Biochemical changes in the aleurone of Rht-containing wheat associated with the low temperature-induced development of sensitivity to GA_3

by

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SUMMARY

The wheat aleurone system was used to examine the possibility of a membrane-based site of gibberellic acid (GA_3) action. Effect of low temperature on the GA_3-sensitivity of aleurone tissue from Rht-containing genotypes and non-Rht-containing genotypes has been examined. Sensitivity of the aleurone tissue de-embryonated seed to GA_3 was monitored by determining the amount of α-amylase produced as a result of 24 hrs incubation at 30°C, with different concentrations of exogenous GA_3. Essentially, a 20 hr pre-incubation at 5°C, as compared with 30°C, of de-embryonated seed/aleurone tissue of Rht-containing genotypes brought about dramatic increases in GA_3-sensitivities. No such low temperature-induced increases were detected in the case of non-Rht-containing genotypes.

Concomitant with the manifestation of this low temperature-induced increase in GA_3-sensitivity the phospholipid and sterol composition of the aleurone tissue containing either the Rht2 or Rht3 dwarfing gene, was monitored to determine whether any gross or specific changes taking place in the membranes of the tissue were synchronized with the increase in GA_3-sensitivity.

While low temperature exposure had no effect on the sterol composition of Kite (Rht2) aleurone tissue, a 20 hr pre-incubation at 5°C resulted in significant changes in the content of lipids, especially phospholipids. Significant low temperature-induced changes in both the head group and acyl contents of two phospholipids, PC and PE, were detected. More importantly, these changes displayed a very close temporal relationship with the low temperature-induced increase in GA_3-

sensitivity. Further, this relationship was paralleled by a highly significant correlation between the changes in the phospholipids and the changes in α-amylase production.

Experiments with Rht3/rht3 F_6 isogenic lines obtained from a cross between Minister Dwarf x Cappelle Desprez revealed that F_6 Rht3 (GA_3-insensitive) grain can be induced to produce similar amounts of α-amylase to that produced by the F_6 rht3 (sensitive) grain as a result of the low temperature treatment referred to above. While this low temperature treatment had a profound effect on the phospholipid composition of the F_6 Rht3 aleurone tissue, an effect quite similar to that evident in the case of aleurone tissue of Kite, it had no effect on either the phospholipids of F_6 rht3 aleurone tissue or its GA_3-sensitivity. In fact, as a result of the low temperature-induced changes the phospholipid profile of the aleurone tissue from the two lines became quite similar. Once again, there was a significant correlation between the low temperature-induced changes in the phospholipids and α-amylase production. Results from the experiments with inhibitors of lipid biosynthesis also support, albeit with certain qualifications, the concept of a link between phospholipid augmentation and subsequent development of GA_3-sensitivity. No effect of the low temperature could be detected on the sterol composition of aleurone tissue of either of the two lines.

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Statement

This thesis has not been previously submitted for a degree at this or any other University, and is the original work of the writer except where due reference is made in the text

(Surinder Pal Singh)

List of Abbreviations

Å	Angstrom (= 10^{-10}m)
BASF 13-338	4-chloro-5(dimethylamino)-2-phenyl-3(2H)-pyridazinone
^{o}C	Degree Celsius
DEGS	Diethylene glycol succinate
Diclofop-Methyl	Methyl 2-[4-(2',4'-dichlorophenoxy) phenoxy] propanate)
Ea	Arrhenius activation energy
Er	Endoplasmic reticulum
16:0	Palmitic acid
18:0	Stearic acid
18:1	Oleic acid
18:2	Linoleic acid
18:3	Linolenic acid
GA_3	Gibberellic acid
Gammexane	Gamma-hexachlorocyclohexane
GLC	Gas-liquid chromatography
IAA	Indole acetic acid
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
PI	Phosphatidyl inositol
PG	Phosphatidyl glycerol
PC	Phosphatidyl choline
PE	Phosphatidyl ethanol amine
LPC	Lyso phosphatidyl choline
DPG	Diphosphatidyl glycerol
µ	Micro

Rht	Reduced height gene
SK & F-7997A_3	Tris-(2-diethylaminoethyl) phosphate hydrochloride
TLC	Thin-layer chromatography

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PREFACE

A portion of the subject matter of this thesis has been published under the titles mentioned below:

1. The low temperature induction of hormonal sensitivity in genotypically gibberellic acid-insensitive aleurone tissue.
   S.P. Singh and L.G. Paleg. Plant Physiol. 74: 437-438.

2. Low temperature induced GA_3-sensitivity of wheat. I. Characterization of the low temperature effect on isolated aleurone of Kite.
   S.P. Singh and L.G. Paleg. Plant Physiol. (in press).

3. Low temperature induced GA_3-sensitivity of wheat. II. Changes in lipids associated with the low temperature induced GA_3-sensitivity of isolated aleurone of Kite.
   S.P. Singh and L.G. Paleg. Plant Physiol. (in press).

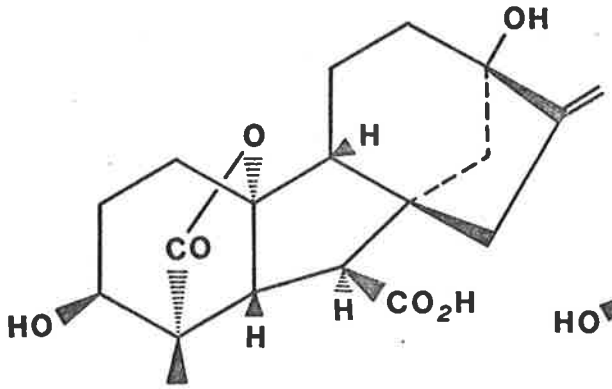
I. INTRODUCTION

1. Gibberellin and the aleurone system

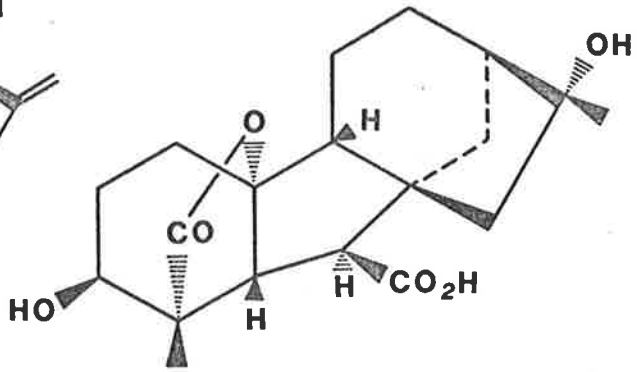
Gibberellin is the generic name for a class of naturally-occuring diterpenoid compounds possessing an ent-gibberellane skeleton (Figure 1). Although evidence of their occurence has been cited in gymnosperms, ferns, fungi, algae and bacteria (Kato et al., 1962 ; Katznelson, 1965 ; Mowat, 1963 ; Schraudolf, 1966), gibberellins are found primarily in angiosperms (Paleg and West, 1972).

Initial evidence suggesting a hormonal nature of gibberellins came from the response of dwarf plants to exogenous gibberellins. Dwarf peas (*pisum sativum*) elongated to 5 times their length in a few days on application of microgram quantities of pure GA_3 (Brian et al., 1954 ; Brian and Hemming, 1955) and the growth rate was equal to those of related tall varieties. In dwarf maize (*Zeamays*), GA_3 restored growth of four single-gene mutants to that of the normal phenotype, but two other mutants were unresponsive (Phinney, 1956). In each instance the applied gibberellin substituted for a natural but unknown growth promoting substance. The absence of marked abnormalities concomitant with the growth induction indicated that the prime effect of gibberellins might be to accentuate metabolism, rather than to cause a gross re-orientation of cellular activity. Endogenous gibberellin, therefore, was recognized as an important growth-promoting phytohormone.

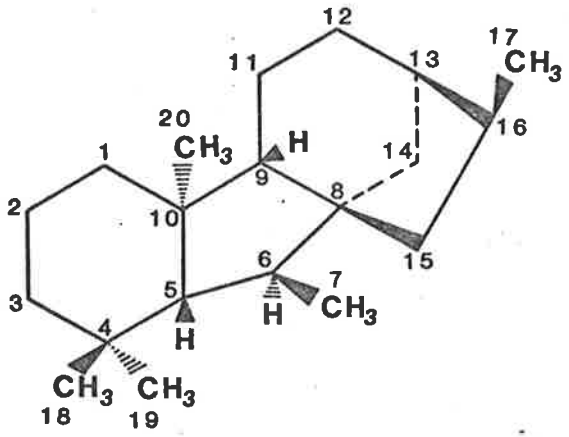
Figure 1: Chemical ring structures and numbering system for the ent-gibberellane skeleton, GA_1, GA_2, GA_3 and GA_4.



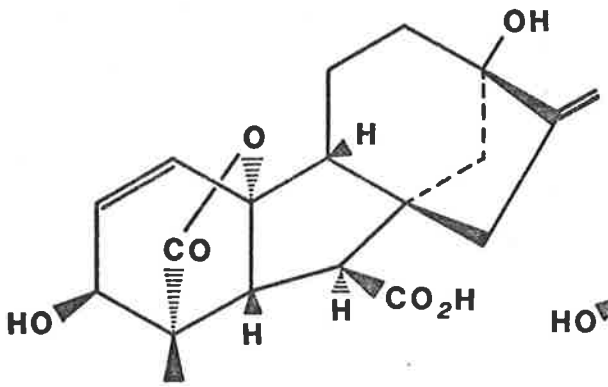
GA_1



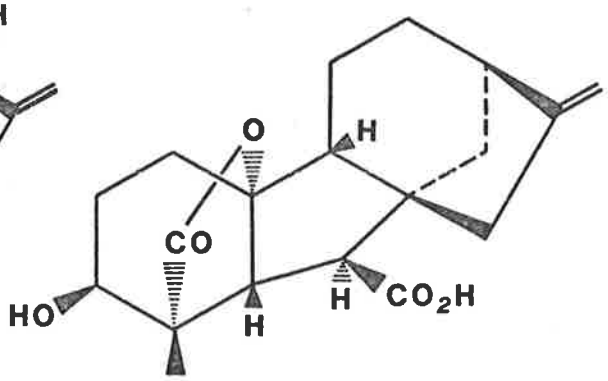
GA_2



ent-gibberellane



GA_3



GA_4

One of the primary problems encountered in elucidating the action/mechanism of a hormone is determining which events are the primary effects and which are the secondary effects. In other words, the initial event concerning the 'mechanism' and the latter series of events representing the 'mode' of hormone action (Paleg, 1965). Compounding the problem is the lack of specificity in the phytohormone responses. Unlike animal systems in which a hormone exerts its effect almost exclusively on a target organ which is responsive only to that hormone, most hormonal responses in plants are under the control of two or more of the known phytohormones: auxins, gibberellins, cytokinins, abscisic acid or ethylene. Hence, a given developmental process or area of metabolism often is a result of the interaction of any of several hormones. There are, however, certain developmental periods and growth processes which are considerably more responsive to a specific hormone.

For gibberellins, the physiological effects are most pronounced in floral induction in rosette plants (Lang, 1956), parthenocarpic fruit induction (Crane, 1964), stem elongation in rosette plants and genetic dwarfs (Lang, 1956 ; Phinney, 1956 ; Brain, 1959) and the breaking of both seed dormancy in monocotyledonous seeds (Paleg, 1961 ; Cohen and Paleg, 1967) and apical bud dormancy (Bukovak and Wittwer, 1961).

An additional problem in elucidating the mechanisms and mode of action of gibberellins is the increasingly large array of physiologically active structural configurations and the considerable specificity of the individual gibberellins in the bio-assay systems (Reeve and Crozier, 1974).

For elucidation of the mode of actions of GA_3, the aleurone layer of cereal grains has classically been the system of choice. The GA_3-induced initiation and control of hydrolytic enzyme activity in the cereal aleurone tissue, first reported in 1960 (Paleg, Yomo) is well established now. The attractiveness of cereal aleurone layers as the system for study of the biochemical basis of GA_3 action derives from several unique features of aleurone tissue: 1) Gibberellins are the natural trigger of enzyme production *in vivo* (Paleg, 1965 ; Briggs, 1973) ; 2) its response is confined to this one hormone class (Coombe et al., 1967 ; Clutterback and Briggs, 1973) ; 3) the target tissue is anatomically and physiologically homogenous (Buttrose, 1963 ; Jones, 1968 ; Yomo and Varner, 1971 ; Stevens, 1973) ; 4) it is a non-growing, non-photosynthetic, non-dividing tissue (Yomo and Varner, 1971 ; Jones, 1973) ; 5) the cells can be isolated free of other tissues and still respond to GA (Paleg, 1964 ; Varner, 1964) and, 6) the response of the isolated aleurone tissue is independent of the addition of substrates (Briggs, 1973 ; Jones, 1973). Furthermore, auxin in particular is without effect (Briggs, 1965 ; Cleland and McComb, 1965 ; Coombe et al., 1967) thereby circumventing the potential problem of whether GA is acting via auxin or acting independently.

One of the easiest measureable responses of aleurone tissue to GA_3 is the synthesis and secretion of α-amylase and because of this α-amylase is widely used as a reference enzyme when studying the biochemical events following GA_3 application. However, apart from amylases other acid hydrolases are also secreted from this tissue in response to GA_3, including proteases (Yomo, 1961 ; Briggs, 1965 ; Jacobsen and Varner, 1967; Sundblom and Mikola, 1972) ribonucleases

(Chrispeels and Varner, 1967 ; Bennett and Chrispeels, 1971), glucanases (MacLeod and Millar, 1962 ; Taiz and Jones, 1970 ; Jones, 1971), hexosidases (Pollard, 1969) and nucleosidases (Shuster and Gifford, 1962).

The synthesis and release of α-amylase following GA_3 application to aleurone layers generally occurs after a characteristic lag period of 4-8 hours in both barley and wheat (Chrispeels and Varner, 1967 ; Goodwin and Carr, 1972a ; Gibson and Paleg, 1982). As α-amylase is a hydrolase, and because of the initiatory action of GA_3, it was earlier suggested that the enzyme might either be a proenzyme (Paleg, 1960) or a pre-formed enzyme located in membrane-bound lysosomes (MacLeod and Millar, 1962). Inhibition of the synthesis of α-amylase both by anaerobiosis and protein synthesis inhibitors (Briggs, 1965 ; Varner, 1964 ; MacLeod, 1963 ; Paleg, 1964 ; Varner and Schidlovsky, 1963) led to the suggestion that the enzyme may arise by de novo synthesis. Incorporation studies using ^{14}C-algal protein hydrolysates (Briggs, 1963) and 1-phenylalanine-^{14}C (Varner and Ramchandra, 1964) indicated that much of the protein secreted from GA_3-treated aleurone tissue arose by protein synthesis. Purification of α-amylase from tissue treated with GA_3 and radioactive amino acids on DEAE-cellulose, and subsequent tryptic digestion of the radioactive α amylase peak, indicated that all but two of the resulting peptides were radioactive, thus giving firm support to the idea of de novo synthesis of GA_3-induced α-amylase (Varner and Ramchandra, 1964). More evidence to support this view comes from experiments using the density labelling technique (Filner and Varner, 1967). The α-amylase produced in tissue treated with GA_3, ^{14}C-lysine and $H_2{}^{16}O$ was highly purified and

used as a marker. Crude α-amylase obtained from aleurone tissue treated with GA_3 and $H_2{}^{18}O$ was mixed with a trace of the ^{14}C-α amylase and subjected to isopycnic centrifugation in a cesium chloride density gradient. Filner and Varner concluded that virtually all of the enzyme was synthesized de novo.

2. Mechanism of action of gibberellins

The GA_3-induced initiation and control of α-amylase synthesis in the cereal aleurone tissue, and the diversity of other responses to GA_3 shown by plant tissues, sparked an era of intense research activity directed towards elucidating the mechanism of action of GA_3. Naturally, due to reasons mentioned above, the aleurone system was the one most extensively employed. Several theories of GA_3 action have emerged as a result. This section of the introduction, thus, is an assessment of these theories of GA_3 action and it is probably a fair summary to say that our knowledge of the primary events involved in GA_3 action is still very slight (Kende and Gardner, 1976).

2.1 GA_3 effect on the cell wall

Hormones, like auxins or gibberellins, which enhance cell enlargement, must either directly or indirectly affect cell extensibility. This effect prompted the suggestion that perhaps the cell wall was the primary site of GA_3 action. Cleland et al., (1968) found that GA_3 caused cell elongation without inducing cell wall plasticiza-

tion. They suggested that GA_3 promoted growth by causing an increase in the osmotic concentration of cells in the growing region. However, increases in osmolarity were shown not to induce growth in *Avena* stem segments. (Adams et al., 1973). Furthermore, treatment of *Avena* stem segments with GA_3 greatly increased internodal plasticity, but had virtually no effect on internodal elasticity (Adams et al., 1975).

There are no measurable differences in the composition or structure of cell walls during growth of the internodes with or without GA_3 (Albersheim et al., 1977). Similarly, there are no significant differences between cell walls that are growing rapidly in response to GA_3, and ones that are growing slowly (Albersheim et al., 1977). All that is required for cell expansion to take place, is synthesis of new cell wall material and its incorporation into the wall. This involves breaking and reforming of bonds in order to insert the new material into the wall. It seems unlikely that a single hydrolase would be the wall loosening factor. For example, cellulase was able to increase wall extensibility, but did not cause any wall extension in *Avena* coleoptile sections (Wada et al., 1968 ; Ruesiuk, 1969). Also, cellulase in pea epicotyls is concentrated in regions of cell division rather than cell elongation (MacLachlan et al., 1970). Taiz and Jones (1970) have demonstrated an enhanced release of β-1,3-glucanase from barley aleurone layers in response to GA_3 treatment. They suggested that glucanase produced by aleurone cells was responsible for the observed cell wall digestion. GA_3 was also shown to stimulate activities of endo-β-1,4-xylanase, β-xylopyranosidase and α-arabinofuranosidase, but had no effect on cellulase activity (Taiz and Honigman, 1976). Thus, it is very likely that several hydrolases are involved in the process of

wall loosening, and these may vary from tissue to tissue. Other studies with GA_3 action in lettuce hypocotyl sections, where the response is entirely confined to cell elongation, have indicated that an increase in cell extensibility occurred on exposure to GA_3 (Silk and Jones, 1975 ; Stuart and Jones, 1977 ; Stuart et al., 1977), but the nature of the wall-softening process was not determined. However, the distribution of ^{3}H-GA, amongst sub-cellular fraction from lettuce hypocotyl sections was examined by differential centrifugation (Stoddart, 1979). Material pelleting at 2000g (2KP) accounted for 2-5% of the total tissue radioactivity and no other significant interactions were detected. The 2KP fraction was found to consist mainly of cell-wall material (Stoddart, 1979).

Overall, if the process of cell wall-loosening constitutes the mechanism of action of GA_3, then it will have to be confined to systems where the ultimate response to GA_3 appears to be related to wall-softening.

2.2 Transcriptional Control : Analogies with animal steroid hormones

Gibberellins, plant sterols and animal steroid hormones have close structural relationships and all are products of the terpenoid pathway (Heftmann, 1963). The structural similarities have prompted suggestions that the gibberellins may have a mode of action which resembles that of animal steroid hormones. In order to examine this possibility it is necessary to outline briefly current views on steroid hormone action.

2.2.1 Steroid hormones

Specific receptors are the key elements in the mechanisms of action of animal steroid hormones. The receptors are soluble cytoplasmic proteins with a sedimentation coefficient of approximately 4S (Ca 90,000 molecular weight) and are recoverable as an 8S dimeric complex in cell extracts (Gorell et al., 1972 ; Sica et al., 1973). These receptors, complex with the hormone and migrate to and across the nuclear membrane. Within the nucleus, the hormone/receptor complex becomes associated with the chromosome by means of a two-point attachment to chromatin and non-histone protein. This association results in the expression of new genetic information. Receptor protein is present in all somatic cells but the level is greatly enhanced in target tissues. Radioactive hormone can be recovered in both the cytosol (hormone/receptor complex) and nuclear (chromatin/receptor complex) fractions, (O'Malley and Means, 1974).

2.2.2 Gibberellins

To what extent can the steroid system, or elements of it, be applied to the action of gibberellins on plant tissues. There is really no firm evidence from plants which would allow any sort of meaningful overall conclusion on this question.

Firstly, the existence of specific receptor proteins for gibberellins in the plant cell cytoplasm has been investigated. The search for such receptors was made possible by the synthesis of

gibberellins with high specific radioactivity. For example, (1,2-^{3}H)-GA_1 at 40-50 Ci/mmol was used to probe the existence of specific receptor proteins in dark-grown pea epicotyls. Epicotyl sections, fed with labelled GA_1 or GA_5, concentrated radioactivity in the growing hook region of the epicotyl and irradiation with red light, which decreases sensitivity to exogenous GA, greatly reduces the extent of accumulation (Pitel and Vining, 1970 ; Nadeau and Rappaport, 1974 ; Musgrave et al., 1969). Stoddard et al., (1974) detected associations between GA_1 and soluble macromolecular components in extracts of the epicotyl hook. Using similar methods, Konjevic et al., (1976) have reported binding of ^{14}C-GA_3 to epicotyl extract components excluded by Sephadex G-10.

Are such associations significant? Cell fractionation after feeding with labelled GA usually results in the recovery of significant amounts of radioactivity in all the major particulate fractions, and this binding is saturable only at high GA levels (Kende and Gardner, 1976). Animal hormones act at extremely low concentrations where as GA gives an increasing growth response over an extended concentration range; perhaps as much as five orders of magnitude of molarity. Thus, as Kende and Gardner (1976) have suggested, it may be more realistic to look for sub-cellular interactions which have the capacity to accept GA over a similar span. A further difficulty is raised by the failure to demonstrate binding effects *in vitro* using equilibrium dialysis in a manner analogous to that employed in animal receptor studies (Stoddart, 1975).

Many studies have demonstrated the influence of gibberellins on the rate of transcription. A clearly defined GA_3-induced change in aleurone

mRNA in a cell-free system from wheat embryos was shown to occur (Higgins et al., 1976). The amount of translatable mRNA for amylase began to increase between 2-3 hours after GA_3 treatment, and over the first 12 hours the level of mRNA correlated well with the *in vivo* rate of amylase synthesis. This rise in mRNA could, however, occur as a result of enhanced synthesis, activation of some pre-existing form of RNA, or a decrease in the rate of mRNA breakdown and more than one of these possibilities may be involved. For example, GA_3 may be involved in the 'capping' of mRNA which has been stored in an 'uncapped' form. 'Capping' of mRNA has been observed in plants (Kennedy and Lane, 1975), and may be necessary to activate the mRNA for translation.

Much of the evidence for involvement of specific mRNA synthesis in GA_3-induced responses rests on results obtained using metabolic inhibitors. Varner and Chandra (1964) used inhibitors of RNA and protein synthesis and concluded that GA_3 caused production of specific mRNA's, thereby controlling the synthesis of α-amylase.

However, many of these inhibitors may be non-specific in their effects (Honig and Rabinovitz, 1965 ; Laszlo et al., 1966 ; Ellis and MacDonald, 1970). This necessitates extreme caution in the interpretation of such results and may well lead to false conclusion. Thompson and Cleland (1972), using DNA/RNA hybridization techniques, showed that GA_3 was able to induce stem growth in dwarf pea *Pisum sativum* without causing detectable changes in hybridizable RNA. Jones and Chen (1976) have applied immunohistochemical techniques to sections of barley aleurone layers and have shown that α-amylase-specific immunofluorescence is localized in the perinuclear region. They have

also noted that GA-stimulated proliferation of ER tends to be similarly concentrated. These data, however, give support to the case of GA-stimulated α-amylase synthesis being dependent upon RNA export from the nucleus, but provide no evidence that this is a primary response of the tissue to the hormone.

Overall, therefore, it is conjectural whether a steroid type of mechanism exists in plants. Cytosol receptors may, or may not, be present and there is little evidence for accumulation of hormone in the nucleus.

2.3 Post-transcriptional site of GA action : Translation

The possibility of translational control of protein synthesis by GA_3 has also been studied. In barley aleurone tissue, the formation of polysomes was reported after a lag period of 3-4 hours (Evins, 1971 ; Evins and Varner, 1972). ABA inhibited GA_3-induced polysome formation and α-amylase synthesis, suggesting that polysome formation could be a pre-requisite for the synthesis of hydrolyzing enzymes (Evins and Varner, 1972). In wheat embryos, also, GA_3 control of protein synthesis appeared to be at the translational level (Chen and Osborne, 1970). However, later work on wheat (Varty and Laidman, 1977) and barley (Jacobsen and Zwar, 1974) aleurone tissue demonstrated little or no stimulation of rRNA synthesis and no stimulation of total RNA content in response to GA_3 treatment. Furthermore, rRNA and tRNA synthesis is unnecessary for normal amylase induction (Jacobsen and Zwar, 1974).

For all GA_3 responses RNA synthesis is probably required for the continued expression of the response in the medium to long term. Although much information has accumulated regarding hormone-induced changes in RNA metabolism the crucial question, as to whether RNA metabolism is involved in the initiation or the perpetuation of the response, remains unanswered. A good example to illustrate this point is the finding that in *Avena* stem segments, protein synthesis is required for GA_3-promoted growth and invertase activity (Kaufman et al., 1968). However, GA_3-enhanced growth occurs before invertase activity increases (Kaufman et al., 1973), suggesting that the intial effect of GA_3 is not on enzyme synthesis, but rather enzyme synthesis is required for continued growth of the segment. This problem of cause and effect exists whether studying DNA synthesis, RNA synthesis, or enzyme synthesis in response to treatment with GA_3.

2.4 Membranes : Site of GA_3 action

The physical intervention of membrane systems between the origin of hormonal signals and cellular responses to these signals has prompted speculation that membranes may be the primary targets for hormone-initiated events. Evidence linking phytohormones with membranes has been obtained from hormone-binding studies with particulate cell fractions (Venis, 1977 ; Lamb, 1978 ; Hertel, 1979) and from studies of their effects on the physical properties of natural and model membranes (Kende and Garderner, 1976 ; Trewavas, 1976). Membrane-hormone interactions have also been inferred from studies of ion fluxes across membranes (Nelles, 1973 ; Marre, 1977 ; Higgins and Jacobsen, 1978), and various

theories for hormone action based on an initial alteration of one or more membrane properties have been proposed (Morre and Cherry, 1976 ; Marre, 1979 ; Trewavas, 1979).

Membrane composition displays an almost infinite variability from tissue to tissue, between organelles, between species and at different ages of the tissue. Further, it has the potential of being modulated by environmental conditions. Not only is there possibility for variation in the proportions of the main components of membranes to one another (ie. phospholipid:sterol:protein), but there is also scope for diversity within the phospholipid fraction depending on the nature and amounts of fatty acids and the nature of the polar head groups. These properties of the membrane components offer adequate scope to encompass the required degrees of specificity to distinguish between one molecular species of hormone and another, and even between different hormones of the same general structural configuration (Wood and Paleg, 1974 ; Pauls et al., 1982).

In view of the diversity of responses of plant tissues to GA_3, as detailed above, the question needs to be asked; is the site of hormone action for these many and varied responses the same in each case? The answer to this question is not clear yet, but certainly if this were the case, one could probably require a receptor which was flexible enough to be altered in configuration by environmental conditions, stage of development of the plant or cell, other hormones and regulating compounds, and any other factors which influenced the activity of GA_3 in a particular situation. Also, the receptor site must achieve and

maintain a high degree of specificity when in its receptive state. As we have already seen, membranes are likely candidates for this role. Further, since hormones are synthesized at a point remote from their site of action, it seems inescapable that in reaching a site of action, membranes would be traversed. At the target, the hormone would penetrate the membrane either passively or actively, or it may bind to a portion of the membrane, hence, initiating its response. Thus, a membrane hypothesis would also be feasible in terms of the timing of the response.

There are several possible ways in which GA_3 could act on a membrane in order to bring about a physiological response. Broadly speaking, the hormone may alter the membrane composition or the membrane structure, or both. Compositional alteration may be brought about by changing the ratios of phospholipid:sterol:protein, or by changing individual phospholipids, sterols and proteins into new or different species (eg. by changing the fatty acid profiles of phospholipids). Membrane structure could thus be changed, altering fluidity or permeability by the presence of GA_3 in the lipid matrix.

2.4.1 GA_3-induced changes in membrane composition

Evidence indicating that the primary events after GA_3 treatment are to do with the synthesis of new membrane components, emanates mainly from studies with the barley aleurone layer. In barley aleurone cells, GA_3 treatment initiated an increased rate of ER synthesis as measured by ^{14}C-choline incorportion into phospholipids (Evins and Varner, 1971).

This increase began about 4 hours after treatment, and after 10 hours GA_3 enhancement was 4-8 times that of control. A similar GA_3 effect was found for total cellular phospholipids when ^{32}Pi was employed as a more general tag for phospholipid labelling (Koehler and Varner, 1973). Johnson and Kende (1971) and Ben-Tal and Varner (1974) pursued this idea by examining the effects of GA_3 on three enzymes of a phospholipid-synthesizing pathway. The first enzyme, choline kinase, was unaffected by the treatment, but the other two, phosphorylcholine-cytidyl transferase and phosphorylcholine-glyceride transferase, increased markedly in the presence of GA_3. After 2 hours of hormone treatment, both enzyme activities were 150% of controls. However, as evidence for the GA_3 stimulation of phospholipid synthesis has been derived from subcellular fractionation experiments where *in vitro* enzyme actions could influence results, and since the phospholipid extraction procedures used were liable to yield contaminated samples, the conclusion derived from these experiments on barley are suspect. Furthermore, the evidence for a GA_3 stimulation of phosphoryl choline-glyceride transferase activity in membrane fractions from barley is also very tenuous because the 1, 2-diglyceride substrate in the assay is itself derived from the membrane. The concentration of the substrate was, consequently, not under experimental control. Indeed, GA_3 stimulation of 1, 2-diglyceride synthesis has been reported with both barley (Firn and Kende, 1974) and wheat (Varty, 1975) aleurone tissue and this would profoundly influence the phosphorylcholine-glyceride transferase assay.

More recently, studies on wheat aleurone tissue failed to show any control of phospholipid synthesis or ER formation by GA_3 (Mirbahar and

Laidman, 1982 ; Varty and Laidman, 1976 ; Laidman et al., 1974). Furthermore, Varty and Laidman (1976) demonstrated that phospholipid synthesis in wheat aleurone tissue was induced by imbibition and the rate of synthesis was unaffected by GA_3. Ultrastructural studies carried out on the wheat aleurone tissue also failed to shown any role of GA_3 on ER formation (Laidman et al., 1974 ; Colborne et al., 1976). Considering the results with both wheat and barley tissue it seems reasonable to conclude that the involvement of GA_3 in phospholipid synthesis in aleurone tissue remains to be clarified. Further evidence contrary to the view that GA_3 might be manifesting its action via an alteration in membrane composition comes from the recent work of Jusaitis et al., (1981b, 1982). They used stem segments isolated from *Avena sativa* plants to explore the mechanism and mode of action of GA_3. Although prolonged treatment of oat plants with GA_3 produced marked changes in phospholipid and sterol composition of stem segments, these changes did not always accompany the GA_3-induced growth response of the segments. Treatment of stem segments with GA_3 for only 20 hours produced a significant growth response with little or no effect on lipid composition, suggesting that neither the mechanism nor the mode (at least in the short term) of hormone action in this system involves an alteration of lipid (membrane) composition.

The suggestion that GA_3 might be affecting the other major component of membranes viz ; sterols is also not well supported. Treatment of *Solanum xanthocarpum* tissue cultures with a mixture of kinetin and GA_3 caused no appreciable change in the steroidal content (Heble et al., 1971). Free sterols of peanut (*Arachis hypogaea*) were altered by treatment with an auxin or GA_3 (Hale et al., 1977). The

concentration (µg/mg lipid) of free sterol decreased in the stems, increased in roots and pods, and remained unchanged in the leaves. Changes in sterol concentration were greater for GA_3 than for auxin-treated plants. All of these effects could have been the result of effects of the hormones on translocation, differential hydrolysis of stored substrates, or on synthesis. In addition, extractions were performed 5 days after treatment was initiated, and it is probable that the initial action of the hormone would have taken place well before this time.

2.4.2 GA_3-induced changes in membrane structure/properties

The possibility that the biological effects of GA_3 may be the consequence of an interaction of GA_3 with membrane components and subsequent modulation of the physical properties of the lipid bilayer has also been suggested.

2.4.2.1 Effects of GA_3 on artificial membrane systems

Interaction of GA_3 with plant membrane components has been directly investigated by Wood and Paleg (1972, 1974), using artificial membrane micelles derived from crude soya bean lecithin (phospholipid). The model was based upon the ability of phospholipids to become arranged in self-ordered concentric membrane structures (liposomes) when dispersed in aqueous media (Bangham et al., 1965). Some of the permeability properties of these aqueous suspensions of phospholipids are similar to

some of the important permeability properties of biological membranes. (Sessa and Weissman, 1968). The liposomes, thus represent an artificial or model membrane.

Wood and Paleg (1972) demonstrated that GA_3 influenced the permeability of model membranes composed of plant constituents. The hormone increased permeability of liposomes to glucose and chromate ions (Wood et al., 1972) and the alterations in permeability induced by GA_3 were not mediated by enzymes, did not involve carrier systems, and did not depend on the presence of charges on the permeating molecules (Wood and Paleg, 1972). The specificity of this particular system was low, since diethylstilbestrol, GA_8 and IAA were also able to affect the liposomes.

Further, it was shown that crude lecithin liposomes were more responsive to the GA_3-induced increase in permeability than the liposomes prepared from purified phospholipid fractions. It is thus possible that the GA_3 response of the crude lecithin liposomes related to some unidentified component not present in the purified phospholipid preparations; an explanation which Wood and Paleg (1972) link with the observation that such liposomes have high initial leakage rates which may mask or preclude a subsequent hormonal response. The possibility that a non-phospholipid impurity may be responsible for the observed effects on permeability can, however, be dismissed on the grounds that steroids also elicit a similar leakage response. Effects on permeability elicited by steroid hormones in similar preparations have been described by Bangham et al., (1965) and Weissman et al., (1965). In animal systems

pharmacologically active steroids have been shown to affect the membranes of lysosomes, erythrocytes, and mitochondria, causing visible disruption of the organelle surface in many instances. These effects were distinguished from those caused by Triton X-100 and digitonin by electron microscope studies which indicated that the surfactants caused complete disruption of the liposome micelles whilst the steroids did not (DeDuve and Wattiaux, 1966 ; Blecher and White, 1960). Mudd and Kleinschmidt (1970) have shown that the 35-carbon polyene antibiotic, filipin, isolated from *Streptomyces griseus*, greatly enhances the efflux of betacyanin from discs of red beet and that this process occurred without any apparent ultrastructural change in either the tonoplast or plasmalemma.

