitated with the addition of 4 volumes of cold 3.9 M ammonium sulphate was collected by centrifugation at 15300 x g for 30 min at 2 C (Bhatty, 1968). The pellet was dissolved in 2.5 ml of 0.05 M sodium citrate adjusted to pH 3.8 with hydrochloric acid (Bhatty, 1968) and dialyzed

overnight against the same buffer.

E. Enzyme assays

Amylase assay

Amylase activity was determined by one of two assay procedures. One technique was based on the cleavage by enzymatic activity of soluble fragments from an insoluble chromogenic substrate. The method was described by RinderKnecht, Wilding & Haverback (1967), except that the substrate recommended by them, Remazolbrilliant Blue bound to starch, was replaced by the same dye covalently bound to amylose. The latter substrate was a commercially available preparation (amylose azure, CalBiochem, San Diego, Calif.). The assay was evaluated at two substrate concentrations, 2% (w/v) and 10% (w/v). The second assay method was a saccharogenic method using the Nelson copper reagent to determine the change in the reducing potential of the substrate solution . The procedure was described in detail by Robyt & Whelan (1968). The substrate (Baker & Adamson soluble starch powder) was obtained from Nichols Chemical Co., Ltd. (Montreal, P.Q.).

In both instances, the assay conditions were adjusted to accomodate barley alpha-amylase. For optimal enzyme activity and stability, the assay buffer contained 20 mM potassium acetate, 20 mM calcium chloride, and was adjusted to pH 5.5 at 25 C with hydrochloric acid (Greenwood & MacGregor, 1965). In accordance with earlier

recommendations of the International Union of Biochemists (1965), the assays were performed at 30 C. The substrate and samples were allowed to equilibrate at 30 C for a minimum of 15 min before the assays were initiated. Digestion of the substrate was allowed to proceed for 10 min before the action of the enzyme was terminated. In all cases, the absorbance of the sample was measured in a double-beam spectrophotometer (Cary 219, Varian Assoc., Inc., Palo Alto, Calif.). The reference was a zero-time control in which the substrate and terminating reagent were added simultaneously.

One Unit of amylase activity was defined as the amount of enzyme required to hydrolyze one microequivalent of glucosidic linkages per minute (International Union of Biochemists, 1965). For the saccharogenic method, the conversion to enzyme Units was achieved by comparing the absorbance of the sample measured at 520 nm to that of a series of maltose standards (Robyt & Whelan, 1968).

Glucanase assay

The assay for glucanase activity was performed essentially as described by Taiz & Jones (1970). The 0.1% (w/v) laminarin (purum grade, Fluka AG, Chemische Fabrik, Buchs, Switzerland) solution was prepared in 20 mM potassium acetate, 20 mM calcium chloride adjusted to pH 5.5 at 25 C with hydrochloric acid. The substrate was dissolved in the buffer by immersing the flask containing the suspension of laminarin into boiling water for 5 min. The

assay was performed at 30 C (International Union of Biochemists, 1965) and the incubation time with enzyme was extended to 60 min. After the final dilution of the sample with 5 ml distilled water, the optical density of the sample was measured at 520 nm as described above.

A standard curve relating absorbance to microequivalents was prepared using a series of maltose standards. One enzyme Unit was as defined for amylase activity.

Xylanase assay

1. Substrate preparation.

Larchwood xylan was obtained from Sigma Chemical Co. (St. Louis, Mo.) and was purified either by the method of MacNeil & Albersheim described by Taiz & Honigman (1976), or by the more classical method of alkaline copper precipitation (Jermyn, 1955). In the latter method, the commercial xylan was regarded as the equivalent of the crude xylan obtained from the first precipitation step. The remainder of the purification followed the published procedure.

The degree of purification of the xylan was evaluated by paper chromatography of the acid hydrolyzate. Initially, hydrolysis was achieved with 1 M sulphuric acid (Taiz & Honigman, 1976); however, significant loss of sample occurred during the precipitation and removal of sulphate with barium carbonate. Subsequent hydrolysis reactions were performed with formic acid (S. Sarkar, personal

communication). Five mg of xylan were suspended in 5 ml 6 N formic acid. A vacuum of 580 mm of mercury was applied to the test tube and the neck was sealed by heating in a flame. The ampule was immersed in boiling water for 18 to 22 h. At this time, the contents of the ampule were transferred to a larger test tube and taken to dryness under vacuum at 30 C. The dried samples were stored overnight at 26 C under vacuum to ensure complete removal of formic acid. The residue was resuspended in 0.5 ml 60% (v/v) ethanol for paper chromatography and the entire solution was applied. In addition, 0.0025 ml of a standard mixture was chromatographed. The standard contained, in 60% ethanol, 0.5% (w/v) each of L-arabinose (CalBiochem, San Diego, Calif.), D-galactose (Matheson, Coleman and Bell, Cinncinati, Ohio), D-glucose and D-xylose (Fisher Scientific Co., Fair Lawn, N.J.). Descending paper chromatography was conducted with the single phase solvent (ethyl acetate:pyridine:water (12:5:4, v/v/v)) described by Menzies & Seakins (1969), in preference to the sequential development suggested by Ray (1963). After development, sugars were detected by dipping the papers in either the alkaline silver oxide reagent (Menzies & Seakins, 1969) or the *p*-anisidine (Eastman-Kodak Co., Rochester, N.Y.) - phthalic acid (J.T. Baker Chemical Co., Phillipsburg, N.J.) reagent (Sherma, 1972).

2. Assay procedure.

The method for the determination of xylanase activity

was essentially as described by Taiz & Honigman (1976) with only slight revision. The pH of the substrate buffer was 5.5. As in the other enzyme assays, the assay was conducted at 30 C with the enzymatic reaction proceeding for 60 min. The absorbance was read versus a zero-time control at 520 nm.

A standard curve was prepared using D-xylose standards and enzyme activity calculated as above.

Protease assay

The assay method for proteolytic activity was that described by Bhatty (1968) for the determination of haemoglobinase. The substrate was prepared as a 1% (w/v) solution of bovine haemoglobin (crude bovine haemoglobin powder, Type II, Sigma Chemical Co., St. Louis, Mo.) in 0.05 M sodium citrate adjusted to pH 3.8 at 25 C with hydrochloric acid. The substrate solution and a 1 ml aliquot of sample were equilibrated at 30 C before the assay began. At time 0, 4 ml of substrate was pipetted into the sample and mixed gently to avoid foaming. Consistent with the recommendations of the International Union of Biochemists (1965), the assay was performed at 30 C and the length of the assay was extended to 120 min to accommodate the lower temperature than used by Bhatty (1968). The assay was terminated by the addition of 5 ml of cold 0.6 M trichloroacetic acid solution. The solution was cleared by centrifugation at 1000 x g for 30 min at 2 C. The absorbance of the supernatant layer was measured at 280 nm

against a reference prepared by adding the trichloroacetic acid to an aliquot of sample before the substrate was added.

The absorbance was related to enzyme Units through a calibration curve prepared by dissolving L-tryptophan (Sigma Chemical Co., St. Louis, Mo.) in a 1:1 mixture of citrate buffer and trichloroacetic acid solution . One enzyme Unit was defined as the amount of enzyme that liberated 1 microequivalent of tryptophan per min at 30 C.

F. Inorganic ion release by barley aleurone layers

Ten aleurone layers were incubated in 2 ml of medium that contained 0.010 mM gibberellic acid and 20 mM sodium succinate adjusted to pH 5.5 with hydrochloric acid. The calcium chloride normally present in the incubation medium was omitted to permit the estimation of calcium release from the isolated aleurone layers. (Jones (1973) has demonstrated that calcium chloride had no effect on the release of magnesium or potassium ions from aleurone layers.) After incubation at 25 C for 24 h in either hydrocarbon-free air or 12.9 nl/ml ethylene in air, the layers were rinsed twice with deionized glass-distilled water. The combined rinse and incubation solutions were cleared by centrifugation at 980 x g for 20 min. The supernatant layer was transferred to a 50 ml volumetric flask and the pellet was resuspended in 8 ml water and centrifuged. The rinse supernatant layer was combined with the first, and the solution was brought to 50 ml. These

solutions represented the samples, and were further diluted for the analysis of each ion as required. Control samples were prepared by incubating isolated aleurone layers in the absence of gibberellic acid, and background samples were prepared as above, but without the addition of aleurone tissue. All glassware had been rinsed with hydrochloric acid or nitric acid to reduce contamination by inorganic ions. The culture tubes were further rinsed thoroughly with deionized, glass-distilled water before use.

Calcium, magnesium, potassium and sodium were quantified using a Varian AA-475 atomic absorption spectrophotometer. Calcium was analyzed at 422.7 nm in a nitrous oxide-acetylene reducing flame. Interference by sodium and potassium in the samples was eliminated by adding 2000 mg/l potassium to samples and standards to supress ionization. The standard stock solution was a commercial preparation of calcium carbonate in dilute nitric acid (Fisher Scientific Co., Fair Lawn, N.J.). Magnesium was determined at 285.2 nm with the assistance of a deuterium continuum lamp. An air-acetylene oxidizing flame was used, and 10000 mg/l lanthanum was added to samples and standards as a releasing agent. Standards were prepared from magnesium metal dissolved in dilute nitric acid (Fisher Scientific Co., Fair Lawn, N.J.). Potassium and sodium were also analyzed in an air-acetylene oxidizing flame. The 589.0 and 766.5 nm lines from the hollow cathode lamps were used for sodium and potassium respectively. To suppress

ionization and to eliminate cross interference, 200 mg/l potassium was added to the sodium samples and standards, while 1000 mg/l sodium was added to potassium samples and standards. The standards for potassium and sodium were the chloride salts dissolved in water (Fisher Scientific Co., Fair Lawn, N.J.).

Phosphate was determined by the method originally described by Fiske & Subbarrow (1925) and modified for inorganic phosphate by Bartlett (1959). Standards were prepared from a stock solution of mohobasic potassium phosphate. The assay was performed spectrophotometrically at 660 nm (Cary 219 spectrophotometer, Varian Assoc., Inc., Palo Alto, Calif.).

G. Scanning electron microscopy

Isolated barley aleurone layers were dehydrated, at 0 C, through a series of ethanol solutions (6.7%, 13.6% and 20.0%). After 2 h in 20.0% ethanol, the aleurone layers were removed and frozen in liquid freon, fractured, then freeze dried. The specimens, mounted on aluminum studs, were coated with a gold layer (150 to 200 Angstroms), and examined with a Cambridge Stereoscan 150 scanning electron microscope. At least four specimens from each set of experimental conditions were examined.

H. Polyacrylamide gel isoelectric focusing

The isoelectric focusing was performed in gels cast in glass cylinders (12 cm x 0.35 cm ID) to yield a gel 9.5 cm in length. The gels (T=7.5%; C=2.5%) contained: 7.31% acrylamide and 0.19% N,N'-methylene-bis-acrylamide (Bio-Rad Laboratories, Richmond, Calif.), 5.0% (v/v) glycerol, 2.0% (w/v) ampholyte (Ampholine, pH [illegible] to 10, 5 to 7 or 7 to 10, LKB, Bromma, Sweden). Usually, 10 ml of gel solution was prepared for each experiment. The solution was cooled on ice and degassed by applying a vacuum line from an aspirator for at least 15 min. To polymerize the gels, 0.01 ml N,N,N',N'-tetramethyl-ethylene diamine (Matheson, Coleman and Bell, Cincinnati, Ohio) and 0.050 ml 10% (w/v) ammonium persulphate were added. The solution was gently swirled and degassed for 1 min, then the solution was pipetted into the glass tubes and overlayed with distilled water. The gels were allowed to set overnight at 2 C before they were used.

For gels used in initial experiments, a 1:1 mixture of pH 5 to 7 and 7 to 9 Ampholines was used. Gels with a lower pH range contained a 1:4 mixture of Ampholines with pH ranges 3.5 to 10 and 5 to 7 respectively (O'Farrell, 1975). In both cases, the electrode compartments were filled with buffers of amino acids as recommended by Nguyen and Chrambach (1977). During focusing of high pH range gels, the anode (lower) reservoir contained 700 ml 0.04 M glutamic acid and the cathode (upper) compartment contained 400 ml

0.1 M ethanolamine. The anodic and cathodic buffers for the low pH range gels were 0.04 M glutamic acid and 0.04 M histidine (free base), respectively. In both cases, the cathodic buffer was placed on ice and degassed for 30 min before focusing was started.

The isoelectric focusing apparatus had a water jacketed lower reservoir (Buchler Instruments, Fort Lee, N.J.). Water at 2 C was circulated through the water jacket during focusing. A magnetic stirring bar circulated the electrode buffer within the lower reservoir. The sample (in 20% v/v glycerol) was applied to the gel and overlayed with 0.025 ml of an aqueous solution containing 1% (w/v) Ampholine, pH 7 to 9, and 5% (v/v) glycerol. The cathodic buffer was introduced slowly and the apparatus was assembled. A d.c. power supply (model 3-1014A, Buchler Instruments, Fort Lee, N.J.) supplied a potential of 180 volts across the gels for 17 h. Focusing was then continued at 710 volts for 0.5 h and 950 volts for an additional hour. After the focusing was terminated, the gel rods were immediately placed on ice to reduce diffusion. Amylase activity was located by the starch-iodine method (Jacobsen *et al.*, 1970). Proteins were located by staining the gels overnight in 0.04% (w/v) Coomassie brilliant blue G250 (Sigma Chemical Co., St. Louis, Mo.) in 3.5% (w/v) perchloric acid (Reisner, Nemes & Bucholtz, 1975). The gels were then soaked in 7% (v/v) acetic acid for 4 h and destained in 7% (v/v) acetic acid-5% (v/v) methanol in a diffusion destainer (model 172A,

Bio-Rad Laboratories, Richmond, Calif.). The pH gradient was determined by placing the gel on a metal plate pre-cooled to -20 C, and freezing the gel. The gel was cut into 0.5 cm sections and each segment was extracted overnight in 1.0 ml distilled water. The samples were cooled in an ice water bath before the pH of the extract was determined.

Amylase samples were prepared by decanting the incubation medium from aleurone layers exposed to gibberellic acid. The aleurone layers were rinsed with 1 ml 20 mM succinic acid, 20 mM calcium chloride, adjusted to pH 5.5 at 25 C with sodium hydroxide. The rinse buffer was added to the original medium, and the sample was centrifuged at 980 x g at 4 C for 20 min. The supernatant layer was collected and placed on ice. Four volumes of cold 3.9 M ammonium sulphate were added slowly with constant stirring. The samples were kept on ice for 30 min, then the precipitate was collected by centrifugation at 15300 x g (14000 rpm, Beckman model JA-20 rotor) at 2 C. The pellet was dissolved in 0.3 ml buffer, and excess ammonium sulphate was removed by gel filtration in small centrifuge columns (described in Appendix). Glycerol and buffer were added to the effluent to obtain a 20% (v/v) solution with respect to glycerol, and a final volume of 0.5 ml.

Samples of coloured marker proteins were also prepared for each run. These provided a visual indication of the progress of the focusing, and served as reference points for

the pH gradient. Chicken egg white conalbumin (type II), whale skeletal muscle myoglobin (type II) and human haemoglobin (type IV) - all from Sigma Chemical Co., St. Louis, Mo. - were used at a concentration of 1.0 mg/ml in 20% (v/v) glycerol; 0.100 ml of standard was applied to each gel. All three standards were used in separate gels during the initial focusing trials, whereas only chicken egg white conalbumin was used as a standard for low pH range gels used in subsequent experiments.

I. Protein determination

Protein concentrations were evaluated by the method of Sedmak & Grossberg (1977) using 0.06% (w/v) Coomassie brillant blue G250 (Sigma Chemical Co., St. Louis, Mo.) in 3% (w/v) perchloric acid. Calibration of the method was performed using standard solutions of bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, Mo.). Since colour development depended on the pH of the solution (Reisner *et al.*, 1975; Sedmak & Grossberg, 1977), the standards and samples were prepared in the same buffered solutions.

IV. RESULTS AND DISCUSSION

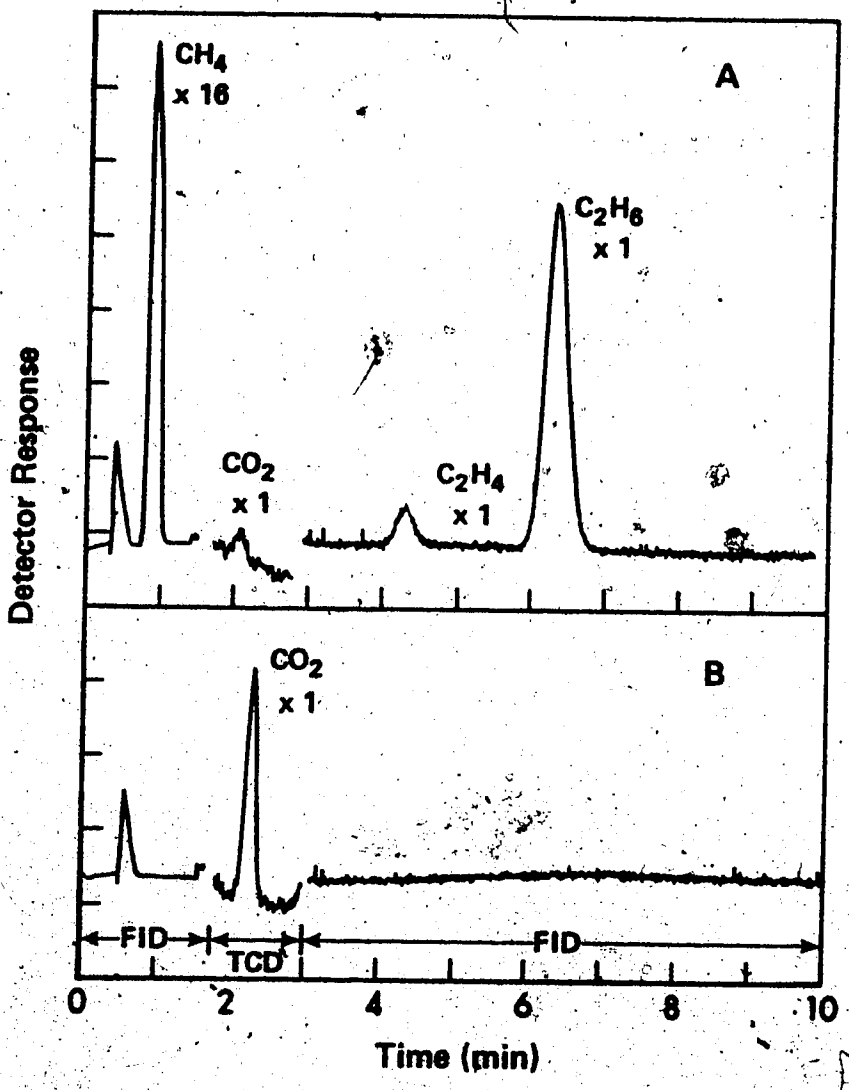
A. Air purification

Analysis of laboratory air and the compressed air from commercially available cylinders revealed appreciable and variable concentrations of low molecular weight hydrocarbons, *viz.* methane, ethylene and ethane (Figure 4A). The occurrence of these impurities in air mixtures for [redacted] of plant growth and metabolism may lead to incorrect [redacted]usions. Although some methods have been developed to selectively reduce the concentration of ethylene in air (for example, by the use of Purafil-Abeles *et al.*, 1971; summary in Abeles, 1973), little effort has been addressed to the elimination of other low molecular weight hydrocarbons from the atmosphere for plant physiological studies.

The relative efficiencies of available purification methods were evaluated. The results are presented in Table I (Methods and Materials). Of the three hydrocarbons considered, methane was found to be the most persistent contaminant in the air stream. Because methane is less reactive and more volatile (boiling point -165 C) than ethane or ethylene, most oxidative and adsorptive techniques failed to remove methane effectively.

The primary limitation of the adsorbent traps was their relatively short service life before reactivation of the adsorbent was required. As the binding capacity of the traps approached saturation there was a loss of efficiency.

Figure 4. Gas chromatograms illustrating the effect of heated catalysts on carbon dioxide and hydrocarbon concentrations in an air stream. The concentrations of methane, ethylene and ethane were 9.16 nl/ml, 0.020 nl/ml and 0.420 nl/ml, respectively, in control air (A), while that of carbon dioxide was less than 4 nl/ml. There were no detectable hydrocarbons in the air that had passed through the platinized asbestos catalyst heated to 650 C (B), but the concentration of carbon dioxide had increased to 17 nl/ml.


CH4
x 16
A
C2H6
x 1
CO2
x 1
C2H4
x 1
Detector Response
CO2
x 1
B
FID
TCD
FID
0
2
4
6
8
10
Time (min)

This situation was exemplified by a silica gel trap maintained at -86 C (method 4). Immediately after the initial flushing period of 10 min (time 0, Table I), the trap was retaining 96.8% of the methane, but after an additional 30 min, the trap had become totally ineffective in this respect. Nevertheless, both ethylene and ethane were completely adsorbed from the air stream throughout the trial. Of the adsorbent traps considered, activated cocoanut charcoal at -196 C (method 3) was the most effective within the time span of these experiments. With extended use, this trap would also be subject to the limitation of a finite binding capacity.

In addition to the adsorption of hydrocarbons from the air stream, the silica gel trap (method 4) also retained carbon dioxide from the room air initially confined within the trap. The carbon dioxide was then gradually released into the air stream. After 70 min of flushing with carbon dioxide-free air, the carbon dioxide concentration of the effluent had declined from an initial value of 393 nl/ml to 9 nl/ml, and had not yet reached equilibrium. Although retention of carbon dioxide was observed in several instances, this effect was generally more pronounced in purification methods that utilized a silica gel solid support. The transient retention of carbon dioxide would be most critical during short-term experiments and carbon dioxide exchange measurements. Carbon dioxide should therefore be carefully monitored while using any of these

systems.

Oxygen (boiling point -183 C) had a tendency to condense out of the air stream in adsorbent traps at -196 C. This has also been reported by Stephens & Burleson (1967). After 10 min, the oxygen concentration of the effluent from the activated charcoal trap immersed in liquid nitrogen had declined to 1.4% from the control concentration of 19.9%. The oxygen concentration of subsequent samples increased rapidly as the trap approached equilibrium. As the liquid oxygen accumulated in the trap, however, the flow of air became very erratic.