There is evidence, therefore, that GA_3 and other compounds can affect the permeability of liposomal membranes without necessarily causing the type of total disruption characteristic of detergents. What are the possible molecular bases for such an action. Phospholipid/cholesterol structures tend to have a highly ordered arrangement based upon the regularity of bonding or charge interactions between arrays of similar molecules (Singer, 1974). Willmer (1961) has suggested that the insertion of dissimilar molecules (such as steroids or gibberellins) into such a matrix would result in discontinuities. These could have effects on the permeability properties of the membrane. Such changes in membrane fluidity, and its attendant alterations of membrane - associated processes, provide an attractive possibility as to how GA_3 is able to invoke an amplified response at characteristically low concentrations. Wood et al., (1974) found a significant perturbation of phospholipid bilayers when treated with a maximum mole ratio of GA_3

to lipid of 1:100. Each molecule undergoing a change in fluidity affects those molecules in its immediate neighbourhood due to the phenomena of conformational cooperativity (Rothman, 1973), and a single molecule may thus influence a domain of 20 molecules or an area of 400 $\mathring{A}^2$ of membrane (Wood et al., 1974).

Kende and Gardner (1976) have drawn attention to the parallels between plant responses to GA_3 and the olfactory response in animals. In both instances the effect is evident over several orders of concentrations and also diverges from Michaelis-Menten saturation kinetics at the higher end of the range. The olfactory process is explained in terms of the stimulating compound partitioning into an epithelial membrane (Mozell, 1970 ; Mozell and Jagodowicz, 1973) and the response occurring when a molecule desorbs from the membrane leaving a "hole" or ionophore through which ions can pass to initiate nervous impulses. There is, thus, the possiblility of a clear parallel with the changes in solute flow observed in the lecithin liposome/GA_3 system described by Wood and Paleg (1972, 1974).

What is the nature of the association of GA_3 with the liposomal membrane? Wood et al., (1974) have studied this problem using proton magnetic resonance spectroscopy. Solutions of phosphatidyl choline (lecithin) in deuterochloroform, showed identifiable resonances for the N,N,N-trimethylamino group and olefinic portions. Complex envelops of signals associated with $-(CH_2)_n-$and-CH-O-protons were also observed. In the presence of GA_3 there was a proportional shift of the trimethylamino group resonance towards higher magnetic field. The authors interpreted

these changes as being a consequence of an electrostatic attraction between the cationic trimethylamine group of lecithin and the carboxyl group of GA_3:

$$\text{Lecithin} + GA_3 \rightleftharpoons \text{Complex}$$

As the observed shifts occurred in a deuterochloroform environment the conclusions cannot be extrapolated to the aqueous state. They are, nevertheless, suggestive of an association of GA_3 with a basic membrane component in the liposome.

Further evidence demonstrating that gibberellins can influence membrane behaviour by modulating its physical properties, comes from a recent work of Pauls et al., (1982). Differential scanning calorimetry and electron spin resonance were used to characterize the association of a GA_4 and GA_7 mixture with phospholipid membranes. The GA_4/GA_7 mixture lowered the temperature and reduced the co-operativity of the main phase transition of liposomes prepared from dipalmitoyl phosphatidylcholine (DPPC), distearyl phosphatidylcholine (DSPC) dipalmitoyl phosphatidyl glycerol (DPPG), and eliminated the pretransition. The reduction in co-operativity of the main transition was not accompanied by a reduction in transition enthalpy, indicating that the gibberellins simply perturb the bilayer rather than complex with the phospholipid molecules. The negatively charged DPPG was less susceptible to perturbation than the Zwitterionic phosphatidylcholines. More importantly, perturbations by

GA_8, a physiologically inert gibberellin, was virtually imperceptible. Electron spin resonance of GA_4/GA_7 - treated liposomes of phosphatidyl choline and phosphatidyl glycerol confirmed the results obtained by differential scanning calorimetery.

2.4.2.2 Effects of GA_3 on natural membranes

GA_3 - induced changes in membrane properties have been reported in natural systems. Gibson and Paleg (1976) purified lysosomes from wheat aleurone cells treated with GA_3, and observed an altered permeability of the lysosomal membranes (Gibson and Paleg, 1977). Temperature-dependent leakage of lysosomal enzyme was a function of the GA_3 concentration used to treat the tissue. Although it was not clear if this was a result of a physical or compositional change in the lysosomal membrane, the altered thermal behaviour suggested that membranes produced in aleurone tissue treated with high GA_3 concentration were more flexible and more fluid at any given temperature than membranes formed in response to lower GA_3 concentrations (Gibson and Paleg, 1977).

Further evidence for a direct action of GA_3 at the membrane was found by Nelles (1977a) who demonstrated that the plasma membrane permeability ratio of sodium:potassium of dwarf corn coleoptile cells is decreased by GA_3 during the first 15 minutes after application. The membrane potential difference was increased within a few minutes of GA_3 application (Nelles, 1977b). A direct action of GA_3 on the membranes was also suggested by the work of Neumann and Janossy (1977a,b). In an electron microprobe study they found that GA_3 could influence ion ratios in plastids, cytoplasm and vacuoles of dwarf maize with no appreciable lag-time. Browning and Saunders (1977) demonstrated two fractions of

extractable gibberellins located within wheat chloroplast membranes' one released from the membranes by methanol, the other by Triton. They suggested that the Triton-extractable gibberellins were intimately associated with non-polar proteins or other molecules located within rather than on the periphery of the membrane.

Overall, the idea that GA_3 action is concerned with membrane properties and that the primary action site is also membrane-located has many attractions. It fits well with the kinetic data for response, provides possible explanations for the diversity of type and timing of response, suggests why intensive efforts to isolate soluble receptor proteins may have failed, and maintains a reasonable analogy with aspects of steroid hormone action in animal systems.

3. Low temperature : A useful tool to modulate membrane lipid composition, structure and function

Studies on the relation between lipid structure and membrane function have depended on the correlation between a measured enzyme or physiological function and the molecular ordering or fluidity of membrane lipids in response to changes induced by some external variable. The most widely used variables altering membrane lipid fluidity are lipid composition and temperature. The lipid composition of bacteria and animals may be changed by diet, or by altering growth temperature. With plants it is more difficult to alter membrane lipid composition by diet. Thus, changing temperatures, in particular low temperature, is by far the most common variable used to alter plant membrane lipids.

It has been pointed out above that there is a possibility of the biological effects of GA_3 being a consequence of interaction of GA_3 with membrane components and the consequent modulation of the physical properties of the lipid bilayer. Therefore, any changes in the membrane components could also be reflected as an altered physiological response of the sytem to GA_3. In fact such a situation has been shown to exist in *Avena* stem segments (Jusaitis et al., 1982), where alterations in the membrane phospholipids and sterols, brought about by low temperature, changed the GA_3 response of the tissue.

The next part of the introduction thus deals with the changes in membrane composition, structure and function induced by low temperature. The interaction of low temperature and GA_3 responses of natural systems will also be discussed.

3.1 Low temperature effects on membrane composition

The membrane can be divided into three major constituents ; acyl lipids (predominantly phospholipids and galactolipids), proteins and sterols. The effect of low temperatures on each of these components will be discussed in turn.

3.1.1 Acyl Lipids

A large body of evidence suggests that in the membrane systems of many plants and ectothermic organisms, the degree of unsaturation of fatty acids is in some manner controlled by the environmental temperature. De La Roche et al., (1972) demonstrated that wheat seedlings grown at a lower temperature synthesize much larger amounts of

phospholipids than those grown at 24°C. Seedlings grown at 2°C exhibited a higher degree of unsaturation of membrane phospholipids than those at 24°C and the increase could be accounted for principally by an increase in linolenic acid (18:3) (De La Roche et al., 1975 ; Willemot, 1975, 1977). A similar increase in 18:3, accompanied by a decrease in the level of linoleic acid (18:2) was found in nine species of wheat and one of rye (*Secale cereale*) (Farkas et al., 1975) after low temperature treatment. Similar trends have been observed in other cereal grasses (Redshaw and Zalik, 1968).

Wilson and Crawford (1974) examined the effects of low temperature on the fatty acid composition of the polar lipids of *Gossypium hirsutum* and *Phaseolus vulgaris*. They observed an increase in the degree of unsaturation of fatty acids associated with the phospholipid fraction, while no effect on the fatty acid composition of the glycolipids was observed. This increase in unsaturation of fatty acids during acclimation to chilling temperatures has been observed in a broad range of plants, including alfalfa (*Medicago sativa*) (Gerloff et al., 1966 ; Grenier and Willemot, 1974), *Brassica napus* (Appelquist, 1971), *Linum spp.*, *Helianthus spp.* (Canvin, 1965), *Atriplex lentiformis* (Percy and Strouse, 1974), *Glycine max* (Rivera and Penner, 1977), Anacystis nidulans (Holton et al., 1964), *Cyanidium caldarum* (Kleinschmidt and McMahon, 1970). The phenomenon is not restricted to higher plants but has also been observed in microorganisms such as *Pseudomonas fluorescens* (Cullen et al., 1971), *Bacillus stearothermophilus* (McElhaney and Souza, 1976), *Clostriduim butyricum* (Khuller and Goldfine, 1974), *Tetrahymena pyriformis* (Wunderlich and Ronai, 1975 ; Fukushima et al., 1976), and non-plant systems such as crustacean plankton (Farkas and Herodek, 1964), and Goldfish (Johnston and Roots, 1964 ; Caldwell

and Vernberg, 1970). It seems the exposure to a chronically cold environment is invariably associated with an increased degree of unsaturation of the fatty acid chains of phospholipids and, in some cases of galactolipids in photosynthetic membranes (Chapman et al., 1983), and in a wide variety of species at all levels of biological organization. A low temperature-induced stimulation of phospholipid levels without alterations in the degree of unsaturation of their fatty acids has also been observed in some plant cases (Willemot, 1975 ; Horvath et al., 1981).

3.1.2 Sterols

Although sterols are important components of membranes (Demel and Dekruyff, 1976 ; Grunwald, 1970 ; Heftmann, 1971), there is a marked paucity of literature showing an effect of low temperature on sterol biosynthesis and incorporation into membranes.

Triticum aestivum plants shifted from 10°C to 1°C experienced a general decrease in sitosterol, stigmasterol and campesterol in shoot tissue, with no significant change in cholesterol (Davis and Finkner, 1973). In yeast cells, sterol synthesis is influenced by temperature (Hunter and Rose, 1972), the maximal rate of synthesis coinciding with the optimal temperature for growth (Starr and Parks, 1962). DeLaRoche (1979) demonstrated a significant increase in the ratio of sitosterol to stigmasterol in a total membrane fraction of winter wheat seedlings grown at 1°C. Jusaitis et al., (1981a) reported that in *Avena* stem segments, stigmasterol decreased markedly with temperature, while the sitosterol/stigmasterol ratio increased significantly.

3.1.3 Proteins

Although considerable work has been published on the effects of freezing treatments on membrane-bound proteins (eg. Gusta and Weiser, 1972 ; Siminovitch et al., 1968 ; Vigue and Oslund, 1974), very little has been done using chilling temperatures (ie. temperatures above freezing). Pomeroy et al., (1975) found no appreciable changes in the levels of mitochondrial protein in wheat and rye seedlings grown at 2°C when compared with those grown at 24°C. Huner and MacDowall (1976), using the same plants, demonstrated that no drastic changes in chloroplast membrane polypeptides occurred during growth at low temperatures. Yamaki and Uritani (1973, 1974a,b) in a series of papers discussing the mechanism of chilling injury in sweet potato (*Ipomoea batatas*), suggested that the protein moiety of mitochondrial membranes underwent a reversible conformational change in the early stage of chilling treatment, which became irreversible with prolonged chilling. They proposed that the membrane protein was bound to the phospholipid by hydrophobic bonds, and that these bonds were broken by chilling treatment, resulting in 'cold-desaturation' which was initially reversible, but eventually became irreversible. A similar reduction of hydrophobic forces was suggested to occur during low temperature vernalization of bolting-resistant plants (Lexander, 1975). Recently, low temperature induced increases in lipid to protein ratios of pea thalakoid membranes have been detected (Chapman et al., 1983). This increase has been postulated as one of the major factors which contribute to regulation of fluidity in this particular membrane.

Indirect changes in membrane-bound protein (enzyme) structure and activity brought about by low temperature-induced alterations of

membrane composition will be discussed later in this review (Introduction 3.3.2).

3.2 Changes in lipid structure at low temperatures

What are the effects of low temperature-induced alterations in membrane composition upon the membrane lipid structure? Fluidity within the lipid bilayer is a central feature of the mosaic model of a biological membrane, and maintaining this fluidity is essential for life processes (Singer and Nicholson, 1972). The effect of low-temperature-induced membrane alteration appears to be the maintenance of a favourable fluidity of the membrane in order to achieve optimal membrane stability and function, regardless of the temperature. Membrane fluidity is a function of many variables, including the nature of membrane lipids (type of phospholipids, fatty acid chain length and degree of unsaturation, head group and sterol type and content), temperature, water content, presence of ions (especially divalent cations), and the total effects of protein-lipid interactions.

That the melting point of a particular fatty acid of specified chain lengths is inversely proportional to the degree of unsaturation is a firmly established fact (Raison, 1980). In mixtures of palmitic and linoleic or linolenic acids, an increase of only 5% in the degree of unsaturation reduces the melting point of the mixture by as much as 15^{o}C (Lyons and Asmundson, 1965 ; Raison, 1973). Small increases in the degree of unsaturation in membrane lipids of cold-treated organisms could thus convert the membrane to a more "fluid" state. The increase in fluidity results from the fact that unsaturated acyl chains have greater kinetic motion and, thus, undergo co-operative motion and occupy a greater area of the bilayer (Phillips et al., 1970).

An increase in the level of unsaturated fatty acids of membrane lipids has been observed in many plants exposed to low temperatures, and this was thought to be related to frost hardening and to be part of the mechanism of avoiding freezing injury (Levitt, 1972). It was generally assumed that such increases in lipid unsaturation would increase membrane fluidity, lower the temperature of order-disorder transitions and allow physiological processes dependent upon a fluid lipid structure to be maintained at lower temperatures. However, the works of Siminovitch et al., (1975) and Yoshida and Sakai (1973) gave rise to the hypothesis that major changes in membrane lipid structure could also arise from alterations in the position of esterification of unsaturated fatty acids on the glycerol moiety of complex lipids. This would result in a variation in inter- and intra-molecular mixing of the acyl chains which would alter the membrane fluidity (Phillips et al., 1970). Recently, Murata (1983) demonstrated that this hypothesis might be true. He found that the molecular composition of phosphatidyl glycerols from chilling - sensitive and chilling - resistant plants exposed to low temperature was responsible for imparting variable fluid characteristics to the membranes in which they occurred.

Further, as low temperature has also been shown earlier to stimulate synthesis of certain phospholipids in plants without altering their fatty acids the architectural differences between the polar head groups of phosphatidyl cholines and phosphatidyl ethanolamines assume significant roles in the development of more fluid membranes. (Phillips et al., 1972 ; Michaelson et al., 1974 ; Horvath et al., 1981).

3.3 Membrane functions affected by low temperature-induced changes in membrane structure

3.3.1 Changes in membrane permeability

Alterations in lipid bilayer structure, of the sort discussed above, can affect both passive diffusion and the active transport of compounds through membranes.

3.3.1.1 Passive diffusion

The permeability of liposomes prepared from synthetic lecithin has provided useful data which help to explain functional changes in membranes in terms of an alteration in bilayer structure. Rates of permeation of electrolytes and non-electrolytes were found to be maximum at, or near, the temperature of the transition where fluid - and solid-phase lipids co-exist, and decreased in both the gel and fluid phases below and above this temperature (Blok et al., 1976). Since the order - disorder transition of lipids is accompanied by a decrease in the area per moelcule, it was postulated that pores developed in the bilayer at the transition temperature and the number and lifetime of these pores was dependent upon the fatty acid chain length of the lipids (Blok et al., 1976).

Murata and Fork (1977) demonstrated that the diffusion of ions through thylakoid membranes is affected by the physical state of the membrane lipids, and that this can be varied by altering the growth temperature of the plant. Nobel (1974) found that the reflection coefficient, a measure of the osmotic response of a solute compared to

sucrose, for erythritol and glycerol was temperature - dependent, increasing with decreasing temperature, ie. membranes becoming more impermeable, for chilling - resistant plants. For chloroplasts of chilling - sensitive plants, however, the reflection coefficient decreased greatly at the temperature of the order - disorder transition of the membrane, about 12^{o}C. Below 12^{o}C membrane of the chilling - sensitive plants were more permeable than those of chilling - resistant plants (Nobel, 1974). The swelling of chloroplasts of chilling - sensitive plants, when plants are maintained in the light at 10^{o}C (Taylor and Craig, 1971), is also consistent with an alteration in the permeability properties at temperature near that of the order - disorder transition. Thus, it is clear that low temperature - induced changes in membrane lipids can influence the permeability passive diffusion properties of the cell membranes.

3.3.1.2 Active transport

In assessing changes in the functional aspects of active transport processes linked to structural changes in membrane lipids it is necessary to distinguish between changes in the actual mechanism of transport and apparent changes in the rate because of an alteration in the rate of ATP production. For example, Arrhenius - type plots of the rate of rubidium uptake and of respiration of excised corn roots show increases in slope below 10^{o}C. In this case the change in Ea of transport might be a reflection of a change in the Ea of respiration (Carrey and Berry, 1978). In contrast, excised roots of barley (a chilling-tolerant plant) show no increase in the Ea of respiration at low temperatures, but the Ea for rubidium uptake increases below about 12^{o}C (Carey and Berry, 1978). Since the membrane lipids of chilling -

tolerant plants do not undergo any order - disorder transition in the range of $0^{o}C$ - $30^{o}C$ (Raison et al., 1977), the results suggest that temperature has a direct effect on the transport enzyme and that the change in the Ea observed in both plants is not mediated by a change in membrane lipid structure.

3.3.2 Changes in membrane-bound enzyme properties

In addition to determining barrier and permeability properties of cells, membranes also provide an appropriate matrix for many membrane - bound enzyme systems. Many enzymes have been shown to have a specific lipid requirement for optimal activity (Charnock et al., 1973 ; Eytan and Racker, 1977 ; Farias et al., 1975) and some require the membrane to be in a fluid state (Kimelberg and Papahadjopoulos, 1972). Phospholipids have been directly implicated both in supporting the activity of enzymes and in restoring their maximum activities (Hallinan, 1974 ; McMurchie et al., 1983).

Arrhenius plots of the activity of many membrane - bound enzymes are usually biphasic, exhibiting one or more discontinuities in slope. The slope of a particular portion of a curve represents the Ea of the enzyme over that particular temperature range (Raison, 1973). A change in activation energy of the enzyme is usually explained as a consequence of a thermal phase change in lipid components of the membranes (McGlasson and Raison, 1973 ; Raison, 1974 ; Raison and McMurchie, 1974), and recently, also due to lipid - protein interactions (McMurchie et al., 1983).

The temperature at which this abrupt change in Ea of enzyme activity occurs is largely dependent on membrane lipid composition, and the degree of fatty acid unsaturation. This has been verified by studies of thermal behaviour of membrane - bound enzymes in fatty acid auxotrophic microorganisms (EsFahani et al., 1972 ; DeKruyff et al., 1973 ; Hsung et al., 1974). The abolition of a phase change in mitochondrial succinate activity of hibernating squirrel (*Citellus lateralis*) has been observed by Raison and Lyons (1971).

In plants, also, there is ample evidence of low temperature - induced membrane alterations affecting membrane - bound enzyme activity. Mitochondria of wheat plants grown at low temperatures showed marked increased in fatty acid unsaturation, and this was correlated with respiratory activity (Miller et al., 1974 ; Pomeroy et al., 1973). For a number of plants the temperatures for the increase in Ea for succinate oxidase activity of mitochondria and for the photo-reduction of NADP from water, catalyzed by chloroplasts, are coincident with the temperatures for the change in the molecular ordering of the membrane lipids detected by spin labelling (Lyons and Raison, 1970 ; Raison et al., 1971 ; Shneyour et al., 1973 ; Chapman et al., 1979). For membranes of chilling - tolerant plants, which do not exhibit changes in molecular ordering of lipids, the Ea for reactions catalyzed by their associated enzyme systems is constant between $0^{o}C$ and $30^{o}C$ (Lyons and Raison, 1970 ; Shneyour et al., 1973). The increase in Ea of succinate oxidase activity was readily abolished by treating mitochondra of chilling - sensitive plants with detergents which abolish the phase transition (Raison et al., 1971). The changes in Ea were, therefore, considered a result of an alteration in the conformation of the active

site induced by the changes in molecular ordering of membrane lipids brought about by low temperature.

3.4 Interaction between low temperature exposure and GA_3 responses

There is ample evidence to indicate that there is a definite interaction between the effects of low temperature exposure of plant systems, and their GA_3 response. An excellent example is the effect of GA_3 on growth of grasses at low temperatures. Much of this work had been aimed at increasing the growth of pasture species during periods of low temperature (winter, early spring) when growth of the pasture is very slow and very little is available for stock to feed on. GA_3 is successful in stimulating low temperature growth of *Poa spp.* (Leben and Barton, 1957 ; Leben et al., 1959), *Lolium* spp. (Wittwer and Bukovac, 1957), *Phalaris spp.* (Scurfield and Bull, 1958 ; Williams and Arnold, 1964), *Paspalum dilatatum* (Lester and Carter, 1970), *Oryza spp.* (Takahashi et al., 1973).

Pangola grass (*Digitaria decumbens*) exposed to $10^{o}C$ night temperatures had increased starch contents and decreased amylolytic activities, dry weights, leaf areas and shoot lengths as compared with control plants at $30^{o}C$ night temperature (Karbassi et al., 1971). However, GA_3 treatment of the $10^{o}C$ plants reversed the effects of the low night temperature, increasing starch-degrading enzyme activity to the level found in $30^{o}C$ untreated controls (Carter et al., 1973). Applegate (1958) found the GA_3 sprayed onto the ornamental *Zinnia elegans* produced a greater percentage increase in stem elongation at lower growth temperatures than at higher ones. Tomato (*Lycopersicon*

esculentum) plants grown at low soil temperature and treated with GA_3 responded with significant increases in stem length over and above untreated plants at high soil temperature (Menhenett and Wareing, 1975).

More evidence along these lines comes from the works of Nakamura and Shibaoka (1980) and Jusaitis et al., (1982). Nakamura and Shibaoka showed that epicotyl segments cut from azuki bean (*Vigna angularis*) seedlings grown at 27°C for 5 days and then treated at 15°C for 2 days displayed an increased response to GA_3 over epicotyl segments which did not receive any 15°C treatment. Similarly, Jusaitis et al., found an alteration of the growth response of *Avena* stem segments to GA_3 as a result of low temperature treatment.

In addition to the above mentioned GA_3 - low temperature interactions, low temperature has been shown to alter numerous other physiological responses of plant tissues ; responses in which the involvement of GA_3 is probable, albeit not completely clear. These are, stratification of seeds, breaking of dormancy in buds of deciduous trees, induction of flower-bud opening, vernalization, flower initiation, sex determination of maize flowers, flower unfolding, stimulation of spores or gametes in algae, fungi and liverworts. (Precht et al., 1973 and references mentioned therein).

Work carried out earlier by Jusaitis et al., (1981a,b, 1982), with *Avena* stem segments was strongly suggestive of a membrane based site of GA_3 action and indicated that certain membrane lipid components were strongly correlated with the growth response of *Avena* stem segments to GA_3. The work embodied in this thesis examines the possibility of a membrane-based site of GA_3 action using the more attractive aleurone

system which is non-photosynthetic, non-meristamatic and non-growing. Further, the possiblity that certain membrane lipid components might be correlated with the GA_3 - response of the aleurone tissue was also examined.

II. MATERIALS AND METHODS

1. Materials

1.1 Plant Materials

Seed material and their sources are listed below.

Seed material (Wheat variety)		Source
Minister Dwarf and Tom Thumb	:	Dr. Rod King, Division of Plant Ind. CSIRO, Canberra.
Tordo, Kite, Aroona, NaiNari 60, Olympic and Halbred	:	Prof. C.J. Driscoll, Dept. of Agronomy Waite Agricultural Research Inst. Adelaide.
Isogenic lines (F_6) of a Minister Dwarf x Capelle Desprez Cross	:	Dr. Michael Gale, Plant Breed Institute, Trumpington, Cambridge.

1.2 Chemicals and reagents

All chemical and reagents used were AR grade. Suppliers are listed below.

Amylose	:	Sigma Chemical Co., Missouri, U.S.A.
BASF 13-338	:	BASF Aktiengesellschaft, FR6
Berberine hydrochloride	:	Sigma Chemical Co., Missouri, U.S.A.
α-Cholestane	:	Sigma Chemical Co., Missouri, U.S.A.
Cholesterol	:	British Drug houses, Poole, England.
Cholesterol linoleate	:	Eastman Kodak Chemical Co., New York, U.S.A.
DEGS (15%) on Chromosorb W-AW (80/100 mesh)	:	Alltech Associates, New South Wales, Australia.
Diclofop-Methyl	:	Hoechst Ltd., Frankfurt.
Fatty acid standards (methyl esters) for G.L.C.	:	Applied Science Laboratories, Pennsylvania, U.S.A.
GA_3	:	Abbot Chemicals, U.S.A.

Gaschrom-Q	:	Applied Science Laboratories, Pennsylvania, U.S.A.
Gammexane	:	Agchem Ltd., Adelaide.
HMDS	:	Applied Science Laboratories, Pennsylvania, U.S.A.
Lanosterol	:	Mann Research Laboratories, N.Y., U.S.A.
Methyl Heptadecanoate	:	Applied Science Laboratories, Pennsylvania, U.S.A.
OV-101	:	Applied Science Laboratories, Pennslvania, U.S.A.
Phospholipid Standards for TLC	:	Sigma Chemical Co., Missouri, U.S.A.
Sephadex G25	:	Pharmacia, Uppsala, Sweden.
Silica Gel G	:	Merck and Co., New Jersey, U.S.A.
Silicic acid	:	Bio-Rad Laboratories, California.
Sk&f 7997-A_3	:	Smith Kline and French Labs, Philadelphia, U.S.A.

β-Sitosterol (with 33% Campesterol)	:	Calbiochem, California, U.S.A.
Stigmasterol	:	Sigma Chemical Co., Missouri, U.S.A.
Trifluoperazine	:	Sigma Chemical Co., Missouri, U.S.A.

All cylinders of gas were purchased from C.I.G., Australia.

1.3 Solvents

All solvents used for extraction procedures and for chromatography were distilled in a glass fractionating column before use and were stored in the dark.

2. METHODS

2.1 Experiments involving de-embryonated seed

All seed were de-embryonated by dissecting the embryo prior to the start of experimentation. The de-embryonated seed were surface sterilized by soaking for 7 minutes in a 50% solution of NaOCl (4% w/v available chlorine). After copious washings with distilled water (10 x 100 ml) the de-embryonated seed were aseptically transferred to a large sterile petri dish containing 2 sheets of filter paper and 10 ml of sterile distilled water and were allowed to imbibe for 12 hours at 30°C unless stated otherwise. All subsequent operations were carried out in a laminar flow cabinet with all equipment sterilized by autoclaving for 20 minutes at 15 psi. All solutions were sterilized by passing through a millipore filter (0.22 µm). Ten de-embryonated seed (unless stated otherwise) were pre-incubated in 125 ml conical flasks containing 5 ml of distilled water and 10 mg/l streptomycin. Pre-incubation for various lengths of time at various temperatures was carried out in a water bath shaking at 50 O.p.m. At the end of the pre-incubation period the ambient solution was poured off and replaced with 5 ml of 20 mM $Ca(NO_3)_2$ with different dosages of GA_3. The flasks were then returned to the water bath and the tissue was further incubated for a period of 24 hours at 30°C. At the end of this period the response of the de-embryonated seed to GA_3 was measured by assaying the α-amylase produced.

When lipid analyses were required, batches of 30 de-embryonated seed were pre-incubated at 5°C or 30°C for various lengths of time. At the end of the pre-incubation period, 10 de-embryonated seed were

incubated with GA_3 according to procedures described above and α-amylase produced measured. Aleurone layers were dissected from the other 20 de-embryonated seed and used for lipid analysis. Dissection of the aleurone layers was carried out with the de-embryonated seed soaking in ice water using a fine spatula and forceps. After removal of the endosperm, the aleurone layers (consisting also of the metabolically dead pericarp and testa) were agitated vigorously on a magnetic stirrer for 2 minutes to wash off any adhering starch granules and were immediately frozen in liquid N_2 and freeze-dried. Where dry seed were involved, the aleurone layers were dissected from de-embryonated seed after boiling them for 5 minutes.

2.2 Experiments with isolated aleurone layers

Aleurone layers were isolated from de-embryonated, imbibed seed essentially according to the method of Gibson and Paleg (1972). A total of 15g (dry wt) of de-embryonated seed were used as starting material for each rolling and they were allowed to imbibe water for 24 hours at 30°C. Imbibed de-embryonated seed were transferred to a roller mill with 80 ml of 5 mM lactic acid and rolled for 3 minutes. The roller mill consisted of a large screw top jar, diameter 12 cm, and an inner polythene bottle filled with fine dry sand, diameter 7 cm, weight 900g, and the glass jar was rotated by means of two motor driven rollers at a speed of 50 revolutions per minute. The resulting slurry was filtered through a sieve (2mM mesh) and the tissue returned to the mill with 70 ml distilled water. The tissue was rolled for a further 2 minutes. At the end of this time the polythene bottle was removed and the tissue shaken vigorously with 80 ml of distilled water. The slurry was then filtered through the mesh as before. The tissue was returned to the mill

for a further 1 minute roll in distilled water and the tissue shaken and filtered as before. Finally, the tissue was washed on the metal sieve with copious amounts of distilled water and allowed to drain between many layers of filter paper for 5 minutes. The aleurone tissue was weighed and 1g samples transferred to 125 ml incubation flaskes for pre-incubation. All procedures were carried out in a laminar flow steril chamber with all equipment and solutions sterilized by autoclaving for 20 minutes at 15 psi. Pre-incubation and subsequent incubation procedures were as described earlier (Methods 2.1). Where lipid analysis of the aleurone tissue was required, batches of 20 isolated aleurone layers were appropriately pre-incubated and dropped into liquid N_2, freeze-dried and used for lipid extraction and analysis.

Aleurone layers were also isolated by dissection from imbibed de-embryonated seed in cases where the starting seed material was scarce. This was done in the case of Minister dwarf and the dwarf selection. The dissection was carried out in the laminar flow sterile chamber and the aleurone layers removed from imbibed, de-embryonated seed. After copious washing with sterile distilled water the isolated aleurone layers were subjected to further pre-incubation and incubations as described earlier (Methods 2.1).

2.3 Measurement of α-amylase activity

At the end of the 24 hour incubation period, the ambient fluid was decanted and the aleurone layers/de-embryonated seed were homogenized with an Ultra turrax in 7 ml of grinding medium containing 0.1M NaCl and 0.02 M calcium acetate. The homogenate was centrifuged at 3,000 g for 10 minutes and the supernatant was combined with the ambient solution from

the incubation flask. This extract was assayed for α-amylase activity according to the method of Gibson and Paleg (1975). Prior to assay, all solutions were heated at 70°C for 20 minutes to inactivate any β-amylase present (Paleg, 1960). The reaction mixture contained 5 ml of substrate and 5 ml of enzyme solution. The substrate was prepared by dissolving 0.201 g amylose in a small amount of ethanol and adding this slurry to a boiling mixture containing 60 ml $CaCl_2$ (0.01 M), 15 ml 16.32% KH_2PO_4 and 225 ml water. The IKI stock solution contained 0.6 g iodine and 6.0 g KI made up to 100 ml with distilled water. For use in the enzyme assay, 2.5 ml of the IKI stock solution was made up to 100 ml with distilled water.

Each enzyme solution was made up to 10 ml before starting the assays. Firstly, each enzyme solution to be tested was diluted with 5mM calcium acetate/50 mM KH_2PO_4 solution so that the reaction was about 50% complete in 6-7 minutes at 30°C. Into each of two tubes containing 1 ml of diluted IKI was added 0.5 ml of the diluted enzyme solution. Into one of the tubes, 0.5 ml of substrate was added and this became the zero time sample, while the other tube was made up to the same volume with water. The latter tube was used as the zero absorbance on the spectrophotometer. The substrate and the diluted enzyme solution were equilibrated at 30°C and 5 ml of each were mixed at zero time. At two minute intervals up to 10 minutes, 1 ml samples were added to 1 ml of diluted IKI solution. The sample tubes were then diluted with 5 ml of distilled water and the O.D. of each sample was determined in a Cecil spectrophotometer Model CE303 at 620 nm.

α-amylase activity is expressed as SIC (starch-iodine colour) units calculated according to the method of Briggs (1967). The O.D. data obtained spectrophotometrically were plotted against time and the time

taken for 50% of the initial O.D. to be reached was calculated from the graph (T_{50}). The reciprocal of this value (i.e. $1/T_{50}$) gave SIC units per 0.5 ml of diluted enzyme. Multiplication of this value by the total volume of the enzyme solution (10 ml), and by any dilution factors, gave total enzyme units.

2.4 Extraction and analysis of lipids

2.4.1 Lipid extractions

Lipids were extracted from weighed, freeze-dried aleurone layers using 3 x 5 ml hot, water-saturated butan-1-ol (Varty and Laidman, 1976). Filtrates from extractions were combined and evaporated to dryness at 50°C in a rotary evaporator. Atmospheric pressure was reinstated with N_2 and the residue was taken up in chloroform : methanol (2:1) and partitioned with 0.05% (W/v) $CaCl_2$. The aqueous phase was kept for the analysis of sterylglycosides and the chloroform phase was taken to dryness under vacuum; the residue was taken up in a small volume of chloroform:methanol (19:1). Water (10 drops) was then added to the chloroform:methanol mixture which was reduced once more to dryness in order to break lipoprotein associations. The residue was again taken up in a small volume of chloroform:methanol (19:1) and purified by partition chromatography on Sephadex G-25.

The lipid fraction from the column was evaported to dryness under N_2 and the last traces of solvent were removed under vacuum. Finally, the lipid was taken up in 5 ml of chloroform and stored under N_2 at -20°C for further analysis. Every effort was made to carry out as many

steps as possible under N_2 atmosphere in the cold and as quickly as possible to minimise lipid oxidation or degradation.

2.4.2 Fractionation of total lipid

The total lipid fraction obtained above was fractionated into neutral lipids, glycolipids and phospholipids by silicic acid column chromatography. Silicic acid (Bio-Rad laboratories Minus 325 mesh) was heated at 120°C for 2 hours before use and packed in a glass column 1.8 cm. (internal diameter) by 40 cm long. Neutral lipids were eluted with chloroform, glycolipids with acetone and phospholipids with methanol.

2.4.3 Separation of lipid fractions into classes

Lipid fractions obtained from silicic acid columns were further separated into individual classes by thin layer chromatography (TLC). For all TLC, 20 x 20 cm glass plates were coated with a 0.3 mm thick layer of silica gel G using a Shandon chromatogram spreader. Plates were allowed to air-dry before being activated at 110°C for 12 hours. Plates were then cooled to room temperature and stored in a dessicator and could be used without re-activation for 4 days. Whenever reactivation was required plates were heated for 1 hour at 110°C before use. Spotting of standards and extracts was performed under a stream of N_2.

Plates were developed in Shandon glass chromatography tanks sealed with ground glass covers, and with solvent-saturated chromatography paper lining the sides of the tank to ensure a vapour-saturated atmosphere. Plates were allowed to equilibrate with the atmosphere within the tank for 5 minutes prior to lowering into the solvent. The solvent-run in all cases was 15 cm.

2.4.3.1 TLC of phospholipids

The phospholipid fractions (Methods 2.4.2) was concentrated and applied to TLC plates which were developed in chloroform: methanol:water (14:6:1.8 v/v/v) (Jusaitis et al., 1981a). The plates were dried under N_2, and bands were visualized by spraying with 0.05% (w/v) berberine-HCl and examining under UV fluorescence. Phospholipid classes were identified by co-chromatography with authentic standards, and by comparison of R_F values with documented values for the same solvent system (Spanner, 1973).

2.4.3.2 TLC of neutral lipids

The neutral lipid fraction was separated into free sterols, free fatty acids, triglycerides and steryl esters on TLC plates developed in n-hexane: diethyl ether: acetic acid (83:16:1 v/v/v). Lipid bands and standard markers were developed on the same plates and were located by spraying with 0.05% (w/v) berberine-HCl and examining under UV fluorescence.

2.4.4 Quantification of lipid classes

All lipid classes were quantified by gas liquid chromatography (GLC) using a Hewlett-Packard HP-5840A gas chromatograph.

2.4.4.1 Quantification of phospholipid and free fatty acids

Phospholipid classes and free fatty acids were quantified on the basis of the fatty acid content, derivatized as fatty acid methyl esters.

The band(s) of silica gel containing the phospholipids or free fatty acids was scraped from the plate into a screw-capped glass vial(s) containing 5 ml of 5% H_2SO_4 in methanol. To this was added 20 ml of methyl-heptadecanoate (1 mg ml^{-1}) as an internal standard. The vials were flushed with N_2, then sealed with teflon-lined screw caps, and the reaction allowed to proceed for 3 hours at 70°C. The tubes were agitated at approximately half hourly intervals, and after 3 hours were cooled to room temperature. Fatty acid methyl esters were extracted by adding water (5 ml) and 5 ml petroleum ether (40-70°C) and shaking vigorously. The upper petroleum ether layer was removed into another vial, to which water (2 ml) was added, and again shaken vigorously. The lower water layer was removed and discarded. Anhydrous sodium sulphate (1-2g) was added to the petroleum ether and the mixture was again shaken. The washed, dried petroleum ether containing the fatty acid methyl esters was decanted into another vial for storage under nitrogen until required for GLC analysis.

2.4.4.1.1 GLC of fatty acid methyl esters

Methyl esters of fatty acids were subjected to GLC on 15% (w/w) DEGS on chromsorb W(AW) (80-100 mesh) in glass columns (2 mm i.d. x 1.84 m). Before packing, glass columns and glass wood used for plugging the ends of columns were silanized by rinsing three times with 3% (v/v)

hexamethyldisilazane in toulene, and heating to 60°C for 15 minutes between rinses.

Columns were packed by plugging one end with silanized glass wool, then connecting the plugged end to a vacuum pump. The precoated packing material was loaded via a glass funnel connected to the other end of the column. To ensure firm and even packing, the column was tapped throughout the loading process. When packing was complete, the other end was plugged with silanized glass wood.

Before using, a freshly packed column was conditioned at 185°C for 4 hours with the carrier gas (N_2) after which the column was disconnected from the detector and held at 180°C overnight without N_2.

2.4.4.1.2 **Operating Conditions**

The GLC was equipped with a flame ionization detector. Temperature conditions were as follows: Column = 175°C isothermal, detector = 270°C and injection port = 170°C. N_2 was used as a carrier gas at a flow rate of 15 ml min^{-1} at 44 p.s.i. Hydrogen at 40 ml min^{-1} at 15 p.s.i. and medical air at 120 ml min^{-1} at 44 p.s.i. were used for the flame ionization detector. Ethyl acetate was the solvent used for injecting fatty acid methyl esters (FAMES).

FAMES were identified by comparing their retention times with those of pure methyl ester standards. Peak areas were measured automatically using a keyboard - controlled digital processor with built-in integration capability. Weights of different FAMES present in the sample were calculated by internal standardization using methyl heptadecanoate as internal standard.

2.4.4.2 Quantification of sterols

The 4-desmethylsterol (Free sterol) and steryl ester regions were scraped separately from the TLC plates into screw-capped glass tubes. Sterols and steryl esters were extracted from the silica gel with several washes of ethyl acetate (3 x 5 ml) and filtering through Whatman N 0.1 filter paper. The combined filtrates were collected in teflon-lined screw-capped glass vials. The 4-desmethylsterol extract was evaporated to dryness at 50°C under vacuum and the residue dissolved in 100 µ l of ethylacetate containing 1 mg/ml of α-cholestane as internal standard, ready for GLC analysis.

The steryl ester extract was also evaporated to dryness and then saponified with 2 ml of 30% (w/v) KOH in 80% aqueous methanol for 1 hour at 60°C. The saponified mixture wad diluted with 2 ml of deionized water and the sterols extracted with petroleum spirit (3 x 5 ml). The combined extract was evaporated to dryness under vacuum and the residue redissolved in 100 µl of ethyl acetate, with 1 mg/ml of α-cholestane as internal standard, ready for GLC of sterols derived from steryl esters.

The aqueous phase obtained from $CaCl_2$ - partitioning of the original extract was taken to dryness in vacuum (60°C) and the residue was hydrolysed with 2 ml of 0.5% (v/v) H_2SO_2 in 95% aqueous ethanol for 12 hours at 60°C. After dilution of the acidic mixture with 2 ml of deionized water the suspension was filtered and extracted with diethyl ether (3 x 5 ml) to extract sterols. The combined ether extracts were washed with water (2 x 1 ml), dried over anhydrous sodium sulphate and filtered.

After removal of the ether under vacuum the residue was redissolved in 100 µl of ethyl acetate with 1 mg/ml α-cholestane as internal standard, ready for GLC of sterols derived from steryl glycosides.

2.4.4.2.1 **GLC of sterols**

Sterols were subjected to GLC analysis on 1.5% (w/w) OV101 on gaschrom Q (100-120 mesh) in glass columns (2 mm i.d. x 1.84 m).

Columns were packed according to procedures described earlier (Methods 2.4.4.1.1) and were conditioned at 260°C for 4 hours with the carrier gas (N_2); the columns were disconnected from the detector and incubated at 250°C overnight without N_2 flush.

2.4.4.2.2 **Operating conditions**

The GLC was equipped with a flame ionization detector. Temperature conditions were as follows: column = 240°C isothermal, detector = 280°C, and injection port = 235°C. N_2 was used as the carrier gas at a flow rate of 15 ml min^{-1} at 44 p.s.i. Hydrogen was used at 40 ml min^{-1} at 15 p.s.i. and medical air at 120 ml min^{-1} at 44 p.s.i. for the flame ionization detector. Ethyl acetate was the solvent used for injecting the sterol mixtures.

Sterols were identified by comparing their retention times with those of authentic sterol standards. Peak areas were measured automatically using a keyboard - controlled digital processor with built-in integration capability. Weights of different sterols present in the sample were calculated by internal standardization using α-cholestane as the internal standard.

2.5 Statistical treatment of data

Lipid data were subjected to analysis of variance as well as multivariate analysis of variance using the Pillai's test statistic, to test the significance of the pre-incubation time and temperature effects, as well as their interaction effects on the fatty acid composition of phospholipids of aleurone tissue. In order to facilitate comparisons, all lipid data in the case of the dwarf and tall selections has been corrected for any dry weight differences in the aleurone tissue of the two lines. The α-amylase data were subjected to analysis of variance to test the significance of pre-incubation time and temperature effects as well as their interaction effects on the GA_3 response of the aleurone tissue. All data are presented as means of three replicates. All statistical analyses were performed using a GENSTAT programme on a Cyber 170 Computer.

III. RESULTS AND DISCUSSION

1. Low temperature-induced alteration of GA_3 - sensitivity of the wheat aleurone system

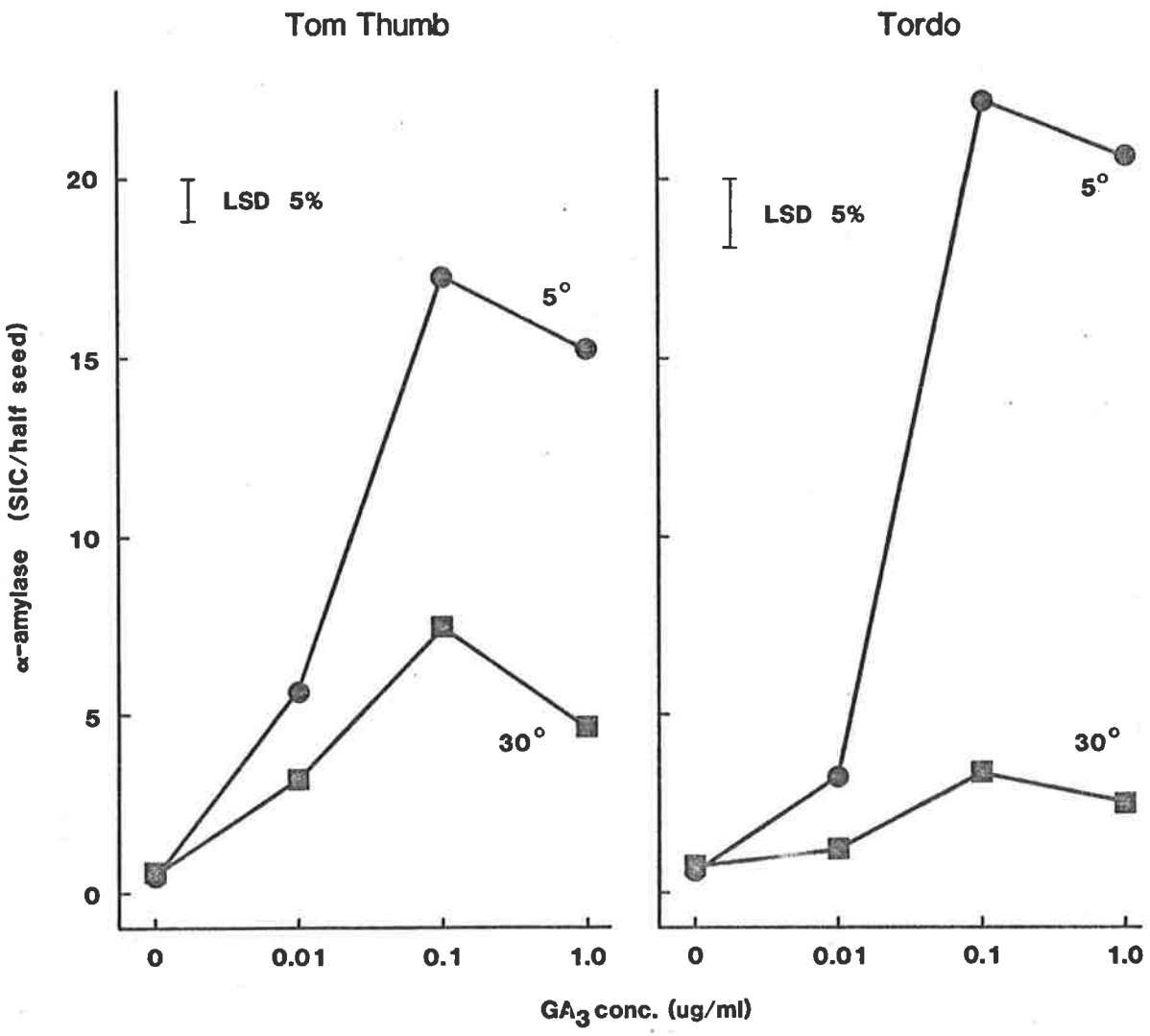
In order to examine the possibility of a membrane-based site of GA_3 action it was decided to examine the feasibility of modulating the GA_3 - sensitivity of the wheat aleurone tissue without adding any chemical agents. The best possibility of doing this was low temperature pre-treatment. This section examines the effects of low temperature pre-incubation on the α-amylase response of the wheat aleurone tissue to GA_3.

1.1 Low temperature-induced increases in GA_3 - sensitivity in wheat aleurone tissue with Rht3 (Tom Thumb) or Rht 1, Rht 2 (Norin 10) dwarfing genes.

Sensitivity of the Rht3 aleurone tissue/de-embryonated seed to GA_3 was monitored by determining the amount of α-amylase produced as a result of 24 hours incubation with different concentrations of exogenous GA_3. GA_3-insensitive seed of Tom Thumb, Tordo and Minister Dwarf, all of which are Rht3 dwarf wheats (Flintham and Gale, 1982 ; Gale and Marshall, 1975; Fick and Qualset, 1975), were used.

A 20 hour pre-incubation at 5^oC, as compared with 30^oC, of de-embryonated seed (designated as half seed in figure legends) of Tom Thumb caused a 2.5-fold increase in the amount of α-amylase detected after a subsequent 24 hour incubation with GA_3, while the increase was 7-fold for de-embryonated seed of Tordo (Figure 2). A similar 3-fold

Figure 2: Effect of 20 hrs pre-incubation at 5°C or 30°C on α-amylase production by de-embryonated seed of Rht3 - containing wheat varieties, Tom Thumb and Terdo.


Tom Thumb
Tordo
LSD 5%
LSD 5%
5°
5°
30°
30°
20
15
10
5
0
0
0.01
0.1
1.0
0
0.01
0.1
1.0
α-amylase (SIC/half seed)
GA3 conc. (ug/ml)

increase was detected for de-embryonated seed of Minister Dwarf (Figure 3a). The levels of α-amylase produced by the de-embryonated seed of Tom Thumb and Minister Dwarf pre-incubated for 20 hours at 30°C (Figures 2, 3a) are essentially the same as those reported in the literature when the two varieties were described as insensitive to GA_3 in terms of their α-amylase response (Gale and Marshall, 1975). Furthermore, low temperature treatment of isolated aleurone tissue produced an equally dramatic increase in GA_3 sensitivity (Figure 3b) and in all cases the magnitude of the low temperature-induced increase in GA_3-sensitivity was GA_3 concentration-dependent (Figures 2, 3). Also, the low temperature pre-incubation had no effect on the α-amylase produced by controls in the absence of GA_3 (Figures 2, 3).

Similar low temperature (5°C) pre-incubation of aleurone tissue/de-embryonated seed of wheats having the Norin 10 dwarfing genes, Rht1 or Rht2, brought about equally dramatic increases in GA_3-sensitivity (Figures 4, 5). The wheat varieties used were Aroona (Rht 1) and Kite (Rht 2). In the case of Aroona a 20 hour 5°C pre-incubation increased the GA_3 response by 1.88-fold and again the magnitude of this increase was GA_3 concentration-dependent. (Figure 4). Lowering the pre-incubation temperture to 20°C did not have any effect on the GA_3 sensitivity of Kite and the first increase in the GA_3 response was detected as a result of a 10°C pre-incubation for 20 hours (Figure 5a). The magnitude of the low temperature-induced increase was GA_3 concentration-dependent and 5°C pre-incubation was more effective than 10°C (Figure 5a). Again, low temperature was able to induce increases in the GA_3 response of aleurone tissue or de-embryonated seed of Kite (Figures 5a, 5b).

Figure 3: Comparison of α-amylase produced by tissues of Rht3 containing wheat variety Minister Dwarf pre-incubated for 20 hrs at different temperatures.

The de-embryonated seed (A) were imbibed for 16 hrs at 30°C prior to pre-incubation. Aleurone layers (B) were dissected from seed which had been imbibed for 16 hrs at 30°C.

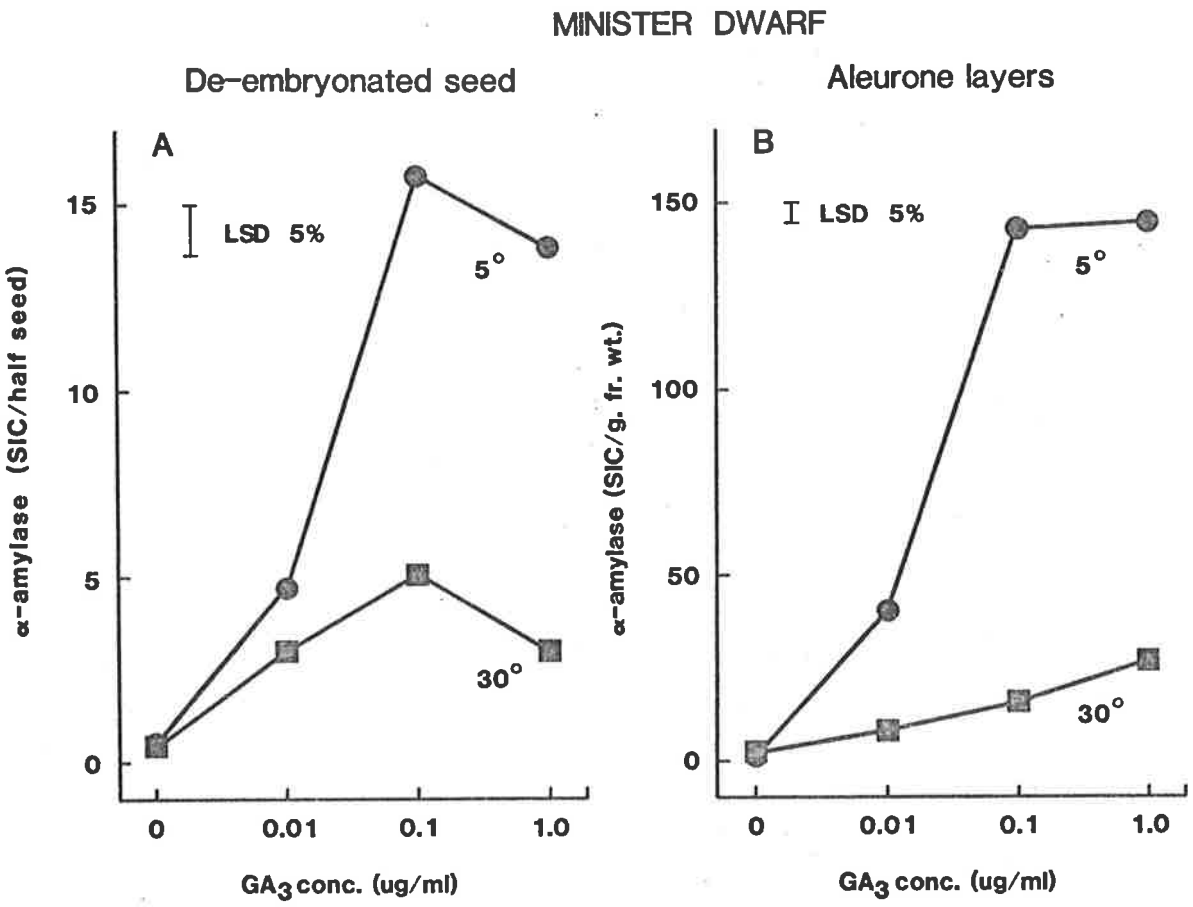

MINISTER DWARF
De-embryonated seed
Aleurone layers
A
B
LSD 5%
LSD 5%
5°
30°
5°
30°
α-amylase (SIC/half seed)
α-amylase (SIC/g. fr. wt.)
15
10
5
0
150
100
50
0
0
0.01
0.1
1.0
0
0.01
0.1
1.0
GA3 conc. (ug/ml)
GA3 conc. (ug/ml)

Figure 4: Effect of 20 hrs pre-incubation at 5°C or 30°C on α - amylase production by de-embryonated seed of Rht1 - containing wheat variety Aroona.

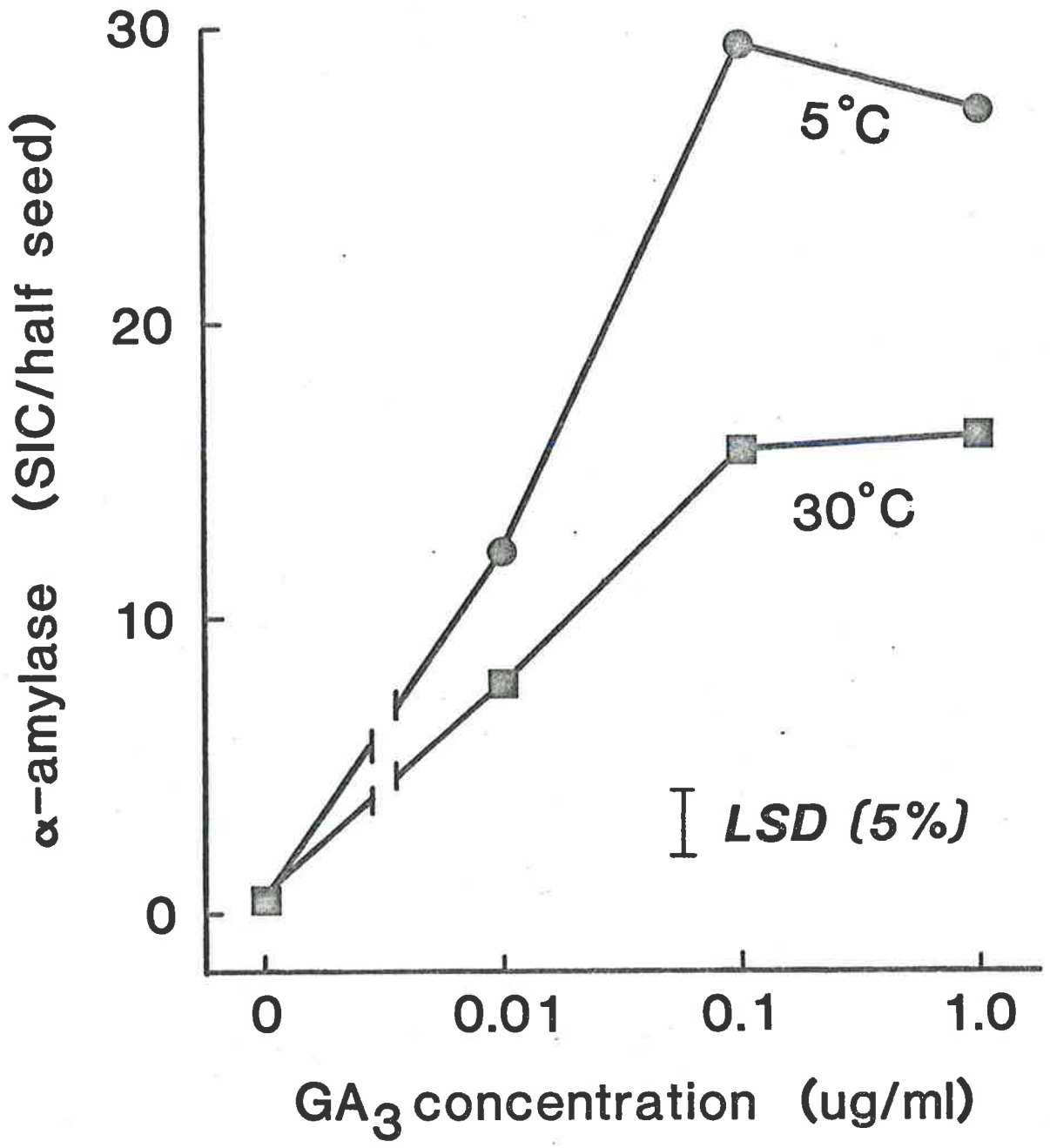
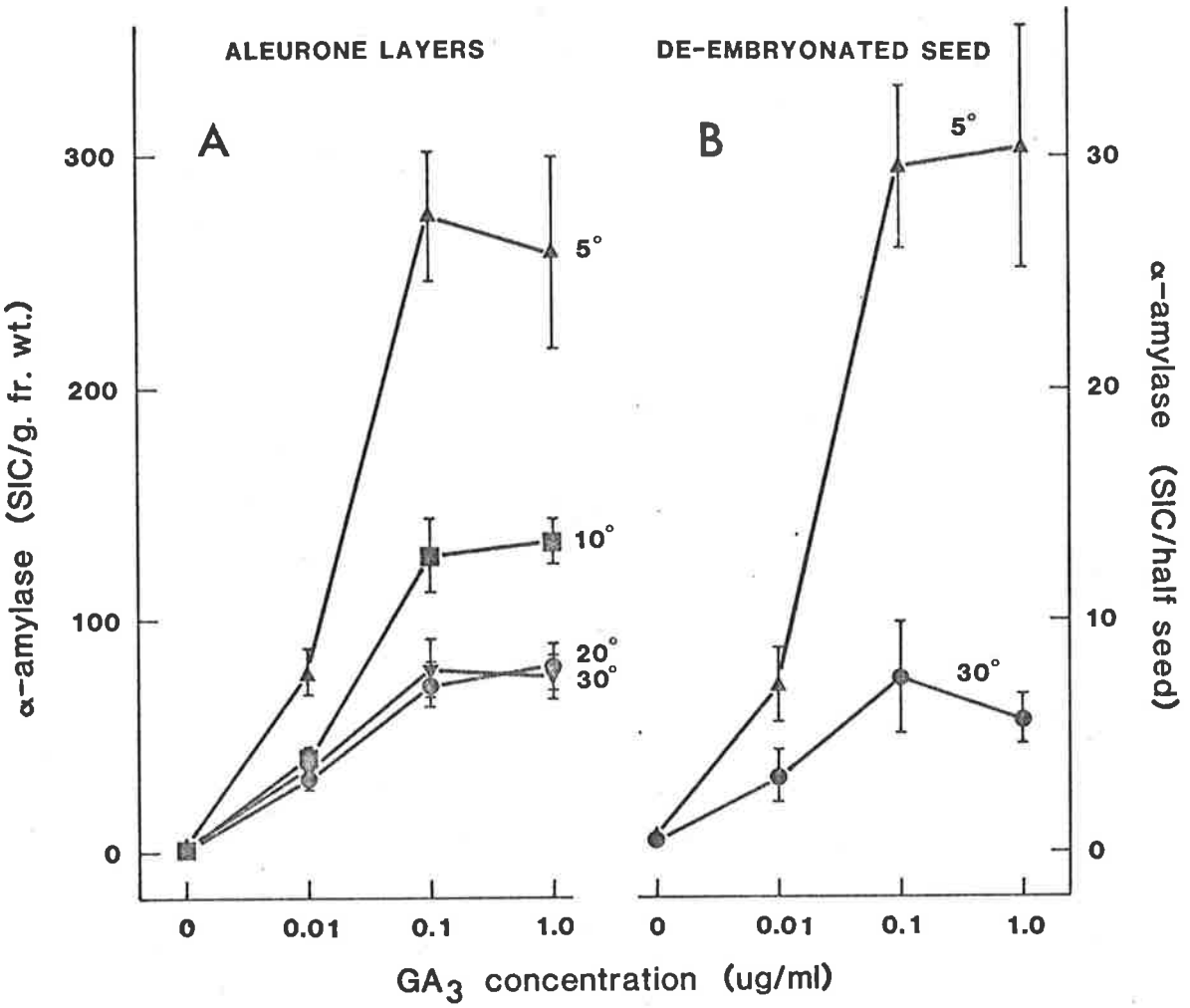

30
20
10
0
α-amylase (SIC/half seed)
5°C
30°C
LSD (5%)
0
0.01
0.1
1.0
GA3 concentration (ug/ml)

Figure 5: Comparison of α-amylase produced by tissues of the Rht2 - containing wheat variety Kite, pre-incubated for 20 hrs at different temperatures.

The aleurone layers (A) were obtained from de-embryonated seed which had been imbibed for 24 hrs at 30°C. The de-embryonated seed (B) were imbibed for 24 hrs at 30°C prior to pre-incubation.


ALEURONE LAYERS
DE-EMBRYONATED SEED
A
B
5°
10°
20°
30°
α-amylase (SIC/g. fr. wt.)
α-amylase (SIC/half seed)
300
200
100
0
30
20
10
0
0
0.01
0.1
1.0
GA3 concentration (ug/ml)

1.2 Characterization of the low temperature effect on the GA_3-sensitivity of aleurone tissue.

The results presented in the previous section (Results 1.1) clearly indicated that it was possible to increase/alter the response to GA_3 of aleurone tissue having one of the dwarfing genes. The results presented in this section characterize in greater detail the low temperature-induced increase in the GA_3 response.

1.2.1 Low temperature-induced increase in GA_3-sensitivity of aleurone tissue of Kite (Rht 2).

Sensitivity to GA_3 of the aleurone tissue of Kite, having the Rht 2 dwarfing gene, pre-incubated at different temperatures (5^oC - 30^oC) and for various lengths of time (0 - 24 hours), was monitored by determining the amount of α-amylase produced as a result of a subsequent 24 hours of incubation at 30^oC with different concentrations of GA_3.

While pre-incubation time or temperature had no effect on the sensitivity of the aleurone tissue in the absence of exogenous GA_3 (Figure 6), there was a definite interaction of pre-incubation time and temperature on the GA_3-sensitivity of Kite aleurone tissue (Figures 7, 8, 9). Low temperature pre-incubation increased the GA_3-sensitivity of the aleurone tissue many fold when compared with pre-incubation at temperatures greater than 10^oC.

The optimum GA_3 concentration (0.1 μ g/ml) for the α-amylase response remained unchanged irrespective of the pre-incubation temperature and time (Figures 7, 8, 9). The largest increase (5.12 fold)

Figure 6: Effect of pre-incubation temperature and time on α - amylase production by isolated aleurone layers of Kite (Rht2) in the absence of GA_3.

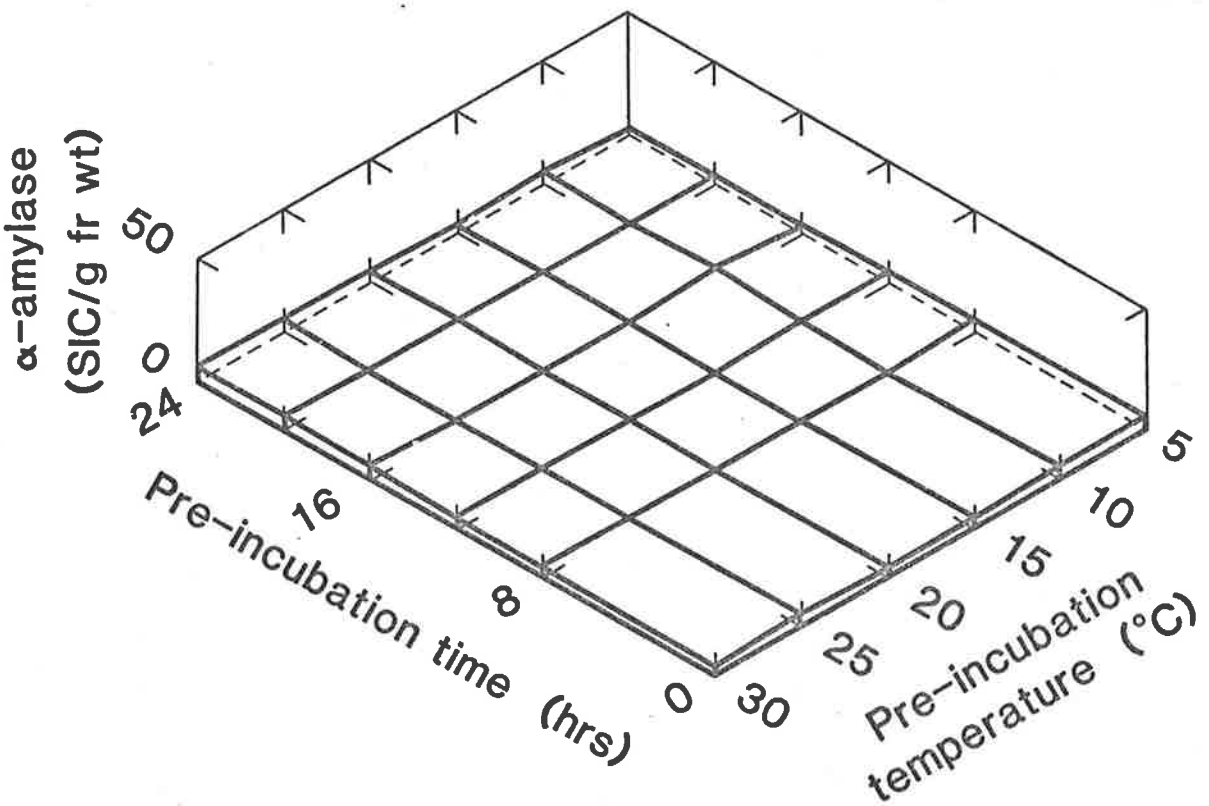

α-amylase
(SIC/g fr wt)
50
0
24
16
8
0
Pre-incubation time (hrs)
5
10
15
20
25
30
Pre-incubation
temperature (°C)

Figure 7: Effect of pre-incubation temperature and time on α - amylase production by isolated aleurone layers of Kite (Rht2) in response to 0.01 μ g/ml GA_3.