All of the chemical reactants (methods 10 to 22) effectively removed ethylene from the air stream with the exception of the methods where sulphuric acid was either mixed with aqueous formaldehyde (methods 10 and 11) or deposited on silica gel (method 18). When concentrated sulphuric acid, originally recommended by Weber (1953), was replaced by fuming sulphuric acid in the aqueous formaldehyde reagent, no ethylene was detected in the samples taken at time 0, although subsequent samples contained appreciable concentrations of ethylene. As evident in Table I, ethylene was totally absent from air that had passed through columns of brominated charcoal (method 12) or potassium permanganate (methods 14 and 15). This is in contrast to previous studies that reported neither brominated charcoal (Forsyth *et al.*, 1967; Southwick & Smock, 1943) nor permanganates (Forsyth *et al.*, 1967)

could completely remove ethylene from air. This contradiction may be the consequence of different surface area to air volume ratios and different initial amounts of ethylene to be removed from the air. Iodine pentoxide (method 16) oxidized ethylene and most of the ethane. The consequent reduction of iodine pentoxide to molecular iodine, which was bright red on the silica gel, could be used as an indicator to assess the progressive exhaustion of the trap. Also, the extent of the red colour development in the iodine pentoxide trap has been used as a quantitative indication of the hydrocarbon content of air (Anonymous, 1957). A consistent limitation of the methods 10 through 19 was their inability to completely remove methane.

Table I clearly shows that platinized asbestos, cupric oxide-ferric oxide pellets and powdered cupric oxide maintained at high temperatures (methods 20 to 22) provided the most efficient means of removing hydrocarbons from a continuous flow of air without creating the problems encountered with adsorbent traps. These catalysts depended upon a constant source of oxygen. In the air supply used for this comparison, oxygen was present at a sufficiently high concentration to maintain the active catalysts. A stoichiometric amount of oxygen was removed from the air stream. In the present study, the oxygen concentration was reduced from 19.9% in the control to 19.1% in the air that had passed through the platinized asbestos column. A similar small reduction in oxygen concentration was observed

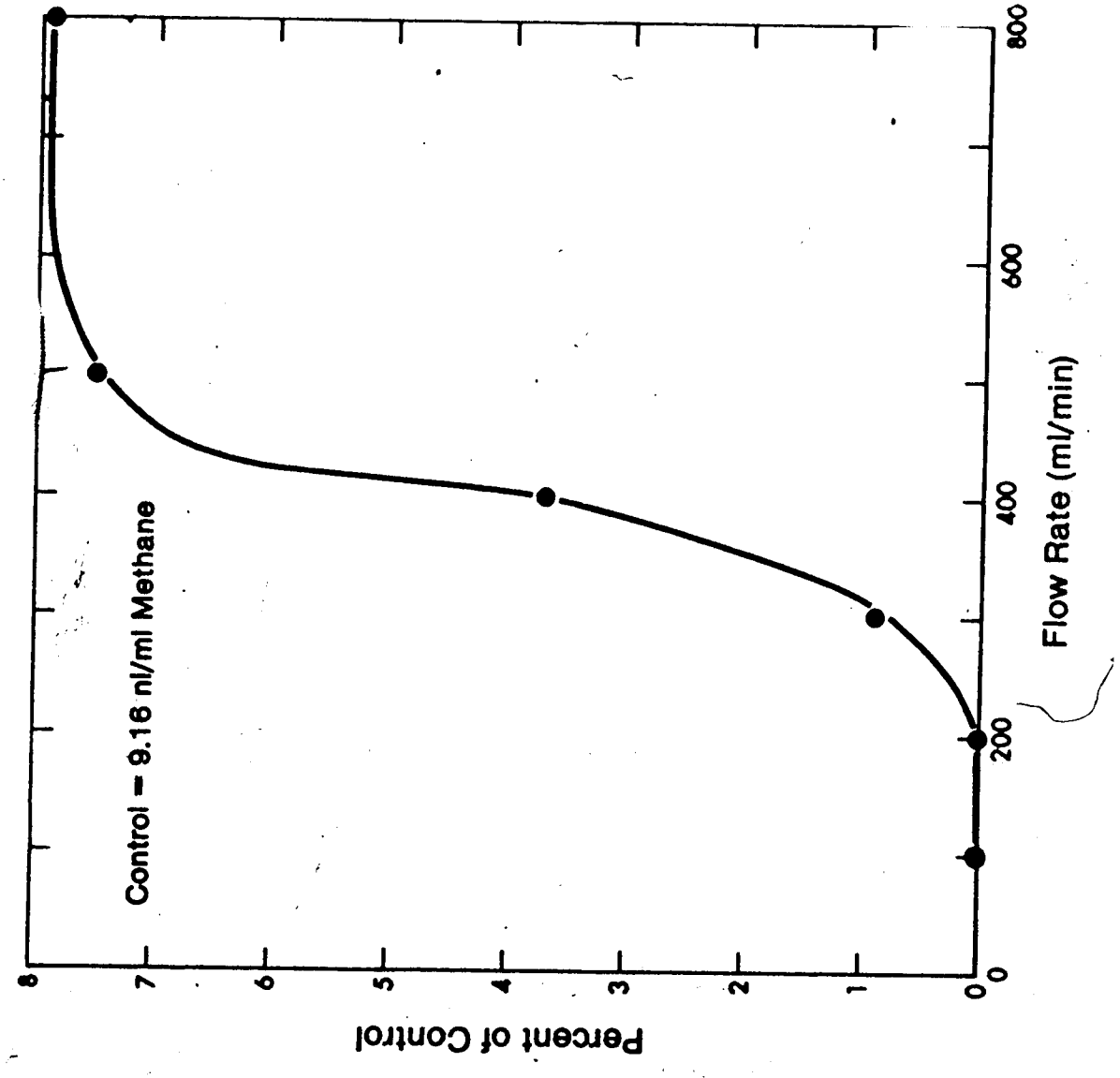
with the cupric oxide catalysts. The amount of carbon dioxide produced by the oxidation of hydrocarbons should also be considered. The effluent from the platinized asbestos catalyst contained 17 nl/ml carbon dioxide, whereas the carbon dioxide concentration of the air supply (Figure 4) was less than 4 nl/ml (the lower limit of integration for a 5 ml injection). The amount of carbon dioxide produced depended on the concentration of hydrocarbon impurities in the original air supply.

The high temperatures required for the operation of the combustion tubes created a potential source of nitrogen oxides. The production of nitrogen oxides is, under certain conditions, associated with high temperature combustion devices operated in the presence of molecular oxygen and nitrogen (Starkman, 1971). The nitric oxide formed by this process reacts spontaneously to produce nitrogen dioxide in the oxygen-rich environment of compressed air (Nash, 1950; Burns, 1970). Although temperatures in excess of 2200 C were required to produce large quantities of nitric oxide, the lower temperature of 650 C present in the combustion tubes could conceivably be sufficient to yield physiologically active amounts of nitrogen dioxide (Butcher & Charlson, 1972). However, analysis of the nitric oxide and nitrogen dioxide content of compressed air, both before and after the air had passed through the catalyst-bed of heated platinized asbestos, yielded identical results; less than 0.5 pl/ml (the lower limit of the analysis) of total

nitrogen oxides were found. These values are at least 3 orders of magnitude less than the concentration of nitrogen dioxide that has been found to elicit a response in plants. Prolonged exposure to 500 pl/ml nitrogen dioxide substantially reduced growth of pinto beans and tomatoes (Taylor & Eaton, 1966), while acute damage was observed only at much greater concentrations (Darley, 1971). Moreover, nitric oxide and nitrogen dioxide would be quantitatively removed from air by absorption in the dilute sodium hydroxide solution used in our system for the removal of carbon dioxide (Burns, 1970). Thus, the potential for the production of nitrogen oxides does not limit the usefulness of the combustion tubes for removing hydrocarbons from air.

The relationship between the air flow rate and the efficiency of a heated catalyst was also investigated. (The efficiency of any catalytic system is dependent on the ratio of surface area to air volume, and the maintenance of the appropriate, uniform temperature within the catalyst bed.) The results obtained with the platinized asbestos at 650 C are presented in Figure 5. As the flow rate was increased beyond 200 ml/min, the concentration of methane in the effluent also increased. No ethane or ethylene was detected up to flow rates of 800 ml/min. The ability of the catalyst to oxidize methane completely at this high flow rate was restored by increasing the operating temperature to 800 C However, this elevated temperature led to extensive deactivation of the platinum catalyst by deplatinization and

Figure 5. Relationship between flow rate and ability of a heated platinum catalyst (650 C) to oxidize methane in a contaminated air supply. A detailed description of the platinum catalyst system is found in Methods and Materials. Under these conditions, no ethylene or ethane were detectable even at 800 ml/min.


Percent of Control
0
1
2
3
4
5
6
7
8
Control = 9.16 nl/ml Methane
0
200
400
600
800
Flow Rate (ml/min)

by the formation of platinum oxides. In related experiments, the capacity of the catalytic system was readily expanded without the demand for higher operating temperatures, by enlarging the volume of the heated catalyst bed.

One of the principle advantages of the catalytic method for air purification was the prolonged service life except where, as noted above, excessively high operating temperatures caused a drastic reduction in efficiency within a 48 h period. The longevity of the catalysts at the lower temperatures has been demonstrated by the continuous operation in our laboratory of a platinized asbestos catalyst system for many months without any appreciable deterioration of the efficiency of the system.

The catalysts tested could be obtained in various forms. Platinized catalysts were available commercially in a range of concentrations on several different solid support materials, in the form of granules, pellets, fibres or powders. Similarly, cupric oxide could be used in any of these forms. Pellets offered the least resistance to air flow, but only at the expense of surface area.

The catalyst systems can be assembled inexpensively. A semimicro-combustion furnace was used initially to provide stability and flexibility in operating temperatures, however, all subsequent work depended on inexpensive refractory half-tubes (Mellen Co., Inc., Penacook, N.H.) to provide the necessary temperatures. The latter were

shielded by copper pipe (150 mm x 62 mm OD) to offer some insulation value, and to protect the ceramic heating core. The addition of a rheostat to the circuitry permitted adequate temperature regulation. The high temperature catalyst tubes could therefore be adapted to suit the needs of most biological systems where a source of hydrocarbon-free air was desirable.

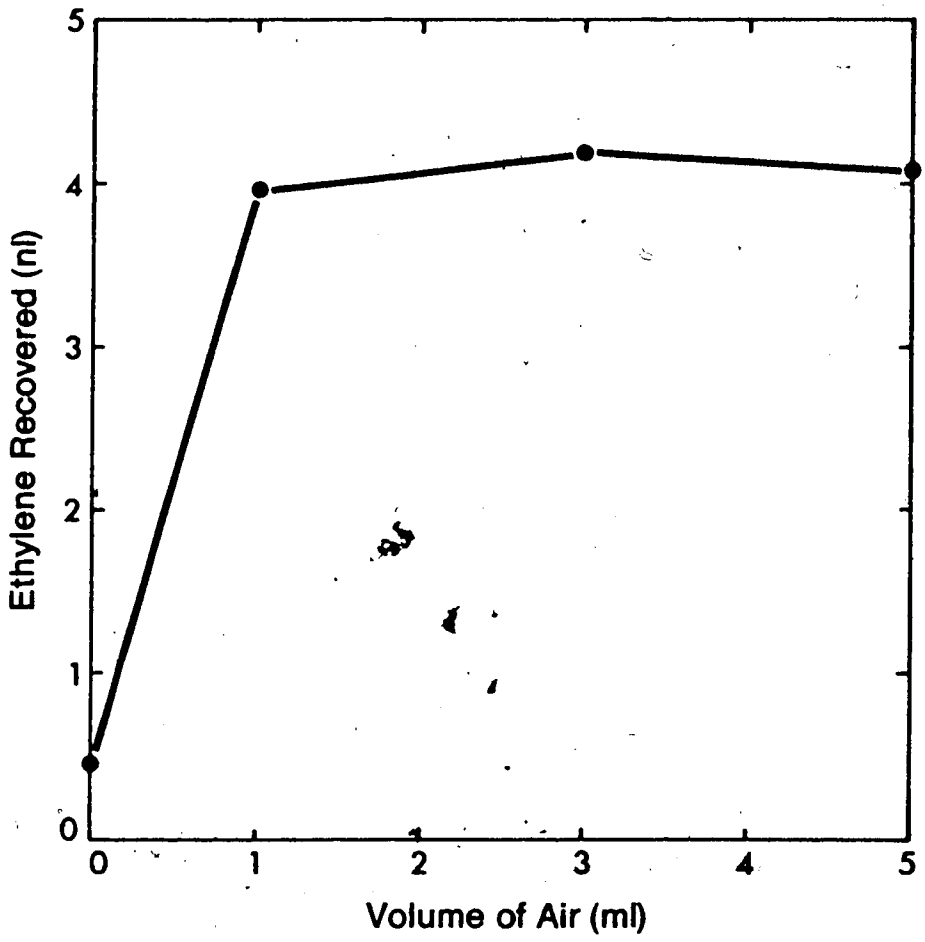
Some of the methods suggested by the literature for removing ethylene from air were not evaluated. Mercuric perchlorate was commonly used to scrub ethylene from air (eg. Young, Pratt & Biale, 1952); this method produced difficulties attendant to the reagent's corrosive nature and to the disposal of heavy metal wastes. Furthermore, the efficiency of this method was more dependent on the degree of foaming and flow rate than was generally realized (Chandra & Spencer, 1963). Other mercuric salts have been shown not to interact with saturated hydrocarbons (summary in Nash, 1950). The slow reaction rates and the amount of equipment required for methods utilizing ozone or X-rays precluded their general use for plant physiological studies (Abeles, 1973).

B. Analysis of dissolved gases

Initial experiments were performed to optimize the two analytical procedures of vacuum extraction and gas stripping. Once the techniques were standardized, the adequacy of the methods could be evaluated.

During the preliminary vacuum extraction runs, the amount of ethylene recovered was very low. Other research groups have improved the efficiency of the vacuum extraction procedure by designing apparatuses that allowed repetitive degassing cycles and refluxing the sample while under vacuum (Bayes, Flook & Graham, 1949). Our simple apparatus (Figure 1A) did not permit these operations, so other means of increasing the recovery of dissolved gases were sought. Figure 6 shows that the introduction of hydrocarbon-free air (Eastwell *et al.*, 1978) greatly improved the efficiency of the extraction procedure. The dilution of the extracted gases by the addition of hydrocarbon-free air minimized errors introduced by variation in withdrawing the extracted gases from the chamber. It also increased the concentration gradient between the hydrocarbons in solution and in the gas phase above the sample. The larger gas volume in the sample chamber permitted more thorough intermixing of the two phases while the apparatus was agitated. Increasing the volume of hydrocarbon-free air introduced into the chamber from 1 ml to 5 ml had no further effect on the efficiency of the method. For all subsequent extractions, 3 ml of hydrocarbon-free air were used. Bayes *et al.* (1949) found that larger samples decreased the effectiveness of their vacuum extraction apparatus; and additional extraction cycles were required to obtain quantitative results. Therefore, the next parameters considered were the sample volume and the duration of the extraction process. When the

Figure 6. Effect of the addition of hydrocarbon-free air on the efficiency of the vacuum extraction technique. The sample solution, at 25 C, was equilibrated with air containing 13.0 nl/ml ethylene. Three ml of solution were analyzed in each instance, and each value is the mean of three separate runs.


Ethylene Recovered (nl)
0
1
2
3
4
5
Volume of Air (ml)
0
1
2
3
4
5

sample had been equilibrated with 13.0 nl/ml ethylene, maximum yield was obtained from small samples (less than 3 ml) after only 1 min of extraction. However, with larger sample sizes, the recovery of ethylene from solution became dependent on the length of the extraction period (Table II). Increasing the time of the extraction from 3 min to 10 min increased the fraction of ethylene recovered from the sample, but extending the length of the extraction period further to 20 min was of no additional benefit to the recovery of ethylene. Therefore, the recovery rate was dependent on both sample volume and on extraction time. Both of the parameters had to be standardized to ensure reproducible results. All subsequent samples were extracted for 10 min, and the sample size was 3 ml.

The concentration of dissolved gas may alter the rate of recovery of solute by changing the concentration gradient between the liquid sample and the dead air space above it. A series of ethylene concentrations between 1.4 nl/ml and 46.1 nl/ml in air was used to establish the dependency of the method on solute concentration. As demonstrated by Figure 7, the response was linear over the range tested, and yielded a mean recovery of 90%. Thus, the vacuum extraction method was used with confidence over this concentration range, once the extraction time and sample volume had been fixed.

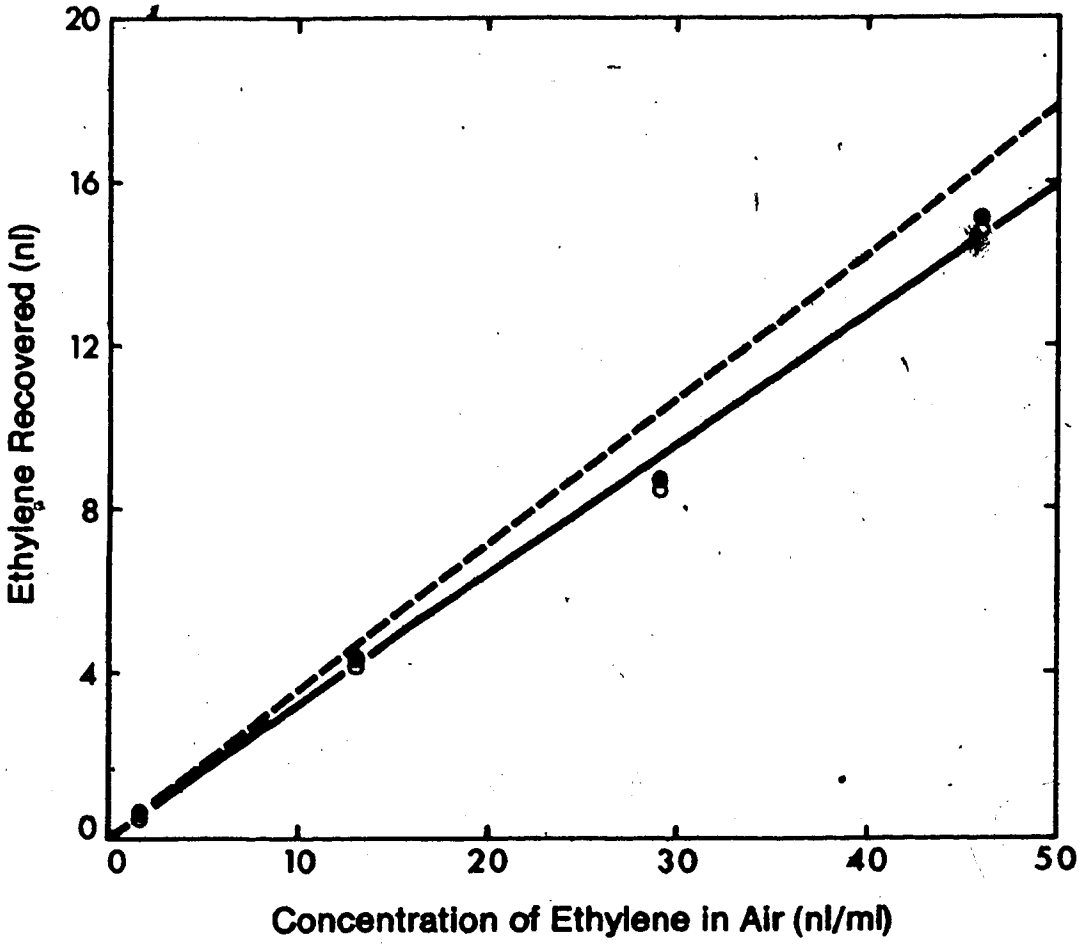
Variables of the gas stripping method were also investigated for their effects on the recovery of dissolved

Table II. Effect of sample size and extraction time on the recovery of dissolved ethylene by vacuum extraction

Glass distilled water was equilibrated with 13.0 nl/ml ethylene at 25 C. The extraction was performed with gentle agitation, and under a vacuum of 508 mm mercury for the indicated time. Three ml of hydrocarbon-free air were introduced to aid the extraction process. Each value is the mean of 3 determinations.

Sample Size	Theoretical Yield	Ethylene Recovery			
		3 min Extraction		10 min Extraction	
(ml)	(nl)	(nl)	(%)	(nl)	(%)
1	1.55	1.43	92	1.42	92
3	4.64	4.15	89	4.12	89
5	7.74	6.18	80	6.52	84
10	15.48	11.01	71	12.69	82
20	30.96	18.10	58	22.35	72

Figure 7. Effect of ethylene concentration on the relative amounts of ethylene recovered from solution by gas stripping (●) and vacuum extraction (O) techniques. The sample volume was 3 ml in each case, and the broken line indicates the theoretical amounts expected as calculated by Henry's Law.


20
16
12
8
4
0
Ethylene Recovered (nl)
0
10
20
30
40
50
Concentration of Ethylene in Air (nl/ml)

gases. The influence of the flow rate of carrier gas on the efficiency of the technique was explored for flow rates ranging from 40 ml/min to 100 ml/min. The gas chromatograph was recalibrated at each flow rate. The results, presented in Table III, indicated that the recovery decreased as the flow rate increased. Although the recovery of dissolved ethylene was superior at 40 ml/min, the resolution between ethane and ethylene deteriorated, and the peaks became relatively diffuse at this lower flow rate. This would affect measurements at low ethylene concentrations, and when other hydrocarbons were present in the sample. For all succeeding experiments, a flow rate of 60 ml/min helium was used.