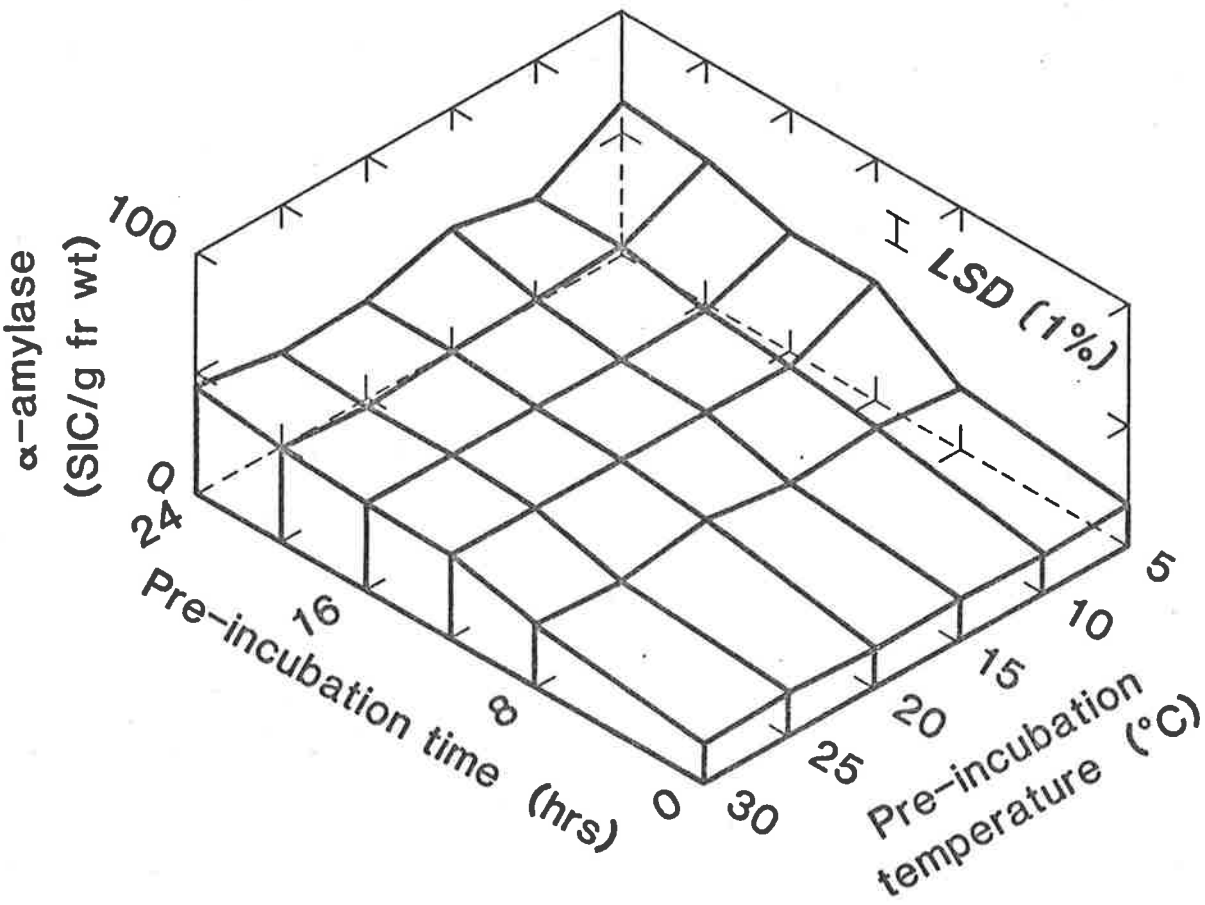

α-amylase
(SIC/g fr wt)
100
0
24
16
8
0
Pre-incubation time (hrs)
30
25
20
15
10
5
Pre-incubation
temperature (°C)
LSD (1%)

Figure 8: Effect of pre-incubation temperature and time on α - amylase production by isolated aleurone layers of Kite (Rht2) in response to 0.1 μ g/ml GA_3.

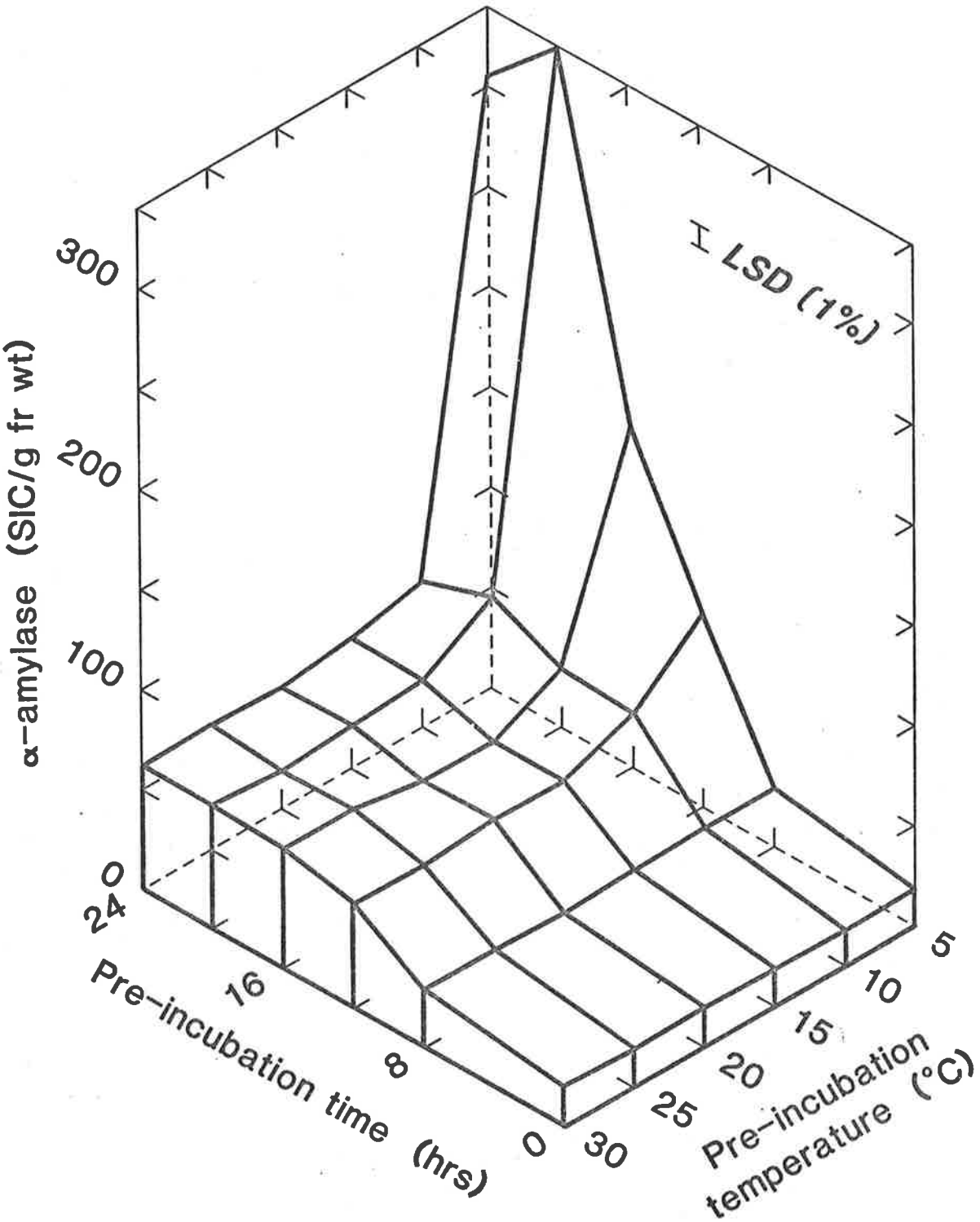
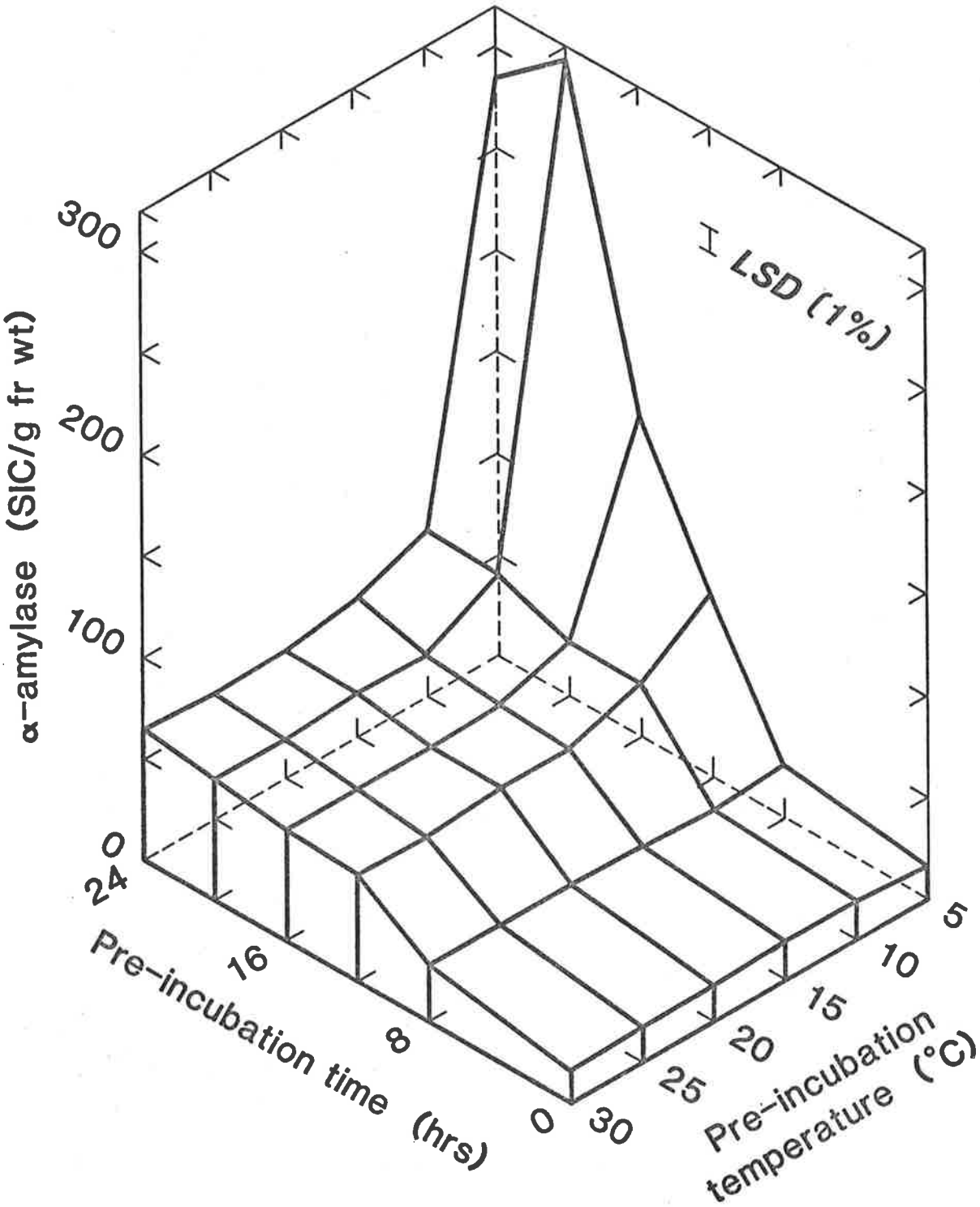

α-amylase (SIC/g fr wt)
300
200
100
0
I LSD (1%)
24
16
8
0
Pre-incubation time (hrs)
30
25
20
15
10
5
Pre-incubation temperature (°C)

Figure 9: Effect of pre-incubation temperature and time on α - amylase production by isolated aleurone layers of Kite (Rht2) in response to 1.0 μ g/ml GA_3.


α-amylase (SIC/g fr wt)
300
200
100
0
LSD (1%)
24
16
8
0
Pre-incubation time (hrs)
5
10
15
20
25
30
Pre-incubation temperature (°C)

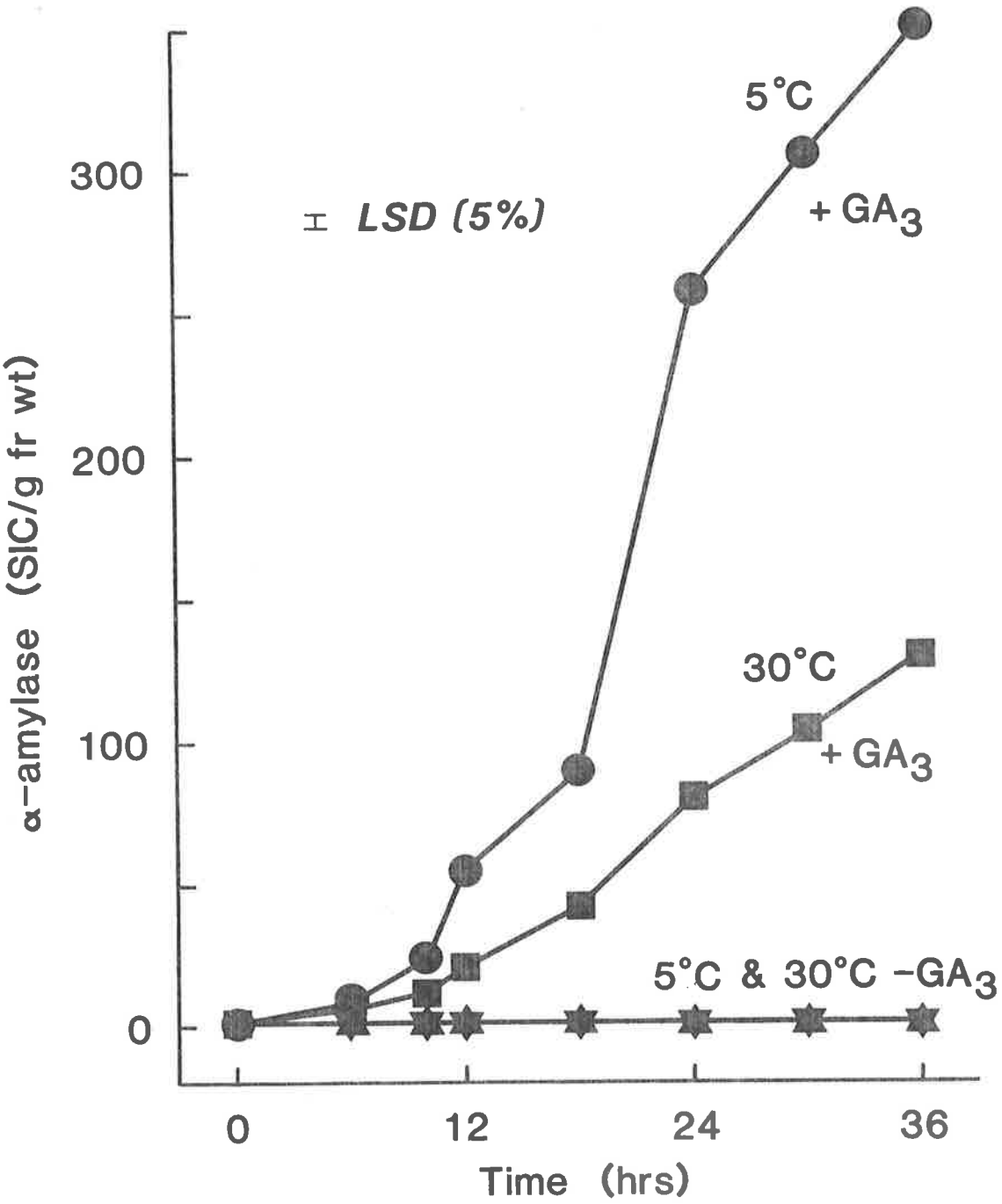
in GA_3-sensitivity was observed as a result of a 20 or 24 hour pre-incubation at 5°C (rather than at temperatures between 15°C and 30°C which are the temperatures usually employed for observing the GA_3 - sensitivity of wheat endosperm (Chrispeels and Varner, 1967; Flintham and Gale, 1982; Gibson and Paleg, 1972). Lowering the pre-incubation temperature from 30°C to 15°C had no effect on the GA_3-sensitivity and the first significant increase occurred at 10°C and 5°C after 12 hours of pre-incubation, when the exogenous GA_3, concentration was 0.1 µg/ml or 1.0 µg/ml (Figures 8 and 9). At a GA_3 concentration of 0.01 µg/ml, the increase in sensitivity was significant only at 5°C after 12 hours pre-incubation, and the low temperature-induced increase at this hormone level was much reduced when compared to that of the higher GA_3 concentrations (Figures 7, 8, 9). The similarity of the effect of low temperature on the GA_3-sensitivity of both the isolated aleurone layers and the de-embryonated seed of Kite, indicated no involvement of the embryo and the endosperm in perceiving or responding to the low temperature-induced GA_3-sensitivity (Figures 5a, 5b).

1.2.1.1 Time course of α-amylase production by isolated aleurone layers of Kite with altered GA_3-sensitivities.

Although pre-incubation for 20 hours at 5°C, as compared with 20 hours at 30°C, increased the GA_3-sensitivity dramatically, it had no effect on the lag-time of α-amylase production (Figure 10). After 6 hours incubation with GA_3, the aleurone tissue pre-incubated at 5°C produced α-amylase at a much higher rate than the tissue pre-incubated at 30°C and this difference in rate was evident even after 36 hours incubation. It can be concluded, therefore, that the increased amount of α-amylase produced by the 5°C - incubated aleurone tissue during the

Figure 10: Time course of α -amylase production by isolated aleurone layers of Kite (Rht2) pre-incubated for 20 hrs at 5°C or 30°C.

α-amylase produced was assayed at the end of incubation periods of different durations ± 0.1 μ g/ml GA_3 at 30°C.


5°C
+ GA3
LSD (5%)
30°C
+ GA3
5°C & 30°C -GA3
α-amylase (SIC/g fr wt)
300
200
100
0
0
12
24
36
Time (hrs)

subsequent 24 hour incubation in GA_3, was due to a higher rate of α-amylase production and not to a shortening of the lag-phase (Figure 10). The lag time of α-amylase production detected in this experiment (Fig. 10), lies in the 4-8 hour range reported earlier in other studies (Chrispeels and Varner, 1967 ; Gibson and Paleg, 1982 ; Goodwin and Carr, 1972a).

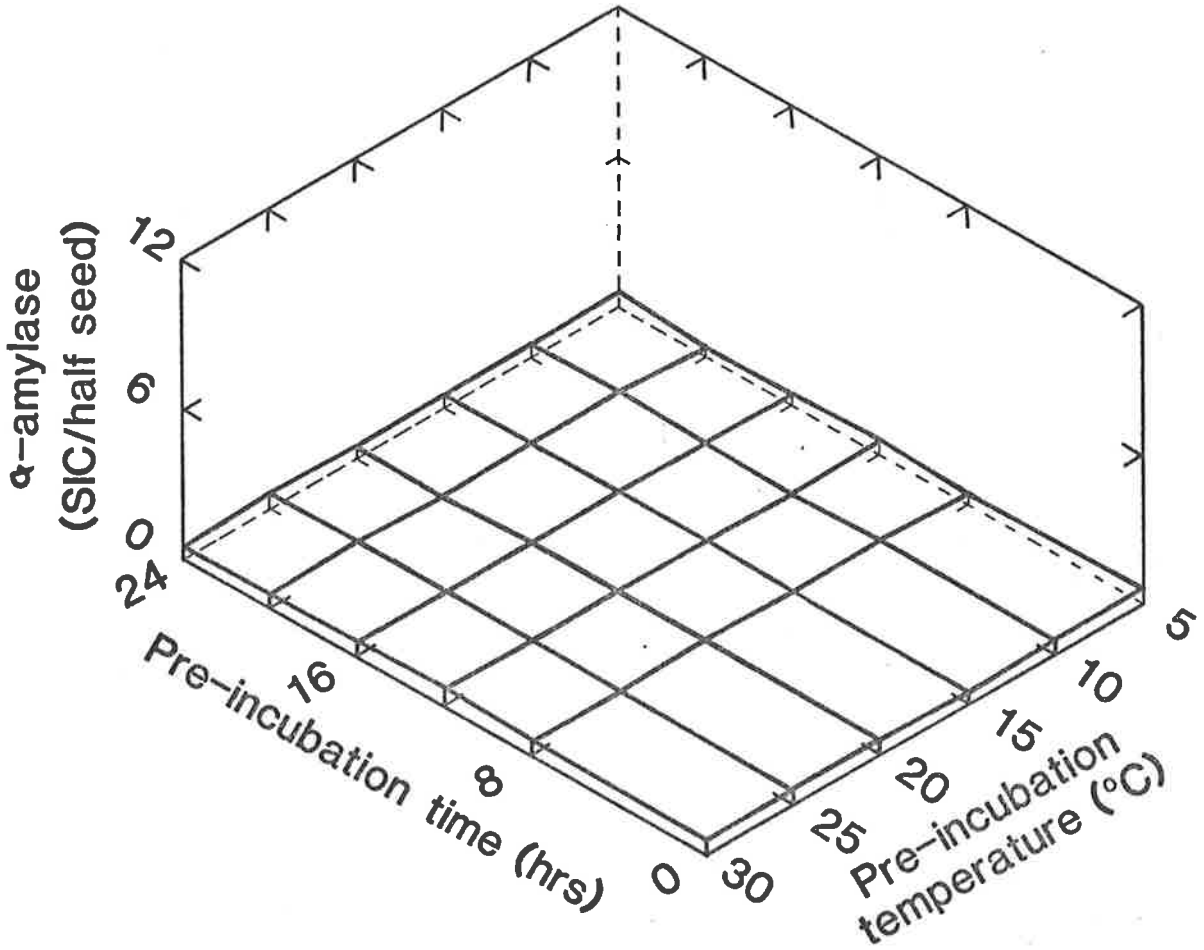
1.2.2 Low temperature effect on the GA_3-sensitivity of de-embryonated seed of F_6 Rht 3/rht 3 isogenic lines.

The isogenic dwarf and tall (F_6 Rht 3/rht 3) lines were obtained from an initial cross between Cappelle-Desprez (rht 3, Tall) x Minister Dwarf (Rht 3, Dwarf) and subsequent segregation and selection until the F_6 generation. A random selection (F_6) of each of the genotypes was used in the following experiments. The dwarf selection has been designated as CAP x MD (Rht 3, dwarf) and the tall selections as CAP x MD (rht3, tall); and both were supplied by Dr. Michael Gale of the Plant Breeding Institude, Cambridge, U.K.

Sensitivity to GA_3 of the de-embryonated seed or aleurone tissue, pre-incubated at different temperatures (5^oC-30^oC) and for various lengths of time (0-24 hours), was monitored by determining the amount of α-amylase produced as a result of 24 hours incubation at 30^oC with different concentrations of exogenous GA_3.

In the case of the dwarf selection CAP x MD (Rht 3), while pre-incubation time or temperature had no effect on the sensitivity of the de-embryonated seed in the absence of exogenous GA_3 (Figure 11), there was a definite interaction of pre-incubation time and temperature on the

Figure 11: Effect of pre-incubation temperature and time on α-amylase production by de-embryonated seed of CAP x MD (Rht3, dwarf) selection in the absence of GA_3.


α-amylase
(SIC/half seed)
12
6
0
24
16
8
0
Pre-incubation time (hrs)
5
10
15
20
25
30
Pre-incubation
temperature (°C)

sensitivity of the aleurone to GA_3 of the de-embryonted seed (Figures 12, 13, 14). Low temperature pre-incubation increased the GA_3-sensitivity of de-embryonated seed of the dwarf selection many-fold when compared with pre-incubation at temperatures greater than 10^oC.

The optimum GA_3 concentration (0.1 µ g/ml) for the α -amylase response remained unchanged irrespective of the pre-incubation temperature and time (Figures 12, 13, 14). The largest increase in GA_3-sensitivity was observed as a result of 20 or 24 hours pre-incubation at 5^oC (rather than at temperatures between 15^oC and 30^oC which are the temperatures usually employed during pre-incubation, for observing the GA_3-sensitivity of wheat endosperm (Ho et al., 1981; Chrispeels and Varner, 1967; Flintham and Gale, 1982; Gibson and Paleg, 1972). Lowering the pre-incubation temperature from 30^oC to 15^oC had no effect on the GA_3-sensitivity and the first significant increase occurred at 10^oC and 5^oC after 16 hours of pre-incubation when the exogenous GA_3 concentration was 0.1 µg/ml or 1.0 µg/ml (Figures 13, 14). At a GA_3 concentration of 0.01 µg/ml, the increase in sensitivity was significant only after 16 hours pre-incubation at 5^oC, and the low temperature-induced increase at this hormone level was much reduced when compared to that at the higher GA_3 concentrations (Figures 12, 13, 14). Furthermore, a comparison of Figures 7, 8, 9 and Figures 12, 13, 14 shows that low temperature pre-incubation had a very similar effect on the GA_3-sensitivities of either Kite (Rht 2) or the dwarf selection (Rht 3).

Similarly, a 20 hour low temperature (5^oC) pre-incubation of isolated aleurone layers of the dwarf selection was able to increase the GA_3-sensitivity many-fold as indicated by increases in α -amylase

Figure 12: Effect of pre-incubation temperature and time on α-amylase production by de-embryonated seed of CAP x MD (Rht3, dwarf) selection in response to 0.01 μg/ml GA_3.

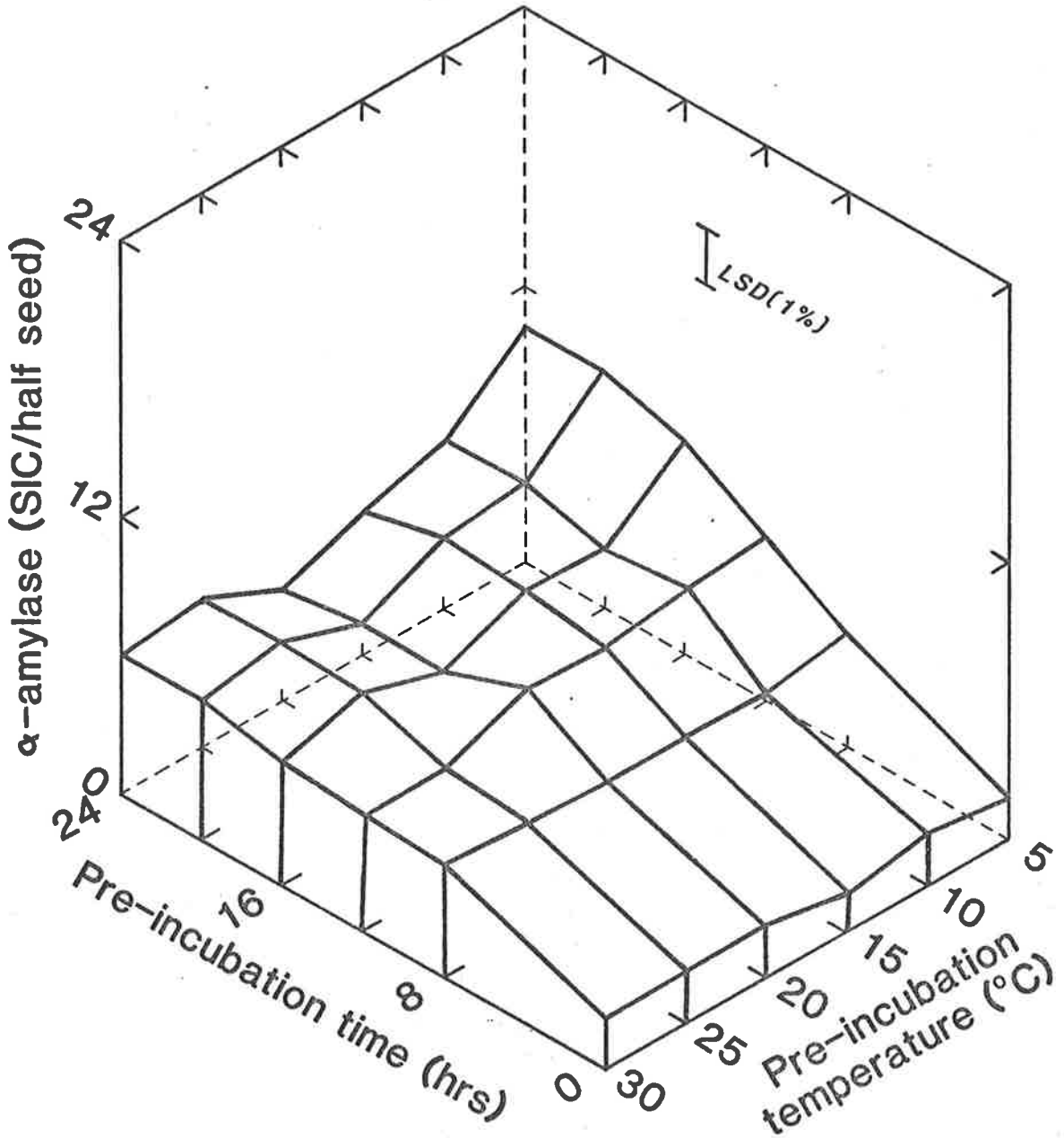

α-amylase (SIC/half seed)
24
12
0
LSD(1%)
Pre-incubation time (hrs)
24
16
8
0
Pre-incubation temperature (°C)
5
10
15
20
25
30

Figure 13: Effect of pre-incubation temperature and time on α - amylase production by de-embryonated seed of CAP x MD (Rht3, dwarf) selection in response to 0.1 μ g/ml GA_3.

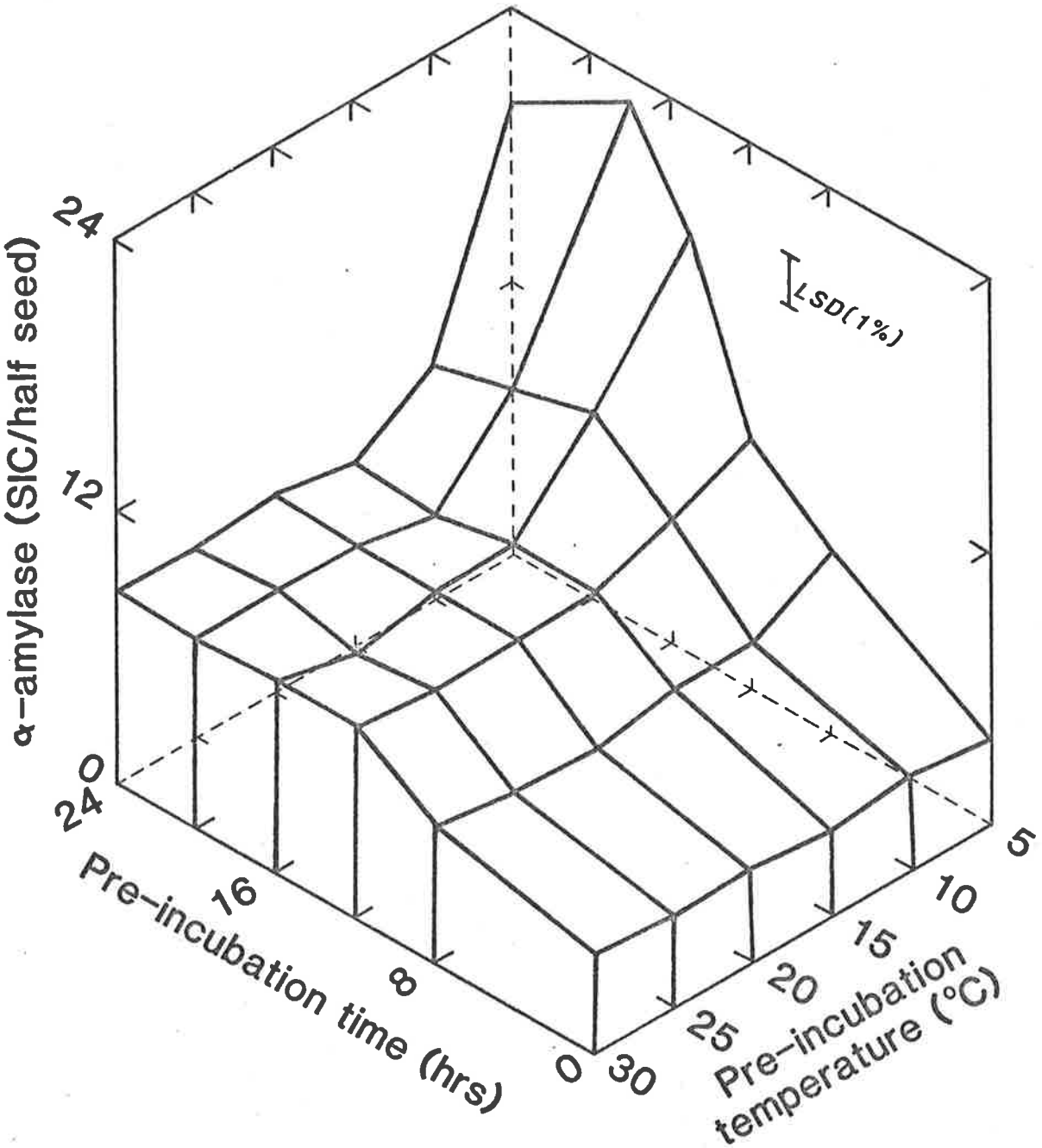
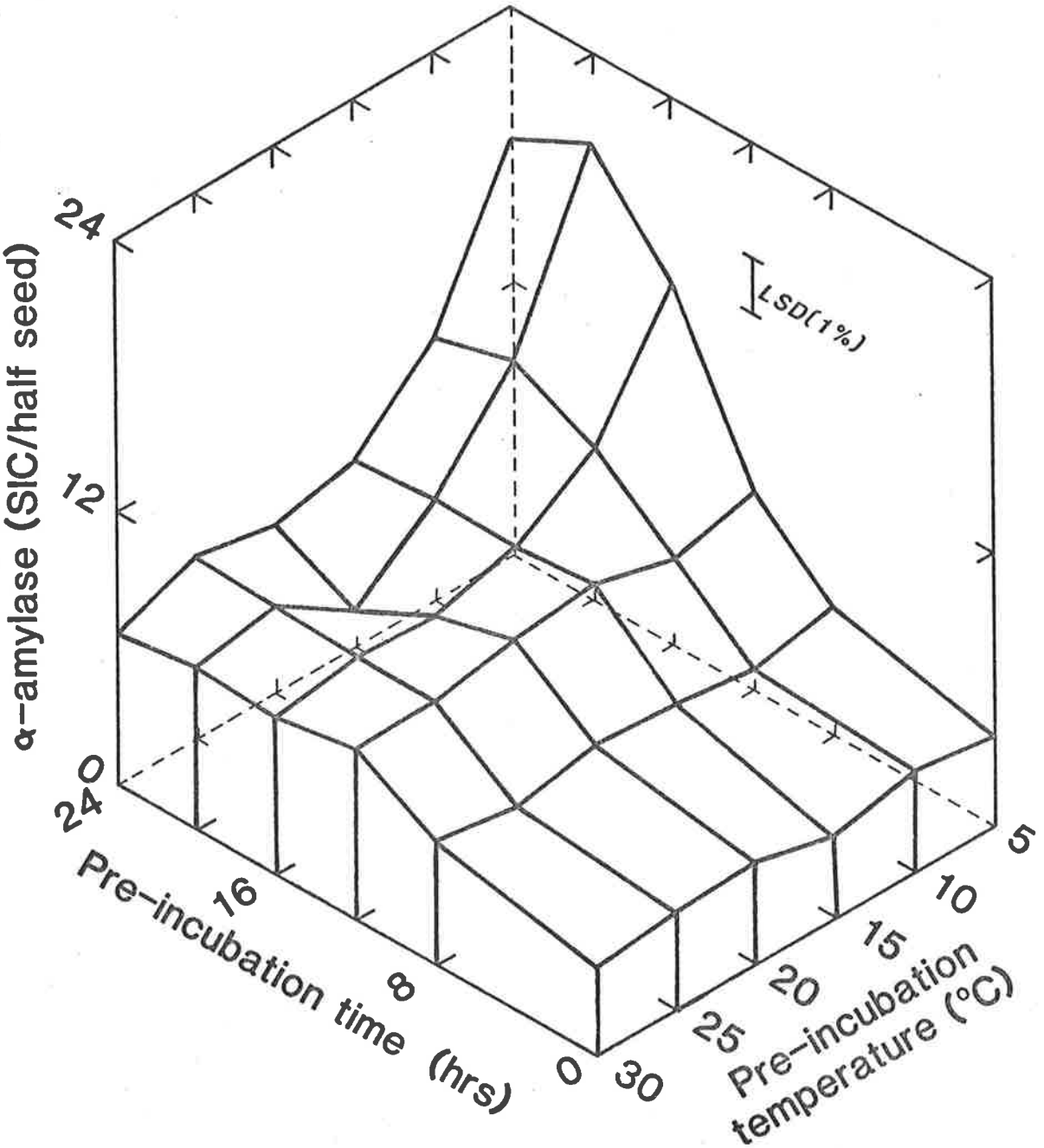

α-amylase (SIC/half seed)
24
12
0
LSD(1%)
24
16
8
0
Pre-incubation time (hrs)
30
25
20
15
10
5
Pre-incubation temperature (°C)

Figure 14: Effect of pre-incubation temperature and time on α-amylase production by de-embryonated seed of CAP x MD (Rht3, dwarf) selection in response to 1.0 µg/ml GA_3.