The efficiency of the gas stripping technique was evaluated with different sample volumes. For sample sizes ranging from 1 to 5 ml, the sample volume had no consistent influence on the recovery of ethylene from solution, and the extraction efficiency varied from 84% to 92% (Table IV). Because the sample was injected while there was no flow of carrier gas, increasing the volume to 5 ml did not lead to the greater dilution of the sample in the carrier gas that had been previously noted (Swinnerton *et al.*, 1962). However, the volume of the sample that could be analyzed was limited. The use of an enlarged cuvette that could accommodate up to 10 ml of liquid resulted in severe deterioration in the quality of the chromatograms, although the proportion of ethylene recovered from the sample

Table III. Effect of carrier gas flow rate on the extraction of dissolved ethylene by the gas stripping method

Glass distilled water at 25 C was equilibrated with 13.0 nl/ml ethylene. Three ml of aqueous sample were analyzed at the appropriate flow rate of helium. Each value is the mean of 3 determinations.

Carrier Gas Flow Rate (ml/min)	Amount of Ethylene Recovered (nl)
40	4.74
60	4.54
80	4.50
100	4.30

Table IV. Effect of sample size on the analysis of dissolved ethylene by the gas stripping method

Glass distilled water at 25 C was equilibrated with 13.0 nl/ml ethylene, and samples were analyzed by the gas stripping method. Each value represents the mean of 3 analyses. The carrier gas flow rate was 60 ml/min.

Sample Size (ml)	Theoretical Yield (nl)	Yield (nl)	% Recovery
1	1.55	1.30	84
2	3.09	2.75	89
3	4.64	4.26	92
4	6.19	5.40	87
5	7.73	7.05	91

remained consistent with the values obtained for the 5 ml cuvette. The sample size limitation imposed by this technqiue has been averted by Swinnerton & Linnenbom (1967). The stripped gases were first passed through a cold collection trap, and the stripped gases were subsequently released onto the analytical column by heating the collection trap.

The efficiency of the gas stripping as a function of sample concentration was also investigated. Throughout the range of ethylene concentration used, the fraction of ethylene recovered from solution was independent of the ethylene concentration (Figure 7). As was the case for the vacuum extraction technique, the mean yield was 90% of the value suggested by Henry's Law.

As a further comparison of the analysis procedures, a much lower concentration of ethylene was used. When the concentration of ethylene in air was reduced to 0.20 nl/ml (this represents a concentration of 0.024 nl/ml in solution), neither method was adequate for samples less than 5 ml. When the sample volume was increased to 10 ml, however, sufficient ethylene was recovered from the sample by vacuum extraction to permit accurate analysis. Once again, the peaks obtained through the gas stripping technique with the 10 ml samples were unsatisfactory for the gas chromatographic determination of ethylene.

For both methods, control runs were also performed, using samples that had been equilibrated with

hydrocarbon-free air. No ethylene could be detected, and indicated that the analytical methods would not contribute any ethylene to the sample.

Special precautions were required when the gas stripping method was used. Certain solutions tended to foam during this procedure. In experiments with dilute bovine serum albumin solutions, the problem was readily overcome by the addition of a drop of Antifoam B (J.T. Baker Chemical Co., Phillipsburg, N.J.). As an extra precaution, a sample line filter was desirable to prevent particulates and mists from entering the analytical column.

The method of analysis also affected the performance of the gas chromatograph. The gas stripping method yielded broader, more asymmetrical peaks than those obtained through vacuum extraction. This decreased the precision of the technique for low concentrations of dissolved gases. The selection of the analytical column was also critical when either of these methods were used. Preliminary experiments using the alumina column resulted in problems such as baseline drift, ghost peaks and changing retention times because of the excessive moisture adsorbed on the column, particularly during the gas stripping procedure. Although heating the column between analyses for a few minutes reduced these difficulties, the column had to be reconditioned overnight after a few runs. The Porapak Q column performed much better under these operating conditions, and the short heating cycle of 5 min between

runs was adequate to yield reproducible chromatograms despite continuous use.

Although both methods of analysis performed equally well in terms of ethylene recovery, the technique selected for routine analysis was the vacuum extraction method. All of the problems related to liquid entering the column during gas stripping were avoided with vacuum extraction since the liquid sample handling was done away from the gas chromatograph. Also, this technique yielded relatively better peaks regardless of the column being used. Analysis was restricted to the Porapak Q column, however, for the reasons stated above.

Alternate methods of analysis of dissolved gases are available, of which, direct liquid injections and head space measurements are the most commonly employed techniques. Direct liquid injections require the use of a fractionator tube to reduce the amount of moisture entering the analytical column (McAuliffe, 1966). The small sample size (0.050 ml) accomodated by his fractionator limits the overall sensitivity of the technique. Larger volumes created technical difficulties such as reduced flow rate of carrier gas as a consequence of excess water in the fractionator tube. Even with small liquid injections, the fractionator required frequent repacking (McAuliffe, 1966). In the absence of the fractionator tube, the adsorption packings commonly used for the separation of low molecular weight hydrocarbons require lengthy heating cycles to

adequately expel the accumulated moisture from the column, and their effectiveness may be irreversibly impaired by frequent liquid injections. Head space analysis is very similar to the vacuum extraction technique, in that liquid samples are injected into sealed flasks, and the dissolved gases are extracted by vigorous shaking (for example, Smith & Restall, 1972). Ultimately, however, only a fraction of the extracted gas is injected onto the analytical column, resulting in reduced sensitivity.

The presence of proteins (Battino & Clever, 1966; Wishnia, 1962), salts and other solutes (Battino & Clever, 1966) seriously affects the solubility of gases in water. Although tables of Henry's Law constants are available for many simple salt solutions, it is very difficult to estimate solubilities for complex solutions. Often, the solutions encountered in biological studies are of unknown composition; inorganic ions and a variety of macromolecules released by the tissue affect the solubility of gases in the surrounding medium. In these situations, it is necessary to determine the solubility of gases experimentally.

It has been demonstrated that isolated aleurone layers from barley release inorganic ions (Jones, 1973) and protein (Melcher & Varner, 1971) in response to gibberellic acid. The presence of these solutes in the medium alters the solubility of ethylene in the medium, and hence changes the concentration of dissolved ethylene during treatment of the aleurone layers. To investigate this possibility,

15 aleurone layers were incubated in 3 ml of incubation buffer containing 0.010 mM gibberellic acid. After 48 h at 25 C, the medium was cleared by centrifugation at 980 x g for 20 min, and the supernatant layer was equilibrated with 17.4 nl/ml ethylene by bubbling the gas mixture through the solution. For comparison, water and a sample of fresh medium were equilibrated with the same gas mixture. The solutions were maintained at 25 C, and the dissolved ethylene was determined in 3 samples of each solution. In order to permit comparison of these values, it was necessary to assume that the viscosity of the aqueous solution had not varied significantly. Viscosity of the sample has been shown to influence the recovery of dissolved gases by vacuum extraction (Bayes *et al.*, 1949). The results indicated that the solubility of ethylene in the buffer did decline during the incubation period (Table V). However, the reduction in dissolved ethylene concentration represented less than 4% of the total ethylene dissolved in the fresh medium. Consequently, the change in the composition of the incubation medium would not affect the interpretation of the results obtained for most ethylene concentrations.

C. Gassing apparatus

The two gassing apparatuses were designed and constructed with the intention of incubating barley half-seeds and isolated aleurone layers in environments that were carefully controlled with respect to the composition of

Table V. Solubility of ethylene in aqueous media

Solvents were equilibrated with 17.4 nl/ml ethylene at 25 C. The medium was 20 mM sodium succinate buffer (pH 5.5) containing 20 mM calcium chloride and 0.010 mM gibberellic acid. "Used medium" was prepared by incubating 15 aleurone layers in 3 ml of medium at 25 C for 48 h. The dissolved ethylene was determined by vacuum extraction of 3 samples of 3 ml each, followed by gas chromatography.

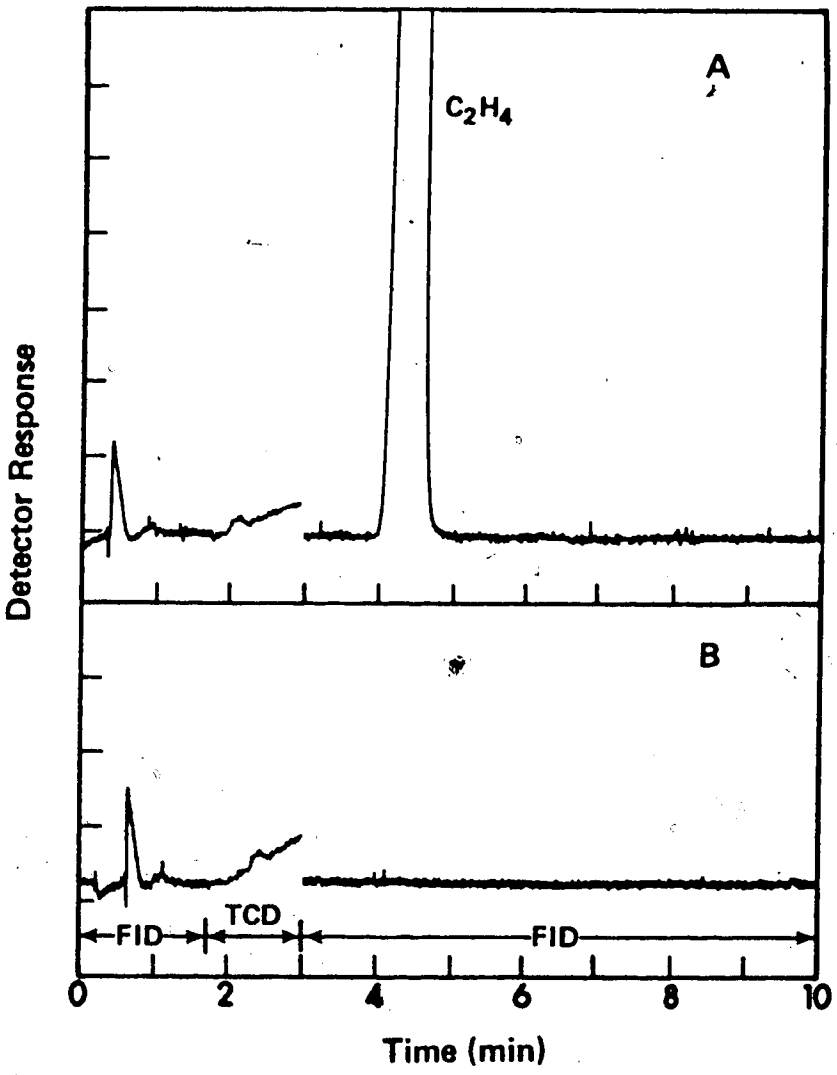
Solvent	Dissolved Ethylene (nl/ml)	Henry's Law Constant (mm Hg/mole fraction) ($\times 10^{-6}$)
Water	1.93	8.67[a]
Medium (Fresh)	1.87	9.00
Medium (Used)	1.79	9.40

[a] literature value from Daniels & Alberty (1966).

the atmosphere surrounding the tissue. As indicated in Methods and Materials, these systems relied on heated platinum catalysts to purify an air stream before the sample is exposed to the air. Analysis by gas chromatography showed that the air for both of the flow through systems was hydrocarbon-free, although they differed significantly with respect to carbon-dioxide content. For the initial 72 h incubation period for half-seeds, the on site compressed air line served as the source of air. Consequently, the carbon dioxide concentration remained similar to normal atmospheric levels (330 nl/ml). The mean carbon dioxide concentration found in the hydrocarbon-free air was 350 nl/ml. In contrast, the air for the treatment of isolated aleurone layers was from commercial compressed air cylinders. The carbon dioxide concentration from this source was greatly reduced compared to normal environmental levels (Figure 4). Any residual carbon dioxide, and carbon dioxide produced by the combustion tubes was removed by passing the air through a 5% (w/v) sodium hydroxide solution. Hydrocarbons and carbon dioxide were undetectable in the final air product as indicated by gas chromatography (Figure 8). It was desirable to have a carbon dioxide-free environment during the ethylene treatment as carbon dioxide served as an inhibitor of ethylene action in other tissues (Abeles, 1973; Kang *et al.*, 1967; Keys *et al.*, 1975).

A mechanism was required to introduce minute quantities of ethylene into the air stream to produce a mixture that

Figure 8. Gas chromatograms illustrating the composition of the air, with respect to low molecular weight hydrocarbons and carbon dioxide, modified for the incubation of isolated barley aleurone layers. Chromatograms were produced from 3 ml injections of air containing 13.0 nl/ml ethylene (A) and of control air (B). The recorder was operated at maximum sensitivity to aid in the detection of trace contaminants. The apparatus is described in the text, and the retention times for methane, carbon dioxide, ethylene and ethane are the same as in Figure 4.


A
C_2H_4
B
Detector Response
FID
TCD
FID
0
2
4
6
8
10
Time (min)

was stable with respect to ethylene concentration over an extended period of time. This was accomplished in the past by means of a variety of apparatuses including mariotte bottles (Pratt *et al.*, 1960), constant pressure devices (Roberts, 1951) or permeation tubes (O'Keeffe & Ortman, 1966). All of these methods are sensitive to temperature fluctuations, and all but the latter technique are affected by atmospheric pressure. A simpler metering system was devised, starting with a diluted mixture of ethylene in air, and using fine glass capillary tubing to help restrict and maintain the flow of gases. Although the flow of gases through capillary tubing is also temperature dependent, the effect of temperature fluctuation was far less severe than the pressure changes that a similar temperature change would induce in the reservoirs of gas employed in the other metering systems. Use of the combination of various lengths of capillary tubing and the low range pressure regulator allowed the ethylene-air mixture to be introduced into the air stream to produce a wide range of final ethylene concentrations. In the current experiments, ethylene concentrations spanning the range from 0.037 nl/ml to 115 nl/ml were readily attained. Moreover, once equilibrium had been reached (approximately 1 h), the concentration of ethylene in the mixture was very stable. During the course of experimentation at 12.8 nl/ml, the variance in measured ethylene concentration was less than 1.2 nl/ml over a 100 day period. Thus, this apparatus allowed the preparation of

well defined ethylene mixtures that remained constant throughout individual experiments.

The addition of ethylene to the air stream must not contribute other hydrocarbons and contaminants to the air. Commercial grade ethylene typically contained impurities that included: 7 nl/ml acetylene, 50 nl/ml hydrogen, 200 nl/ml propane, 200 nl/ml methane and 2500 nl/ml ethane (analysis courtesy of Canadian Liquid Air, Edmonton, Alberta). Recently, a gas chromatographic method for the purification of ^{14}C-labelled ethylene has appeared (Boyer, 1975). However, once the ethylene has been purified by this method or any other method, it must be added to the air stream in very small but carefully regulated amounts. This usually requires the employment of the more elaborate metering systems cited above. The use of a dilute commercial mixture as the source of ethylene eliminated the need for complex apparatus. To avoid the contaminants found in commercial grade air (Figure 4) and ethylene, mixtures of either 113 nl/ml or 219 nl/ml research grade ethylene in ultra zero air were purchased (Matheson of Canada Ltd., Whitby, Ont.). When these mixtures were further diluted with hydrocarbon-free air to yield the appropriate ethylene concentrations, no hydrocarbons other than ethylene (Figure 8) were detected in the samples (the limit of detection was 0.05 ng of each hydrocarbon in the 3 ml samples). Thus, no further purification of ethylene was required to achieve the desired level of purity for these

studies.

The remainder of the gas mixing system required careful planning as well. The materials used in constructing equipment for plant physiological studies should be chosen cautiously. Plastics and rubbers are known to release ethylene, particularly when exposed to heat or light. Common sealants have also been found to release substantial amounts of hydrocarbons (Bassi & Spencer, 1979). Therefore, to prevent contamination of the air stream, the entire system, including tubing and unions, was constructed of glass, metal or teflon. Consequently, no extraneous hydrocarbons were found in the air of the culture tubes (Figure 8).

D. Enzyme assays

Amylase assay

The determination of amylase is of great importance for both industrial and clinical applications. For this reason, a wide variety of assay procedures has been developed. However, each method is subject to limitations dependent on the nature of the material to be assayed. Therefore, the most suitable assay procedure for the present investigation was not immediately evident, and preliminary exploration of the methods was required. Searcy, Wilding & Berk (1967) summarized the classical methods of amylase determination with respect to accuracy and reliability. Of the approaches evaluated by them, which included the viscosimetric,

turbidimetric, amyloclastic and saccharogenic methods, the determination of reducing sugars with the 3,5-dinitrosalicylic acid reagent offered the greatest sensitivity and relative freedom from interferences. This conclusion was later supported by Hall *et al.* (1970). The general application of this method was restricted by limitations of the technique. It was observed that the colorimetric response of the reagent was dependent on the chain length of the dextrin bearing the reducing terminal (Robyt & Whelan, 1965). As the degree of polymerization increased, so did the extent of colour development in response to an equivalent number of reducing terminals. This aberration was rectified by the introduction of the Nelson copper reagent (Nelson, 1944). However, because both of these assay procedures measure the appearance of reducing sugars in solution, the saccharogenic methods were also very sensitive to contaminating reducing groups found in the sample (Hall *et al.*, 1970) and substrate (Strumeyer, 1967). Several other substances have been shown to interfere with determination of reducing potential. These materials include amino acids, salts and chelating agents (Briggs, 1967).

In an effort to avoid the interferences inherent with the saccharogenic assay, a number of artificial substrates have been developed. The majority of these are either chromogenic moieties covalently bound to an insoluble polysaccharide (Klein, Foreman & Searcy, 1969; Rinderknecht

et al., 1967) or a dyed soluble polysaccharide that was ultimately rendered insoluble by the addition of solvents after incubating the substrate with the enzyme solution (Sax, Bridgwater & Moore, 1971). The release of soluble fragments from the dyed substrate was not a direct measure of enzyme activity, however, since the further hydrolysis by amylase of a solubilized fragment would not be reflected in the final measurement of the soluble dyed material. In addition, the solubilities of these fragments were sensitive to other solutes in the sample (Sax *et al.*, 1971) and reduced the reliability of the methods if samples of diverse composition were encountered. Under controlled conditions, however, the assays employing chromogenic substrates responded linearily to enzyme concentration (Klein *et al.*, 1969; Rinderknecht *et al.*, 1967; Sax *et al.*, 1971).

Of all the possibilities considered, the Nelson photometric determination of reducing sugars as described by Robyt & Whelan (1968), and the solubilization of a dyed derivative of amylose (Rinderknecht *et al.*, 1967) offered the most promising methods. In order to establish the suitability of each of these two assay procedures, their response to amylase prepared from barley aleurone layers (Appendix) was compared. The assay buffer and the temperature were the same for each assay.

In the absence of substrate and enzyme, the response of the Nelson copper reagent to standard maltose solutions remained linear up to an absorbance of 0.989, or 2.92 mM

maltose. The Nelson copper determination of amylase activity yielded a straight response that passed through the origin but remained linear only to an absorbance of 0.767 at 520 nm (Figure 9). This corresponded to an enzyme concentration of 0.48 Units/ml. The upper limit on the enzyme assay may have been imposed by either the depletion of suitable substrate during the assay period, or the total consumption of reagents during the subsequent analysis of reducing terminals. The marginal background absorbance (only 0.028) that resulted from the inclusion of the starch substrate could not account for the reduction in the useable range of the Nelson copper method. Strumeyer (1967) found that the reducing terminals of the starch substrate led to elevated absorbance by blanks, thereby suppressing the range of the assay by expending the arsenomolybdate reagent. But, since the assay remained linear over a sufficient range of enzyme concentrations, the preliminary reduction of the substrate by sodium borohydride (Strumeyer, 1967) was not pursued.

The amylose-azure assay created a two-phase curve, (Figure 10), similar to that obtained by Rinderknecht *et al*. (1967). According to the original publication, the lower region exhibited a linear relationship between absorbance at 595 nm and enzyme concentration. The final absorbance of the assay performed under the present conditions was too low to offer reliable results below 0.2 Units/ml. Above 0.5 Units/ml, the assay remained linear up to 3.0 Units/ml, producing an absorbance of 0.323 at the

Figure 9. The linearity of the saccharogenic assay with respect to amylase activity. Nelson copper reagent was used to determine the increase in the reducing potential of the substrate solution in response to enzyme activity. Assay conditions are as given in Methods and Materials.