α-amylase (SIC/half seed)
24
12
0
LSD(1%)
24
16
8
0
Pre-incubation time (hrs)
30
25
20
15
10
5
Pre-incubation temperature (°C)

production. Furthermore, this low temperature-induced GA_3-sensitivity was once again GA_3 concentration-dependent (Figure 15).

In the case of the tall selection CAP x MD (rht 3), pre-incubation temperature had no effect whatsoever on the GA_3 - sensitivity of the de-embryonated seed (Figures 16, 17, 18, 19). While pre-incubation time had no effect on the α-amylase produced by the de-embryonated seed in the absence of GA_3 (Figure 16), a pre-incubation of 8 hours or more did increase the α-amylase produced to some extent, at all three GA_3 concentrations irrespective of the pre-incubation temperature (Figures 17, 18, 19). Once again the optimum GA_3 concentration was 0.1 μ g/ml and the α-amylase produced by the tall selection in response to this GA_3 concentration was quite similar to that produced by the de-embryonated dwarf seed, pre-incubated at 5^oC (Figures 13, 18). Furthermore, as in the dwarf selection, the magnitude of the α-amylase response of the de-embryonated seed of the tall selection was GA_3 concentration-dependent (Figures 17, 18, 19).

1.2.2.1 Time course of α-amylase production by de-embryonated seed of F_6 Rht 3/rht 3 isogenic lines, pre-incubated at 5^o or 30^oC

A 20 hour pre-incubation at 5^oC, as compared to 30^oC, of de-embryonated seed of the dwarf selection increased GA_3-sensitivity dramatically. Thus, a study of the time course of α-amylase production at these two pre-incubation temperatures was made (Figure 20) to find out the effect, if any, that the low temperture pre-incubation was having on the lag-time of α-amylase production. Obviously, low temperature pre-incubation had no effect on the lag-time of α -amylase production by de-embryonated seed of the dwarf selection. (Figure 20).

Figure 15: Effect of 20 hrs pre-incubation at 5°C or 30°C on α-amylase production by isolated aleurone layers of CAP x MD (Rht3, dwarf) selection.

Aleurone layers were dissected from de-embryonated seed which had been imbibed for 16 hrs at 30°C.

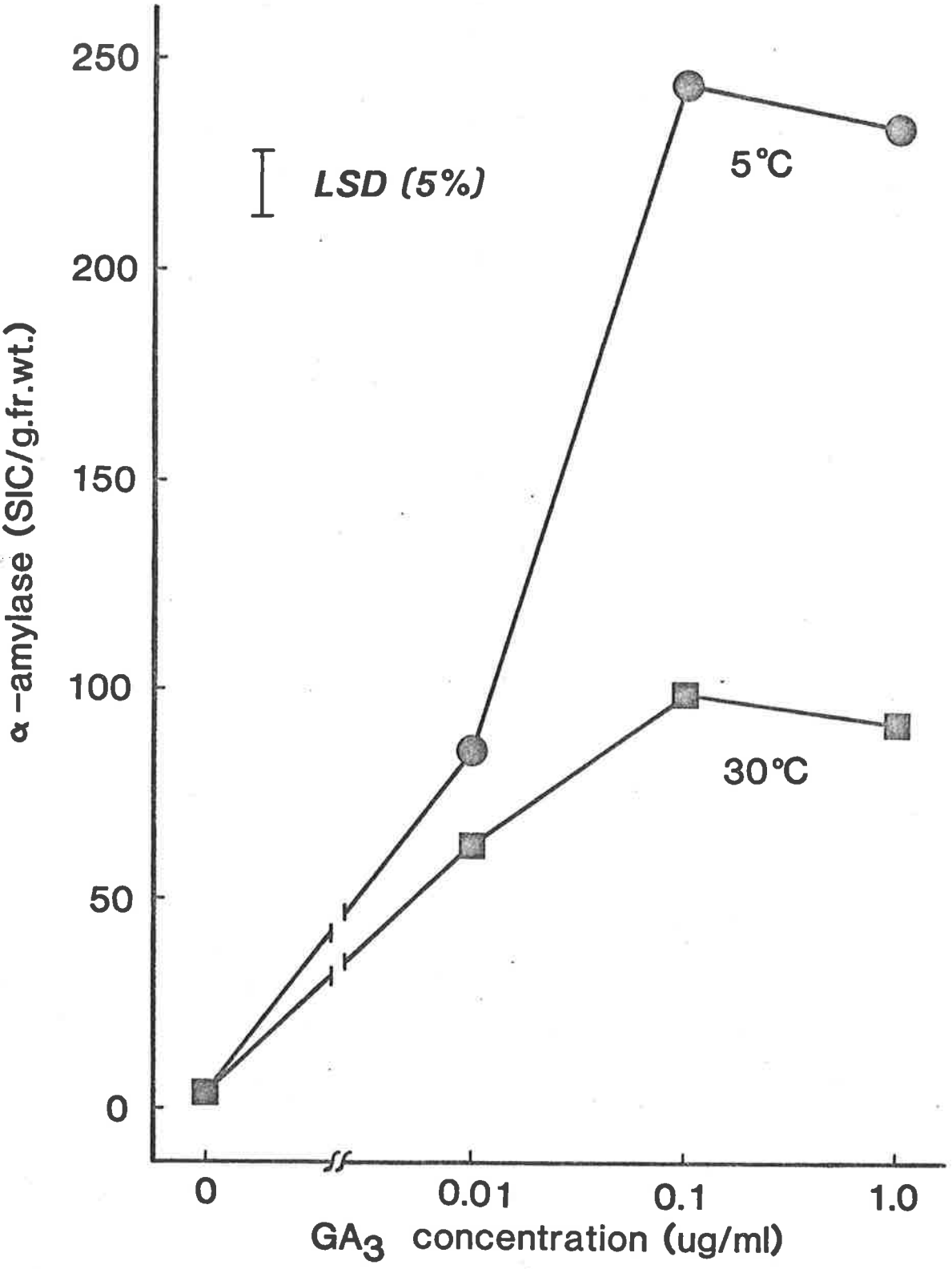

250
200
150
100
50
0
α -amylase (SIC/g.fr.wt.)
LSD (5%)
5°C
30°C
0
0.01
0.1
1.0
GA3 concentration (ug/ml)

Figure 16: Effect of pre-incubation temperature and time on α-amylase production by de-embryonated seed of CAP x MD (rht3, tall) selection in the absence of GA_3.

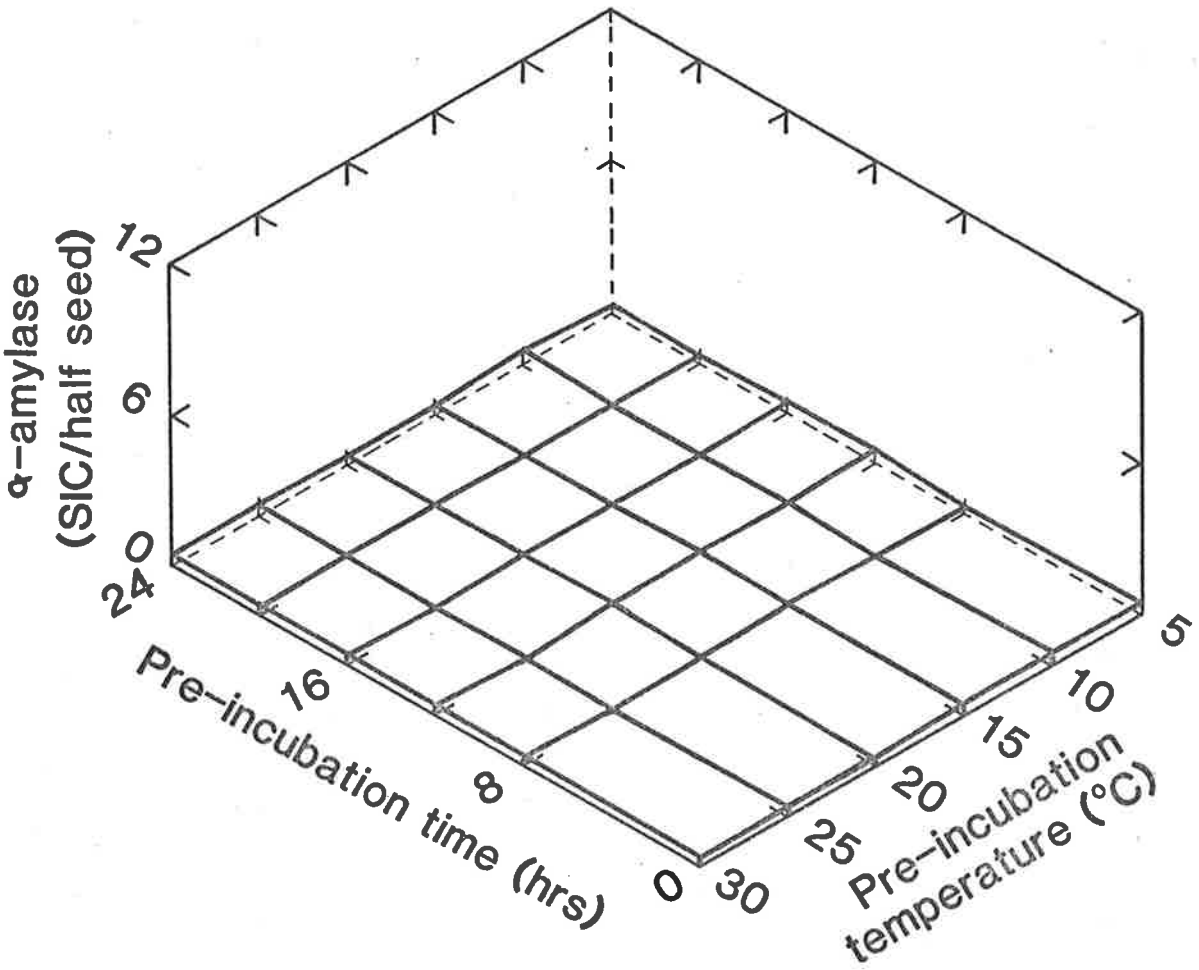

α-amylase
(SIC/half seed)
12
6
0
24
16
8
0
Pre-incubation time (hrs)
5
10
15
20
25
30
Pre-incubation
temperature (°C)

Figure 17: Effect of pre-incubation temperature and time on α - amylase production by de-embryonated seed of CAP x MD (rht3, tall) selection in response to 0.01 µ g/ml GA_3.

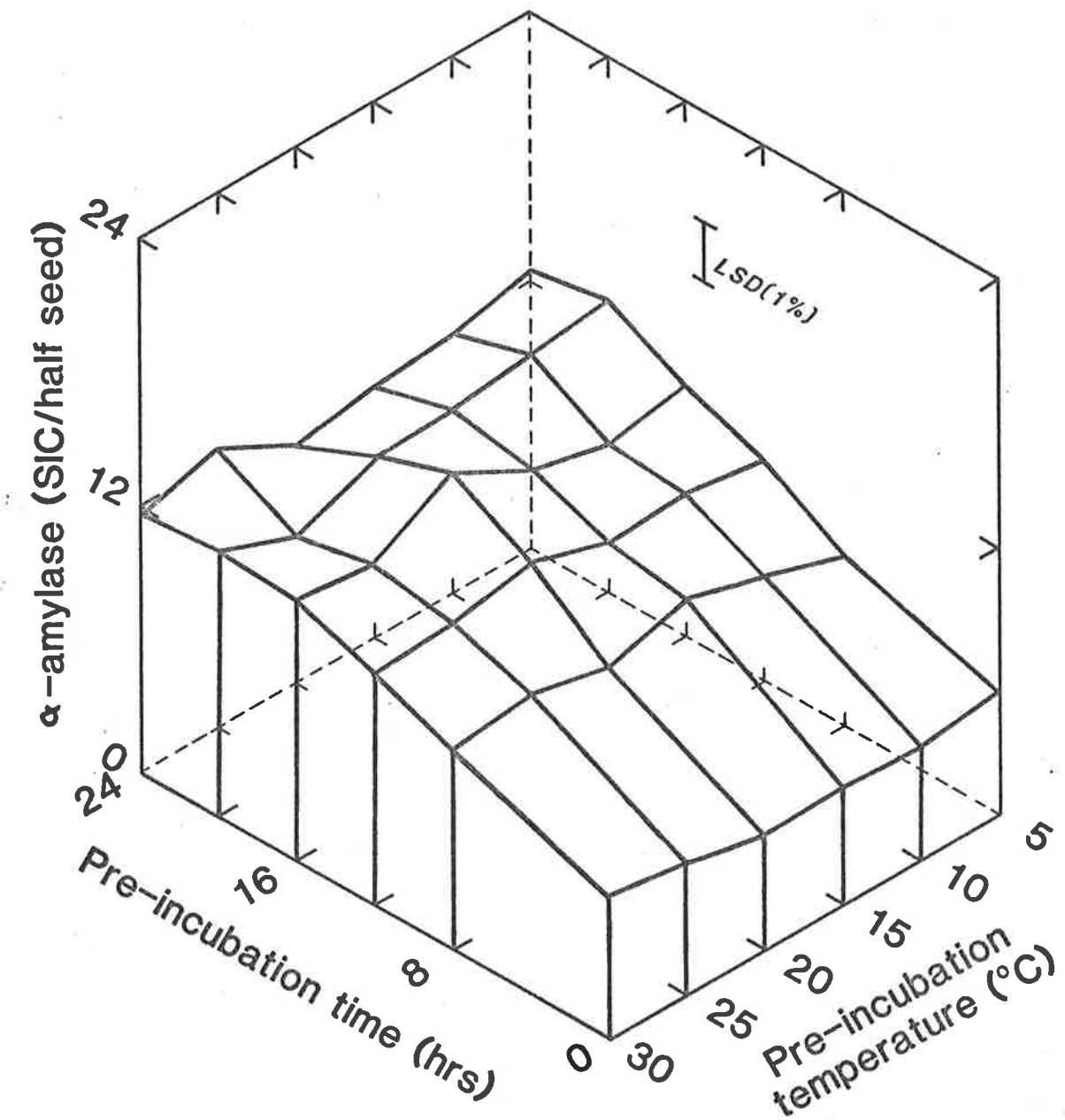

α-amylase (SIC/half seed)
24
12
0
LSD(1%)
240
16
8
0
Pre-incubation time (hrs)
30
25
20
15
10
5
Pre-incubation temperature (°C)

Figure 18: Effect of pre-incubation temperature and time on α - amylase production by de-embryonated seed of CAP x MD (rht3, tall) selection in response to 0.1 µg/ml GA_3.

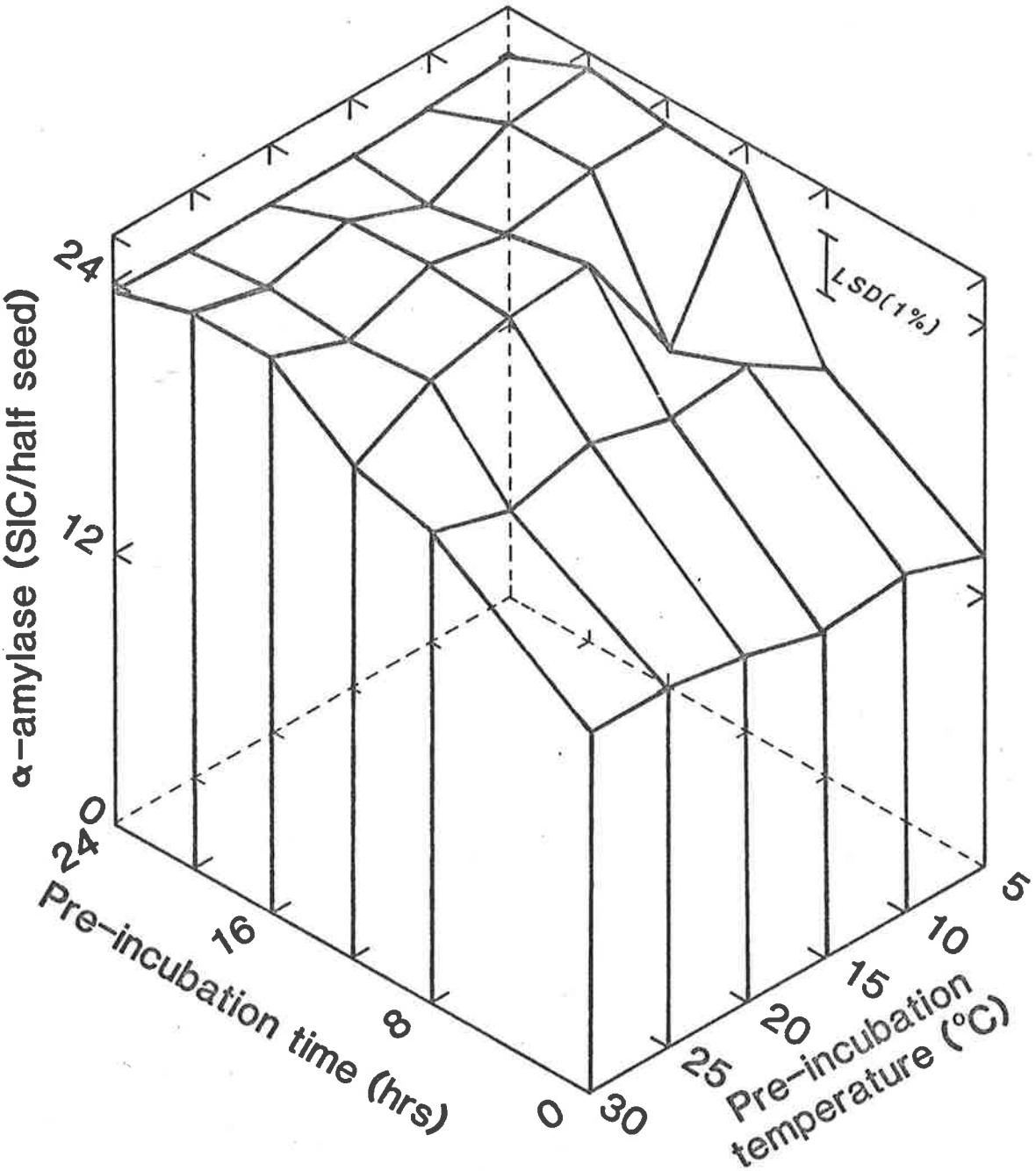

α-amylase (SIC/half seed)
24
12
0
LSD(1%)
24
16
8
0
Pre-incubation time (hrs)
5
10
15
20
25
30
Pre-incubation temperature (°C)

Figure 19: Effect of pre-incubation temperature and time on α - amylase production by de-embryonated seed of CAP x MD (rht3, tall) selection in response to 1.0 μ g/ml GA_3.

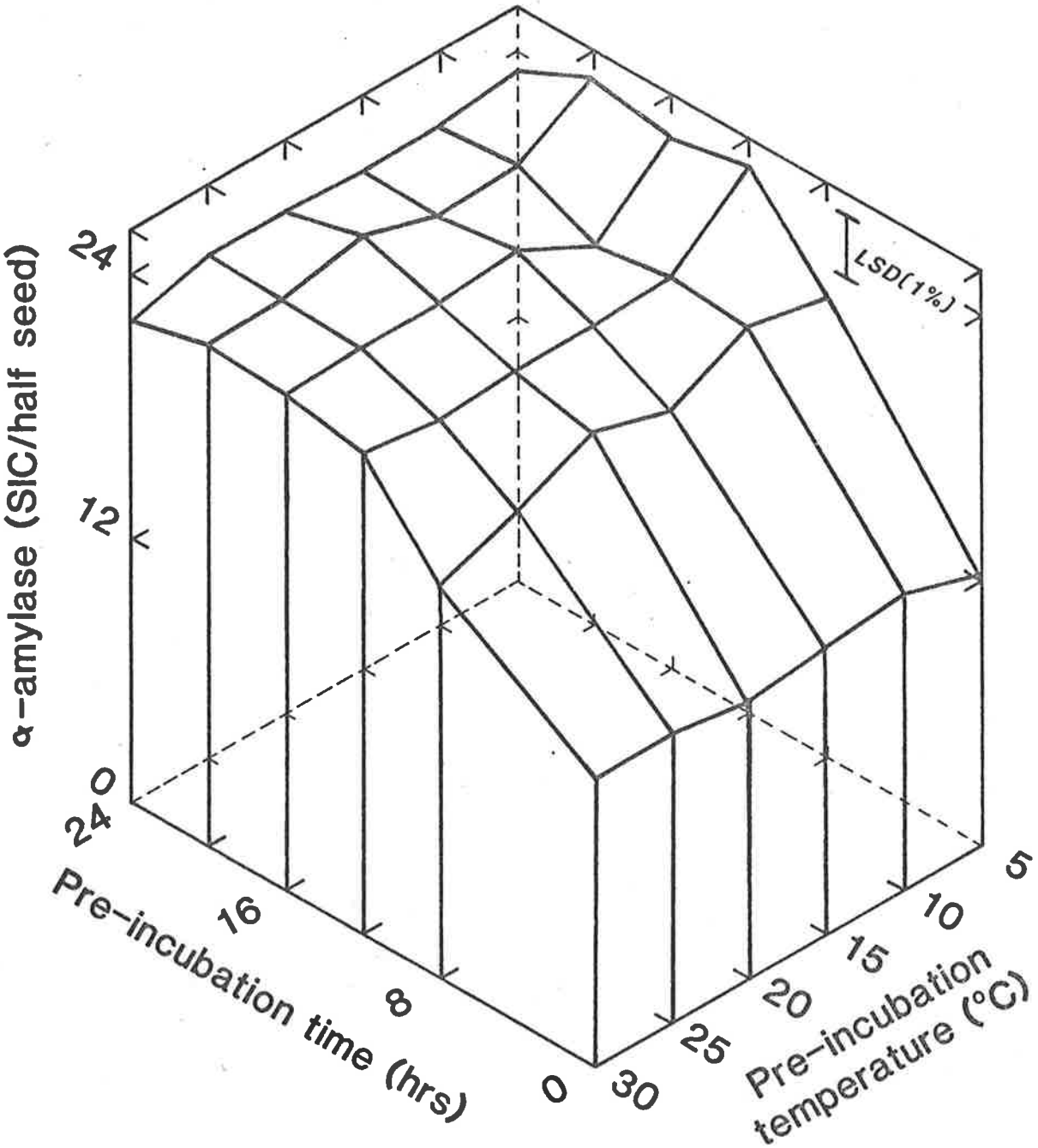
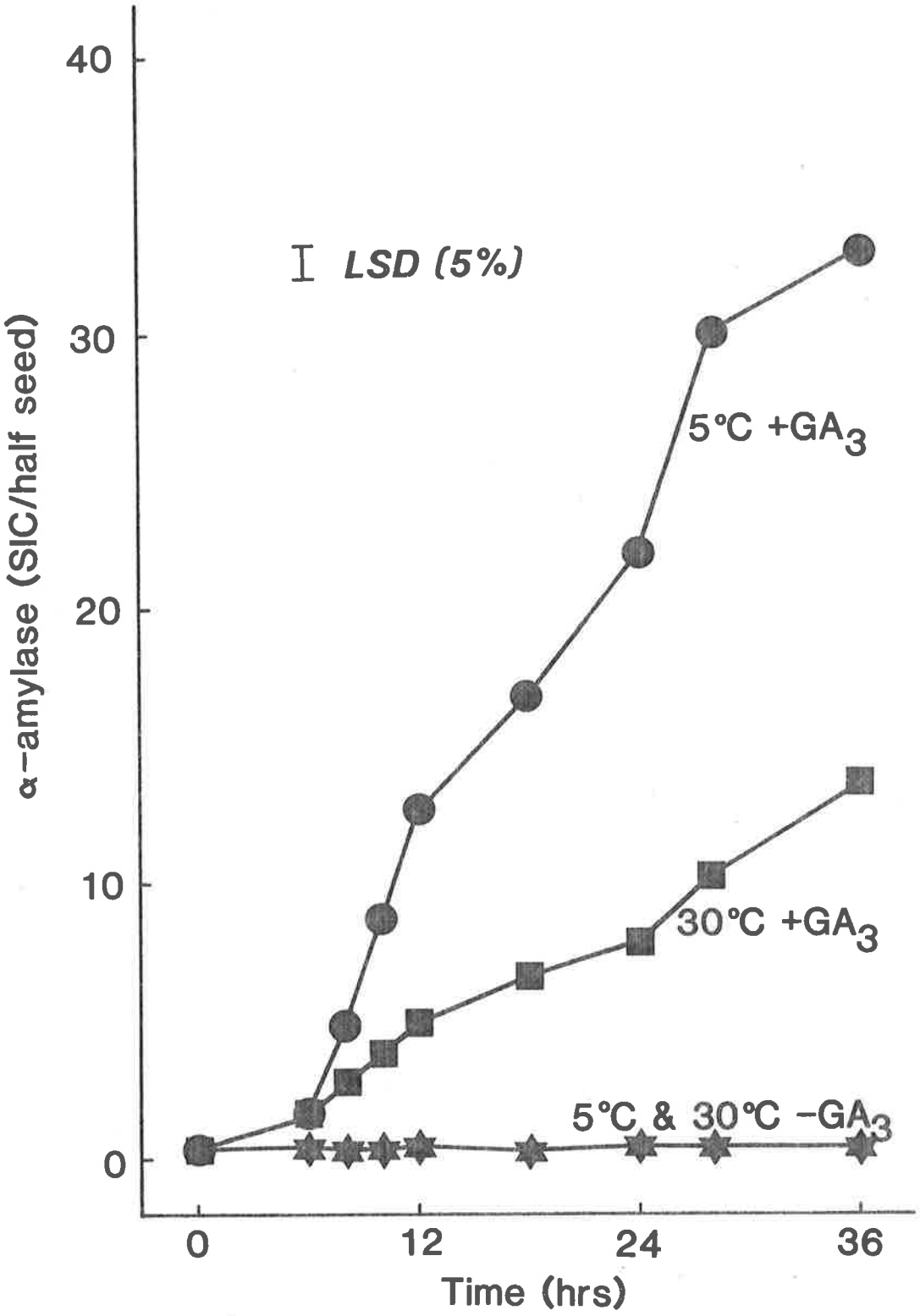

α-amylase (SIC/half seed)
24
12
0
LSD(1%)
24
16
8
0
Pre-incubation time (hrs)
5
10
15
20
25
30
Pre-incubation temperature (°C)

Figure 20: Time course of α-amylase production by de-embryonated seed of CAP x MD (Rht3, dwarf) selection, pre-incubated for 20 hrs at 5°C or 30°C.

α-amylase produced was assayed at the end of incubation periods of different durations ±0.1 μ g/ml GA_3 at 30°C.


40
30
20
10
0
α-amylase (SIC/half seed)
I LSD (5%)
5°C +GA3
30°C +GA3
5°C & 30°C -GA3
0
12
24
36
Time (hrs)

After 6 hours incubation with GA_3, the de-embryonated seed pre-incubated at 5^oC produced α-amylase at a much higher rate than the tissue pre-incubated at 30^oC and this difference in rates was evident even after 36 hours incubation. It can be concluded, therefore, that the increased amount of α-amylase produced by the 5^oC - incubated de-embryonated seed of the dwarf selection, during the subsequent 24 hours incubation in GA_3, was due to a higher rate of α-amylase production and not to a shortening of the lag-phase (Figure 20).

In the case of the tall selection, the time-course of de-embryonated seed pre-incubated either at 5^oC or 30^oC, was identical (Figure 21). This again bore out the results cited earlier (Figures 17, 18, 19) i.e. pre-incubation temperature had no bearing on α-amylase production by the seed of the tall selection. Further, a comparison of the time course of α-amylase production by seed of the dwarf selection (Figure 20) and that of the tall selection (Figure 21) showed that low temperture pre-incubation raised the rate of enzyme production of the dwarf selection to the same level as that of the tall selection. The lag time of α-amylase production detected in these experiments (Figures 20, 21), lies in the 4-8 hour range reported earlier in other studies (Chrispeels and Varner, 1967 ; Gibson and Paleg, 1982 ; Goodwin and Carr, 1972a).

Examining the effect of low temperature pre-incubation on the GA_3 - sensitivities of grain of various wheat genotypes, it can be seen that low temperature pre-incubation was able to increase, to a greater or lesser extent, the GA_3-sensitivity of grain having one of the three Rht alleles. No such effect was detected in grain without the Rht alleles (Table 1).

Figure 21: Time course of α-amylase production by de-embryonated seed of CAP x MD (rht3, tall) selection, pre-incubated for 20 hrs at 5°C or 30°C.

α-amylase produced was assayed at the end of incubation periods of different durations ±0.1 µg/ml GA_3 at 30°C.

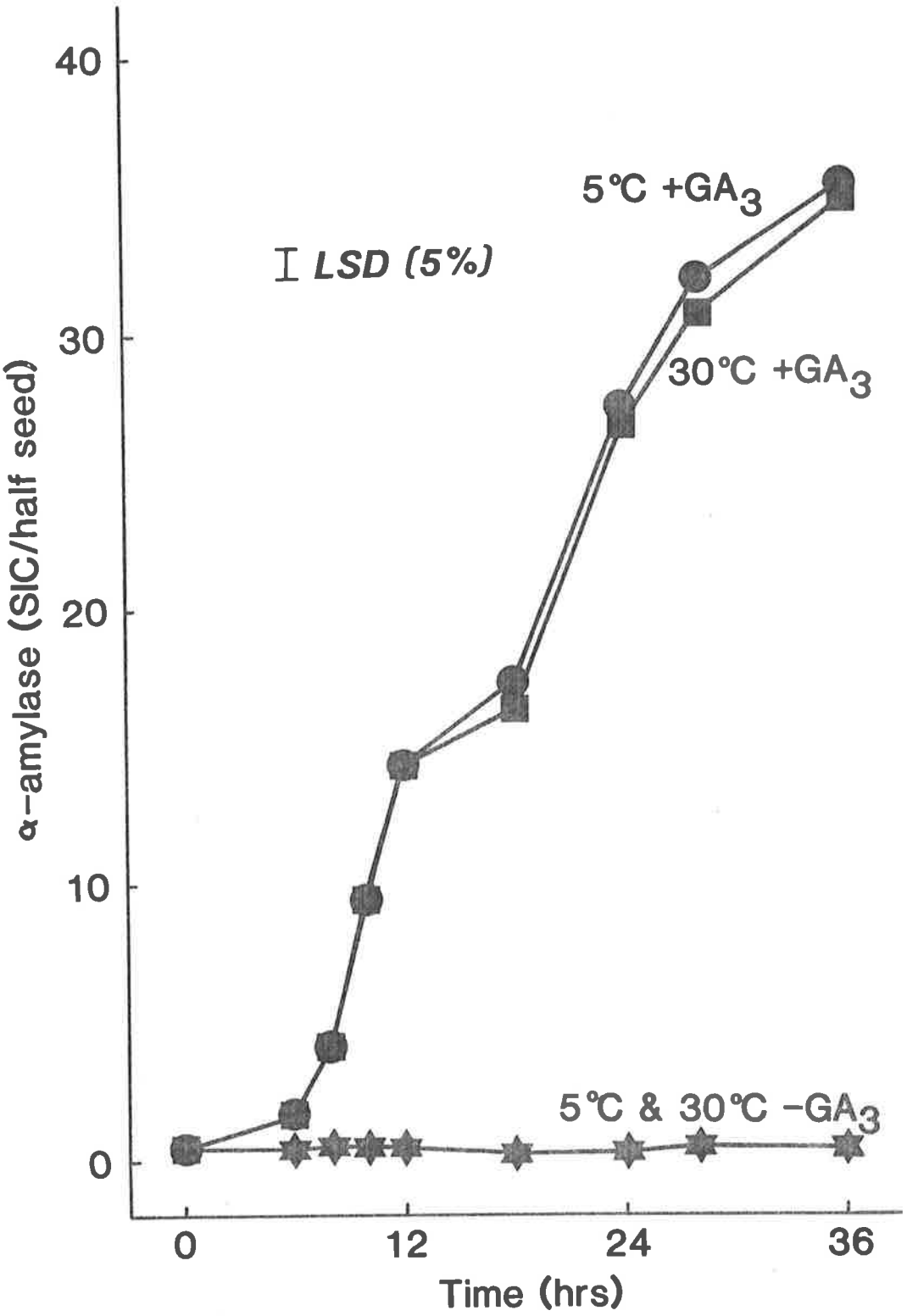

40
30
20
10
0
α-amylase (SIC/half seed)
I LSD (5%)
5°C +GA3
30°C +GA3
5°C & 30°C -GA3
0
12
24
36
Time (hrs)

Table 1 Effect of low temperature pre-incubation on the GA_3-sensitivity of wheat grain with various genetic backgrounds.

De-embryonated seed were pre-incubated for 20 hours at 5°C or 30°C and their GA_3-sensitivity was monitored by measuring the amount of α-amylase produced after 24 hours incubation at 30°C with 0.1µg/ml GA_3. Increases in GA_3-sensitivity are expressed as % increase in the amount of α-amylase produced as a result of 5°C pre-incubation over 30°C pre-incubation.

Variety		% increase
Olympic	(No Rht allele)	nil*
Nainari 60	(no Rht allele)	nil*
Halbred	(No Rht allele)	nil*
Aroona	(Rht1)	88.36
Kite	(Rht2)	279.64
Tom Thumb	(Rht3)	132.34
Tardo	(Rht3)	555.62
Minister Dwarf	(Rht3)	207.22

* 5°C pre-incubation treatment not significantly different from 30°C pre-incubation.

1.3 Discussion

The Japanese variety Norin 10 was introduced into the U.S.A. in 1946 by Dr. S.C. Salmon. Its value as a genetic source of short, stiff straw, compatible with the higher levels of artificial fertilizer which were beginning to be used at that time, was first recognized by Dr. O.E. Vogel at Washington State University. Vogel started to use lines derived from the cross Norin 10 x Brevor in his breeding programme in 1949, from which came the first Norin 10 semi-dwarf, Gaines, released in 1961. The popularity of Norin 10 with wheat producers and breeders world wide was greatly enhanced by its incorporation into the Mexican wheat programme by Dr. Borlaug in 1954. Pitic 62 was the first of many semi-dwarf varieties to be released from CIMMYT. Varieties and breeder's lines from these two programmes have been the basis of most semi-dwarfs bred for most wheat growing areas of the world (Dalrymple, 1978). In addition to the dwarfing genes contributed by Norin 10 one other independent dwarfing gene was found to be carried by wheat varieties like Minister Dwarf from Belgium and Tom Thumb from Tibet (Chaudhry, 1973 ; Fick and Qualset, 1973 ; Gale and Marshall, 1973).

The genetic system underlying Norin 10 dwarfism is now understood to consist of two partially recessive independent genes which act additively together. Rht1 is caried on chromosome 4A (Gale and Marshall, 1976) and Rht 2 maps at a homoeologous position on the short arm of chromosome 4D (Gale et al., 1975 ; McVittie et al., 1978). The gene responsible for dwarfism in Tom Thumb and Minister dwarf is Rht 3 and both Rht 1 and Rht 3 alleles are carried at the same locus. (Gale and Marshall, 1976); Rht 3 being the stronger, partially dominant gene (Zeven, 1979 ; Morris et al., 1972). Rht 1, 2 and 3 alleles impart GA_3 -

insensitivity to the aerial tissues of wheat and Gale and Law (1977) concluded that insensitivity to GA_3 and reduced height are pleiotropically expressed phenotypes of the same genes.

In addition to rendering the aerial tissues insensitive to GA_3, the Rht 3 gene also imparts GA_3-insensitivity to the aleurone tissue, in that the aleurone does not respond to GA_3, by the release of α-amylase (Fick and Qualset, 1975; Flintham and Gale, 1982; Gale and Marshall, 1973, 1975; Ho et al., 1981). Thus, the low GA_3-sensitivity detected in the grain not pre-incubated at 5^oC or 10^oC, of Minister Dwarf, Tom Thumb, Tordo and the dwarf selection CAP x MD (Rht 3), can be ascribed to the presence of the Rht 3 gene.

In the case of Rht 1 and Rht 2 genes, although it is not yet certain that GA_3-insensitivity from these genes operates fully in the aleurone tissue, it is clear that Norin 10 - Brevor 14 (Rht 1 + Rht 2) carries a gene or genes which does render the aleurone tissue insensitive. (Gale and Marshall, 1975). Since the pedigrees of both Kite (Rht 2) and Aroona (Rht 1) contain Norin 10 - Brevor 14, it seems most probable that the GA_3-insensitivity of their aleurone tissue also can be attributed to this genetic component. Supporting evidence for such a conclusion, at least in the case of Kite, is the finding that this variety is resistant to pre-harvest sprouting and that one of the main probable causes for this is the lower rate of α-amylase production (Mares, 1983).

It is highly unlikely that the low temperature treatment in our experiments is effective via an increase in the levels of endogenous gibberellins since there is no effect on GA_3-sensitivity in the absence

of exogenous GA_3. It has been shown that the genetic disfunction of a Rht 3 - containing dwarf wheat was located in the aleurone tissue since GA_3 release from the embryos of insensitive varieties during germination was similar to that of sensitive genotypes (Gale and Marshall, 1975). Moreover, the similarity of the effect of low temperature on the GA_3-sensitivity of both isolated aleurone and de-embryonated seed of Minister Dwarf (Figures 3a, 3b), Kite (Figures 5a, 5b) and the dwarf selection (Figures 12, 13, 14, 15), rules out the involvement of the embryo and the endosperm in perceiving or responding to the low temperature-induced GA_3-sensitivity. Furthermore, genetically-induced GA_3-insensitivity in the aleurone tissue of Rht 3 mutants did not operate via an alteration of α-amylase structural genes, the starch liquifaction capacity of the enzyme, the uptake and metabolism of GA_3 or by increases in the levels of endogenous inhibitors (Flintham and Gale, 1982; Ho, et al., 1981). Lastly, lag-times of GA_3 - induced enzyme production were similar regardless of the pre-incubation temperature (Figures 10, 20, 21). Thus, it is possible to conclude that the low temperature-induced increase in GA_3-sensitivity detected in aleurone tissue/de-embryonated seed from wheat genotypes having the Rht 3 gene, is unlikely to be the result of low temperature on any of the above mentioned factors. Given the remarkable similarity in the low temperature-induced increase in GA_3-sensitivity in the aleurone tissue of Rht 3 mutants to that of the Rht 1 and Rht 2 - containing varieties (Figures 4, 7, 8, 9, 10, 12, 13, 14, 20), it seems possible to conclude that the low temperature-induced increase in GA_3 - sensitivity in the Rht 1 and Rht 2 mutants is also unlikely to be the result of low temperature on the above mentioned factors.

2. Changes in lipids associated with the low temperature-induced increase in GA_3-sensitivity of isolated aleurone tissue of Kite (Rht2)

Low temperature has been shown to increase the GA_3-sensitivity of otherwise GA_3-insensitive aleurone tissue of several wheat varieties containing the Rht allele (Results 1). More specifically, a 20 hours pre-incubation at 5^oC of Kite aleurone tissue was able to effect a strong increase in GA_3-sensitivity when compared with a similar length pre-incubation at 30^oC (Results 1.2.1).

Low temperature has also been shown to alter the lipid composition, especially of membrane phospholipids and fatty acids, of plants, micro-organisms and animals (Kuiper, 1970 ; Yoshida, 1973 ; Willemot, 1975 ; Singh, 1975 ; Siminovitch, 1975 ; Horvath, 1979 ; Hunter, 1972 ; Kates, 1973). As the involvement of the endomembrane system in the synthesis and secretion of α-amylase is quite well established (see Laidman, 1982) and as this work examines the possibility of a membrane-based site of GA_3 action, the following section examines the changes brought about in the lipids, especially the phospholipids and sterols since they are the major component of membranes, as a result of the low temperature pre-incubation of Kite aleurone tissue. The possibility of establishing correlations between any changes in the lipid composition and the low temperature-induced changes in GA_3-sensitivity of the aleurone tissue will also be examined.

2.1 Stimulation of PC and PE synthesis by low temperature

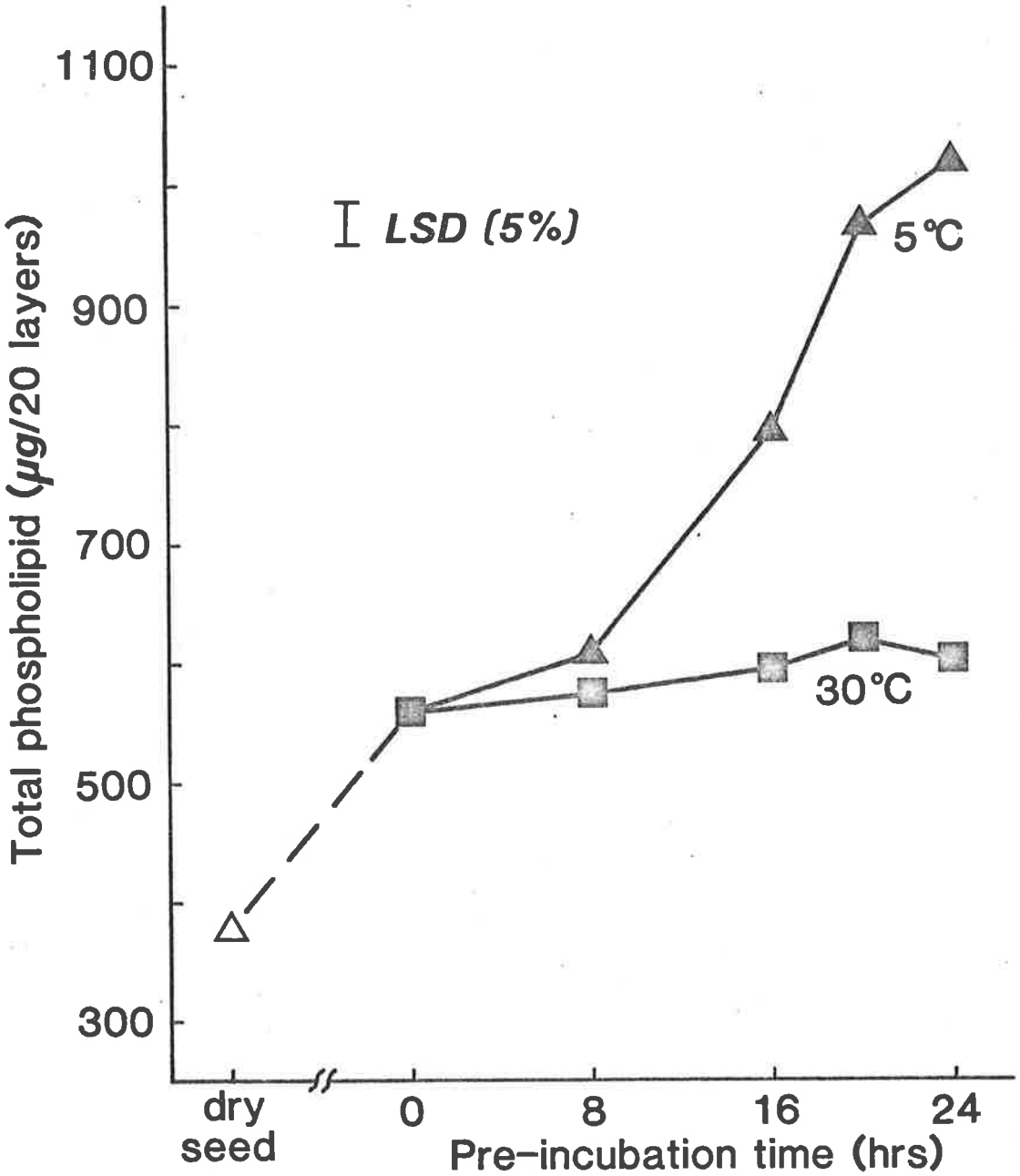
Isolated aleurone layers of Kite were subjected to pre-incubation at 5^oC or 30^oC for various lengths of time (0-24 hours) and the

phospholipids and their component fatty acids were analysed. The two temperatures, 5°C and 30°C, were selected on the basis of the results reported earlier (Results 1.2.1), as after a pre-incubation at these two temperatures the aleurone tissue displayed different sensitivities to GA_3. Six different phospholipids were detected viz; PI, PG, PC, LPC, PE and DPG. The other phospholipid commonly found in plant tissues, PS, was not detected.

Only the imbibition of water was required to initiate phospholipid synthesis and an imbibition of 24 hours at 30°C brought a significant increase in the level of total phospholipids in the Kite aleurone tissue (Figure 22). Furthermore, pre-incubating the aleurone tissue for periods of 16 hours or longer at 5°C, as compared to 30°C, brought about more significant increases in the level of the total phospholipids; the first increase being detected as a result of 16 hours of pre-incubation (Figure 22).

The significant increase detected in the levels of total phospholipid during 24 hours of imbibition (Figure 22), was entirely due to increases in the levels of PI and PG (Table 2). Although this was quite similar to earlier findings (Varty and Laidman, 1976 ; Mirbahar and Laidman, 1982) there existed an important, major difference. The levels of PC and PE did not change as a result of the 24 hours imbibition. Although a further pre-incubation for 16 hours or longer at 30°C did increase the level of PC, the magnitude of the increase was small and the PC concentration remained considerably below that of PI. The level of PE did not alter significantly during any of the pre-incubation periods at 30°C, and the levels of the other two phospholipids, LPC and DPG, did not alter during any of the experimental

Figure 22: Effect of imbibition and pre-incubation temperature and time on the total phospholipid content of aleurone tissue of Rht2 -containing wheat variety Kite.


1100
900
700
500
300
Total phospholipid (μg/20 layers)
LSD (5%)
5℃
30℃
dry seed
0
8
16
24
Pre-incubation time (hrs)

treatments (Tables 2, 3). While 24 hours imbibition did initiate synthesis of PI and PG, subsequent pre-incubations, at either of the two temperatures, had no effect on their levels (Tables 2,3).

On the other hand, if the tissue was pre-incubated at $5^{o}C$, the levels of both PC and PE increased dramatically within 16 hours (Table 3). This low temperature-induced stimulation was greater in the case of PC in which an increase of 4.5-fold over control was detected, while in the case of PE it was 1.5-fold. With both PC and PE, the increase occurred as a result of 16 hours pre-incubation at $5^{o}C$ and continued until 20 hours of pre-incubation after which it leveled out (Table 3), and this supports the trend presented in Figure 22. More importantly, the increase in phospholipid levels had a close temporal relationship with the increase in GA_3-sensitivity brought about in the Kite aleurone tissue as a result of $5^{o}C$ pre-incubation (Table 3 and Results 1.2.1).

As a result of the low temperature-induced stimulation of PC synthesis, after 20-24 hours of pre-incubation, PC comprised 47% of the total phospholipid of the aleurone tissue which had the greatest GA_3 - sensitivity, and this observation was similar to that reported earlier (Table 3 ; Varty and Laidman, 1976 ; Mirbahar and Laidman, 1982). In insensitive aleurone, the level of PC did not exceed 23%. Overall, low temperature pre-incubation was able to produce aleurone tissue with significantly altered phospholipid composition and increased GA_3 - sensitivity (Tables 2, 3 and Results 1.2.1).

Table 2. Phospholipid composition of isolated aleurone tissue of Kite pre-incubated at 30°C for various lengths of time.

Experimental Treatment	Phospholipid (µg lipid/20 layers)					
	PI	PG	PC	LPC	PE	DPG
Dry Seed	34**	25*	100	59	100	59
	(9.1)[a]	(6.7)	(26.5)	(15.5)	(26.5)	(15.7)
24 hrs imbibition	180	58	106	53	112	50
(Control)	(32.2)	(10.4)	(19.0)	(9.4)	(20.0)	(9.0)
Pre-incubation time (hr)						
8	172	61	111	55	120	54
	(30.0)	(10.7)	(19.4)	(9.6)	(20.9)	(9.4)
16	174	67	131*	59	108	55
	(29.3)	(11.3)	(22.1)	(10.0)	(18.1)	(9.2)
20	188	70	139*	69	104	50
	(30.3)	(11.3)	(22.5)	(11.2)	(16.7)	(8.0)
24	185	68	138*	65	95	50
	(30.8)	(11.3)	(22.9)	(10.7)	(15.9)	(8.4)

* Significantly different from control at P = 0.05, ** P = 0.01

[a] Figures in parenthesis represent % of the phospholipid.

Table 3. Phospholipid composition of isolated aleurone tissue of Kite pre-incubated at 5°C for various lengths of time.

Experimental Treatment	Phospholipid (µg lipid/20 layers) PI	PG	PC	LPC	PE	DPG
Dry Seed	34**	25*	100	59	100	59
	(9.1)[a]	(6.7)	(26.5)	(15.5)	(26.5)	(15.7)
24 hrs imbibition (Control)	180	58	106	53	112	50
	(32.2)	(10.4)	(19.0)	(9.4)	(20.0)	(9.0)
Pre-incubation time (hr)						
8	168	63	126	71	113	66
	(27.6)	(10.4)	(20.8)	(11.7)	(18.7)	(10.9)
16	186	71	342**	52	141**	53
	(22.0)	(8.4)	(40.5)	(6.2)	(16.6)	(6.3)
20	173	74	456**	56	159**	46
	(17.9)	(7.7)	(47.4)	(5.8)	(16.4)	(4.8)
24	185	67	479**	65	161**	62
	(18.2)	(6.5)	(47.1)	(6.3)	(15.8)	(6.1)

* Significantly different from control at P = 0.05, ** P = 0.01

a Figures in parenthesis represent % of the phospholipid.

2.1.1 Effect of low temperature pre-incubation on the acyl content and composition of PC and PE

Table 4 presents the fatty acid profiles of phospholipids in aleurone tissue from dry seed of Kite and a comparison of these fatty acid profiles with those after 24 hours of imbibition (the values represented as controls in Tables 5 and 6) shows the effect of imbibition on the acyl content of the phospholipids. Significant increases in the 16:0, 18:1 and 18:2 component fatty acids of PI and 16:0 and 18:2 fatty acid component of PG, were detected as a result of 24 hours of imbibition (Table 4). In the case of the other phospholipids, 24 hours of imbibition had no effect on their fatty acid complement (Table 4).

Tables 5 and 6 present the fatty acid content of the phospholipids of the Kite aleurone tissue pre-incubated at $5^{o}C$ or $30^{o}C$. Essentially, the effects of pre-incubation time and temperature on the acyl components of phospholipids, mirrored the effect that these temperatures had on the individual phospholipid levels (Tables 2, 3, 5 and 6) with certain important differences.

With the exception of PC and PE, neither the pre-incubation temperature nor the pre-incubation time had any effect on the fatty acid content of any of the phospholipids of the aleurone tissue of Kite (Tables 5 and 6). Low temperature pre-incubation had a stimulatory effect on the amounts of certain fatty acids of both PC and PE and this stimulation was time-dependent (Table 6). Although significant increases in the levels of 16:0 and 18:2 acids of PC were detected as a result of pre-incubation at $30^{o}C$ for 16 hours or more, these increases were quite

Table 4. Fatty acid profiles of phospholipids of aleurone tissue from dry seed of Kite. Control tissue was imbibed for 24 hrs at 30°C (0 hr pre-incubation).

Phospholipid	Fatty acid (μg lipid/20 layers)				
	16:0	18:0	18:1	18:2	18:3
PI	16**	2	0.2*	15**	1
PG	8**	1	1	14*	1
PC	40	6	2	49	3
LPC	24	4	1	29	1
PE	39	5	5	48	4
DPG	21	2	2	30	3

* Significantly different from control at P = 0.05, ** P = 0.01

Table 5. Fatty acid profiles of phospholipids of isolated aleurone tissue of Kite pre-incubated at 30°C for various lengths of time.
Control tissue was imbibed for 24 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	Fatty acid (µg lipid/20 layers)				
		16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	87	6	3	80	2
	8	80	6	2	81	2
	16	81	10	5	75	2
	20	84	11	6	82	5
	24	80	13	6	82	4
PG	0 (Control)	25	3	3	25	1
	8	26	2	2	29	2
	16	25	4	2	30	5
	20	21	6	6	33	3
	24	22	4	6	35	1
LPC	0 (Control)	18	4	2	26	3
	8	21	1	3	29	2
	16	21	3	5	25	6
	20	25	4	6	30	3
	24	26	5	6	22	6

Table 5 (continued)

DPG	0 (Control)	16	3	3	25	4
	8	19	1	2	29	3
	16	22	3	6	17	6
	20	18	2	4	20	6
	24	21	3	6	17	3
PC	0 (Control)	38	5	6	50	7
	8	39	5	4	56	7
	16	44	6	6	69*	7
	20	49*	6	6	73*	5
	24	47*	8	6	71*	6
PE	0 (Control)	38	6	6	56	6
	8	43	5	7	58	6
	16	40	4	9	49	5
	20	36	5	8	51	4
	24	32	4	9	46	4

* Significantly different from control at P = 0.05

Table 6. Fatty acid profiles of phospholipids of isolated aleurone tissue of Kite pre-incubated at 5°C for various lengths of time.
Control tissue was imbibed for 24 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	Fatty acid (µg lipid/20 layers)				
		16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	87	6	3	80	2
	8	76	7	4	80	1
	16	85	10	6	82	1
	20	75	13	7	75	2
	24	82	13	6	80	3
PG	0 (Control)	25	3	3	25	1
	8	28	4	2	29	1
	16	28	3	4	32	3
	20	25	5	6	36	2
	24	21	6	4	32	3
LPC	0 (Control)	17	4	2	26	3
	8	24	2	3	37	4
	16	18	3	6	19	6
	20	19	4	6	23	3
	24	24	6	5	26	4

Table 6 (continued)

DPG	0 (Control)	16	3	3	25	4
	8	22	3	2	36	3
	16	20	2	6	18	6
	20	17	3	7	15	4
	24	21	7	5	24	4
PC	0 (Control)	38	5	6	50	7
	8	22**	3	7	73*	21**
	16	116**	16*	18**	180**	13*
	20	153**	15*	30**	244**	15*
	24	164**	16*	27**	257**	16*
PE	0 (Control)	38	6	6	56	6
	8	22**	3*	10	68*	11*
	16	49*	8	7	71**	6
	20	52*	8	13*	80**	6
	24	50*	7	16*	84**	3

* Significantly different from control at P = 0.05, ** P = 0.01

small, and hence, resulted in only small increases in the levels of PC (Tables 2 and 5). On the other hand, larger increases in 16:0, 18:0, 18:1 and 18:2 acids were detected as a result of 16 hours or more pre-incubation at $5^{o}C$, resulting in a large increase in the level of PC (Tables 3 and 6). While pre-incubation time at $30^{o}C$ had no effect on the fatty acid content of PE, pre-incubation at $5^{o}C$ for 16 hours or more resulted in significant increases in 16:0, 18:1 and 18:2 acids of PE also causing increases in the level of PE (Tables 3, 5 and 6).

The effect of 8 hours of low temperature pre-incubation on the fatty acids of both PC and PE (Table 6) deserves a special mention here as it was different from the picture presented above for the other pre-incubation times. Low temperature pre-incubation for this duration brought about an increase in the levels of the polyunsaturated 18:2 and 18:3 acids of both PC and PE, with a concomittant decrease in the saturated 16:0 acid in the case of PC and 16:0 as well as 18:0 saturated acids in the case of PE. More importantly, these changes in fatty acid levels, at 8 hours of pre-incubation at $5^{o}C$, occurred without any change in the absolute amounts of the two phospholipids (Tables 3 and 6), and before any changes occurred in GA_3-sensitivity (Results 1.2.1). This response of the aleurone tissue acyl content of phospholipids to low temperature was quite similar to responses of other tissues reported in the literature, in which exposure to low temperature seems invariably associated with an increase in the de-saturation of the fatty acid chains of phospholipids of a wide variety of species at all levels of biological organization (Wilson and Crawford, 1974 ; Farkas et al., 1975).

Tables 7, 8 and 9 provide information regarding the effect of pre-incubation treatments on the percentage composition of fatty acids of component phospholipids of the aleurone tissue. The significance levels of the multivariate analysis of variance statistic in Table 7 indicate the effect that various pre-incubation factors had on the fatty acid percentage composition of the individual phospholipids. While pre-incubation time, temperature and their interaction had no effect on the percentage composition of fatty acids of PI, PG, LPC, and DPG, the fatty acid composition of PC and PE was significantly affected by these factors (Tables 7, 8 and 9).

Pre-incubation at 30^{o}C, for any length of time examined, had no effect on the percentage composition of fatty acids comprising PC and PE (Table 9). On the other hand, as a result of 8 hours pre-incubation at 5^{o}C, the fatty acid complement of both PC and PE became significantly more de-saturated (Table 8). This low temperature-induced de-saturation, as mentioned earlier, was no doubt brought about as a result of increases in the levels of polyunsaturated fatty acids and concomitant decreases in saturated fatty acids without any alteration in the levels of the phospholipids (Tables 3, 6 and 8). This more de-saturated state of PC and PE, however, was short-lived for, as a result of 16 hours pre-incubation at 5^{o}C, there was a large increase in the percentage of 16:0 accompanied by concomittant decreases in the percentages of 18:2 and 18:3. As a result of this, the fatty acid percentage composition of PC and PE, after 16 hours pre-incubation at 5^{o}C, became similar to that of the control (Table 8).

Table 7. Effect of pre-incubation time and temperature on the fatty acid percentage composition of various phospholipids of the isolated aleurone tissue of Kite.

Treatment	Phospholipid[a]					
	PI	PG	PC	LPC	PE	DPG
24 hrs imbibition (1)[b]	8.43	8.79	9.28	8.18	9.34	8.32
Pre-incubation time (3)	19.16	18.86	24.62*	17.92	22.38*	20.64
Pre-incubation temp (1)	8.43	9.04	14.63**	9.13	14.79**	9.24
Pre-incubation time x temp. (3)	20.34	19.65	29.73**	19.89	28.64**	20.31

* Treatment significant at P = 0.05 level, ** P = 0.01

[a] Numerical values represent Pillai's test-statistic obtained from a multivariate analysis of variance of the percentage fatty acid composition of individual phospholipids.

[b] Values in parentheses represent the degrees of freedom of the treatment.

Table 8. Fatty acid percentage composition of phospholipids of isolated aleurone tissue of Kite pre-incubated at 5°C for various lengths of time. Control tissue was imbibed for 24 hours at 30°C.

Phospho-lipid	Pre-incubation time (hrs)	% Fatty acid				
		16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	48.7	3.5	1.8	44.8	1.2
	8	45.2	4.2	2.2	47.6	0.8
	16	46.0	5.5	3.5	44.4	0.6
	20	43.5	7.7	4.2	43.3	1.3
	24	44.6	7.0	3.2	43.5	1.7
PG	0 (Control)	43.4	5.7	6.0	43.2	1.7
	8	44.7	5.9	2.5	45.2	1.7
	16	39.7	4.8	5.8	45.2	4.5
	20	33.5	6.5	8.5	48.4	3.1
	24	32.1	8.5	5.8	48.5	5.1
LPC	0 (Control)	33.6	7.1	4.7	48.6	6.0
	8	33.6	3.3	4.7	52.5	5.9
	16	34.2	5.8	11.1	36.8	12.1
	20	34.0	6.8	11.5	42.0	5.7
	24	37.6	8.7	9.0	39.7	5.0

Table 8 (continued)

DPG	0 (Control)	31.0	5.5	6.4	49.9	7.2
	8	32.5	4.7	3.4	54.4	5.0
	16	38.2	4.4	11.6	34.7	11.1
	20	37.8	6.1	14.5	32.2	9.4
	24	34.6	11.1	8.6	39.5	6.2
PC	0 (Control)	36.4	4.7	5.2	47.5	6.2
	8	17.4**	2.5	5.3	58.3**	16.5**
	16	33.9	4.5	5.1	52.7	3.8
	20	33.5	3.2	6.6	53.3	3.4
	24	34.0	3.3	5.6	53.4	3.7
PE	0 (Control)	34.3	5.6	5.2	49.9	5.0
	8	19.4**	2.3*	8.5	60.0**	9.8**
	16	34.5	5.7	5.3	50.1	4.4
	20	32.5	5.2	8.0	50.6	3.7
	24	31.3	4.6	9.7	52.4	2.0

* Significantly different from Control at P = 0.05, ** P = 0.01

Table 9. Fatty acid percentage composition of phospholipids of isolated aleurone tissue of Kite pre-incubated at 30°C for various lengths of time. Control tissue was imbibed for 24 hours at 30°C.

Phospho-lipid	Pre-incubation time (hrs)	% Fatty acid				
		16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	48.7	3.5	1.8	44.8	1.2
	8	46.4	3.6	1.4	47.3	1.3
	16	46.8	5.8	3.0	43.1	1.3
	20	45.0	5.6	3.4	43.5	2.5
	24	43.0	7.2	3.4	44.1	2.3
PG	0 (Control)	43.4	5.7	6.0	43.2	1.7
	8	41.7	3.5	3.6	47.4	3.8
	16	38.0	6.2	3.5	45.4	6.9
	20	30.5	8.4	9.0	47.1	5.0
	24	32.8	6.3	9.1	51.0	0.8
LPC	0 (Control)	33.6	7.1	4.7	48.6	6.0
	8	37.5	1.7	4.8	53.0	3.0
	16	35.0	4.6	7.8	42.6	9.8
	20	36.7	5.5	9.1	43.7	5.0
	24	40.0	7.2	9.5	34.6	8.7

Table 9 (continued)

DPG	0 (Control)	31.0	5.5	6.4	49.9	7.2
	8	35.4	2.3	3.4	54.0	4.9
	16	41.0	6.2	10.6	31.7	10.5
	20	36.8	3.6	8.6	39.3	11.7
	24	42.3	5.6	11.1	34.5	6.5
PC	0 (Control)	36.4	4.7	5.2	47.5	6.2
	8	35.3	4.4	4.0	50.2	6.1
	16	33.1	4.8	4.6	52.5	5.0
	20	35.5	4.1	4.0	52.5	3.9
	24	33.7	5.8	4.3	51.5	4.7
PE	0 (Control)	34.3	5.6	5.2	49.9	5.0
	8	36.0	4.3	5.9	48.5	5.3
	16	37.5	3.9	8.0	45.6	5.0
	20	34.4	5.3	7.4	49.5	3.5
	24	33.4	4.5	9.0	48.6	4.5

* Significantly different from Control at P = 0.05, ** P = 0.01

2.1.2 Correlations between low temperature-induced alteration in lipid components and the low temperature-induced increase in GA_3-sensitivity of Kite aleurone tissue

In order to assess which parameters of the lipid composition were most closely associated with GA_3-sensitivity of the aleurone tissue, the K. Pearson correlation coefficient (r) was calculated for the amount of α-amylase produced after 24 hours incubation with GA_3 (0.1 μ g/ml) vs. the concentration of individual lipids in the tissue prior to treatment with GA_3 (Table 10).

The low temperature-induced increase in the levels of PC and PE and their component fatty acids, was very highly correlated with the similar low temperature-induced increase in GA_3-sensitivity. This was indicated by the highly significant values of r found for both PC and PE totals, as well as for their component fatty acids, while all other phospholipids and their component fatty acids showed low, insignificant values of r. Some correlations, such as the 18:1 and 18:2 fatty acid components of PC and PC itself, with correlation coefficients of 0.99 or above are extraordinary (Table 10). The case of 18:3 deserves special mention as it was the only fatty acid constituent of PC and PE to have a non-significant r value. This was similar to the results reported by Jusaitis et al., (1982) in which 18:3 was always poorly correlated with the GA_3-induced growth response of *Avena* stem segments. The fact that 18:3 seems to be poorly correlated with two different responses of two different tissues to GA_3 is quite remarkable.

Table 10 Correlation coefficients of α amylase production by aleurone tissue of Kite against (A) component fatty acids of individual phospholipids, or (B) individual phospholipid or fatty acid totals.

(A) Fatty acid		Pearson correlation Coeff. (r)	(B) Phospholipid or fatty acid	Pearson correlation Coeff. (r)
PI	16:0	-0.269 n.s.	PI	0.109 n.s.
	18:0	0.637 n.s.		
	18:1	0.620 n.s.	PG	0.634 n.s.
	18:2	-0.304 n.s.		
	18:3	-0.112 n.s.	PC	0.990** n.s.
PC	16:0	0.977**	LPC	-0.162 n.s.
	18:0	0.784*		
	18:1	0.990**	PE	0.916** n.s.
	18:2	0.997**		
	18:3	0.563 n.s.	DPG	-0.050 n.s.
PG	16:0	-0.126 n.s.	16:0	0.912**
	18:0	0.339 n.s.	18:0	0.782*
	18:1	0.424 n.s.	18:1	0.922**
	18:2	0.607 n.s.	18:2	0.969**
	18:3	0.285 n.s.	18:3	0.235 n.s.
LPC	16:0	-0.152 n.s.		
	18:0	0.452 n.s.		
	18:1	-0.164 n.s.		
	18:2	-0.408 n.s.		
	18:3	0.024		
PE	16:0	0.745*		
	18:0	0.754*		
	18:1	0.813**		
	18:2	0.875**		
	18:3	-0.230 n.s.		
DPG	16:0	0.011 n.s.		
	18:0	0.531 n.s.		
	18:1	0.615 n.s.		
	18:2	-0.353 n.s.		
	18:3	0.125 n.s.		

* Correlation coeff. significant at P = 0.05, ** P = 0.01

ns Correlation coeff. not significant.

2.2 Sterol conversions during imbibition in the Kite aleurone tissue

The sterol composition of Kite aleurone tissue pre-incubated at 5°C or 30°C for various lengths of time (0-24 hours) was also monitored to see if low temperature pre-incubation had any affect on it. The four sterols detected were cholesterol, campesterol, stigmasterol and sitosterol and they were found to be in both derivative and free form. The derivative forms were acylated steryl esters and steryl glycosides, with the former being more abundant than the latter.

Figure 23 clearly shows that in aleurone tissue from dry seed, most of the sterols were in the esterified and glycosidic form, and during the 24 hours imbibition (0 hours pre-incubation time) most of the derivatized sterols were hydrolysed to the free form. This gave rise to an equivalent complement of free sterols in the 24 hour imbibed aleurone tissue. Further pre-incubation at 5°C or 30°C for any lengths of time examined, had no effect whatsoever on the levels of either free, esterified or glycosidic sterols.