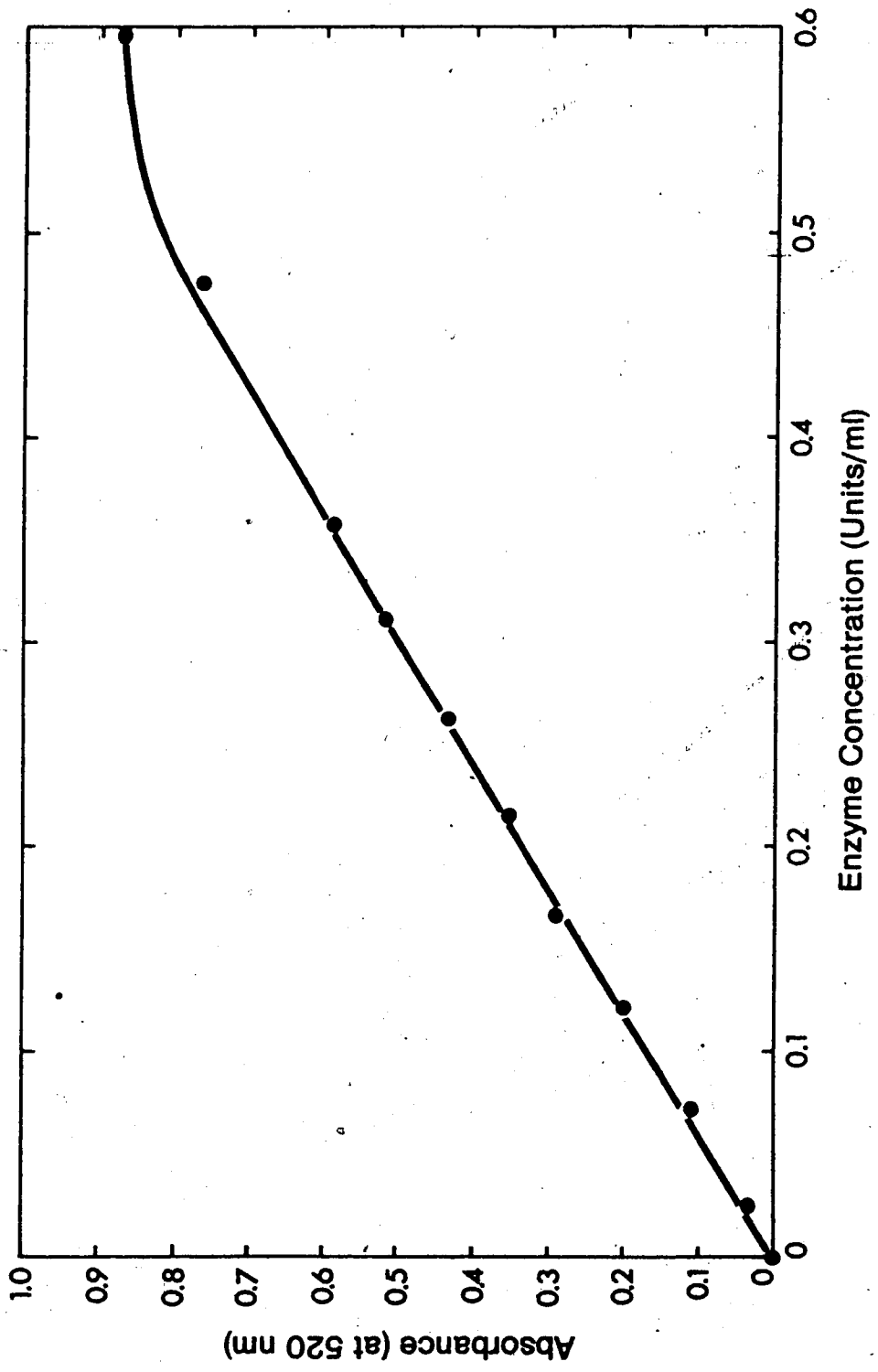
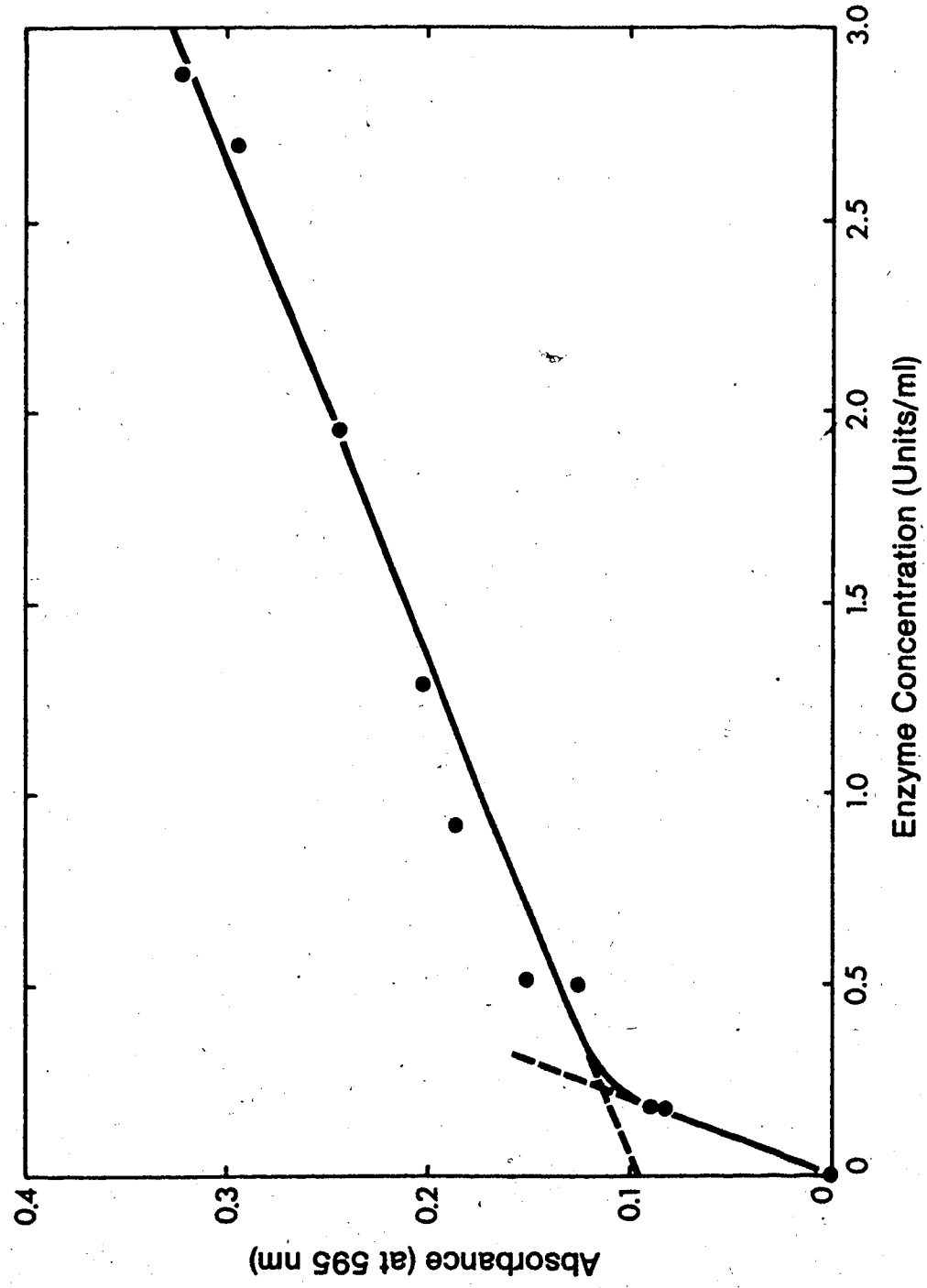

Absorbance (at 520 nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.1
0.2
0.3
0.4
0.5
0.6
Enzyme Concentration (Units/ml)

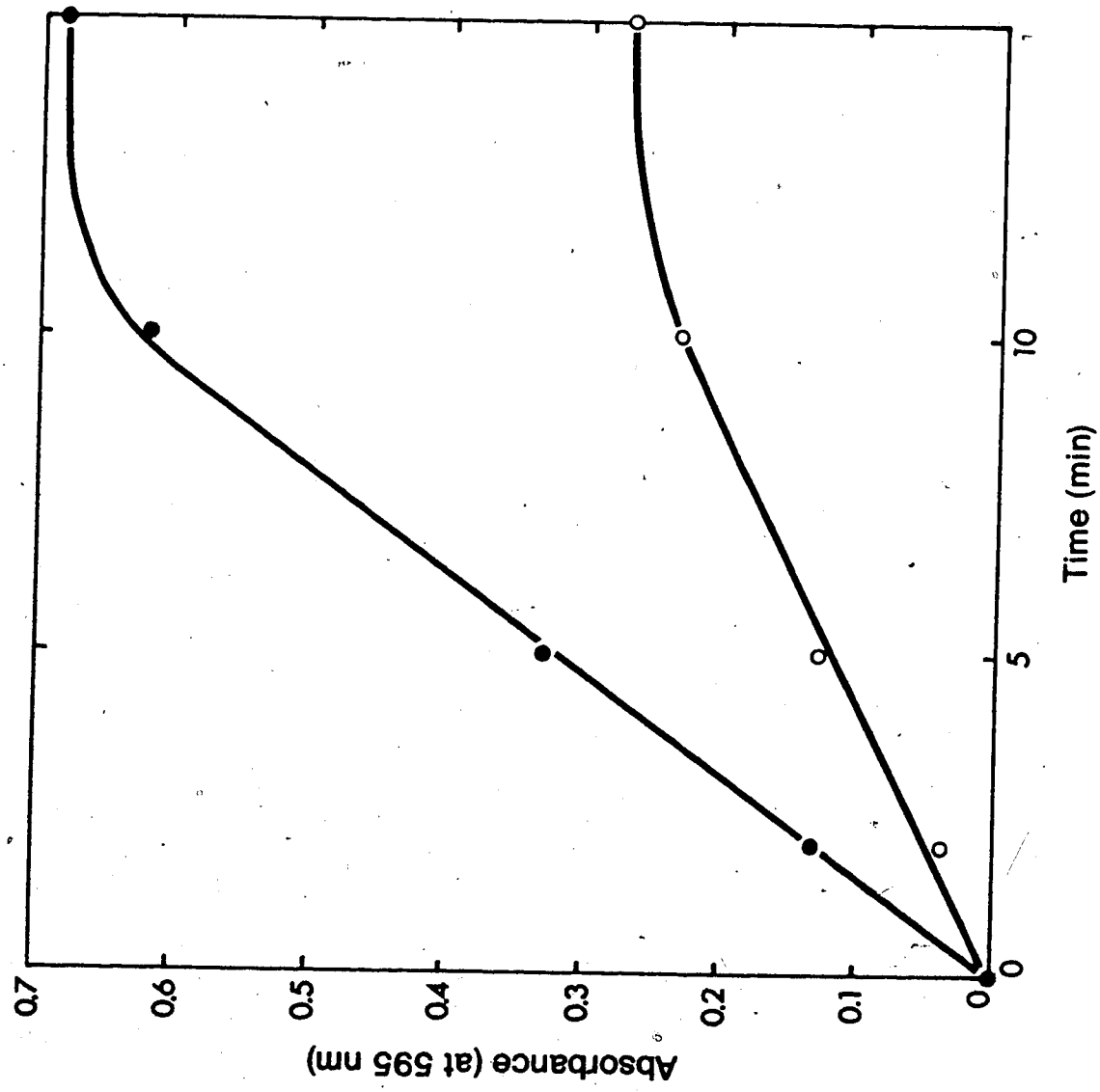
Figure 10. Relationship of amylase assay results to enzyme activity using an insoluble chromogenic substrate. The quanitity of substrate solubilized was dependent on enzyme concentration. The substrate concentration is 2% (w/v) and the assay conditions are specified in Methods and Materials.


Absorbance (at 595 nm)
0.4
0.3
0.2
0.1
0
0
0.5
1.0
1.5
2.0
2.5
3.0
Enzyme Concentration (Units/ml)

upper limit. Nevertheless, the overall sensitivity of this assay did not compare favourably with the Nelson copper assay. Since assays using insoluble substrates depend on high substrate concentration to overcome the insensitivity of the methods (Sax *et al.*, 1971), the substrate concentration was increased from the original value of 2% (w/v) to 10% (w/v). This produced a uniform enhancement of absorbance by a factor of 2.8, but the sensitivity of the assay was still below that attained by the Nelson copper assay. The elevated substrate concentration did not improve the linearity of the assay. Increasing the length of the assay was not feasible at either concentration of substrate because the rate of solubilization decreased sharply after the initial 10 min (Figure 11). This indicated a deviation from the zero-order kinetics preferred for enzyme assays. The stratification of substrate concentration within the sample during the assay period (Hall *et al.*, 1970) may have prevented the increased substrate concentration from extending the period of time during which the rate of substrate solubilization was constant.

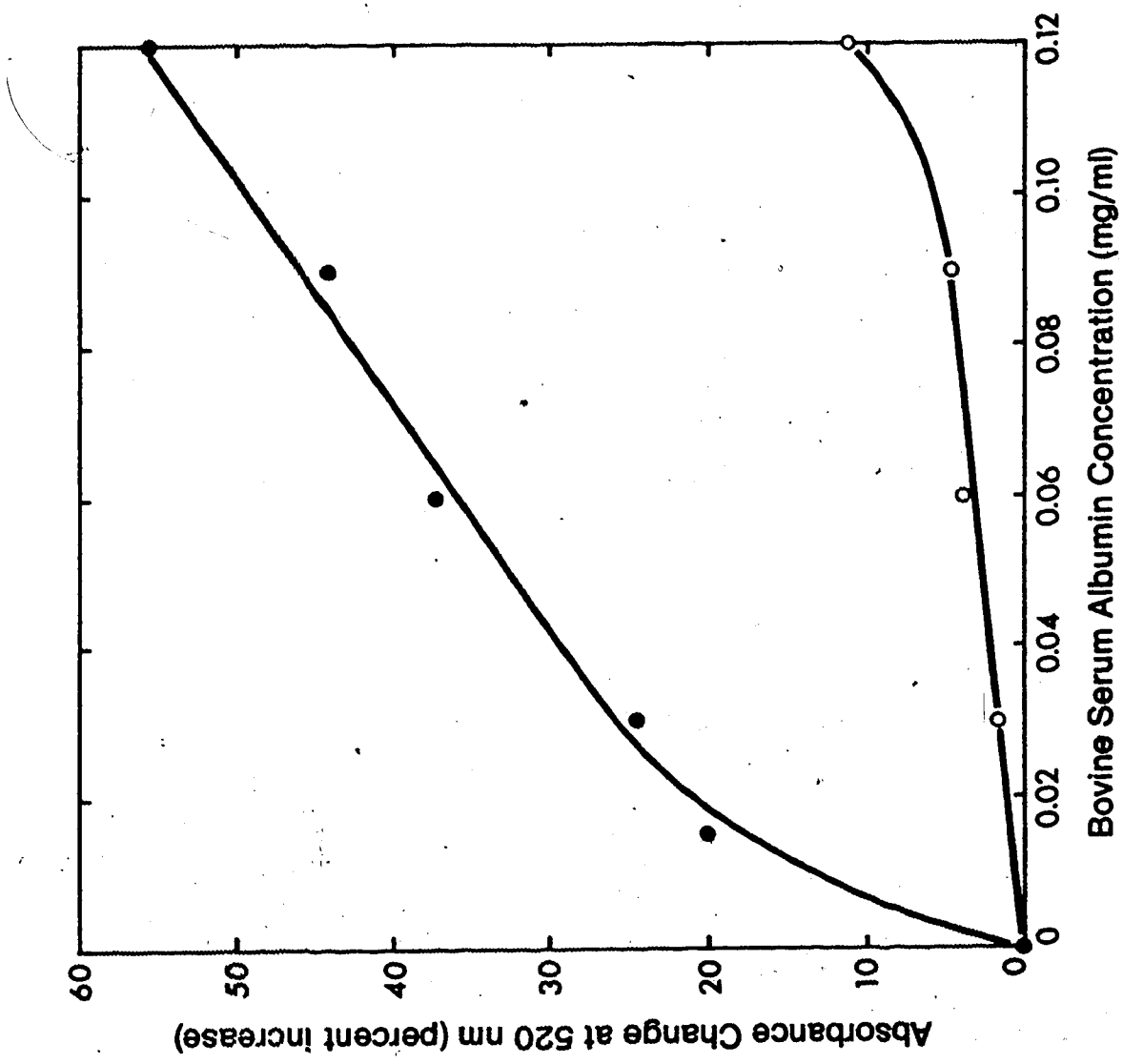
On the basis of these observations, the Nelson copper reducing value method appeared to be superior for the determination of amylase activity. However, the possible interference by several cellular constituents required further consideration. The potential effect of proteins on amylase activity and on amylase assays has been revealed (Bernfeld, Berkely & Bieber, 1965; O'Donnell & McGeeney,

Figure 11. Effect of substrate concentration on the time course and sensitivity of the amylase assay. Enzyme activity was determined with the use of the insoluble substrate, Amylose Azure, at 2% w/v (O) and at 10% w/v (●). Other assay conditions are presented in Methods and Materials.


Absorbance (at 595 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
Time (min)

1974). Purified barley amylase was assayed by the Nelson copper method in the presence of various concentrations of bovine serum albumin. The addition of inert protein produced an increase in apparent activity of up to 56% at 0.120 mg/ml bovine serum albumin (Figure 12). The effects of the protein on amylase activity and on the response of the Nelson copper assay were differentiated by measuring the absorbance produced by maltose standards in the presence of the same range of protein concentrations. The concentration of maltose was adjusted to approximate the concentration of reducing equivalents released by the enzyme during the assay period. As evident from Figure 12, protein had very little effect on the colour development in response to maltose. Therefore, the enhanced response of the assay to amylase was likely an effect of the activity of the enzyme rather than interference with the normal colour development in response to reducing sugar terminals. Although the original Nelson copper method for the determination of serum glucose used zinc sulphate and barium hydroxide to deproteinize the sample (Nelson, 1944), Henry & Chiamori (1960) found that the omission of the protein precipitation had no effect on the absorbance produced by various samples. At very high protein concentrations, some turbidity was noted but no effect on colour development was detected. These observations by Henry & Chiamori (1960) are corroborated by the data in Figure 12. Moreover, the enhancement of amylase activity by protein has been observed with amylases from

Figure 12. Effect of inert protein on the determination of amylase activity by the Nelson copper assay. Bovine serum albumin was added either to maltose standards (O) or during the assay of amylase activity (●). Each aliquot of partially purified amylase contained 69 mUnits of activity and 0.001 mg protein, and the maltose standards contained 730 nmole maltose. The absorbances of the amylase assay and maltose standards without added protein were 0.228 and 0.205 respectively.


Absorbance Change at 520 nm (percent increase)
0
10
20
30
40
50
60
0
0.02
0.04
0.06
0.08
0.10
0.12
Bovine Serum Albumin Concentration (mg/ml)

various sources. Bernfeld *et al*. (1965) found that 5 mg/ml bovine serum albumin or ovalbumin increased the apparent activity of porcine pancreatic amylase in very dilute solutions. The addition of inert proteins prevented the loss of specific activity upon dilution of the enzyme to 1 ng/ml, and Bernfeld *et al*. (1965) presumed that the inert proteins had prevented the reversible denaturation of the amylase. O'Donnell & McGeeney (1974) showed that a number of proteins stimulated the activity of canine pancreatic amylase by up to 32%. This effect was observed at a relatively high enzyme concentration (26 ng/ml) so the mechanism may have been distinct from the dilution effects observed by Bernfeld *et al*. (1965). In their study of barley malt alpha-amylase, Greenwood & MacGregor (1965) found that the addition of inert barley malt proteins to amylase solutions stabilized the enzyme preparation over a wide range of pH, but no direct stimulation of activity was reported.

In reference to the current study, samples of medium and extract prepared for the determination of amylase activity routinely contained 900 ng/ml and 400 ng/ml protein respectively. Inspection of Figure 12 indicated that these very low protein concentrations would have minimal effect on the determination of amylase by the saccharogenic method.

Samples of biological origin may also contain chelating agents and reducing agents that alter the colour yield during the determination of reducing sugars (Briggs, 1967;

Hall *et al.*, 1970). To examine this potential source of error, samples of medium and extract from barley aleurone layers were autoclaved, then centrifuged to remove the fine precipitate that formed. Portions of these solutions were added to samples of partially purified amylase, and the enzyme activities were determined. Although the addition of the autoclaved solutions elevated the background absorbance by values in the order of 0.01, they had no affect on the apparent activity of amylase, provided that a separate zero-time blank was prepared for each sample. The necessity for individual blanks may have limited the acceptance of this assay in clinical applications, but did not restrict the use of the Nelson copper reducing value assay for present research. This assay was adopted for all subsequent amylase determinations.

Xylanase assay

1. Substrate preparation.

The repeated dialysis steps required for the purification of xylan by the method of McNeil & Albersheim (Taiz & Honigman, 1976) were found to be very time consuming for the production of sufficient xylan. An alternate method was sought that would yield larger amounts of product in a relatively short period of time; the precipitation of xylan by alkaline copper proved to be very effective (Jermyn, 1955). Paper chromatography of hydrolysis products indicated that both techniques reduced contamination of xylan by other carbohydrates and their derivatives

(Figure 13). (For the purposes of this experiment, the use of *p*-anisidine-phthalic acid detection reagent was superior to the alkaline silver oxide reagent. Although the latter was more sensitive, it did not offer any colour differentiation among the types of carbohydrates encountered, whereas *p*-anisidine-phthalic acid did.) A major constituent in all xylan samples was a hydrolysis product with a low Rg-value. (Rg-value is the distance that a component has migrated relative to glucose.) The brown colour produced with this product in response to the *p*-anisidine-phthalic acid reagent and the low Rg are characteristic of uronic acids. This component of xylan is likely the 4-O-methyl-glucuronic acid found by Aspinall & McKay (1958) in larchwood xylan. Other components of low Rg-value also appeared in the hydrolyzate, but were not identified. When the standard sugars were subjected to the hydrolysis conditions and the products chromatographed, the unknown constituents of low Rg-value were absent, suggesting that they were not artifacts of the hydrolysis procedure.

When duplicate xylanase samples were assayed using the xylan purified by either method, the results were identical. In light of the ease of preparation by alkaline copper precipitation, the method of Jermyn (1955) was used for the purification of xylan required for all successive xylanase assays.

2. Xylanase assay.

The xylanase assay, as used for these investigations,

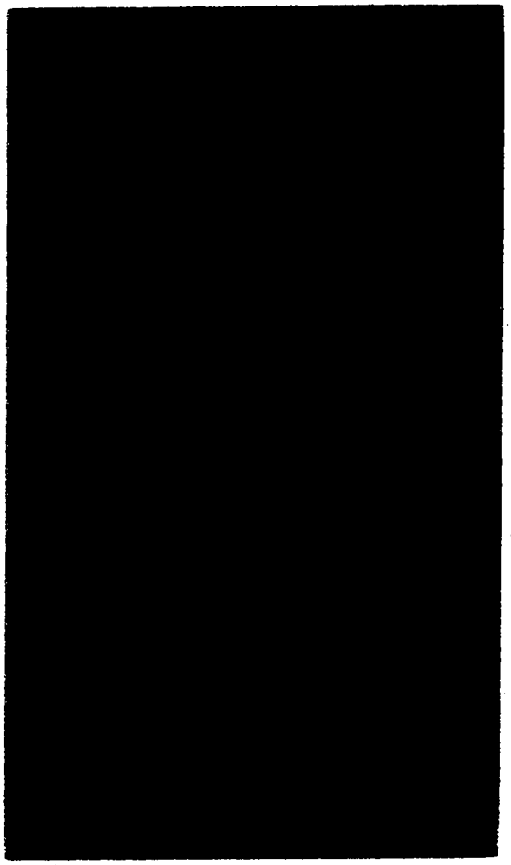


Figure 13. Paper chromatograms of the products of xylan hydrolysis with 6 N formic acid. The standard (STD) consisted of 0.013 mg each of (from top) xylose, arabinose, glucose and galactose. The samples were the hydrolysis products of 5 mg crude commercial xylan (A), or commercial xylan purified by the methods described by Taiz & Honigman, 1976 (B) or by Jermyn, 1955 (C).

was linear up to an enzyme concentration of 33 mUnits/ml. This corresponded to an absorbance of 0.62 at 520 nm. Since xylan forms an insoluble complex with copper in alkaline solutions (Jermyn, 1955), it was thought that the presence of the substrate in samples might affect the determination of reducing potential by the Nelson copper method. However, the addition of xylan solution to xylose standards produced no change in the absorbance of standards against reference solutions containing the equivalent amount of xylan.

Glucanase assay

As was the case for the xylanase assay, the glucanase assay was linear to an enzyme concentration of 33 mUnits/ml. At this level of enzyme activity, the absorbance was 0.53 at 520 nm.

Protease assay

The protease assay was linear over the entire range of enzyme concentration tested. The maximum enzyme concentration considered, 5.8 mUnits/ml, yielded an absorbance value of 0.41 at 280 nm. This level of activity was never exceeded by samples prepared for routine analysis.

E. Tissue preparation

In order to avoid the necessity of addition of antibiotics to the incubation medium, particular care was used to exclude microbial contamination from the tissue preparations. During preliminary experiments, it was found that the common sterilization practice of soaking half-seeds

in 1% sodium hypochlorite for 20 min (Chrispeels & Varner, 1967) was not sufficient to prevent microbial growth on the half-seeds. However, adequate control of bacterial and fungal infections was attained by rinsing the half-seeds in 75% ethanol followed by sterilization in undiluted commercial bleach as described in Methods and Materials. The concentration of total available chlorine in the bleach was found to be 4%. Monitoring the concentration of available chlorine in the rinsing solutions revealed that 5 rinses with 25 ml glass distilled deionized water were required to reduce the chlorine concentration to 1.2 mg/l. Prepared in this manner, microbial contamination of half-seeds was kept to a minimum. Periodically, flasks containing half-seeds were retained for extended periods of time to allow microbial contaminations to become more easily detected. If no contamination could be observed after the normal 72 h incubation period, no contamination was ever observed in flasks within the succeeding four days. This offered a means of eliminating contaminated half-seeds before isolated aleurone layers were prepared. In spite of these precautions, however, microbial contaminations occasionally developed during the incubation of aleurone layers. In the buffered incubation medium, the infection developed very quickly and contamination was readily apparent within the first 24 h. Such samples were discarded. If no symptoms developed during the initial 24 h period, the samples of isolated barley aleurone layers

typically remained free of any detectable contamination for the remainder of the incubation period.

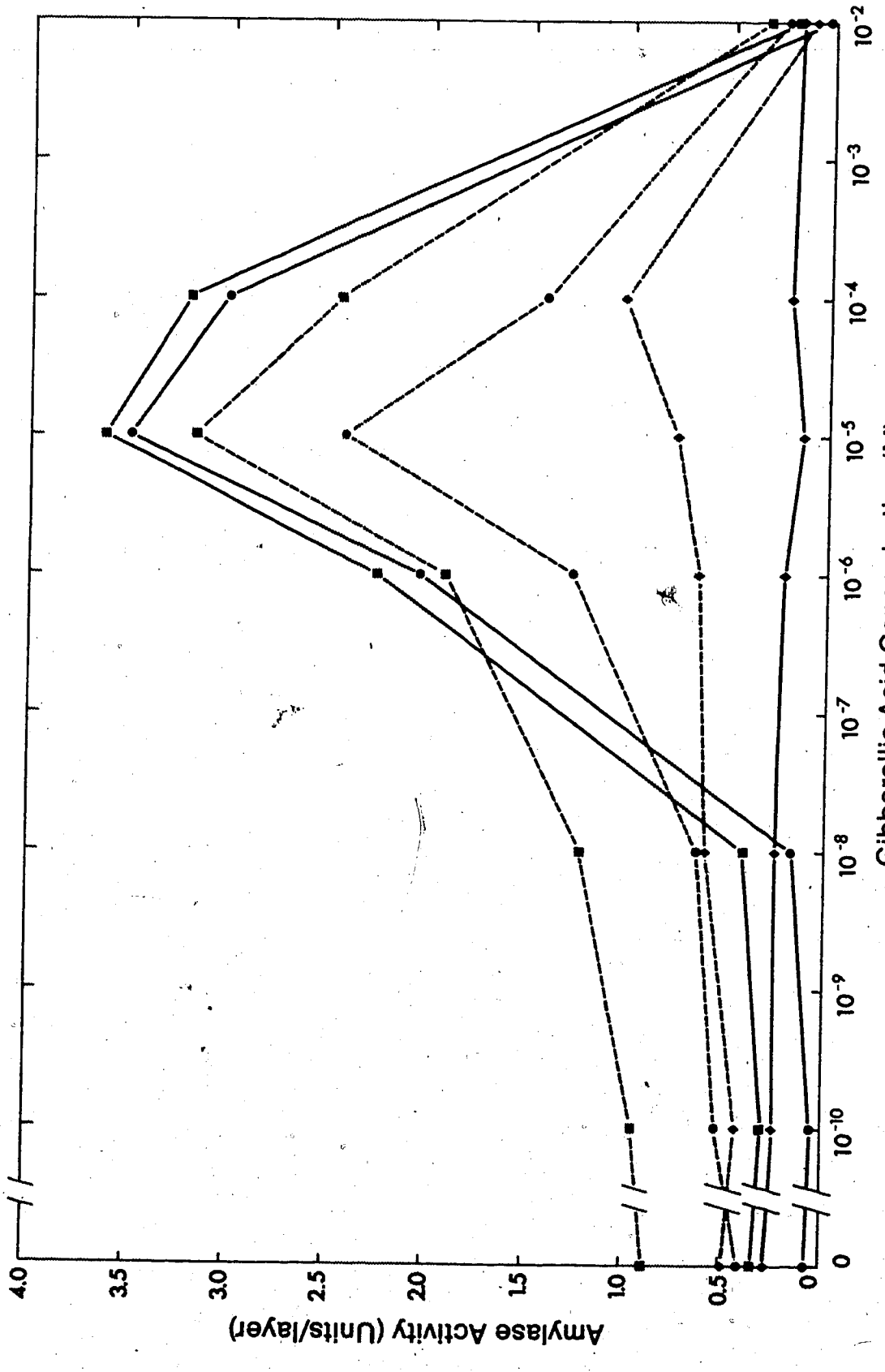
The introduction of an air flow through the flasks containing the half-seeds greatly reduced the solubilization of the endosperm during the 72 h incubation period. This may be a reflection of a greater rate of evaporation from the half-seeds, or it may be the consequence of biological processes. These possibilities were not resolved. Under these conditions, however, the replacement of the water in the flasks with a succinate-calcium chloride buffer (Firn & Kende, 1974) became impractical. The addition of the buffer further reduced the liquification of the endosperm to such an extent that aleurone layers could not be removed intact. The initial incubation of half-seeds was therefore continued on filter paper moistened with water.

F. Selection of incubation medium for isolated aleurone layers

Since Chrispeels & Varner (1967) published their early work on the gibberellic acid-enchanced synthesis of amylase by isolated barley aleurone layers, most studies have been conducted in an acetate buffer medium similar to theirs. This tendency was reinforced by the prior findings of Briggs (1963) that the use of acetate as the buffering agent increased the sensitiviy of amylase assays. However, Firn & Kende (1974) noted that replacing acetate with succinate in the incubation medium improved the response of aleurone

layers to 0.001 mM gibberellic acid. This effect was investigated in greater detail. Isolated aleurone layers were incubated for 48 h in buffers containing a wide range of gibberellic acid concentrations and the amylase activity produced was measured. The concentration of the buffering agent (20 mM), concentration of calcium chloride (20 mM) and the pH (5.5) were kept constant in both buffers. The results are presented in Figure 14. The production of amylase was maximum at 0.010 mM gibberellic acid, and declined sharply as the concentration was changed in either direction. The pattern was similar for both the succinate and acetate buffers, although at 0.010 mM gibberellic acid, total amylase activity produced in the succinate buffer exceeded that in the acetate buffer by 14%. In the absence of gibberellic acid, activity in the acetate buffer was 2.6 times greater than in the succinate buffer. The net result was a 10.6-fold increase in total amylase activity in response to 0.010 mM gibberellic acid when aleurone layers were incubated in succinate buffer, but only a 3.6-fold increase in activity in the acetate buffer. Furthermore, the nature of the buffer and the concentration of gibberellic acid also determined the fraction of the amylase activity that was released into the medium from the aleurone layers. The proportion of amylase released reached a maximum at 0.010 mM gibberellic acid. At this concentration, 97% of the amylase activity had been released into the succinate buffer, while in the acetate buffer, only

Figure 14. Dependence of amylase synthesis and release by isolated barley aleurone layers on buffer composition and gibberellic acid concentration. Aleurone layers were incubated in either a succinate (——) or an acetate (– – –) buffer containing the appropriate concentration of gibberellic acid. After 48 h, amylase activity in the medium (●) and in the extract from the layers (◆) was determined. Total activity (■) was the sum of the activity found in the medium and in the extract.


Amylase Activity (Units/layer)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Gibberellic Acid Concentration (M)

76% of the activity appeared in the medium. Therefore, the use of succinate as the buffering agent in the incubation medium greatly improved the sensitivity of amylase production by aleurone layers to the presence of gibberellic acid.

In a separate series of experiments, the effect of buffer selection on other enzyme systems was observed. The data presented in Table VI demonstrated that the magnitudes of the gibberellic acid-enhanced synthesis and/or release of xylanase and glucanase were also governed by the nature of the buffer. Since the sodium succinate buffer amplified the response of the aleurone layers to gibberellic acid, it was used for all further studies.

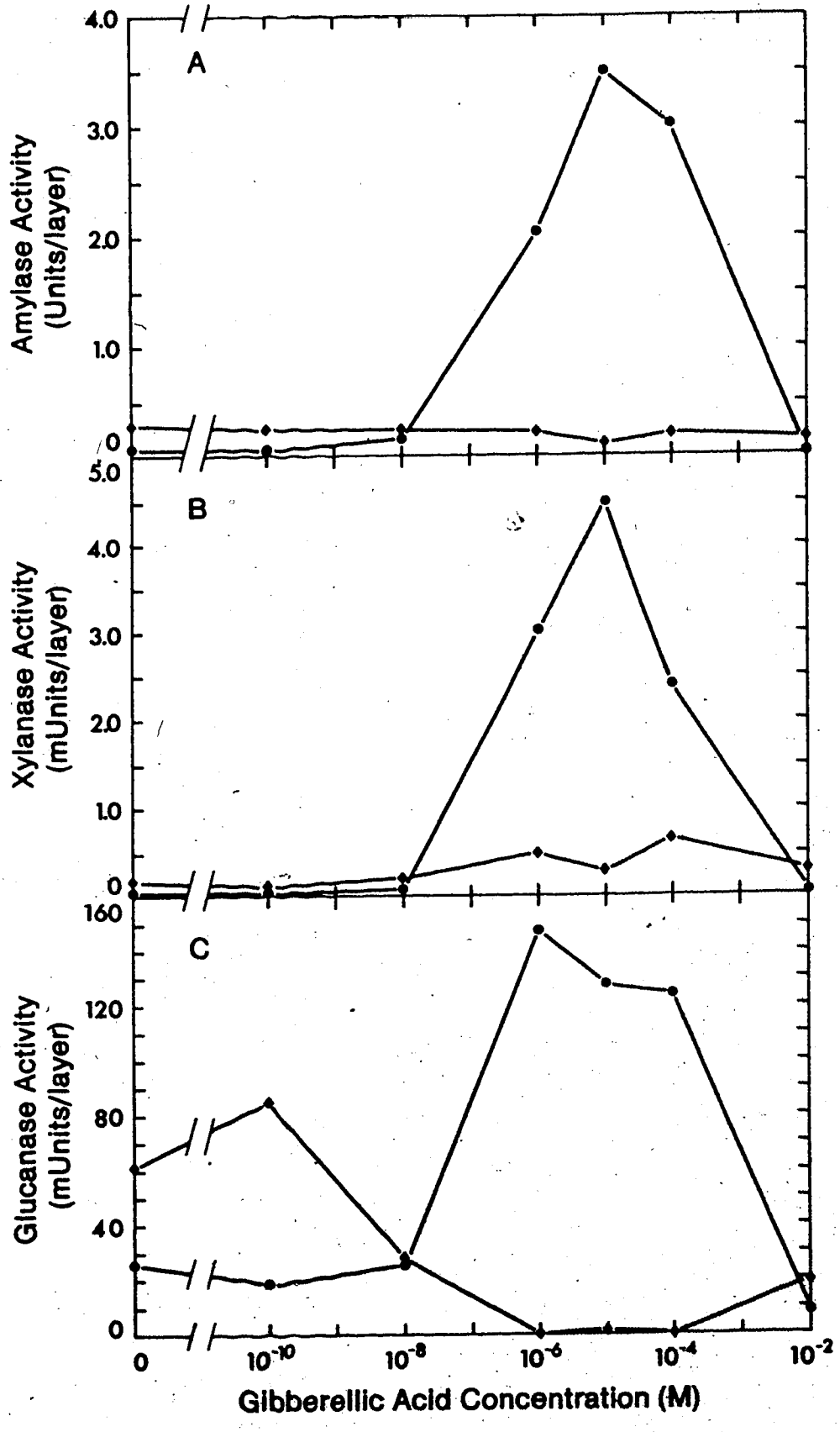
The dose response curve for amylase synthesis and secretion promoted by gibberellic acid indicated that the maximum response to gibberellic acid occurred at 0.010 mM (Figure 15A). At higher concentrations, the production of amylase was greatly curtailed. Although most studies have not extended beyond the maximum (for example, Jacobsen & Varner, 1967; Jones & Varner, 1967), the depression of amylase synthesis by cereal aleurone tissue in response to excessive gibberellic acid has been observed. Okuda *et al*. (1967) found that a broad maximum for the production of amylase by barley half-seeds was centred at 0.010 mM gibberellic acid. Above 0.010 mM the synthesis of amylase decreased abruptly. Similarly, the optimal concentration of gibberellic acid for amylase synthesis by isolated wheat

Table VI. Response of aleurone layers to gibberellic acid in the presence of acetate or succinate buffers

Ten aleurone layers were incubated in either 20 mM sodium succinate or 20 mM potassium acetate buffers. Both were adjusted to pH 5.5, and contained 20 mM calcium chloride. The isolated aleurone layers were incubated in the media for 48 h at 25 C. The values are the means of triplicate samples.

Gibberellic Acid Concentration (mM)	Buffer	Enzyme Activity (mU/layer)			
		Xylanase		Glucanase	
		Extract	Medium	Extract	Medium
0	Acetate	0.09	0.18	57	60
0	Succinate	0.16	0.02	61	26
0.010	Acetate	1	0.32	13	80
0.010	Succinate	.33	2.58	8	90

Figure 15. Effect of gibberellic acid concentration on the synthesis and release of hydrolytic enzymes. Aleurone layers were incubated in succinate buffer containing various concentrations of gibberellic acid. After 48 h at 25 C, amylase activity (A), xylanase activity (B), and glucanase activity (C) were determined in the medium (●) and in the extract from the layers (O). Each value represents the mean of two samples.


A
B
C
Amylase Activity (Units/layer)
Xylanase Activity (mUnits/layer)
Glucanase Activity (mUnits/layer)
4.0
3.0
2.0
1.0
0
5.0
4.0
3.0
2.0
1.0
0
160
120
80
40
0
0
10^{-10}
10^{-8}
10^{-6}
10^{-4}
10^{-2}
Gibberellic Acid Concentration (M)

aleurone layers was found at 0.028 mM (Collins, Jenner & Paleg, 1972). Higher concentrations reduced amylase activity. No explanation for the inhibition of amylase synthesis by super-optimal gibberellic acid concentrations has been offered.

The synthesis and release of other hydrolytic enzymes were also sensitive to the concentration of gibberellic acid. The effect of gibberellic acid concentration on the appearance of xylanase activity was determined (Figure 15B). The dose response of xylanase paralleled that of amylase very closely. These enzymes required the addition of gibberellic acid to promote their synthesis and secretion. The enzyme, glucanase, was dependent on gibberellic acid for secretion, but not for its synthesis (Taiz & Jones, 1970; Jones, 1971; Bennet & Chrispeels, 1972). This relationship was also sensitive to high concentrations of gibberellic acid (Figure 15C). In 10 mM gibberellic acid, the total glucanase activity was 32% of the activity produced in the total absence of gibberellic acid. Other enzymes that have shown a concentration dependency on gibberellic acid included ribonuclease (Bennett & Chrispeels, 1972; Chrispeels & Varner, 1967) and proteases (Jacobsen & Varner, 1967).

Unless otherwise stated, 0.010 mM gibberellic acid was the final concentration in the incubation medium of all further experiments. This concentration elicited the maximum response in amylase and xylanse activity. Although

the synthesis and secretion of glucanase activity was greater at 0.001 mM gibberellic acid, the response of glucanase was generally much less sensitive to gibberellic acid concentration than were the other enzyme systems.

G. Production of volatiles by isolated aleurone layers

The enclosure of a living tissue in a sealed vessel would have led to a changing gaseous environment within that vessel as a result of various metabolic processes. The severity of the changes that occurred during the incubation of isolated aleurone layers in closed systems was examined. Twenty aleurone layers were incubated in 28 ml erlenmeyer flasks containing 4.0 ml of 0.010 mM gibberellic acid, in the succinate-calcium chloride buffer. The opening of each flask was a ground glass joint that accepted a hollow glass stopper containing an injection port. Teflon-faced septa (Microsep F-145, Alltech Associates, Inc.) were used to prevent contamination of the flask by hydrocarbons released by the silicone rubber septa. The flasks were flushed with carbon dioxide-free, hydrocarbon-free air for 2 h through hypodermic needles inserted through the septa, then incubated at 25 C on a gyrotary shaker. As 1 ml samples of air were withdrawn for analysis, 1 ml of carbon dioxide-free, hydrocarbon-free air was injected into the flask to maintain constant pressure. Table VII shows the dramatic changes that developed within the sealed flask.

Jacobsen (1973) found that sealing aleurone tissue in

Table VII. Production of volatiles by barley aleurone layers

Twenty aleurone layers were incubated in sealed 28 ml flasks containing 4.0 ml of 0.010 mM gibberellic acid, 20 mM sodium succinate and 20 mM calcium chloride adjusted to pH 5.5 with hydrochloric acid. The flasks were incubated at 25 C on a gyrotary shaker in darkness. The values are the average of duplicate samples.

Incubation Time (h)	Gas Concentration (nl/ml)		
	Carbon Dioxide	Methane	Ethylene
1	60	0.22	0.00
3	2438	0.30	0.00
24	18094	1.54	0.00
48	65075	2.34	0.01

flasks did not affect the production of amylase. However, carbon dioxide has been an effective inhibitor of ethylene action in many experimental systems (Abeles, 1973), and may suppress a response to ethylene that would have been otherwise observed. Furthermore, the high level of carbon dioxide in the flasks increased the concentration of dissolved carbon dioxide. This would have been reflected by a drop in pH of the incubation medium.

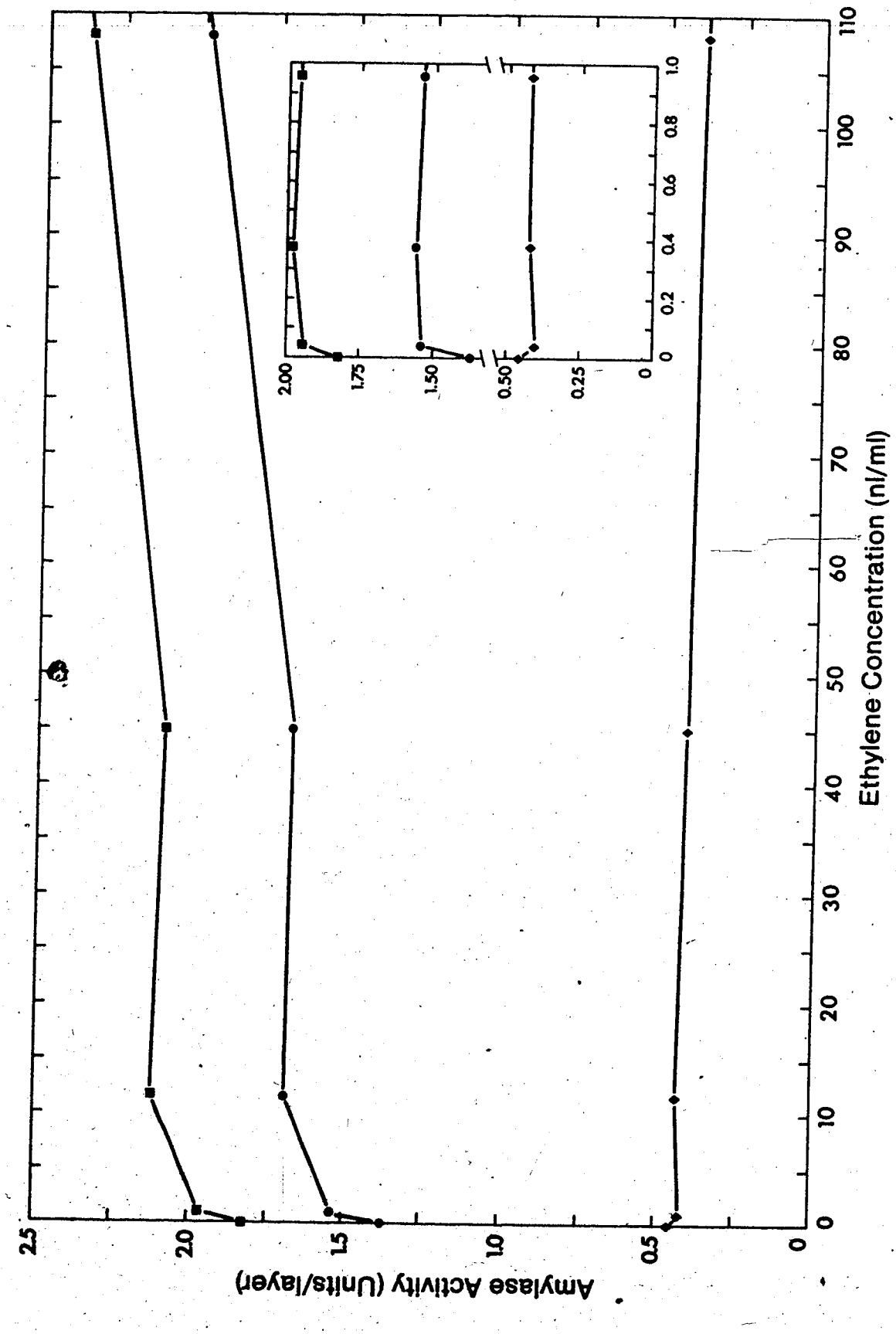
Although the amount of ethylene that accumulated within the flasks was small, 0.01 nl/ml after 48 h, it was similar to the level found by Jacobsen (1973) under similar conditions. In contrast to his results, however, there was no ethane or propylene observed in the enclosed samples.