Tables 11, 12 and 13 present profiles of individual sterols, glycosidic, esterified and free, during the above mentioned pre-incubation conditions. Obviously, the individual sterols follow the trend represented for their totals in Figure 23. Aleurone tissue from dry seed of Kite was more abundant in both steryl glycosides and steryl esters. In both cases, the esterified and glycosidic form of sitosterol was most abundant followed by campesterol, stigmasterol and cholesterol (Tables 11 and 12). As a result of 24 hours imbibition, both steryl glycosides and steryl esters were hydrolysed and, thus, their levels were reduced drastically (Tables 11 and 12). Further pre-incubation had no effect on their levels.

Figure 23: Effect of imbibition and pre-incubation temperature and time on the total sterol content of aleurone tissue of Rht2 - containing wheat variety Kite.

○ and ● derivative sterols (SG and SE)

□ and ■ free sterols

[mean values not significantly different for 5°C or 30°C pre-incubation temperature]

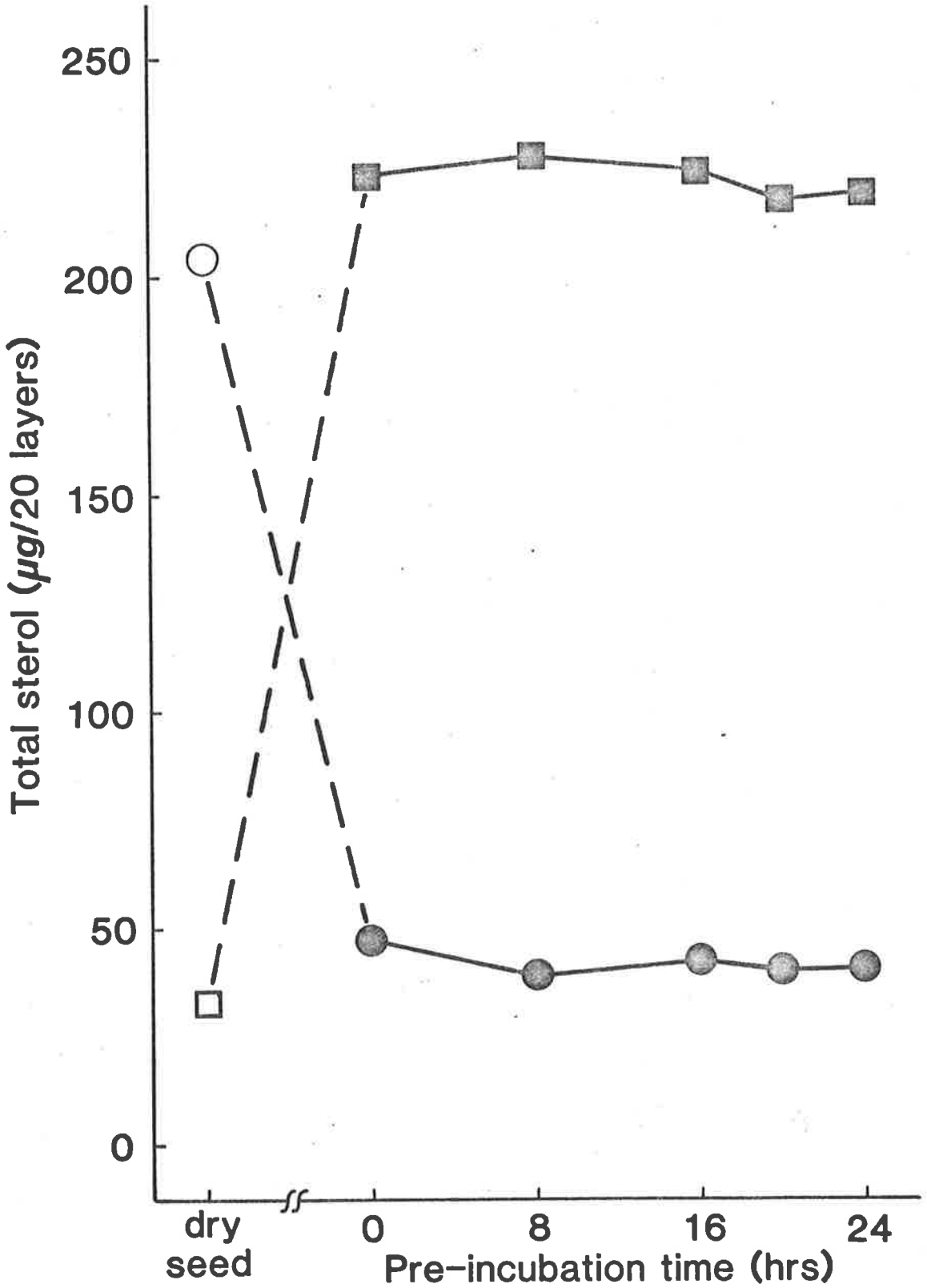

250
200
150
100
50
0
Total sterol (μg/20 layers)
dry seed
0
8
16
24
Pre-incubation time (hrs)

Table 11. Steryl glycoside composition of isolated aleurone tissue of Kite pre-incubated at 30°C or 5°C for various lengths of time.

Experimental Treatment	Sterol (μg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	3.4*	23.7**	6.8*	34.3**
24 hr imbibition (Control)	1.0	5.6	0.4	13.4
Pre-incubation time (hrs) at 30°C				
8	1.0	3.2	1.8	9.4
16	1.0	2.0	0.6	10.7
20	1.1	3.0	1.2	6.4
24	0.8	1.7	2.0	11.4
Pre-incubation time (hrs) at 5°C				
8	1.2	3.1	1.8	9.6
16	0.6	2.4	1.0	10.6
20	1.3	5.1	2.0	13.2
24	0.9	4.2	0.7	11.8

* Significantly different from control at P = 0.05, ** P = 0.01

Table 12. Steryl ester composition of isolated aleurone tissue of Kite pre-incubated at 30°C or 5°C for various lengths of time.

Experimental Treatment	Sterol (µg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	5.6*	44.2**	10.4**	75.8**
24 hr imbibition (Control)	2.1	7.2	1.8	15.6
Pre-incubation time (hrs) at 30°C				
8	1.1	6.4	2.3	10.4
16	2.6	8.2	3.4	14.7
20	1.0	7.4	1.5	9.8
24	1.9	6.1	3.7	13.4
Pre-incubation time (hrs) at 5°C				
8	2.7	9.1	4.8	9.8
16	0.6	7.7	3.7	14.7
20	1.6	6.8	4.0	13.1
24	2.0	5.8	2.1	11.8

* Significantly different from control at P = 0.05, ** P = 0.01

Table 13. Free sterol composition of isolated aleurone tissue of Kite pre-incubated at 30°C or 5°C for various lengths of time.

Experimental Treatment	Sterol (µg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	2.8*	7.4**	1.9**	20.6**
24 hr imbibition (Control)	6.0	64.8	15.4	136.2
Pre-incubation time (hrs) at 30°C				
8	7.2	66.4	20.4	125.4
16	5.1	70.4	18.6	130.7
20	6.7	63.7	17.4	140.8
24	7.5	68.4	14.1	120.1
Pre-incubation time (hrs) at 5°C				
8	6.4	75.6	21.8	131.4
16	5.1	71.8	17.4	128.7
20	7.1	66.8	15.8	115.6
24	6.8	63.7	16.4	140.2

* Significantly different from control at P = 0.05, ** P = 0.01

In the case of free sterols, of the four types detected, the most abundant was sitosterol followed by campersterol (Table 13). The free sterols were least abundant in aleurone tissue from dry seed of Kite but there was a dramatic rise in their levels after 24 hours of imbibition coinciding with the disappearance of the esterified and glycosidic sterols (Tables 11, 12 and 13). Further pre-incubation at any of the conditions examined had no effect on their levels (Table 13).

2.3 Discussion

It is generally accepted now that imbibition of water is the principle event responsible for initiating the synthesis of phospholipids in general in wheat aleurone tissue, and that this synthesis of phospholipids, and its associated development of membranes, are probably pre-requisites for the GA_3 response of the aleurone tissue (Varty and Laidman, 1976 ; Laidman et al., 1974 ; Colborne et al., 1976 ; Mirbahar and Laidman, 1982 ; Laidman, 1982).

Although 24 hours of imbibition did initiate phospholipid synthesis in the Kite aleurone tissue, this synthesis was confined only to PI and PG (Table 2) and, since hormonal sensitivity is not influenced during this period (Results 1.2.1), this change may be required for the subsequent development of GA_3 sensitivity but, by itself, is not sufficient to confer such sensitivity.

There is an almost complete lack of synthesis of the other two major phospholipids of the aleurone tissue, i.e. PC and PE, during either imbibition or subsequent pre-incubation unless, of course, the tissue is exposed to low temperature. This lack of synthesis of PC and

PE may possibly be related to the presence of the GA_3-insensitive gene/s referred to earlier (Discussion 1.3), one of which (Rht 2) is present in the genetic make-up of Kite. Some evidence to support this hypothesis has already been obtained. Suty and Vincent (1980) have shown the presence of the Rht1 and Rht2 genes to have a quantitative effect on the lipid content of six day old etiolated wheat seedlings. The lipid components affected in that case were the phospholipids and their constituent fatty acids. If the hypothesis stated above is true, then it seems possible that the presence of the Rht gene results in an aberration in the phospholipid metabolism of the tissue which can be rectified, at least in the case of aleurone tissue, by low temperature treatment. Clarification of this hypothesis will be one of the objects of experiments presented later on in this work (Results 3 and 4).

The first significant change associated with low temperature treatment of Kite aleurone, and which occurs even before an increase in sensitivity, was an increase in the proportions of 18:2 and 18:3 and a decrease in 16:0 fatty acid constituent of PC. In the case of PE, there was an increase in the proportions of 18:2 and 18:3 and a decrease in 16:0 and 18:0 fatty acid constituents. (Tables 6 and 8). These changes occurred in the absence of changes in the relative amounts of the phospholipids, and could certainly drastically alter the physical properties of the membranes they comprise (Raison, 1973). Such alterations in the physical state of the membranes could be an important factor in the subsequent stimulation of PC and PE biosynthesis (Table 3). This suggestion is based on the facts that the vast majority (>90%) of the enzymes catalyzing the terminal steps of PC and PE biosynthesis are membrane-bound (Jelsema et al., 1982a, b), and that phospholipid unsaturation plays an important role in influencing the functioning of

many membrane-bound enzymes (Jurtshuk et al., 1961 ; Jones and Wakil, 1967 ; Sartorelli, 1967).

The next significant change induced by low temperature was in the amounts of PC and PE at and after 16 hours of pre-incubation (Table 3). The levels of these phospholipids increased through 20 hours pre-incubation at 5°C demonstrating a very close temporal relationship with hormone sensitivity (Results 3.1). This relationship was paralleled by a highly significant correlation between the changes in PC and PE and α - amylase production (Table 10). In addition, although the proportion of the individual fatty acids do not change, the amounts of the acyl components of PC and PE (except 18:3) also increased as the amounts of PC and PE increased, and, thus, both the acyl and the head group components (of PC and PE) were highly correlated with hormone sensitivity.

None of the pre-incubation conditions examined had any effect on the sterol metabolism of the aleurone tissue. The dramatic nature of the disappearance of the esterified and glycosidic sterols of the Kite aleurone tissue during imbibition and the appearance of free sterols, in equivalent amounts at the same time, is consistent with the recent findings of Garg (1982) in which a storage role is ascribed to the derivative sterols. The high levels of steryl esters detected in the aleurone tissue from dry Kite seed, and their subsequent conversion during imbibition/germination, also indicates that the steryl esters are acting as a reserve pool for steroidogenesis (Bush and Grunwald, 1972 ; Torres et al., 1976). The appearance of the free sterols in the Kite aleurone tissue coincides with the time when a great deal of membrane biogenesis would be going on (Colborne et al., 1976) and, since free

sterols are an imporant constituent of all plant membranes (Grunwald, 1970), this also supports the suggestion that steryl esters and glycosides are converted to free sterols during imbibition.

3. Changes in lipids associated with the low temperature-induced increase in GA_3-sensitivity of isolated aleurone tissue/de-embryonated seed of CAP x MD(Rht3, dwarf) selection.

In the previous section (Results 2), extremely close relationships between phospholipid composition and hormone sensitivity was detected in Kite aleurone tissue. This gave support to the suggestion of a membrane-based site of GA_3 action in the aleurone tissue. Given the similarity between the low temperature-induced GA_3-sensitivity of Kite (Rht2) and of the dwarf selection (Rht3) (Results 1.2.1 and 1.2.2), experiments presented in this section are a logical extension of those presented previously (Results 2) and could help to clarify the hypothesis that the presence of any of the three alleles of the Rht gene results in an aberration in the phospholipid metabolism of the tissue which can be rectified, at least in the case of aleurone tissue, by low temperature treatment.

In view of the lack of involvement of the embryo and the endosperm of the dwarf selection in perceiving or responding to the low temperature-induced GA_3-sensitivity (Results 1.2.2), it was felt necessary to ascertain if the same could be said for any low temperature-induced lipid changes in the aleurone tissue. Hence, both de-embryonated seed as well as isolated aleurone tissue, of the dwarf selection, have been employed in these experiments. Results obtained from de-embryonated seed do not exclude the direct or indirect influence of the endosperm on any low temperature-induced lipid changes, while results obtained from isolated aleurone tissue are bereft of the influence of the endosperm.

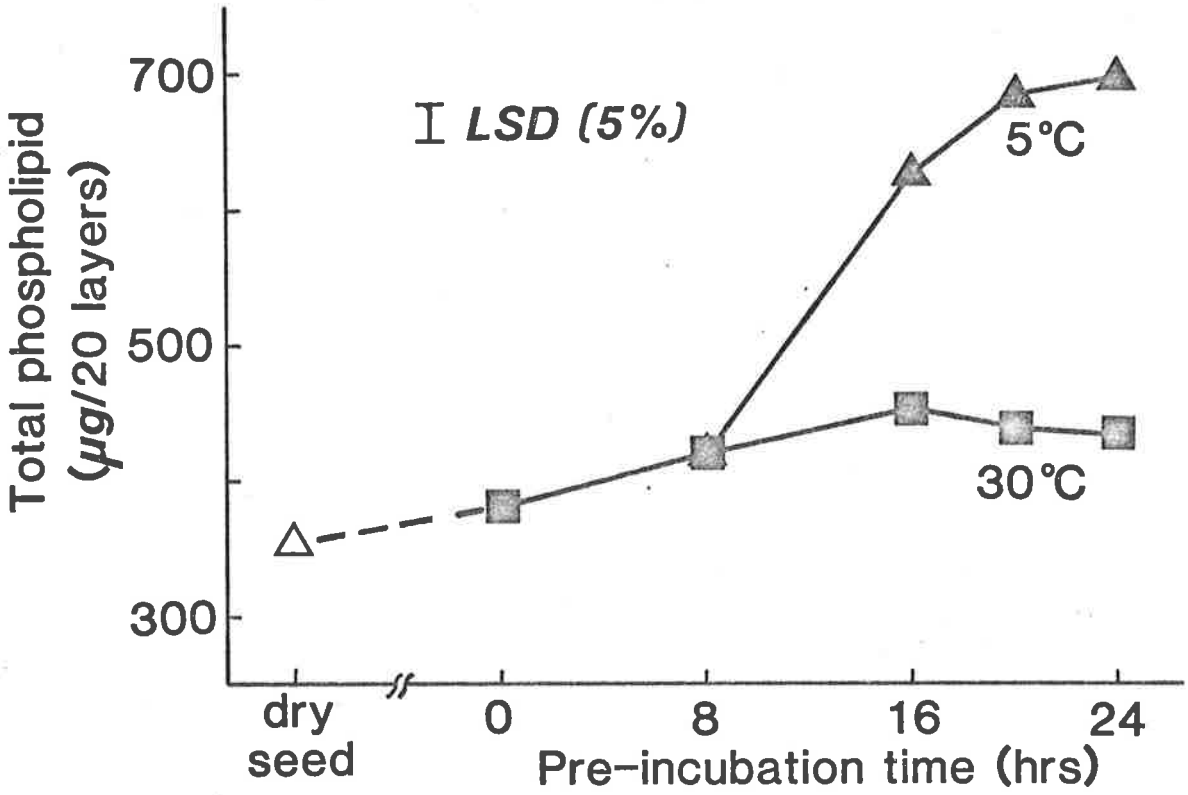
3.1 Experiments with de-embryonated seed: Stimulation of PI, PC and PE synthesis by low temperature.

De-embryonated seed of the dwarf selection were subjected to a 12 hours imbibition followed by pre-incubation at $5^{o}C$ or $30^{o}C$ for various lengths of time (0 - 24 hours). The aleurone tissue was separated and the phospholipids and their component fatty acids were analysed. The two temperatures, $5^{o}C$ and $30^{o}C$, were selected on the basis of the results reported earlier (Results 1.2.2), as after a pre-incubation at these two temperatures the de-embryonated seed displayed different sensitivities to GA_3. Five different phospholipids were detected viz. PI, PG, PC, LPC, PE. The other phospholipid detected in Kite aleurone, DPG, was not found in this tissue.

Imbibition of water for 12 hours at $30^{o}C$ had no significant effect on the total phospholipids of the aleurone tissue (Fig. 24). However, the level of PG was significantly enhanced. This was in contrast to the situation found earlier in the case of Kite aleurone tissue (Results 2.1) where imbibition initiated the synthesis of two of the phospholipids (PI and PG). However, a further pre-incubation of the de-embryonated seed of the dwarf selection, for periods of 16 hours or longer at $5^{o}C$ as compared to $30^{o}C$, brought about significant increases in the level of the total phospholipids the first increase being detected as a result of 16 hours pre-incubation (Fig. 24).

The complete lack of synthesis of the phospholipids, with the exception of PG in which a small increase was detected, during imbibition was at variance with earlier findings of Varty and Laidman (1976) and Mirbahar and Laidman (1982). However, a further pre-

Figure 24: Effect of imbibition and pre-incubation temperature and time on the total phospholipid content of aleurone tissue of CAP x MD (Rht3, dwarf) selection.


Total phospholipid
(μg/20 layers)
700
500
300
I LSD (5%)
5°C
30°C
dry
seed
0
8
16
24
Pre-incubation time (hrs)

incubation at 5°C caused a great deal of stimulation of the synthesis of PI, PC and PE (Tables 14 and 15). Although a pre-incubation for 16 hours or longer at 30°C did increase the level of PC, the magnitude of the increase was small and it did not alter in its relative amount (Table 14). Pre-incubation at 30°C, for any of the periods examined, had no effect on the levels of any of the other constituent phospholipids of the aleurone tissue of the dwarf selection (Table 14).

On the other hand, if, after imbibition, the de-embryonated seed were pre-incubated at 5°C, the levels of PI, PC and PE increased dramatically within 16 hours (Table 15). This low temperature-induced stimulation was greatest in the case of PI in which an increase of 4.5 fold over control was detected; in the case of PC it was 1.8 fold, and in the case of PE it was 1.3 fold. In all three cases, the increase occured as a result of 16 hours pre-incubation at 5°C and, in the case of PI, this increase continued until 20 hours of pre-incubation after which it levelled out (Table 15). This supports the trend observed earlier (Fig. 23). More importantly, the increase in phospholipid levels, again, had a close temporal relationship with the increase in GA_3-sensitivity brought about as a result of 5°C pre-incubation. (Table 15 and Results 1.2.2).

Obviously, the most profound effect of low temperature pre-incubation, in the case of de-embryonated seed of the dwarf selection, was on the relative amount of PI. As a result of a 20 - 24 hour pre-incubation at 5°C, PI rose to 18% of the total phospholipids of that aleurone tissue having maximal GA_3-sensitivity (Table 15). In insensitive aleurone tissue, the level of PI did not exceed 9% (Table 14). Overall, the phospholipid composition of the low temperature pre-

Table 14. Phospholipid composition of aleurone tissue of CAP x MD (Rht3, dwarf) de-embryonated seed pre-incubated at 30°C for various lengths of time.

Experimental Treatment	Phospholipid (µg lipid/20 layers) PI	PG	PC	LPC	PE
Dry Seed	26 (7.4)[a]	19* (5.4)	197 (55.6)	43 (12.2)	69 (19.4)
12 hrs imbibition (Control)	26 (6.9)	32 (8.3)	205 (53.7)	45 (11.8)	73 (19.2)
Pre-incubation time (hr)					
8	34 (8.3)	35 (8.6)	223 (55.1)	41 (10.1)	72 (17.9)
16	40 (8.8)	35 (7.8)	260* (57.4)	43 (9.4)	76 (16.6)
20	32 (7.5)	38 (8.7)	257* (58.5)	39 (9.0)	72 (16.3)
24	35 (8.0)	36 (8.3)	249* (57.4)	39 (9.0)	75 (17.3)

* Significantly different from control at P = 0.05.

a Figures in parenthesis represent % composition of the phospholipids.

Table 15. Phospholipid composition of aleurone tissue of CAP x MD (Rht3, dwarf) de-embryonated seed pre-incubated at 5°C for various lengths of time.

Experimental Treatment	Phospholipid (μg lipid/20 layers) PI	PG	PC	LPC	PE
Dry Seed	26	19*	197	43	69
	(7.4)[a]	(5.4)	(55.6)	(12.2)	(19.4)
12 hrs imbibition	26	32	205	45	73
(Control)	(6.9)	(8.3)	(53.7)	(11.8)	(19.2)
Pre-incubation time (hr)					
8	32	38	233	42	70
	(7.8)	(9.2)	(56.0)	(10.2)	(16.8)
16	95**	35	357**	39	100**
	(15.2)	(5.6)	(57.0)	(6.2)	(16.0)
20	124**	36	380**	44	100**
	(18.2)	(5.3)	(55.5)	(6.4)	(14.6)
24	129**	39	382**	42	105**
	(18.5)	(5.6)	(54.8)	(6.0)	(15.1)

* Significantly different from control at P = 0.05, ** P = 0.01

a Figures in parenthesis represent % composition of the phospholipids.

incubated aleurone tissue of de-embryonated seed of the dwarf selection and Kite aleurone tissue was quite comparable. Equally important, the low temperature effects on their GA_3-sensitivities were also comparable (Table 15, results 2.1). Thus, once again, the results implicate an alteration of phospholipid composition coincident with an alteration in GA_3-sensitivity of the aleurone tissue.

3.1.1 Effect of low temperature pre-incubation on the acyl content and composition of PI, PC and PE.

Table 16 presents the fatty acid profiles of phospholipids in aleurone tissue from dry seed of the dwarf selection. A comparison of these fatty acid profiles with those after 12 hours of imbibition (presented as controls in Tables 17 and 18) shows the effect of imbibition on the acyl content of the phospholipids. Significant increases in 16:0, and 18:2 component fatty acids of PG were detected as a result of imbibition. In the case of all other phospholipids, 12 hours of imbibition had no effect on their fatty acid component (Table 16).

Tables 17 and 18 present the fatty acid contents of the phospholipids of the aleurone tissue of de-embryonated seed of the dwarf selection pre-incubated at $5^{o}C$ or $30^{o}C$. Essentially, the effect of pre-incubation time and temperature on the acyl components of phospholipids, mirrored the effect of these factors on the individual phospholipid levels (Tables 14 and 15).

With respect to PG and LPC, neither pre-incubation temperature nor pre-incubation time had any effect on the fatty acid content of these two phospholipids (Tables 17 and 18). Low temperature pre-incubation had

Table 16. Fatty acid profiles of phospholipids of aleurone tissue of CAP x MD (Rht3, dwarf) dry seed. Control tissue was imbibed for 12 hrs at 30°C (0 hr pre-incubation)

Phospholipid	Fatty acid (µg lipid/20 layers)				
	16:0	18:0	18:1	18:2	18:3
PI	12	1	1	11	1
PG	7*	1	1	11*	1
PC	73	8	9	102	5
LPC	18	3	1	22	1
PE	20	2	3	40	3

* Significantly different from control at P = 0.05.

Table 17 Fatty acid profiles of phospholipids of aleurone tissue of CAP x MD (Rht3, dwarf) de-embryonated seed pre-incubated at 30°C for various lengths of time. Control tissue was imbibed for 12 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	Fatty acid (µg lipid/20 layers) 16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	11	1	1	13	0.4
	8	14	2	0.4	16	1
	16	18	3	1	17	1
	20	13	3	1	15	1
	24	15	2	1	15	1
PG	0 (Control)	14	2	0.4	15	0.4
	8	15	2	1	17	1
	16	17	2	1	13	1
	20	15	5	1	17	1
	24	13	6	0.4	16	0.5
PC	0 (Control)	72	9	9	110	4
	8	79	13	9	117	4
	16	99*	13	11	131*	7
	20	94*	6	15*	137*	6
	24	86*	12	11	132*	7

Table 17 (continued)

LPC	0 (Control)	16	2	2	21	3
	8	15	1	2	22	1
	16	16	1	2	21	2
	20	14	1	3	19	2
	24	13	1	3	17	5
PE	0 (Control)	27	2	3	39	2
	8	26	2	2	40	3
	16	28	3	1	39	4
	20	27	2	3	36	3
	24	26	4	3	39	3

* Significantly different from control at P = 0.05

Table 18. Fatty acid profiles of phospholipids of aleurone tissue of CAP x MD (Rht3, dwarf) de-embryonated seed pre-incubated at 5°C for various lengths of time. Control tissue was imbibed for 12 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	Fatty acid (µg lipid/20 layers) 16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	11	1	1	13	0.4
	8	14	2	1	15	1
	16	41**	5*	2*	45**	2
	20	56**	5*	3*	59**	1
	24	59**	4*	5*	61**	1
PG	0 (Control)	14	2	0.4	15	0.4
	8	15	2	1	19	1
	16	15	4	0.4	15	1
	20	14	3	1	18	1
	24	17	4	1	16	1

Table 18 (continued)

PC	0 (Control)	72	9	9	110	4
	8	44**	5*	19*	147**	19**
	16	121**	12	14*	191**	18**
	20	127**	12	21*	206**	13*
	24	133**	10	24**	203**	12*
LPC	0 (Control)	16	2	2	21	3
	8	17	1	1	21	2
	16	15	0.4	1	21	1
	20	15	1	2	23	3
	24	15	2	2	20	3
PE	0 (Control)	27	2	3	39	2
	8	11**	2	6*	45*	7*
	16	37*	3	2	55**	3
	20	35*	6	5*	49**	5
	24	38*	3	7*	53**	4

* Significantly different from control at P = 0.05, ** P = 0.01

a stimulatory effect on the amounts of certain fatty acids of PI, PC and PE and this stimulation was time-dependent in the case of PI and PC (Tables 17 and 18). In the case of PI, pre-incubation at 30°C for any length of time had no effect on its fatty acids (Table 17). On the other hand, pre-incubation at 5°C for 16 hours or more resulted in large significant increases in all fatty acids (except 18:3) of PI accompanying similar increases in the total PI level (Tables 15 and 18). Although significant increases in the levels of 16:0 and 18:2, and 18:1 (at 20 hours only) acids of PC were detected as a result of pre-incubation at 30°C for 16 hours or more, these increases were quite small and only small increases in the level of total PC were found (Tables 14 and 17). On the other hand, larger increases in 16:0, 18:1 and 18:2 acids were detected as a result of 16 hours or more pre-incubation at 5°C concomitant with a large attendant increase in the level of PC (Tables 15 and 18). In the case of PE, while pre-incubation at 30°C had no effect on fatty acid content, pre-incubation at 5°C for 16 hours or more resulted in significant increases in 16:0, 18:1 and 18:2 acids of PE, and again, similar increases in the level of PE (Tables 15, 17 and 18).

The effect of 8 hours of low temperature pre-incubation on the fatty acids of both PC and PE (Table 18) was remarkably similar to that occurring in the case of Kite (Results 2.1.1). Low temperature pre-incubation of this duration brought about an increase in the levels of the unsaturated fatty acids 18:1, 18:2 and 18:3, with a concomitant decrease in the saturated fatty acids 16:0 and 18:0 in the case of PC, and 16:0 in the case of PE. More importantly, these changes in fatty acid levels, at 8 hours of pre-incubation at 5°C, occurred without any change in the absolute amounts of the two phospholipids (Tables 15

and 18), and before any changes occurred in GA_3-sensitivity (Results 1.2.1). Overall, this response of the acyl content of the phospholipids to low temperature was quite similar to that discussed earlier (Results 2.1.1) and resulted in an increase state of de-saturation of the phospholipids.

Tables 19, 20 and 21 provide information regarding the effect of imbibition and pre-incubation treatments on the percentage composition of fatty acids of component phospholipids of the aleurone tissue. The significance levels of the multivariate analysis of variance statistic in Table 19 indicate the effect that various pre-incubation factors had on the fatty acid percentage composition of the individual phospholipids. While pre-incubation time, temperature and their interaction had no effect on the percentage composition of fatty acids of PI, PG, LPC, the fatty acid composition of PC and PE was significantly affected by these factors (Tables 19, 20 and 21).

Pre-incubation at 30°C, for any length of time examined, had no effect on the percentage composition of fatty acids comprising PC and PE (Table 21). On the other hand, as a result of 8 hours pre-incubation at 5°C, the fatty acid complement of both PC and PE became significantly more de-saturated (Table 20). This situation again is quite similar to that discussed for Kite (Results 2.1.1).

The low temperature-induced de-saturation was no doubt brought about as a result of increases in the levels of de-saturated fatty acids and concomitant decreases in saturated fatty acids without any alteration in the total levels of the phospholipids (Tables 15, 18 and 20). This more de-saturated state of PC and PE, however, was short-lived

Table 19. Effect of pre-incubation time and temperature on the fatty acid percentage composition of various phospholipids of aleurone tissue of CAP x MD (Rht3, dwarf) de-embryonated seed.

Experimental Treatment	Phospholipid[a] PI	PG	PC	LPC	PE
12 hrs imbibition (1)[b]	9.11	8.35	8.79	8.14	8.56
Pre-incubation time (3)	19.01	19.56	23.72*	18.26	21.64*
Pre-incubation temp. (1)	8.41	8.75	17.47**	8.21	14.28**
Pre-incubation time x temp. (3)	20.17	20.58	33.96**	16.84	28.24**

* Treatment significant at P = 0.05, ** P = 0.01

[a] numerical values represent the Pillai's test-statistic obtained from a multivariate analysis of variance of the percentage fatty acid composition of individual phospholipids.

[b] values in parenthesis represent the degrees of freedom of the treatment.

Table 20. Fatty acid percentage composition of phospholipids of aleurone tissue of CAP x MD (Rht3, dwarf) de-embryonated seed pre-incubated at 5°C for various lengths of time. Control tissue was imbibed for 12 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	% Fatty acid 16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	43.0	3.7	2.8	49.0	1.5
	8	44.1	4.9	2.0	47.1	1.9
	16	43.8	5.9	1.8	46.5	2.0
	20	45.2	4.2	2.2	47.6	0.8
	24	45.4	2.9	3.8	47.2	0.7
PG	0 (Control)	43.5	7.3	1.4	46.3	1.5
	8	38.2	5.7	3.9	49.4	2.8
	16	44.0	10.7	1.1	41.8	2.4
	20	38.8	7.7	3.3	48.0	2.2
	24	43.0	9.6	4.4	41.2	1.8

Table 20 (continued)

PE	0 (Control)	36.9	3.3	3.7	52.9	3.2
	8	15.4**	2.3	8.5*	64.0**	9.8**
	16	36.5	3.5	2.4	54.4	3.2
	20	34.7	5.6	5.2	49.5	5.0
	24	36.3	3.0	6.3	50.5	3.9
PC	0 (Control)	35.0	4.6	4.4	53.8	2.2
	8	19.0*	2.0	8.0*	63.0**	8.0**
	16	34.0	3.5	4.0	53.5	5.0
	20	33.5	3.2	5.6	54.2	3.5
	24	34.8	2.6	6.3	53.1	3.2
LPC	0 (Control)	35.5	5.0	5.5	47.0	7.0
	8	39.8	1.2	3.4	50.0	5.6
	16	38.3	1.0	3.1	54.0	3.6
	20	33.6	3.3	4.7	52.4	6.0
	24	34.5	4.6	5.6	48.3	7.0

* Significantly different from control at P = 0.05, ** P = 0.01

Table 21. Fatty acid percentage composition of phospholipids of aleurone tissue of CAP x MD (Rht3, dwarf) de-embryonated seed pre-incubated at 30°C for various lengths of time. Control tissue was imbibed for 12 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	% Fatty acid 16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	43.0	3.7	2.8	49.0	1.5
	8	42.6	6.3	1.2	47.8	2.1
	16	45.1	8.2	2.5	41.8	2.4
	20	40.7	9.8	1.8	44.4	3.3
	24	44.3	5.2	4.9	42.6	3.0
PG	0 (Control)	43.5	7.3	1.4	46.3	1.5
	8	43.4	5.4	1.7	48.0	1.5
	16	49.4	7.0	2.7	38.3	2.6
	20	39.1	13.0	2.2	44.0	1.7
	24	36.5	15.7	1.3	45.1	1.4

Table 21 (continued)

PE	0 (Control)	36.9	3.3	3.7	52.9	3.2
	8	36.6	2.2	3.0	54.7	3.5
	16	36.7	4.4	1.5	51.5	5.9
	20	37.9	2.9	4.1	50.5	4.6
	24	35.5	4.7	3.4	52.4	4.0
PC	0 (Control)	35.0	4.6	4.4	53.8	2.2
	8	35.6	5.7	4.2	52.8	1.7
	16	38.1	4.8	4.1	50.4	2.6
	20	36.5	2.1	5.8	53.3	2.3
	24	34.9	4.9	4.3	53.0	2.9
LPC	0 (Control)	35.5	5.0	5.5	47.0	7.0
	8	37.4	1.8	4.8	53.0	3.0
	16	37.0	2.0	5.3	50.5	5.2
	20	36.1	1.4	8.4	47.8	6.3
	24	34.1	2.1	7.3	44.4	12.1

* Significantly different from control at P = 0.05, ** P = 0.01

for, as a result of 16 hours pre-incubation at 5°C, there was a large increase in the percentage of 16:0 accompanied by concomitant decreases in the percentages of 18:2 and 18:3. As a result of this, the fatty acid percentage composition of PC and PE, after 16 hours pre-incubation at 5°C, returned to that of the control (Table 20).