The accumulation of volatiles within the sealed flasks demonstrated the need for a flow-through system. Although the enclosure of aleurone tissue did not affect normal amylase synthesis (Jacobsen, 1973), responses to various conditions may be modified by the retention of volatiles normally released by aleurone layers. In the open system described (Methods and Materials) for the incubation of isolated aleurone layers, no hydrocarbons could be detected in the effluent air after it had bubbled through the incubation medium containing aleurone layers. The absence of ethylene was confirmed by concentrating any ethylene in the air with a collection trap. After making the collection for 30 min at 25 ml/min, no detectable amount of ethylene had been retained by the collection system.

H. Effect of ethylene concentration on amylase synthesis and release

Initial trials were conducted to reveal the effect of ethylene on amylase synthesis and release, and also to define the threshold and saturating concentrations of ethylene for this effect. Jones (1968) and Jacobsen (1973) observed effects of ethylene on amylase release from aleurone tissue after 18 h and 24 h, respectively. On the basis of these reports, the dose response curve was determined by incubating isolated aleurone layers in the buffered gibberellic acid solution for 24 h in the presence of various ethylene concentrations; the results are presented in Figure 16. Throughout the concentration range examined, the addition of ethylene to the air stream induced an increase in the absolute amount of amylase activity released into the medium. Although this was accompanied by a slight reduction in extractable activity remaining in the aleurone layers, a net increase in total activity was observed. As a result, the final percentage of total amylase activity that is released into the medium increased in response to ethylene. This would indicate that ethylene enhances the release of amylase. Because amylase is continuously synthesized and secreted from the cells during this time (Varner & Mense, 1972), the activity in the extract from the layers represents a dynamic pool of amylase although the level of enzyme activity remained relatively

Figure 16. Effect of ethylene concentration on amylase synthesis and release by aleurone layers. Amylase activity was determined in the medium (●) and in the extract (◆) from the layers incubated in buffered 0.010 mM gibberellic acid for 24 h. Total activity (■) was the sum of the activity found in the extract and in the medium. The insert indicates the changes in amylase activity induced by very low ethylene concentrations. Details of the apparatus and incubation conditions are found in Methods and Materials.


Amylase Activity (Units/layer)
Ethylene Concentration (nl/ml)
0
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40
50
60
70
80
90
100
110
0
0.2
0.4
0.6
0.8
1.0
0
0.25
0.50
1.50
1.75
2.00

stable. An overall acceleration of the synthetic processes alone would lead to an increase in the amylase activity retained by the layers at any given time, rather than the slight reduction observed in response to ethylene. Therefore, all further research was based on the assumption that a change in the percentage of total amylase activity released into the medium does reflect an increase in the ability of barley aleurone layers to release amylase.

The results of this experiment (Figure 16) closely parallel the observations made by Jacobsen (1973). In contrast, others have found that ethylene enhanced the release of amylase, but had no significant effect on total amylase activity (Ho *et al.*, 1977; Jones, 1968). Jacobsen (1973) suggested that these anomalous results may be a reflection of residual abscisic acid in the seed. Furthermore, he found that the response to ethylene in the presence of gibberellic acid was dependent on the year in which the seed was harvested. This latter variable determined the magnitude of the increase in total amylase activity in response to ethylene, and whether a decline in extractable amylase was also induced by ethylene. However, differences in methodology may also make a significant contribution to the disparities among the results obtained by various researchers. The source of air surrounding aleurone layers influences the response of this tissue to plant growth substances (Jacobsen, 1972). This important aspect of the implementation of ethylene studies differed in

each of the cases cited above. Jones (1968) incubated the treated and control samples of isolated aleurone layers in sealed flasks, and introduced ethylene through vaccine caps. Jacobsen (1972; 1973), in an effort to reduce effects of ethylene produced by the tissue, incubated the control samples in flasks stoppered with cotton wool to permit free air exchange with the external environment. For ethylene treatment, however, the tissue was contained in sealed flasks. Ho, Abroms & Varner (personal communication) reduced endogenous ethylene of control samples by enclosing a tube containing mercury perchlorate in the sealed vessel. Aleurone layers were also treated with ethylene in sealed flasks. In no instance was the concentration of oxygen, carbon dioxide or other volatiles determined or controlled. Each of the three studies cited above would expose aleurone layers to a gaseous environment with a different composition. The present study eliminated these concerns by the introduction of the flow-through system of purified air. This produced a stable and well defined source of air for both the control and treated samples of isolated aleurone layers.

Our current research also emphasized the extreme sensitivity of some plant tissues to ethylene. The threshold limit of ethylene concentration required to elicit a response in amylase synthesis and release occurred at a very low level. At the lowest concentration of ethylene tested, 0.041 nl/ml, the amount of amylase activity released

by the layers increased by 13% while a 6% increase in total amylase activity was observed. The magnitude of the ethylene effect continued to increase as the ethylene concentration was elevated through the entire range of concentrations. When isolated aleurone layers were exposed to 108 nl/ml ethylene, the amylase activity released into the medium exceeded the activity in the absence of ethylene by 45%. This was associated with a 21% increase in total amylase activity. There was no evidence to suggest that 108 nl/ml had saturated the response of aleurone layers to ethylene.

I. Release of particulate amylase by Triton X-100

Locy & Kende (1978) established that a large proportion of amylase extracted from aleurone layers was confined to a particulate fraction. This large pool of amylase activity was inaccessible during the normal assay procedures, and may have lead to an inaccurate assessment of the role of ethylene in amylase synthesis and release. Treatment of the homogenate with 0.1% Triton X-100 was an effective means of liberating the latent amylase activity (Locy & Kende, 1978), and was an aid for the determination of amylase retained by aleurone layers.

Preliminary trials were conducted to establish the effect of Triton X-100 directly on amylase activity. This precaution was necessary as non-ionic detergents have been shown to stimulate canine pancreatic amylase (O'Donnell &

McGeeney, 1974). Samples of partially purified amylase (Appendix) containing 61 mUnits of activity were prepared in the presence or absence of 0.002% (v/v) Triton X-100. This concentration of detergent corresponds to levels expected when a sample containing 0.1% (v/v) Triton X-100 was diluted for the enzyme assay. The addition of Triton X-100 produced a 40% increase in the measured activity of purified amylase. Therefore, the distribution of amylase between medium and the aleurone extract could not be accurately determined by adding detergent to the extraction buffer alone. This practice would have artifically inflated the apparent proportion of amylase activity within the aleurone layers. To circumvent this irregularity, Triton X-100 was added to the samples of extract and medium to determine the fraction of amylase activity released from the tissue.

Triton X-100 was used to distinguish between soluble and particulate amylase produced by isolated aleurone layers, and to disclose the effect of ethylene on this relationship. Aleurone layers were incubated for 24 h at 25 C in the incubation medium. Duplicate samples were equilibrated with hydrocarbon-free air, while the other duplicate samples were exposed to 14.0 nl/ml ethylene. The medium and homogenized extracts were diluted to 5 ml before centrifugation. A 2 ml aliquot of each sample was centrifuged directly, while another 2 ml aliquot was incubated for 1 h with 0.1% (v/v) Triton X-100 prior to centrifugation. The addition of Triton X-100 to the medium

produced a 40% increase in amylase activity in samples from both the control and ethylene treatments. This 40% increase in apparent activity corresponded to the increase in activity observed for the partially purified enzyme. The action of detergent on the extract was much more pronounced. There was no significant difference between the change in activity of control and ethylene-treated samples; in both cases, the detergent resulted in a 150% increase in apparent amylase activity. This increase was in part, a consequence of the stimulation of amylase by non-ionic detergents, although an increase in activity of 110% beyond that expected for the direct stimulation of the enzyme suggested a substantial population of latent activity. This particulate fraction of amylase constituted 52% of the total activity within the aleurone layer and falls within the range of 30% to 60% particulate amylase cited by Locy & Kende (1978). However, ethylene had no effect on the distribution of amylase between the soluble and particulate fractions.

The release of amylase activity from the extract by detergents would have reduced the apparent effect of ethylene on total amylase activity. The slight reduction in the amylase activity of extracts in response to ethylene would have been amplified by a factor of 2.1 when detergents were used to liberate bound amylase. This effect was insufficient to account for the increased activity in the medium, and an increase in total activity was still

indicated. Therefore, the exposure of aleurone tissue to ethylene increased the total amylase activity after incubation for 24 h in 0.010 mM gibberellic acid.

J. Effect of ethylene on amylase release, and cell wall degrading enzymes

It is evident from Figure 16 that ethylene enhanced the release of amylase by isolated barley aleurone layers. In the absence of exogenous ethylene, 75% of the total amylase activity was released into the medium. With the addition of ethylene, however, the fraction of amylase released increased as the concentration of ethylene was increased. At 108 nl/ml ethylene, the highest concentration investigated, 84% of the total amylase activity was released. Thus, ethylene promoted the release of amylase, in addition to the stimulation of amylase production.

Since cell wall-degrading enzymes have been implicated in the promotion of amylase release from barley aleurone layers, the effect of ethylene on the synthesis and release of these enzymes was examined over the same range of ethylene concentrations. Glucanase activity, as well as xylanase activity, was determined after 24 h of incubation in buffered gibberellic acid solutions. Even though the total amount of xylanase activity after the first 24 h was very small (0.36 mUnits/layer) relative to the level of enzyme activity ultimately attained, ethylene treatment resulted in an elevation of xylanase activity. There was a

consistent 31% increase in activity in the extract and a 25% increase in total measured activity in response to ethylene. (These increases above the control value were significant at the 2.5% level as indicated by the t-test.) At this point during the gibberellic acid-induced synthesis and release of xylanase, very little enzyme activity (0.04 mUnits/layer) had been released into the medium.

The response of aleurone layers to ethylene reflected by changes in glucanase activity exhibited a different pattern. Ethylene had no significant effect on the total amount of glucanase activity found after 24 h (rejected at the 10% level of significance), but there was a very small promotion by ethylene of glucanase release (significant at the 0.5% level). Control layers retained 27% of the total glucanase activity, whereas the ethylene-treated layers retained only 22% of the activity. Unlike xylanase, a substantial fraction of glucanase activity was found in the medium 24 h after exposure to gibberellic acid.

These observations are consistent with the hypothesis that ethylene stimulates the release of amylase from aleurone layers, and that this enhanced enzyme release was facilitated by the increased production of the cell wall-degrading enzyme, xylanase. It has been shown that the release of amylase through the cell wall is the rate limiting step during enzyme release, rather than secretion through the plasmalemma (Varner & Mense, 1972). In addition, the increased production of enzyme activity

induced by ethylene was a selective event, not a general stimulation of protein synthesis. Although the production of amylase and xylanase was escalated by ethylene, the amount of glucanase activity was unaffected by exposing isolated aleurone layers to ethylene.

The effect of ethylene directly on the activity of preparations of partially purified amylase and xylanase was also investigated. The enzyme solutions were equilibrated with either hydrocarbon-free air or 113 nl/ml ethylene for 2 h at 25 C. In neither case did ethylene affect the activity of the enzyme preparation. The majority of reports in the literature indicate the ethylene is inactive with respect to isolated enzymes (Abeles, 1973), in spite of the observation that the hydrophobic regions of proteins interact with hydrocarbon gases in solution (Wishnia, 1962).

K. Effect of ethylene on the time course of gibberellic acid-enhanced enzyme synthesis and release

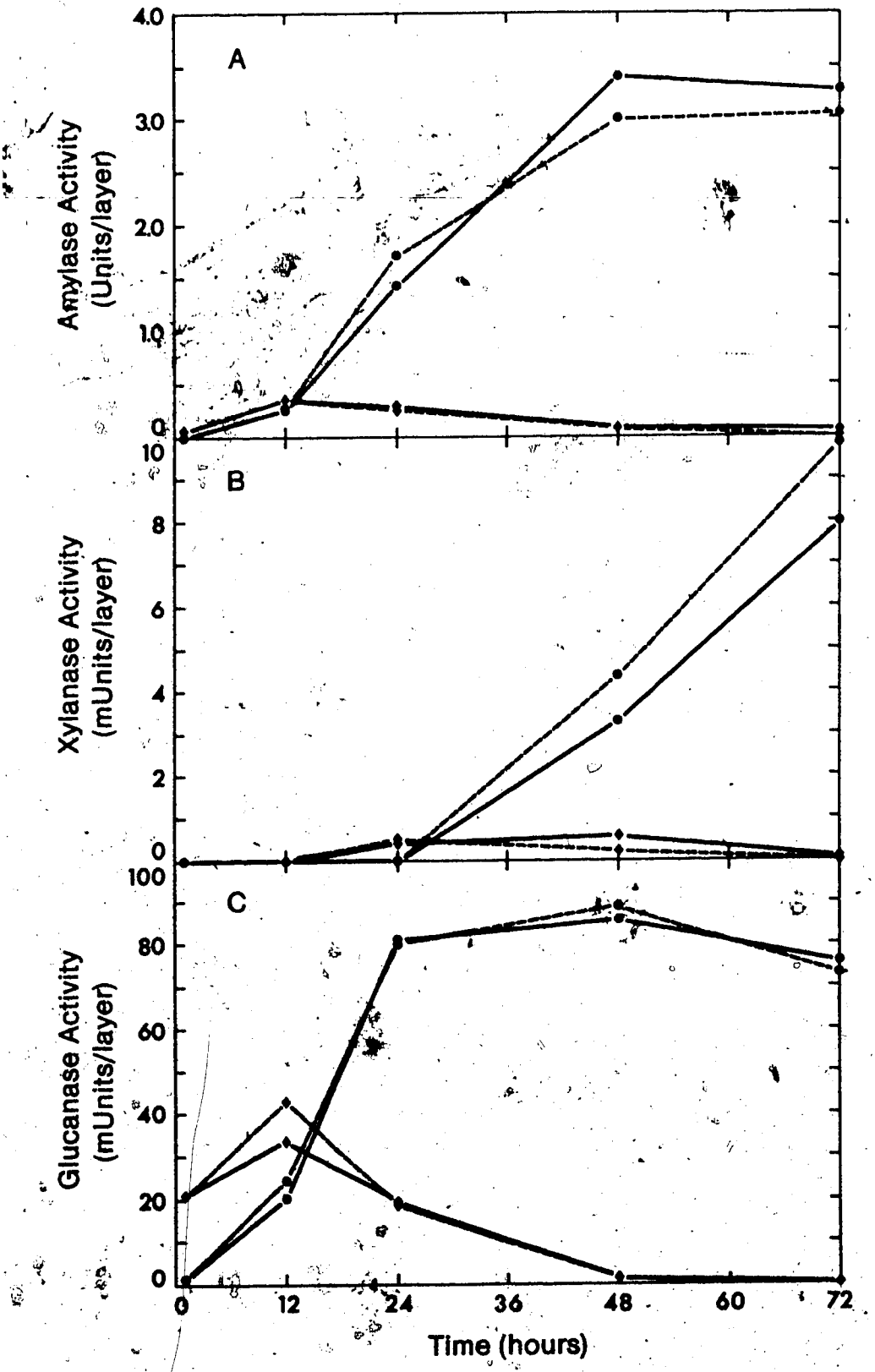
From the measurements made at one time interval, it would have been invalid to draw conclusions on the effect of a plant growth regulator. Clutterbuck & Briggs (1973) have shown, for example, that the effect of kinetin on amylase release from isolated aleurone layers was transient. Initially, kinetin stimulated the release of amylase activity into the medium by up to 67% within the first 24 h. However, subsequent determinations indicated that kinetin ultimately suppressed by 55% the concentration of amylase

found in the medium. Furthermore, Jacobsen (1973), found that gibberellic acid, abscisic and ethylene altered both the rate of amylase synthesis and the duration of the initial lag period. In view of these previous observations, the effect of ethylene on the time course of gibberellic acid-enhanced enzyme synthesis and release was determined.

In each case, 10 aleurone layers were incubated in 2 ml of medium at 25 C for the specified length of time. For ethylene treatment, 30 nl/ml was used. This concentration fell within the plateau region of the dose response curve (Figure 16). In the range of ethylene concentrations from 0.041 nl/ml to 35 nl/ml, changes in ethylene concentration had little additional effect on the release of amylase so any minor fluctuations in ethylene concentration over the period of weeks required to complete these experiments would have had a minimal effect on the results.

The time course for gibberellic acid-enchanced amylase synthesis and release is shown in Figure 17A. As other reports have indicated (Clutterbuck & Briggs, 1973; Jacobsen & Varner, 1967; Varner & Chandra, 1964), the rate of amylase synthesis increased abruptly after an initial lag period, then eventually declined again. Maximum activity was attained after 48 h, followed by a slight decrease in amylase activity when the incubation period was extended to 72 h. The effect of 30 nl/ml ethylene on the time course of amylase synthesis and release is also presented in Figure 17A. Consistent with previous observations (Figure 16),

Figure 17. Effect of 30 nl/ml ethylene on the time course of the synthesis and release of amylase (A), xylanase (B), and glucanase (C). Enzyme activity was determined in the medium (●) and extract (◆) after aleurone layers had been incubated in buffered 0.010 mM gibberellic acid at 25 C for the indicated period of time. Each point represents the average of four samples that were either incubated in hydrocarbon-free air (——) or in air containing 13.0 nl/ml ethylene (— — —).


A
B
C
Amylase Activity (Units/layer)
Xylanase Activity (mUnits/layer)
Glucanase Activity (mUnits/layer)
4.0
3.0
2.0
1.0
0
10
8
6
4
2
0
100
80
60
40
20
0
0
12
24
36
48
60
72
Time (hours)

ethylene enhanced both the production of total amylase activity and the release of amylase into the medium during the first 24 h. However, determinations of amylase activity after 48 h and 72 h indicated that the development of amylase activity was reduced in samples treated with ethylene. Exposure of aleurone layers to 30 nl/ml ethylene for 48 h reduced amylase activity by 12% compared to layers equilibrated with hydrocarbon-free air. The stimulation of amylase production and release by ethylene was therefore a cursory effect.

The progressive increase in activity, and release of xylanase by aleurone layers in response to gibberellic acid was also observed (Figure 17B). Only trace quantities of xylanase had been released into the medium during the first 24 h, although some activity had accumulated within the layers. In this same time period, 49% of the maximum amylase activity had been produced. This contradicted the observations by Taiz & Honigman (1976), who found that xylanase release closely paralleled the release of amylase. Also, they found that the accumulation of xylanase activity ceased after 36 h, and then declined. In the present studies, xylanase activity continued to increase throughout the duration of the experiment, up to 72 h. This disparity may be a consequence of the improvements in incubation techniques introduced in the current investigation. The flow through air supply would prevent the accumulation of carbon dioxide and other volatiles (refer to Table VII) and

the concurrent depletion of oxygen during the incubation period.

The effects of ethylene on the synthesis and release of xylanase were unlike the effects of ethylene described for amylase synthesis and release. As previously noted, ethylene caused an increase in the accumulation of xylanase within the tissue during the first 24 h of the response to gibberellic acid. Samples of media assayed 48 h and 72 h after exposure to gibberellic acid, showed that ethylene promoted a 32% and a 23% increase in activity, respectively. This was concomitant with only a small reduction of xylanase activity in the tissue. Thus, a net increase in total xylanase activity was evident in reponse to 30 nl/ml ethylene, even after prolonged incubation.

The release of glucanase from aleurone layers was also subject to regulation by gibberellic acid. Samples of tissue extract assayed 1 h after the addition of gibberellic acid contained glucanase activity (Figure 17C). This substantiates the observation that formation of glucanase proceeds in the absence of gibberellic acid (Taiz & Jones, 1970). However, activity was quickly released into the medium. Ethylene had no persistent effect on the synthesis or release of glucanase from isolated aleurone layers. The variability in glucanase activity recovered after 12 h obscured the apparent effect of ethylene on the induction of glucanase synthesis and release at this early stage of the response of aleurone layers to gibberellic acid.

Of three hydrolytic enzymes considered, only the synthesis and release of xylanase were affected by ethylene in an unidirectional manner throughout the time course of the experiment. At every time interval, ethylene led to an increase in total xylanase activity. On the other hand, the effect of ethylene on amylase was irregular. In the early stages of the response to gibberellic acid, up to 24 h ethylene stimulated the release of amylase as well as the total amount of activity formed. Further measurements after 48 h indicated a substantial negative effect of ethylene on enzyme activity. Both phases of the regulation of amylase activity by ethylene were investigated further.

L. Ethylene effects on amylase synthesis and release by isolated aleurone layers in the presence of exogenously applied xylanase activity.

As indicated (Figure 17), ethylene promotes the release of hydrolytic enzymes from aleurone layers. The rate of release is limited by the diffusion of the enzyme through the cell wall of the aleurone layer (Varner & Mense, 1972), and thus would be dependent on the activity of cell wall degrading enzymes. Therefore, the application of xylanase activity to the aleurone layers should reduce the restriction of amylase release imposed by the cell wall. Table VIII shows that the addition of xylanase to the medium inhibited the amount of amylase synthesized in 24 h. However, in the presence of exogenous xylanase, ethylene had

Table VIII. Effect of ethylene on amylase synthesis and release in the presence and absence of exogenously applied xylanase

Ten aleurone layers were incubated as described in Methods and Materials. Partially purified xylanase (see Appendix) was added where indicated. Samples were equilibrated with either hydrocarbon-free air or 12.4 nl/ml ethylene. Values are the mean of 4 replicates.

Treatment	Xylanase Added (mU/layer)	Amylase Produced (U/layer)	
		Extract	Medium
Control	0	0.48	1.43[a]
Ethylene	0	0.43	1.69[a]
Control	1.1	0.51	1.03
Ethylene	1.1	0.46	0.91

a difference between control and ethylene-treatment is significant at the 5% level.

no significant effect on amylase synthesis and release.

A series of preliminary experiments were conducted in which the isolated aleurone layers were pre-incubated in a xylanase preparation without gibberellic acid. After 36 h, the layers were washed thoroughly with buffer for 2 h, then tranferred to incubation medium containing gibberellic acid. This treatment reduced the total amylase activity recovered from xylanase treated samples to 0.42 Units/layer, compared to 1.43 Units/layer from samples that were not given the pre-incubation treatment. If the pre-incubation step was performed in buffer alone, the effect on amylase synthesis was minimal, and 1.16 Units/layer were recovered from the sample. This was anticipated as it has been found that the length of the incubation period for half-seeds had no effect on subsequent amylase synthesis or release (Varner, 1964). The addition of an osmoticant (1.0 M or 0.2 M sorbitol) during the pre-incubation period did not reduce the inhibition of amylase synthesis. These concentrations of osmoticant are comparable to those used during the preparation of barley aleurone protoplasts (Taiz & Jones, 1971). Prolonging the dialysis of the xylanase preparation and introducing several buffer changes during dialysis failed to prevent the inhibition of amylase synthesis by the pre-incubation with xylanase.

The extent of cell wall modification that occurred in aleurone layers exposed to gibberellic acid alone or in combination with exogenous xylanase or ethylene, was

examined directly by scanning electron microscopy. In the absence of these additions, the cell wall appeared very smooth (Figure 18 A,B,C). This was consistent with the observations made in previous studies (Pomeranz, 1973). With exposure to gibberellic acid, however, the cell wall became extensively furrowed (Figure 18 D,E,F). In addition, there was an accumulation of material between the plasmalemma and the cell wall. The globular material was likely protein secreted by the aleurone cells. This made direct observation of the cell wall more difficult, but the deposit had been sloughed off in many places along the fractured surface to expose the cell wall (Figure 18 E). The exposure of the tissue to either ethylene or xylanase in combination with gibberellic acid had no visible effect on cell wall structure beyond the changes induced by gibberellic acid alone (Figure 18 G to L). Thus, if the addition of either ethylene or xylanase to gibberellic acid-treated aleurone layers did facilitate the degradation of the cell wall material, it could not be detected by scanning electron microscopy.

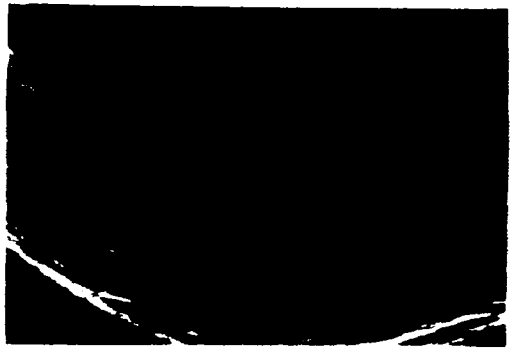
M. The relationship between gibberellic acid concentration and the effect of ethylene on hydrolase synthesis and release by aleurone layers.

A series of experiments were conducted to determine the effect of ethylene on the synthesis and release of hydrolytic enzymes by aleurone layers exposed to a range of

Figure 18. Scanning electron micrographs of isolated barley aleurone layers. Isolated aleurone layers were incubated in incubation buffer for 24 h at 25 C, and the medium was equilibrated with hydrocarbon-free air except as noted. A,B,C) no gibberellic acid; D,E,F) 0.010 mM gibberellic acid. On the following page, G,H,I) 0.010 mM gibberellic acid plus 1.1 mUnits/layer xylanase; J,K,L) 0.010 mM gibberellic acid plus 12.4 nl/ml ethylene. The approximate magnification of the samples was: A,D,G,J) 580 x; B,E,H,K) 2000 x and C,F,I,L) 5900 x.

A

B

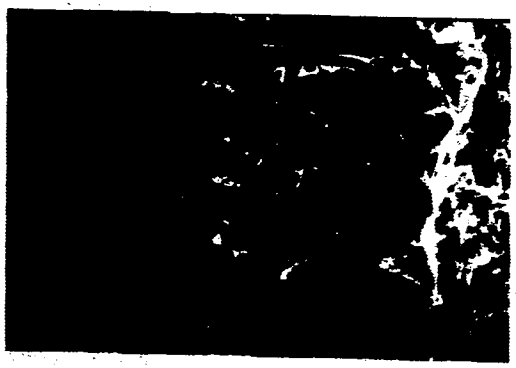


C

D

E

F



G

H

I

J

K

L

gibberellic acid concentrations. The results are compiled in Table IX. The enhancement of amylase and xylanase synthesis in response to ethylene was optimal at 0.010 mM gibberellic acid. The stimulation by ethylene of amylase release was also maximum at this concentration of gibberellic acid. Ethylene did not alter the rate of appearance of total glucanase in a statistically significant way, regardless of the gibberellic acid concentration.

Experimental work has already shown that ethylene was capable of modifying both the gibberellic acid-induced synthesis, and release of amylase from barley aleurone layers (Figure 16). These processes were also sensitive to the concentration of gibberellic acid supplied in the incubation medium (Figure 14). These circumstances suggested that ethylene may be exerting its control by regulating the uptake of gibberellic acid by the aleurone cells. However, since the ethylene effect was reduced at a sub-optimal concentration of gibberellic acid, it cannot be presumed that ethylene increased the availability of gibberellic acid to its primary site of action within the aleurone tissue. The increased variability in the response of aleurone layers to gibberellic acid at other than optimal concentrations makes interpretation of the data more difficult and speculative.

In the complete absence of gibberellic acid, ethylene had no significant effect on the rate of amylase synthesis. This contradicts the previous studies that found that in the

Table IX. Influence of gibberellic acid concentration on the effect of ethylene on barley aleurone layers

Succinic acid - calcium chloride buffer was prepared containing different concentrations of gibberellic acid. Isolated aleurone layers were incubated in the presence of either hydrocarbon-free air or 12.4 nl/ml ethylene for 24 h at 25 C. Each value is the mean of 6 samples.

Gibberellic Acid Concentration (M)	Ethylene Concentration (nl/ml)	Enzyme Activity								
		Amylase (U/layer)			Xylanase (mU/layer)			Glucanase (mU/layer)		
		Extract	Medium	Total	Extract	Medium	Total	Extract	Medium	Total
10^{-6}	0.0	0.32	0.95[a]	1.27[a]	0.49	0.04	0.53	36[a]	72	108
10^{-6}	12.4	0.44	0.80[a]	1.24[a]	0.42	0.05	0.47	41[a]	60	101
10^{-5}	0.0	0.48	1.43[b]	1.91[b]	0.33[b]	0.06	0.39[a]	28	77	105
10^{-5}	12.4	0.43	1.69[b]	2.12[b]	0.39[b]	0.04	0.43[a]	22	76	97
10^{-3}	0.0	0.30	1.33	1.63	0.13	0.03	0.16	22	65	87
10^{-3}	12.4	0.25	1.40	1.65	0.23	0.02	0.25	15	70	85

a difference between samples incubated in hydrocarbon-free air and 12.4 nl/ml ethylene is significant at the 2.5% level, or

b at the 5.0% level.

absence of gibberellic acid, ethylene would inhibit (Jones, 1968), or stimulate (Jacobsen, 1973) amylase synthesis.

N. Control of inorganic ion flux from isolated aleurone layers

The quantity of selected inorganic ions released into the medium by aleurone layers in response to gibberellic acid, alone, or in combination with ethylene, was determined. The movements of all five ionic species were dependent on the presence of gibberellic acid in the medium (Table X). With the exception of the influx of sodium into the tissue, these dependencies support the previous observations of Jones (1973). In contrast to the general dependency on gibberellic acid, of the four cations and one anion considered, the change in the distribution of calcium induced by ethylene was much more pronounced relative to the minor changes imparted on the release of the remaining ions. The addition of 13.1 nl/ml ethylene to the air in contact with the tissues resulted in a 101% increase in the amount of calcium released into the medium. The concentrations of calcium and phosphate encountered in these samples were insufficient to cause the precipitation of calcium phosphate under these experimental conditions.

The influences of plant growth regulators on calcium, magnesium and potassium release are of particular importance. Calcium has been assigned a central role in the accumulation of amylase activity in barley malt. The

Table X. Effects of gibberellic acid and ethylene on the release and adsorption of inorganic ions by isolated barley aleurone layers

Ten aleurone layers were incubated in 2 ml of 20 mM sodium succinate adjusted to pH 5.5 at 25 C with hydrochloric acid. The layers were exposed to 13.1 nl/ml ethylene and/or 0.010 mM gibberellic acid (GA) for 24 h at 25 C. The amounts of the individual ions released were determined by atomic absorption analysis with the exception of phosphate, which was determined spectrophotometrically. Each value is the mean of 4 samples.

Treatment	Change in the Ion Concentration of the Medium (micromoles/layer)				
	Sodium[a]	Potassium	Calcium	Magnesium	Phosphate
Control	-0.26	0.53	0.020	0.028	0.11
+ GA	-1.94	1.16	0.081	0.624	1.14
+ GA + C_2H_4	-2.10	1.26	0.163	0.632	0.98

[a] A negative value indicates adsorption from the medium. The incubation medium initially contained 40 mM sodium, or 8.00 micromoles/layer.

presence of calcium confers stability to barley alpha-amylase (Greenwood & MacGregor, 1965). Furthermore, it has more recently been recognized that in addition to protecting the alpha-amylase, calcium also enables the synthesis and release of hydrolytic enzymes (Carr & Goodwin, 1972; Varner & Mense, 1972). Magnesium and potassium are also active in the latter capacity (Varner & Mense, 1972). Therefore, the ability of ethylene to promote the redistribution of any of these cations, as it markedly does for calcium, may be important in regulating the process of enzyme synthesis and release.

Although the consequence of the release of calcium induced by ethylene may be prominent during the development of alpha-amylase activity within intact barley, it may be of less significance in the current studies on isolated aleurone layers. In the presence of both gibberellic acid and ethylene, only 0.163 micromoles calcium per layer were released, whereas, under standard experimental conditions, 4.0 micromoles calcium per layer were present in the incubation buffer. This relatively high concentration is expected to overshadow any effect that the release of calcium may have on the synthesis and release of alpha-amylase.

The primary source of calcium and phosphate is from the hydrolysis of phytin by phytase (Srivastava, 1964). It is conceivable that ethylene is promoting the release of soluble calcium by stimulating the development of phytase

activity in the aleurone layers. In this regard; it is surprising that ethylene has opposing influences on the liberation of these ions, although ethylene may also stimulate the metabolism of phosphate.

In addition to the release of various ionic species, it was also noted that there was a gibberellic acid-dependent uptake of sodium from the medium (Table X). This may be a counterion effect, to maintain electrical neutrality within the tissue.

O. Roles of proteolytic enzymes in the responses of aleurone layers to ethylene

During the extended exposure of aleurone layers to gibberellic acid, two related processes occur that tend to dissipate amylase activity: increasing amounts of protease are produced (Jacobsen & Varner, 1967), and there is a decline in pH of the surrounding medium (Clutterbuck & Briggs, 1973). The latter event would destabilize alpha-amylase (Greenwood & MacGregor, 1965) and stimulate barley haemoglobinase (Bhatty, 1968; Enari & Mikola, 1968). Since ethylene has been shown to reduce amylase activity in the medium surrounding aleurone layers after long periods (Figure 17A), the effect of ethylene on pH of the medium and haemoglobinase activity was explored.

Preliminary experiments were conducted to measure the change in pH of the medium during the response of the layers to gibberellic acid; the results are summarized in Table XI.

Table XI. Effect of ethylene on the pH of incubation medium surrounding isolated aleurone layers

Ten aleurone layers were incubated for 24 h or 48 h at 25 C in 2 ml of buffered medium. The medium contained 0.010 mM gibberellic acid, 20 mM succinic acid, and 20 mM calcium chloride, adjusted to pH 5.50 at 25 C with sodium hydroxide. Each value is the mean of duplicate samples. Either control air (hydrocarbon-free; carbon dioxide-free) or ethylene (13.0 nl/ml ethylene in control air) was bubbled through the samples for the duration of the incubation period.

Ethylene Concentration (nl/ml)	Incubation Time (h)	pH of Medium
0.0	24	5.29
13.0	24	5.31
0.0	48	4.59
13.0	48	4.56

The pH of the incubation medium dropped appreciably from its initial value of 5.50. After 48 h, the pH of the medium was 4.59. (This is distinctly different from the results of Briggs (1968a) and Clutterbuck & Briggs (1973) who reported that 20 mM succinate buffer had a sufficient buffer capacity to prevent the pH of the medium from dropping when barley half-seeds and isolated aleurone layers, respectively, were exposed to gibberellic acid.) Barley amylase is subject to partial inactivation at pH 4.59, even in the presence of calcium chloride (Greenwood & MacGregor, 1965). In addition, the sulphydryl proteases of barley show maximum activity at pH 3.9 (Bhatty, 1968; Enari & Mikola, 1968), so proteolytic activity would be stimulated by this lower pH. However, there was no significant difference between the pH of control and ethylene-treated samples. Ethylene did not limit the expression of amylase activity in samples by accelerating the decline in the pH of the surrounding medium.

The direct relationship of protease activity to the ethylene response was then investigated. Because the sulphydryl proteases predominate in barley malt (Enari & Mikola, 1968), only this class of proteases was considered in these trials. This included the haemoglobinase of barley characterized by Bhatty (1968). At the low pH of the haemoglobinase assay medium, the less prevalent proteases were essentially inactive (Enari & Mikola, 1968). The results are summarized in Table XII. Although these data

Table XII. Effect of ethylene on the gibberellic acid-enhanced formation of haemoglobinase activity by isolated aleurone layers

Ten barley aleurone layers were incubated in a buffered solution of 0.010 mM gibberellic acid for 48 h at 25 C. Either hydrocarbon-free air or 13.0 nl/ml ethylene was bubbled through the incubation medium. Each value is the mean of 6 replicates, and the standard error of the means are given.

Sample	Haemoglobinase Activity (mU/layer)	
	Control	Ethylene-treated
Extract	0.11 ± 0.02	0.09 ± 0.02
Medium	0.35 ± 0.06	0.46 ± 0.04
Total	0.46 ± 0.05	0.55 ± 0.03

would suggest that ethylene stimulated the synthesis and release by barley aleurone layers of haemoglobinase, statistical evaluation of the data indicated considerable variability within each treatment that precluded drawing a decisive conclusion about the effect of ethylene on haemoglobinase biosynthesis.

An alternative approach was used to study the role of proteolytic enzymes in the reduction of amylase activity induced by ethylene. The aleurone layers were incubated in media containing inhibitors of proteolytic activity. In such instances, a source of free amino acids was required to permit the normal amount of amylase synthesis (Ho & Varner, 1978; Melcher & Varner, 1971). Potassium bromate has been used extensively in commercial applications to prevent the solubilization of protein nitrogen during the malting process (Macey & Stowell, 1961). We extended the studies on barley proteases by including another inhibitor of sulphydryl enzymes, N-ethylmaleimide. Preliminary trials were also conducted using 10 mM *p*-chloromercuriphenyl sulphonic acid (Sigma Chemical Co., St. Louis, Mo.). Although this sulphydryl inactivator essentially eliminated amylase synthesis (0.5% of the amylase activity found in the control samples), further experimentation was abandoned as calcium chloride caused the precipitation of the inhibitor from the incubation medium.

The effects of ethylene and protease inhibitors on the synthesis of hydrolytic enzymes are presented in Table XIII.

Table XIII. Effects of ethylene on the synthesis of hydrolytic enzymes by aleurone layers treated with sulphydryl reagents

Ten aleurone layers were incubated in 2 ml of 0.010 mM gibberellic acid, 20 mM succinic acid, 20 mM calcium chloride adjusted to pH 5.5 at 25 C with sodium hydroxide. In addition, either 5 mM potassium bromate or 0.1 mM N-ethylmaleimide (NEM) was present in the medium of some of the samples. Addition of the amino acid supplement yielded a final concentration of 5 mg/ml casamino acids plus 0.25 mg/ml L-tryptophan. Where applicable, the concentration of ethylene was 13.0 nl/ml. The samples were incubated for 48 h at 25 C under the prescribed conditions. Each value is the mean of 4 samples. Total activities in the control (100%) for amylase, xylanase and glucanase were 2.56 U/layer, 8.27 mU/layer and 91.5 mU/layer, respectively.

Inhibitor	Amino Acids	Ethylene	Enzyme Activity (Percent of Control Total Activity)								
			Amylase			Xylanase			Glucanase		
			Extract	Medium	Total	Extract	Medium	Total	Extract	Medium	Total
None	-	-	2	98	100	4	96	100	3	97	100
None	-	+	2	82	84	4	103	107	2	95	98
Bromate	-	-	5	45	51	7	3	10	10	88	98
Bromate	+	-	8	80	89	8	1	9	12	86	99
Bromate	+	+	10	78	88	7	1	8	18	84	102
NEM	-	-	5	26	31	6	24	30	1	77	78
NEM	+	-	15	37	52	14	4	18	28	74	102
NEM	+	+	20	59	79	14	9	23	31	75	106

In spite of the lower concentration of casamino acids and tryptophan than previously used (Ho & Varner, 1978), the addition of the amino acid supplement effectively negated the effect of potassium bromate on amylase synthesis. The inhibitory action of N-ethylmaleimide on amylase synthesis was not as effectively reversed by the addition of amino acids. When sulphydryl reagents were absent from the medium, ethylene reduced the total amount of amylase activity compared to control samples. However, in the presence of protease inhibitors plus amino acids, the addition of ethylene either had no effect on amylase activity or completely reversed its former role and resulted in increased activity.

The addition of potassium bromate or N-ethylmaleimide severely reduced the activity of xylanase found. On the other hand, glucanase activity was reduced by N-ethylmaleimide only. The inhibition of glucanase production was totally relieved by the addition of amino acids. Both sulphydryl reagents lessened the degree of glucanase release from the aleurone layers.

It was necessary to establish to what extent these additions altered the formation of proteolytic activity. The reduced activity of proteases recovered from samples treated with sulphydryl reagents is evident from Table XIV. The addition of 0.1 mM N-ethylmaleimide and 5 mM potassium bromate reduced total proteolytic activity by 49% and 76% respectively.

Table XIV. Effect of sulphydryl reagents on the haemoglobinase activity recovered from samples

The incubation conditions were as described in Table XIII.

Treatment	Haemoglobinase Activity (mU/layer)		
	Extract	Medium	Total
Control	0.106	0.346	0.455
Bromate + Amino Acids	0.054	0.053	0.107
NEM + Amino Acids	0.064	0.166	0.230

The direct interaction of these reagents with other enzymes was also considered. N-ethylmaleimide and potassium bromate had no inhibitory effect on amylase or glucanase (Table XV), wherease protease and xylanase activities were drastically inhibited by these compounds. The addition of amino acids to the medium offered no protection to protease or xylanase from the sulphydryl reagents. In view of these observations, it was impossible to conclude whether protease inhibitors had limited xylanase synthesis or had inhibited the enzyme after it had been produced. Any influence of protease inhibitors on amylase or glucanase activity would have been on their synthesis or degradation as the inhibitors of proteolytic enzymes did not alter the activity of either of these two enzymes directly.

It was interesting to note that potassium bromate did inhibit xylanase activity. In addition to restricting the solubilization of protein, the addition of bromate during the malting process has been shown to limit the loss of dry weight and extractable components from the malt (Macey & Stowell, 1957). It is conceivable that these losses were reduced by the action of bromate on xylanase. With the loss of cell wall degrading activity, much more material would be retained by the aleurone tissue of malted barley.

The difference between the activity of potassium bromate and N-ethylmaleimide in the gibberellic acid-enhanced synthesis of hydrolytic enzymes may have been the consequence of characteristics of inhibitor action

Table XV. Effects of N-ethylmaleimide and potassium bromate on enzyme activity

Potassium bromate or N-ethylmaleimide (NEM) were added to samples of isolated enzyme to yield final concentrations of 5 mM and 0.1 mM, respectively. The samples were incubated for 2 h at 22 C after the addition of the inhibitors before the assays were started.

Inhibitor	Enzyme Activity (Percent of Control)			
	Haemoglobinase	Amylase	Xylanase	Glucanase
None	100	100	100	100
Bromate	54	103	33	103
NEM	91	99	78	101

unique to each compound. While they both interact with sulphydryl groups, bromate functions as an oxidant, and N-ethylmaleimide reacts to form the N-ethylsuccinamate derivative of the protein (Webb, 1966). Furthermore, N-ethylmaleimide reacts with only the most active or exposed sulphydryl groups. It has also been demonstrated that N-ethylmaleimide will react with other groups, including the alpha-amino group of the terminal amino acid (Smyth, Blumenfeld & Konigsberg, 1964). The uncharged nature of N-ethylmaleimide permits easy entry into many cells.

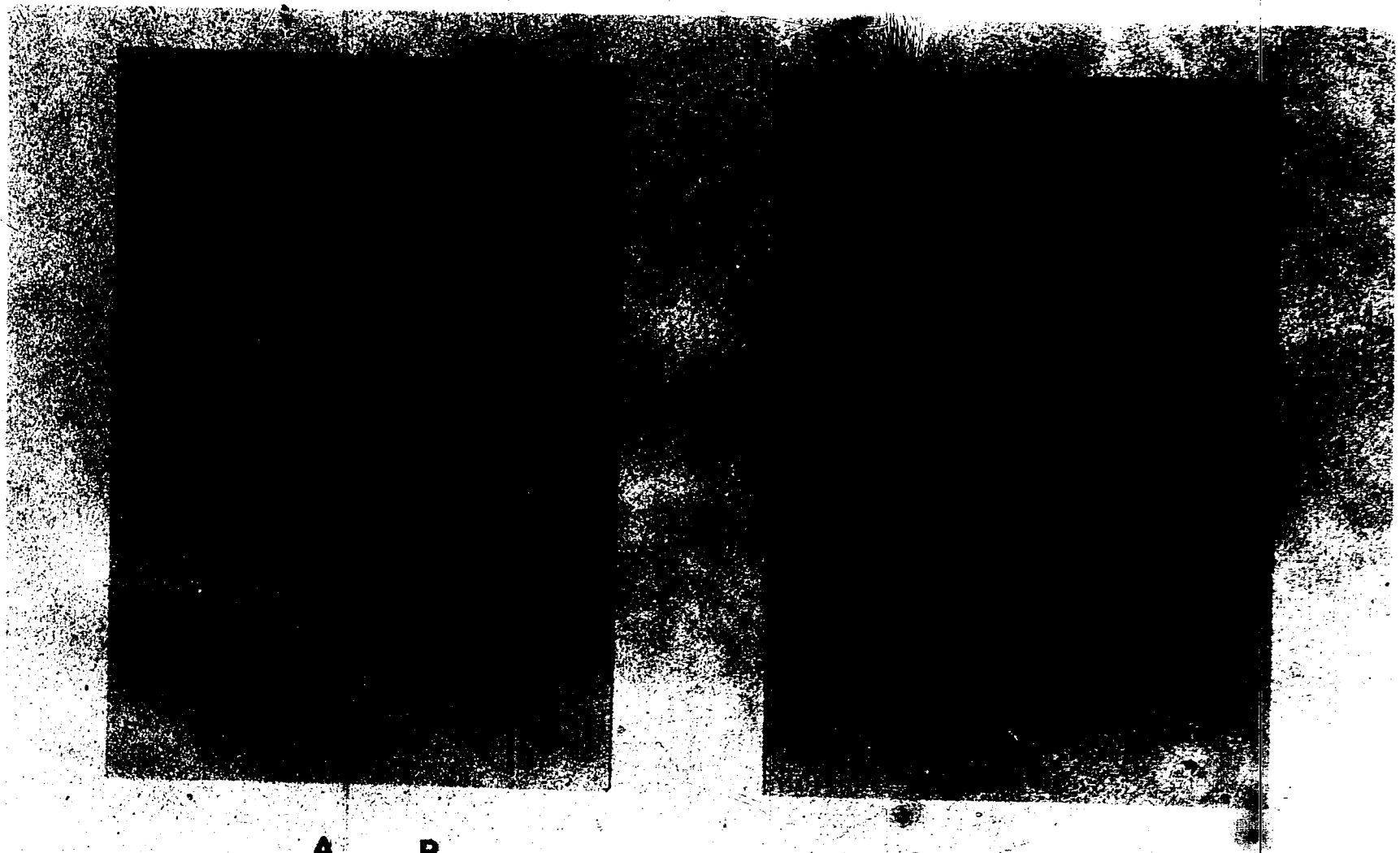
In summary, without the addition of inhibitors of proteolytic activity, ethylene repressed the levels of amylase attained in 48 h of incubation with gibberellic acid. With the reduction of proteolytic activity by sulphydryl reagents, ethylene failed to reduce the gibberellic acid-enhanced synthesis of hydrolytic enzymes up to 48 h. These results have strengthened the hypothesis that ethylene limits the development of amylase activity by increasing the proteolytic activity in samples after long incubation periods. This conclusion must be tempered with the knowledge that potassium bromate and N-ethylmaleimide are non-specific inhibitors. It has been shown that potassium bromate reduced respiration during the malting process of barley (Macey & Stowell, 1957) and that N-ethylmaleimide also reduced respiration, but in sunflower stem segments (Niedergang-Kamien & Leopold, 1957).

P. Effect of ethylene on the multiple forms of amylase produced by aleurone layers in response to gibberellic acid

The isoelectric patterns of the proteins released by aleurone layers treated with gibberellic acid, were examined by polyacrylamide gel isoelectric focusing. Preliminary experiments with gels that contained a gradient from pH 5.0 to pH 8.5 indicated that all of the amylase activity recovered from the medium had focused at values lower than pH 7.0. On this basis, all further experiments were performed using lower pH range gels. The buffers of amino acids assisted in the formation of a uniform gradient from pH 4.0 to pH 7.5, spanning the length of the gel. The reference protein, chicken egg white conalbumin, produced two coloured bands visible during the isoelectric focusing, a major band at pH 6.06 and a minor band at pH 5.91.

By staining the gels for amylase activity, two major regions of amylase were detected (Figure 19 A,B). This separation into two major fractions was very similar to the results obtained with alpha-amylase isolated from half-seeds of *Hordeum vulgare* cv Musashino mugi treated with gibberellic acid (Tanaka & Akazawa, 1970). In the work of Tanaka & Akazawa, and in the present study, minor bands of activity were associated with the major fractions. When duplicate gels were stained for protein (Figure 19 C,D,E), several bands of protein were found to correspond with each of the major regions of amylase activity. More than one form of amylase may have been associated with each major

Figure 19. Polyacrylamide gel isoelectric focusing of proteins released from aleurone layers treated with gibberellic acid. Of the total sample volume of 0.500 ml, 0.025 ml were applied to the gels used for the localization of amylase activity (A,B), and 0.200 ml were applied to gels for protein staining (C,D,E). Bands of amylase activity appeared as colourless regions against a dark background, while the proteins appeared as dark bands against a clear background. The aleurone layers were incubated in either hydrocarbon-free air (A,C) or 11.9 nl/ml ethylene (B,D). Sample E is a 1:1 mixture of samples C and D. The gradients extended from pH 4.0 (bottom of photo) to pH 7.5.


A
B
C
D
E

fraction.

There was no difference in either the isoelectric patterns of amylase activity or the protein staining, from samples of aleurone layers that were incubated with hydrocarbon-free air as compared with 11.9 nl/ml ethylene. These results indicated that ethylene had no effect on the qualitative aspect of amylase synthesis by barley aleurone layers.

V. GENERAL DISCUSSION

Extensive developmental work was required to permit accurate quantification of ethylene effects, both in terms of the administration of ethylene, and in the detection of responses to ethylene. A system was devised that offered excellent flexibility in the concentrations of ethylene that could be accomodated, and also provided well defined parameters for control tissues, and for the treatment of samples with ethylene.

Present research has shown that ethylene enhanced the production of amylase activity by aleurone layers isolated from *Hordeum vulgare* cv Himalaya that had been treated with gibberellic acid for 24 h. It was shown that this effect of ethylene was exerted on the synthesis of amylase and not directly on enzyme activity, and not on the release of amylase activity from membrane-bound amylase. In addition to amylase synthesis, ethylene also promoted the release of amylase from the isolated aleurone layers. These ethylene effects were detected at the lowest concentration used, 0.041 nl/ml, indicating a very low threshold level.

Reports have shown that ethylene could modify the movement of plant growth regulators (for example, Beyer & Morgan, 1969; Rudich, Sell & Baker, 1976). The latter investigators found that ethylene, applied as 2-chloroethylphosphonic acid, stimulated the passage of ^{3}H-gibberellin A1 through cucumber stem segments. In the aleurone system, however, ethylene does not function through

enhanced movement of gibberellic acid to its active site. At sub-optimal concentrations of gibberellic acid, where the concentration of gibberellic acid limits amylase synthesis, the effect of ethylene was no greater than at higher concentrations of gibberellic acid.

Several distinct processes are required for the eventual release of amylase from aleurone layers. These include enzyme synthesis, secretion across the plasmalemma and release from the cell wall (Varner & Mense, 1972). Ethylene may have been acting on one or more of these systems.

Although ethylene has given rise to qualitative differences in the activity of chromatin (reflected by alterations in the RNA synthesized in soybean hypocotyl segments that were exposed to ethylene (Holm *et al.*, 1970)) this did not appear to be the case in gibberellic acid-enhanced amylase synthesis. The isoelectric patterns of proteins released by control and ethylene-treated aleurone layers were identical. Therefore, the effect of ethylene was likely on the overall synthetic and secretory systems, rather than direct activation of genes specific for one or more forms of amylase.

From early work on senescence and fruit ripening, it was generally assumed that ethylene may cause increased membrane permeability. This would account for the enhanced release of amylase from gibberellic acid-treated aleurone layers exposed to ethylene. However, the relevance of

direct effects of ethylene on membrane permeability to the biological effects of ethylene is uncertain. Although ethylene did increase the permeability of model membranes, other hydrocarbon gases also increased permeability. The biological activity of the gases could not be correlated to their effects on model membranes (Mehard *et al.*, 1970). It is worth noting, in this respect, that ethylene did not affect the amount of membrane-bound amylase in barley aleurone layers.

Ethylene could be affecting the release of amylase from the cell wall, rather than secretion through the plasmalemma. The diffusion through the cell wall is the rate limiting step in amylase release (Varner & Mense, 1972). In suspension cultures of *Acer pseudoplatanus*, a large amount of activity of a number of hydrolytic enzymes could be washed from the cell walls by solutions with high salt concentration (Keegstra & Albersheim, 1970). Salts are also important in the release of amylase from aleurone layers (Varner & Mense, 1972; Carr & Goodwin, 1972). The present work has shown that ethylene promoted the release of calcium from aleurone layers. Although amylase release, as well as stability, is dependent on calcium, the stimulation of calcium release by ethylene may not be critical to these processes under the experimental conditions of the current study. A much greater amount of calcium was supplied in the incubation medium than was released by the aleurone layers. However, if the enhanced release of calcium in response to

ethylene reflected an increased availability of soluble calcium within the cells, other biochemical or physiological properties may have been altered. These included membrane structure and integrity, flux of other ionic species and the activities of other enzymes (Rains, 1976). The redistribution of ions could be examined within the aleurone layers by X-ray analysis in conjunction with scanning electron microscopy.

Cell wall degrading enzymes also play a role in enzyme release from aleurone layers. As suggested above, the cell wall imposes a barrier to enzyme release. The importance of xylanase activity was implicated by the finding that inhibitors of xylanase activity reduced the release of both amylase and glucanase. Also, ethylene promoted the appearance of xylanase activity and the concomitant release of amylase. Determining the xylanase activity in extracts from aleurone layers did not offer any insight into the localization of the enzyme. Hydrolytic enzymes are trapped in the cell wall, and in the lytic compartments of aleurone cells (review in Matile, 1975). Isolated barley aleurone protoplasts may be useful in resolving these questions. A method for the preparation of aleurone protoplasts has appeared in the literature (Taiz & Jones, 1971). However, the concentration of osmoticants currently used to maintain the integrity of the individual aleurone cells inhibits the response of cereal aleurone cells to gibberellic acid (Eastwood, 1977; Jones, Armstrong & Taiz, 1973).

In addition to the stimulatory effect of ethylene on amylase synthesis at 24 h, ethylene also reduced amylase activity at subsequent time intervals. Results from the use of haemoglobinase inhibitors suggested that the enhancement of proteolytic activity by ethylene contributed to the eventual loss of amylase activity. However, the enhancement by ethylene of amylase production prior to 48 h was not likely the result of increased availability of amino acids through increased haemoglobinase activity. Melcher & Varner (1971) have shown that the availability of free amino acids did not limit amylase synthesis. The addition of free amino acids to the medium did not increase the production of amylase activity, and, in fact, the amino acids impeded the release of amylase from aleurone layers (Melcher & Varner, 1971).

Through the methodology developed and used in the current study, the effects of ethylene on the synthesis and release of amylase from gibberellic acid-treated barley aleurone layers have been quantified. These effects were then related to other events that occur during the induction and continuation of amylase synthesis. The release of amylase was correlated with xylanase activity. In addition, the expression of haemoglobinase activity limited the ultimate level of amylase activity attained. Both of these enzyme systems appeared to be regulated by ethylene to a modest extent.

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APPENDIX

A. Partial purification of amylase

A source of amylase was required for the evaluation and characterization of enzyme assay procedures that were to be used throughout the current research project. The ideal source of enzyme would be the same as the amylase to be investigated - the amylase secreted from barley aleurone layers.

The initial enzyme solution was prepared by incubating 10 aleurone layers in each of 4 flasks containing 2 ml of incubation medium (Methods and Materials). After incubation for 48 h at 25 C on a gyrotary shaker, the medium was decanted into conical centrifuge tubes and centrifuged at 980 x g for 20 min at 4 C. The supernatant layers were combined to yield the crude preparation.

The remainder of the partial purification of amylase was based on the isolation procedure presented by Loyter & Schramm (1962). The method was dependent on the formation of an insoluble glycogen-amylase complex in 40% ethanol. This permitted separation by centrifugation of amylase from other components.

The glycogen for complex formation was oyster glycogen (type II, Sigma Chemical Co., St. Louis, Mo.) that had been purified. The commercial product contained 2.0 mg protein per g of glycogen. Purification was achieved by passing a 0.5% (w/v) solution of glycogen through two cellulose ion

exchange columns (3 cm x 5.5 cm ID, Cellex D, Bio-Rad Laboratories, Richmond, Calif.) in succession. The filtrate was lyophilized, then stored dessicated at 2 C. The protein content of the glycogen was reduced to 0.024 mg/g by this procedure.

Initial attempts to isolate amylase as a glycogen complex failed. Further investigation revealed that 72% of total amylase activity was precipitating from solution with the addition of ethanol, prior to the addition of glycogen. The formation of a bulky white precipitate suggested that polysaccharides in the original enzyme preparation may have removed amylase from solution by forming a complex analagous to the complex formed with glycogen. A preliminary purification step was required to reduce this loss of activity. A procedure similar to that described by Tanaka & AKazawa (1970) was used in the present study, but alternate methods have also been used (Kruger & TKachuk, 1969; MacGregor, Thompson & Meredith, 1974). Four volumes of cold 3.9 M ammonium sulphate were added to the crude preparation and the resulting solution was stirred, in an ice-water bath, for 30 min before the precipitate was collected by centrifugation at 15300 x g (14000 rpm, Beckman model JA-20 rotor) for 30 min at 2 C. The pellet was drained and resuspended in 2 ml of 20 mM potassium acetate, 20 mM calcium chloride adjusted to pH 5.5 at 25 C with hydrochloric acid. The suspension was dialyzed overnight against 500 ml of the same buffer. The addition of ethanol

to the enzyme solution after ammonium sulphate precipitation removed only 0.5% of the amylase activity.

The ammonium sulphate precipitate, after dialysis, was diluted to 10 ml, and placed in an ice-salt-water slurry. Ethanol (95%) at -20 C was added dropwise to the enzyme solution until a final concentration of 40% was attained. The solution was stirred during the addition of ethanol, and then for an additional 5 min. The sample was centrifuged at 11400 x g (12000 rpm) at -4 C for 20 min. The supernatant layer was decanted into another centrifuge tube and the tube was immediately placed in the ice-salt-water slurry. In succession, 0.3 ml of a 2% (w/v) solution of glycogen and 0.2 ml 95% ethanol were added dropwise while the solution was stirred. After 10 min, the suspension was centrifuged at 6400 x g (9000 rpm) for 20 min at -4 C. The pellet was dissolved in 1.2 ml potassium acetate-calcium chloride buffer (pH 5.5) and set in a water bath at 30 C for 1 h to digest the glycogen.

To remove the digested glycogen without further dialysis and dilution of the sample, the small column gel filtration procedure detailed by Penefsky (1977) was used with only slight modification. The gel filtration material was Bio-Gel P-6 (100 to 200 mesh, Bio-Rad Laboratories, Richmond, Calif.) that had been hydrated in the potassium acetate-calcium chloride buffer. Preliminary experiments with various mixtures of chromium (II) nitrate, bovine serum albumin and barley amylase indicated that the optimum sample

volume for the 1 ml columns was 0.3 ml. This volume allowed recovery of 80% of the protein applied to the column, while no salt could be detected spectrophotometrically in the effluent from the column. When the glycogen digest eluted through the column, the microequivalents of reducing sugars were reduced from 0.26 to 0.00. The results of the purification procedure are given in Table XVI.

B. Preparation of xylanase

Experiments were performed in which the isolated barley aleurone layers were incubated for 24 h in incubation medium containing exogenous xylanase. This experimental design required the preparation of a xylanase sample with a very low background level of amylase activity. Xylanases from various sources have been purified through a number of procedures summarized in Dekker & Richards (1976). Based on these, the following method was developed.

To obtain a crude preparation of xylanase, 10 aleurone layers were incubated in each of 8 flasks containing 2 ml of incubation medium. The layers were incubated for 96 h at 25 C on a gyrotary shaker. The medium was decanted from each flask and combined in centrifuge tubes. The solution was centrifuged at 980 x g for 20 min at 4 C. The supernatant layers were combined.

To eliminate amylase activity in the samples, the enzyme solution was stirred constantly while 0.1 M hydrochloric acid was added dropwise to the solution . The

Table XVI. Partial purification of barley amylase

Details of the purification procedure are given in the text.

Purification Step	Volume (ml)	Amylase Activity (U)	Specific Activity (U/mg Protein)
Initial Solution	10.0	90.9	35
Ammonium Sulphate ppt.	10.0	55.6	46
Glycogen Complex	1.2	9.5	136

pH of the solution was carefully reduced to 3.0. and then stirred for 20 min at room temperature. The pH was returned to 5.0 by the addition of 0.1 M sodium hydroxide. The final solution was then cooled in ice. All subsequent steps were performed at 0 to 4 C. While stirring constantly, 0.25 volumes of cold 3.9 M ammonium sulphate were slowly added to the sample. Stirring was continued for 30 min before the precipitate was removed by centrifugation for 30 min at 15300 x g (14000 rpm, Beckman model JA-20 rotor). The supernatant layers were collected and another 3.75 volumes of 3.9 M ammonium sulphate were added and the suspension centrifuged as above. The pellet thus obtained was resuspended in 2 ml of 0.001 M sodium phosphate buffer, pH 6.2 at 25 C, and dialyzed against 500 ml of the same buffer. Dialysis continued overnight at 4 C. The sample was applied to a column (15 cm x 0.9 cm ID) packed with hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 0.001 M sodium phosphate buffer, pH 6.2 at 25 C. The sample was washed into the column with 2 bed volumes of 0.001 M sodium phosphate buffer (pH 6.2) followed by 2 bed volumes of 0.04 M sodium phosphate buffer (pH 6.2). The xylanase was eluted from the column with 0.16 M sodium phosphate buffer (pH 6.2). After the void volume had passed through the column, the next 2 bed volumes were collected. Xylanase was precipitated from the column effluent by the addition of 4 volumes of cold 3.9 M ammonium sulphate and the precipitate was collected as

previously described, and resuspended in 20 mM succinic acid, 20 mM calcium chloride, adjusted to pH 5.5 at 25 C with sodium hydroxide. The suspension was dialyzed against 1 l of the same buffer overnight at 4 C. The results of the purification procedure are given in Table XVII.

Initial purification trials did not include the inactivation of amylase by the treatment at pH 3.0. The xylanase from this preparation still contained 21% (40 Units) of the original amylase activity. Although the low pH treatment reduced the final yield of xylanse from 90% to 12%, it very effectively eliminated amylase activity from the sample.

The xylanase prepared in this manner was evaluated to determine whether endo- or exo-xylanase predominated in the sample. The measurement of reducing value of the substrate solution would not differentiate between the endo- and exo-enzymes, and both forms of xylanase are produced by barley aleurone layers (Taiz & Honigman, 1976). To distinguish between the xylanases, the products of xylan hydrolysis at time intervals were separated by paper chromatography (Figure 20). The relative concentration of substrate, buffer and enzyme, as well as the incubation conditions were identical to the normal assay procedure. At time 0, all of the material remained on the origin. After 1 h, however, most of the material that reacted with the detection reagent had migrated along the paper, indicating a reduction in chain length. Very little free xylose was

Table XVII Partial purification of xylanase from isolated barley aleurone layers

Details of the purification procedure are given in the text.

Purification Step	Volume (ml)	Amylase Activity (U)	Glucanase Activity (U)	Xylanase Activity (U)	Xylanase Activity (U/mg Protein)
Initial Solution	16	191	11.2	0.573	0.13
Ammonium Sulphate ppt.	2	0	----	0.079	----
Final Product	1	0	0.1	0.071	0.39

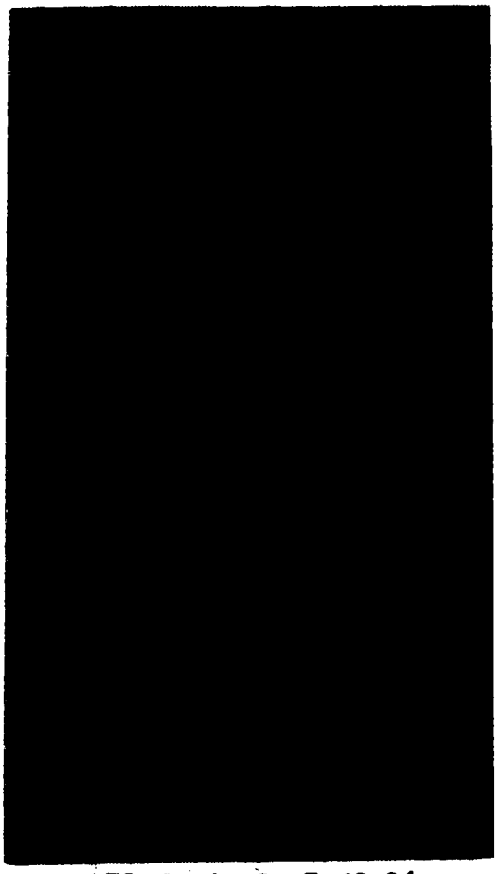

Figure 20. Paper chromatograms of the products of enzymatic hydrolysis of xylan. The standard (STD) consisted of 0.013 mg each of (from top) xylose, arabinose, glucose and galactose. The reaction mixture contained 0.58 mg/ml xylan and 12.9 mUnits/ml xylanase in 20 mM succinic acid, 20 mM calcium chloride, adjusted to pH 5.5 at 25 C with sodium hydroxide. The solution was incubated at 30 C, and 1.5 ml portions were withdrawn at the indicated intervals.

produced during the first hour, although the enzyme assay indicated that 1.15 microequivalents or reducing terminals had been exposed. These results suggested that the endo-xylanase was the prevalent enzyme in this preparation. Further incubation of the enzyme substrate mixture showed that free xylose did accumulate during the following 2

C. References

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