3.1.2 Correlations between low temperature-induced alteration in lipid components and the low temperature-induced increase in GA_3-sensitivity of aleurone tissue from de-embryonated seed of the dwarf selection.

In order to assess which parameters of the lipid composition were necessary for, or most closely associated with, the GA_3-sensitivity of the aleurone tissue, the K. Pearson correlation coefficient (r) was calculated for the amount of α-amylase produced after 24 hours incubation with GA_3 (0.1 μg/ml) vs the concentration of individual lipids in the tissue prior to treatment with GA_3 (Table 22).

The low temperature-induced increase in the levels of PI, PC and PE and their component fatty acids, was very highly correlated with the low temperature-induced increase in GA_3-sensitivity. This was indicated by the highly significant values of (r) found for PI, PC and PE totals as well as for some of their component fatty acids, while all the other phospholipids and component fatty acids showed low, insignificant values of r. Obviously, as was also found in the case of Kite, the low temperature-induced changes in the lipids of aleurone tissue of the dwarf selection were very highly correlated with the low temperature-induced increase in GA_3-sensitivity in the same tissue. Once again, 18:3 was consistently poorly correlated with the increased GA_3-response of the aleurone tissue.

Table 22 Correlation coefficients of α-amylase production by aleurone tissue of CAP x MD (Rht3, dwarf) against, (A) component fatty acids of individual phospholipids, (B)individual phospholipids or fatty acid totals

(A) Fatty acid	Pearson correlation Coeff. (r)	(B) Phospholipid or fatty acid	Pearson correlation Coeff. (r)
PI		PI	0.977**
16:0	0.932**		
18:0	0.829**	PG	0.521 n.s.
18:1	0.871**		
18:2	0.983**	PC	0.935**
18:3	0.346 n.s.		
		LPC	0.062 n.s.
PC		PE	0.846**
16:0	0.891**		
18:0	0.293		
18:1	0.771*	16:0	0.893**
18:2	0.963**	18:0	0.737*
18:3	0.491 n.s.	18:1	0.405 n.s.
		18:2	0.886**
PG		18:3	0.148 n.s.
16:0	0.208 n.s.		
18:0	0.151 n.s.		
18:1	0.444 n.s.		
18:2	-0.001 n.s.		
18:3	0.179 n.s.		
LPC			
16:0	-0.357 n.s.		
18:0	0.277 n.s.		
18:1	-0.238 n.s.		
18:2	0.251 n.s.		
18:3	-0.076 n.s.		
PE			
16:0	0.771*		
18:0	0.710*		
18:1	0.529 n.s.		
18:2	0.853**		
18:3	0.158		

* Correlation coefficient significant at P = 0.05, ** at P = 0.01

3.1.3 Sterol conversions in the aleurone tissue during imbibition of de-embryonated seed of the dwarf selection.

The sterol composition of the aleurone tissue, from de-embryonated seed of the dwarf selection pre-incubated at 5°C or 30°C for various lengths of time (0 - 24 hours), was also monitored to ascertain if low temperature pre-incubation had any affect on it. The four sterols detected were cholesterol, campesterol, stigmasterol and sitosterol and they were found in both derivative and free forms. The derivative forms comprised acylated steryl esters and steryl glycosides with the former being more abundant than the latter.

Fig. 25 clearly shows that in aleurone tissue from the dry seed, most of the sterols were in the derivative form and, during the 12 hour imbibition (0 hours pre-incubation), most of them were hydrolysed to the free form. This gave rise to an equivalent complement of free sterols in the aleurone tissue of 12 hour imbibed de-embryonated seed. Further pre-incubation at 5°C or 30°C, for any length of time examined, had no influence whatsoever on the levels of free or esterified and glycosidic sterols. In general, the behaviour of the sterols was quite similar to that found in the case of Kite (Results 2.2).

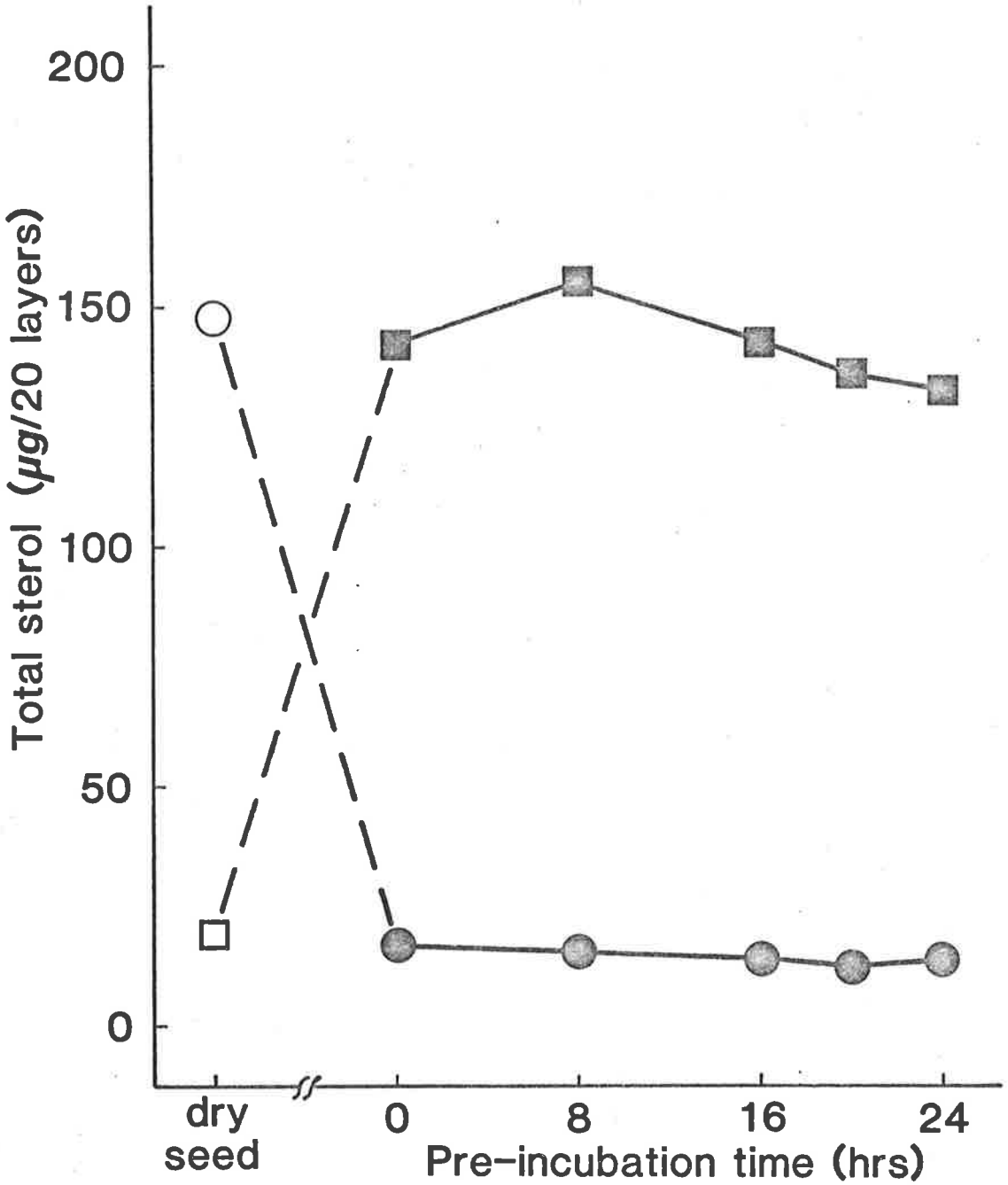
Tables 23, 24 and 25 present profiles of individual sterols in glycosidic, esterified and free forms during imbibition and pre-incubation. Obviously, the individual sterols follow the trend represented for their totals in Fig. 25. Aleurone tissue from dry seed of the dwarf selection was more abundant in both steryl glycosides and steryl esters. In both cases, the esterified and glycosidic forms of sitosterol were most abundant, followed by campesterol, stigmasterol and

Figure 25: Effect of imbibition and pre-incubation temperature and time on the total sterol content of aleurone tissue of CAP x MD (Rht3, dwarf) selection.

○ and ● derivative sterols (SG and SE)

□ and ▩ free sterols

[mean values not significantly different for 5°C or 30°C pre-incubation temperature]


200
150
100
50
0
Total sterol (μg/20 layers)
dry seed
0
8
16
24
Pre-incubation time (hrs)

cholesterol (Tables 23 and 24). As a result of 12 hours imbibition, both steryl glycosides and steryl esters were probably hydrolysed and, thus, their levels were reduced drastically (Tables 23 and 24). Further pre-incubation had no influence on their levels.

In the case of free sterols, of the four types detected, the most abundant was sitosterol followed by campesterol (Table 25). The free sterols were least abundant in aleurone tissue from dry seed of the dwarf selection but there was a dramatic rise in their levels after 12 hours of imbibition, coinciding with the disappearance of the esterified and glycosidic sterols (Tables 23, 24 and 25). Further pre-incubation, at all the conditions examined, had no influence whatsoever on the levels of the free sterols (Table 25).

3.2 Experiments with isolated aleurone tissue: Stimulation of PI, PC and PE synthesis by low temperature.

In experiments with isolated aleurone layers, unlike those with the de-embryonated seed, the aleurone layers were dissected prior to pre-incubation from, de-embryonated seed imbibed for 16 hours. Table 26 represents the phospholipids of the isolated aleurone layers after 8 or 20 hours pre-incubation at 30°C or 5°C.

A pre-incubation for 20 hours at 30°C increased the level of PC only and had no effect on any of the other phospholipids. On the other hand, a pre-incubation at 5°C for 20 hours caused significant stimulation in the levels of PI, PC and PE, similar to that described for de-embryonated seed (Results 3.1.1). As a result of this low temperature-induced increase in the level of PI, its % composition rose

Table 23. Steryl glycoside composition of aleurone tissue of CAP x MD (Rht3, dwarf) de-embryonated seed pre-incubated at 30°C or 5°C for various lengths of time.

Experimental Treatment	Sterol (µg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	1.4	12.0**	3.2*	28.3**
12 hr imbibition (Control)	0.4	1.4	0.8	3.1
Pre-incubation time (hrs) at 30°C				
8	0.1	1.8	0.3	2.6
16	0.3	1.5	1.0	3.3
20	0.3	1.0	1.1	2.0
24	0.5	2.4	0.6	3.2
Pre-incubation time (hrs) at 5°C				
8	0.3	2.3	0.4	3.6
16	0.2	2.1	0.8	2.7
20	0.8	2.0	0.6	3.3
24	0.3	1.7	0.6	2.3

* Significantly different from control at P = 0.05, ** P = 0.01

Table 24. Steryl ester composition of aleurone tissue of CAP x MD (Rht3, dwarf) de-embryonated seed pre-incubated at 30°C or 5°C for various lengths of time.

Experimental Treatment	Sterol (μg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	2.9*	28.7**	5.3**	65.8**
12 hr imbibition (Control)	0.5	3.6	0.7	6.3
Pre-incubation time (hrs) at 30°C				
8	0.4	4.2	0.4	3.9
16	0.5	3.6	1.0	4.2
20	0.6	1.5	1.0	3.4
24	0.2	1.2	1.5	3.5
Pre-incubation time (hrs) at 5°C				
8	0.6	4.2	1.0	5.0
16	0.4	2.4	0.8	3.1
20	0.7	2.0	1.8	3.3
24	0.3	2.8	1.5	4.8

* Significantly different from control at P = 0.05, ** P = 0.01

Table 25. Free sterol composition of aleurone tissue of CAP x MD (Rht3, dwarf) de-embryonated seed pre-incubated at 30°C or 5°C for various lengths of time.

Experimental Treatment	Sterol (µg lipid/20 layers) Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	0.4**	4.8**	0.8**	13.3**
12 hr imbibition (Control)	4.6	38.7	5.8	93.2
Pre-incubation time (hrs) at 30°C				
8	6.5	46.3	3.9	103.1
16	5.3	42.3	6.3	92.1
20	5.3	46.8	6.3	85.6
24	6.0	43.4	6.5	71.4
Pre-incubation time (hrs) at 5°C				
8	5.1	44.4	6.9	95.1
16	6.6	51.3	4.8	75.9
20	5.7	45.3	5.5	71.6
24	6.1	39.4	5.4	86.3

* Significantly different from control at P = 0.05, ** P = 0.01

from 9.3% in the control to 17.6% and this increase was very similar to that detected for the de-embryonated seed (Table 26, Results 3.1.1).

3.2.1 Effect of low temperature pre-incubation on the acyl content and composition of PI, PC and PE in isolated aleurone tissue.

Tables 27 and 28 present the fatty acid profiles of the phospholipids of the isolated aleurone layers of the dwarf selection, pre-incubated at 30°C or 5°C. Essentially, the effect of pre-incubation time and temperature on the acyl component of phospholipids (Table 27 and 28) mirrored the effect that these factors had on the individual phospholipid levels (Table 26). Overall, the effect of low temperature pre-incubation on the fatty acids of phospholipids of the isolated aleurone layers was identical to that in the case of the de-embryonated seed. Table 27 represents the fatty acid profile of phospholipids pre-incubated at 30°C for 8 or 20 hours. Only the 16:0 and 18:2 fatty acid components of PC increased as a result of a 20 hour pre-incubation at 30°C while pre-incubation at this temperature had no influence on any other fatty acid component of the remaining phospholipids of the isolated aleurone layers of the dwarf selection. Table 28 represents the fatty acid profiles of phospholipids of the isolated aleurone layers pre-incubated at 5°C for 8 or 20 hours. Once again, as in the case of the de-embryonated seed, a low temperature pre-incubation for 20 hours caused a stimulation in the levels of fatty acid components of PI, PC and PE. And, once again, an 8 hour pre-incubation at 5°C caused a decrease in the saturated fatty acids and an increase in the de-saturated fatty acids of both PC and PE without effecting the amounts of the phospholipids (Tables 26 and 28, Results 3.1.1).

Table 26. Phospholipid composition of isolated aleurone tissue of CAP x MD (Rht3, dwarf) seed pre-incubated at 30°C or 5°C for 8 or 20 hrs.

Experimental Treatment	Phospholipid (µg lipid/20 layers)				
	PI	PG	PC	LPC	PE
Dry Seed	26	19*	197	43	69
	(7.4)[a]	(5.4)	(55.6)	(12.2)	(19.4)
16 hrs imbibition	39	39	212	47	79
(Control)	(9.3)	(9.4)	(50.8)	(11.5)	(19.0)
Pre-incubation time (hrs) at 30°C					
8	37	41	222	46	89
	(8.5)	(9.4)	(51.0)	(10.5)	(20.6)
20	37	46	305*	48	81
	(7.2)	(8.9)	(58.9)	(9.4)	(15.6)
Pre-incubation time (hrs) at 5°C					
8	41	43	227	51	70
	(9.5)	(9.8)	(52.7)	(11.8)	(16.2)
20	145*	56	434**	60	119*
	(17.7)	(6.9)	(53.3)	(7.4)	(14.7)

* Significantly different from control at P = 0.05, ** P = 0.01

[a] Figures in parenthesis represent % composition of the phospholipids.

Table 27 Fatty acid profiles of phospholipids of isolated aleurone tissue of CAP x MD (Rht3, dwarf) selection pre-incubated at 30°C for 8 or 20 hours. Control tissue was imbibed for 16 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	Fatty acid (µg lipid/20 layers) 16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	16	3	1	18	1
	8	16	3	1	16	1
	20	17	2	1	17	1
PG	0 (Control)	17	2	1	18	1
	8	17	3	1	20	1
	20	19	4	1	21	1
PC	0 (Control)	82	17	9	98	5
	8	92	15	8	102	5
	20	127*	15	7	149*	6
LPC	0(Control)	17	1	2	26	2
	8	18	1	4	22	1
	20	18	1	4	24	1
PE	0 (Control)	29	4	3	40	3
	8	37	4	4	40	5
	20	28	2	4	43	4

* Significantly different from control at P = 0.05

Table 28. Fatty acid profiles of phospholipids of isolated aleurone tissue of CAP x MD (Rht3, dwarf) selection pre-incubated at 5°C for 8 or 20 hours. Control tissue was imbibed for 16 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	Fatty acid (µg lipid/20 layers) 16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	16	3	1	18	1
	8	19	2	1	19	1
	20	63**	7**	2*	67**	4**
PG	0 (Control)	17	2	1	18	1
	8	16	3	2	21	1
	20	22	5	1	27	1
PC	0 (Control)	82	17	9	98	5
	8	30**	5**	24**	145**	24**
	20	151**	16	27*	219**	21*
LPC	0(Control)	17	1	2	26	2
	8	19	1	3	27	2
	20	25	2	4	26	3
PE	0 (Control)	29	4	3	40	3
	8	9**	1*	7*	44*	8**
	20	40*	3	5	61**	10**

* Significantly different from control at P = 0.05, ** P = 0.01

Tables 29 and 30 present the percentage composition of the fatty acids of the phospholipids of isolated aleurone layers of the dwarf selection pre-incubated at 5°C or 30°C. Again it was found that low temperature pre-incubation had a similar effect on the fatty acids of PE and PC as it had in the case of the de-embryonated seed (Results 3.1.1). Essentially, a low temperature pre-incubation for 8 hours caused a decrease in the relative amounts of the saturated fatty acids and a concomitant increase in the de-saturated fatty acids of both PE and PC. Furthermore, this de-saturated state of PE and PC, as in the case of de-embryonated seed, was short lived for after 20 hours pre-incubation at 5°C, the percentage composition of the fatty acids of PE and PC returned to levels similar to those of the control (Table 29). Once again, pre-incubation at 30°C for short (8 hours) or long (20 hours) durations had no effect on the fatty acid percentage of any of the phospholipids of the isolated aleurone tissue (Table 30).

3.2.2 **Sterol conversions during imbibition of the isolated aleurone tissue of the dwarf selection.**

The behaviour of the sterols in the isolated aleurone tissue of the dwarf selection, during imbibition and pre-incubation, was identical to that described for the aleurone from the de-embryonated seed (Tables 31, 32 and 33). Both the steryl glycosides and the steryl esters decreased during the 16 hours of imbibition and further pre-incubation at 30°C or 5°C for 8 or 20 hours had no influence on their levels. Furthermore, four steryl glycosides and esters were detected and the derivative forms of esters were more abundant than the glycosides (Tables 31 and 32). Similarly, in the case of free sterols, a dramatic rise in their levels occurred at the end of the imbibition period (Table 33) coinciding with

Table 29. Fatty acid percentage composition of phospholipids of isolated aleurone tissue of CAP x MD (Rht3, dwarf) selection pre-incubated at 5°C for 8 or 20 hours. Control tissue was imbibed for 16 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	Fatty acid (µ g lipid/20 layers) 16:0	18:0	18:1	18:2	18:3
PI	0(Control)	42.0	6.6	1.9	47.1	2.4
	8	45.2	4.2	2.2	46.6	1.8
	20	43.7	5.2	1.5	46.6	3.0
PG	0 (Control)	44.0	5.8	1.3	47.0	1.9
	8	38.2	6.0	4.1	48.5	3.2
	20	40.0	8.2	2.0	48.2	1.6
LPC	0 (Control)	36.1	2.0	4.5	53.6	3.8
	8	36.7	1.8	5.2	53.3	3.0
	20	41.3	2.5	7.1	43.6	5.5
PE	0 (Control)	36.9	4.8	4.0	50.0	4.3
	8	13.2**	1.8*	9.4**	63.5*	12.3*
	20	33.7	2.1	4.3	51.2	8.7
PC	0 (Control)	38.7	8.1	4.4	46.3	2.5
	8	13.0**	2.2*	10.4**	64.0**	10.4**
	20	34.7	3.8	6.2	50.4	4.9

* Significantly different from control at P = 0.05, ** P = 0.01

Table 30. Fatty acid percentage composition of phospholipids of isolated aleurone tissue of CAP x MD (Rht3, dwarf) selection pre-incubated at 30°C for 8 or 20 hours. Control tissue was imbibed for 16 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	% Fatty acid 16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	42.0	6.6	1.9	47.1	2.4
	8	43.8	8.7	2.5	42.7	2.3
	20	44.2	5.3	1.5	46.0	3.0
PG	0 (Control)	44.0	5.8	1.3	47.0	1.9
	8	42.3	6.3	1.8	48.2	1.4
	20	40.8	9.0	1.6	45.0	3.6
LPC	0 (Control)	36.1	2.0	4.5	53.6	3.8
	8	39.0	2.0	8.4	47.5	3.1
	20	37.5	1.8	8.4	49.7	2.6
PE	0 (Control)	36.9	4.8	4.0	50.0	4.3
	8	41.0	4.0	4.2	45.1	5.7
	20	34.0	2.5	5.2	53.0	5.3
PC	0 (Control)	38.7	8.1	4.4	46.3	2.5
	8	41.5	6.9	3.4	45.9	2.3
	20	41.8	4.9	2.4	49.0	1.9

the disappearance of the glycosides and esters. Further pre-incubation at the conditions studied had no influence on the levels of the free sterols. Once again, four free sterols were detected viz. sitosterol, campesterol, stigmasterol and cholesterol, with sitosterol the most abundant followed by campesterol (Table 33).

3.3 Discussion

Although 12 hours of imbibition initiated an increase in PG in the aleurone tissue of the dwarf selection, there was a complete lack of increase in amounts of the other phospholipids during this period. This situation, while being akin to that observed at least with respect to PG, for Kite aleurone tissue, is in contrast to the findings of various workers who found that imbibition of water is the principal event responsible for initiating the synthesis of phospholipids in wheat aleurone tissue (Varty and Laidman, 1976 ; Laidman et al., 1974 ; Colborne et al., 1976 ; Mirbahar and Laidman, 1982 ; Laidman, 1982). As hormonal sensitivity during imbibition of the aleurone tissue from the de-embryonated seed of the dwarf selection was not influenced, the increase in PG levels during this period may be required for the subsequent expression of GA_3 sensitivity, but, by itself, is not sufficient to confer such sensitivity.

The striking similarity of the low temperature-induced stimulation in phospholipids of two Rht-containing [Kite (Rht2) and the dwarf selection (Rht3)] genotypes, underlies the possibility that low temperature, at least in the case of the aleurone tissue, is able to rectify the aberration in phospholipid metabolism probably caused by the presence of either of the two Rht alleles, Rht2 and Rht3. There is a

Table 31. Steryl glycoside composition of isolated aleurone tissue of CAP x MD (Rht3, dwarf) selection pre-incubated at 30°C or 5°C for 8 or 20 hrs. Control tissue was imbibed for 16 hrs at 30°C.

Experimental Treatment	Sterol (µg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	1.4	12.0**	3.2**	28.3**
16 hrs imbibition (Control)	0.8	2.8	0.2	1.8
Pre-incubation time (hrs) at 30°C				
8	0.5	1.8	0.3	2.7
20	0.5	2.7	0.3	1.8
Pre-incubation time (hrs) at 5°C				
8	0.2	2.1	0.2	2.5
20	0.6	1.8	0.2	2.5

* Significantly different from control at P = 0.05, ** P = 0.01

Table 32. Steryl ester composition of isolated aleurone tissue of CAP x MD (Rht3, dwarf) selection pre-incubated at 30°C or 5°C for 8 or 20 hours. Control tissue was imbibed for 16 hrs at 30°C.

Experimental Treatment	Sterol (µg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	4.0*	28.7**	5.3**	65.8**
16 hrs imbibition (Control)	1.4	5.8	0.8	7.8
Pre-incubation time (hrs) at 30°C				
8	1.8	4.3	1.0	6.4
20	1.7	4.1	1.0	7.4
Pre-incubation time (hrs) at 5°C				
8	1.0	5.1	0.8	8.2
20	1.8	4.8	0.7	7.6

* Significantly different from control at P = 0.05, ** P = 0.01

Table 33. Free sterol composition of isolated aleurone tissue of CAP x MD (Rht3, dwarf) selection pre-incubated at 30°C or 5°C for 8 or 20 hours. Control tissue was imbibed for 16 hrs at 30°C.

Experimental Treatment	Sterol (µg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	0.4**	4.8**	0.8**	13.3**
16 hrs imbibition (Control)	7.8	51.2	4.5	118.6
Pre-incubation time (hrs) at 30°C				
8	8.2	48.2	3.2	115.6
20	7.7	52.8	2.8	112.8
Pre-incubation time (hrs) at 5°C				
8	9.1	45.6	4.1	113.6
20	9.2	47.3	4.3	117.2

* Significantly different from control at P = 0.05, ** P = 0.01

complete lack of increase in levels of PI, PC and PE in the aleurone tissue of the dwarf selection during either imbibition or subsequent pre-incubation unless, of course, the tissue is exposed to low temperature (Tables 14 and 15). This, again, supports the previously discussed hypothesis (Discussion 2.3) ie. that the GA_3-insensitive gene/s (Rht) are responsible for the observed incongruity in the phospholipid metabolism.

The first significant change associated with low temperature treatment of the aleurone tissue from de-embryonated seed of the dwarf selection, and which occurred even before an increase in GA_3-sensitivity was observed, was an increase in the proportions of the de-saturated fatty acids and a decrease in the saturated fatty acids of PC and PE (Tables 18 and 20). These changes occurred in the absence of changes in the relative amounts of the phospholipids, and could certainly drastically alter the physical properties of the membranes they comprise (Raison, 1973). Such alterations in the physical state of the membranes could be an important factor in the subsequent stimulation of PI, PC and PE biosynthesis (Tables 15 and 18).

Further support for the suggestion that alterations in the physical state of membranes of the aleurone tissue of the dwarf selection may result in the low temperature-induced stimulation of phospholipids, comes from the remarkable similarity to the low temperature effect on the phospholipids of the aleurone tissue of the Kite aleurone tissue. In the Kite aleurone tissue, as in the case of the dwarf selection, an 8 hour pre-incubation caused an increase in the de-saturated and a decrease in the saturated fatty acid constituents of PC and PE without any changes in the relative amounts of these phospholipids (Results

2.1.1). Further, as in the case of the dwarf selection, pre-incubation for periods longer than 8 hours at 5°C, caused a stimulation in the synthesis of phospholipids in the Kite aleurone tissue (Results 2.1).

The next significant change induced by low temperature was the increase in amounts of PI, PC and PE at and after 16 hours of pre-incubation (Tables 15, 17 and 18), and this low temperature-induced increase in the phospholipids showed a very close temporal relationship with hormone sensitivity (Results 1.2.2). This relationship was paralleled by a highly significant correlation between the changes in PI, PC and PE on the one hand and α-amylase production on the other (Table 22). In addition, although the proportions of the individual fatty acids do not change, the amounts of certain acyl components of PI, PC and PE also increased as the amounts of the phospholipids increased, and, thus, both the acyl and the head group components (of PI, PC and PE) were highly correlated with hormone sensitivity.

The remarkable similarity of the low temperature-induced changes in phospholipids, irrespective of whether de-embryonated seed or isolated aleurone tissue was used in the pre-incubations, underlines the conclusion that the endosperm plays no role in perceiving or responding to the low temperature stimulus.

With respect to the sterols, once again, as in the case of the aleurone tissue of Kite, none of the pre-incubation factors examined had any specific effect on their metabolism. The dramatic nature of the disappearance of the esterified and glycosidic sterols during imbibition, and the appearance of free sterols in equivalent amounts, underlines a storage role for the derivative sterols in the mature wheat

aleurone tissue. The appearance of the free sterols in the aleurone tissue of the dwarf selection, coincides with the time when a great deal of membrane biogenesis would be going on (Colborne et al., 1976) and, as free sterols are an important constituent of all plant membranes (Grunwald, 1970), this also supports the suggestion that esters and glycosides are converted to free sterols during imbibition.

4. Effect of imbibition, pre-incubation time and temperature on the lipids of aleurone tissue from de-embryonated seed of CAP x MD (rht3, tall) selection.

In the previous section (Results 3), low temperature treatment was shown to have a profound influence on the phospholipid composition of the aleurone tissue of the dwarf selection. More importantly, the low temperature-induced changes in the phospholipids of the aleurone tissue, displayed a very close temporal relationship with the low temperature-induced increase in GA_3-sensitivity of the tissue.

In this section, the behaviour of the lipids of the aleurone tissue of the tall selection was studied after subjecting the de-embryonated seed to similar conditions of imbibition and pre-incubation as was done in the case of the dwarf selection. The objects of this excercise were manifold:-

a) A simultaneous examination of the effect of pre-incubation factors on the F_6Rht3/rht3 isogenic lines would afford a more rigorous exploration of the relationship between the low temperature-induced lipid changes and the presence of the Rht gene. It would also throw some light on the suggestion that the presence of the Rht gene causes an aberration in the phospholipid metabolism of the aleurone tissue.

b) It would yield useful comparisons with respect to the lipid composition of the aleurone tissue of the two lines, in different and comparable states of GA_3-sensitivity.

c) It would throw some light on the role of phospholipid synthesis in the aleurone tissue during imbibition/germination.

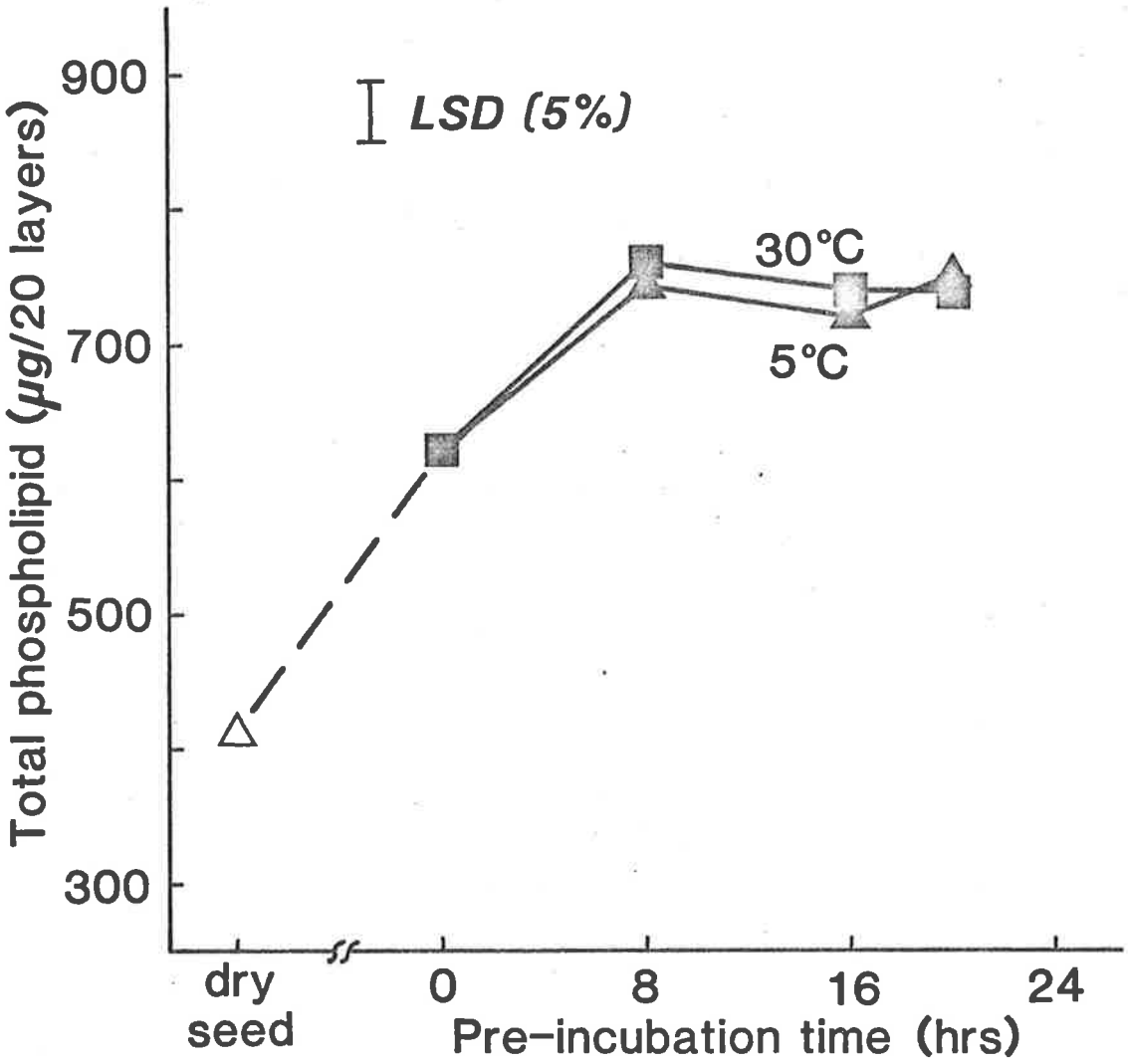
4.1 Stimulation of phospholipid synthesis in the aleurone during imbibition of CAP x MD (rht3, tall) de-embryonated seed

De-embryonated seed of the tall selection were subjected to a 12 hour imbibition followed by pre-incubations at 5°C or 30°C for various lengths of time (0 - 20 hours). Five different phospholipids were detected in the aleurone tissue viz. PI, PG, PC, LPC and PE. These were indentical to the phospholipids found in the dwarf selection tissue (Results 3.1).

Only the imbibition of water was required to initiate phospholipid synthesis, and an imbibition of 12 hours at 30°C brought about a large increase in the total phospholipid content of the aleurone tissue from the de-embryonated seed of the tall selection (Fig. 26). Furthermore, this increase in total phospholipid content continued for 8 hours during the subsequent pre-incubation at either 5°C or 30°C. Obviously, pre-incubation temperature had no effect on the phospholipid levels (Fig. 26) and this was in complete contrast to the effects that imbibition or pre-incubation temperature had on the phospholipids of the dwarf selection (Fig. 24) or of Kite (Fig. 22).

Tables 34 and 35 present the effect of imbibition and pre-incubation on the individual phospholipids of the aleurone tissue of the tall selection. Essentially, the behaviour of the individual phospholipids confirm the trend found for the total phospholipid in Fig. 26. Significant increases were found in the levels of PI, PG, PC

Figure 26: Effect of imbibition and pre-incubation temperature and time on the total phospholipid content of aleurone tissue of CAP x MD (rht3, tall) selection.


LSD (5%)
30°C
5°C
900
700
500
300
Total phospholipid (µg/20 layers)
dry seed
0
8
16
24
Pre-incubation time (hrs)

and PE during imbibition, and these increases continued, with the exception of PG, during the subsequent pre-incubation for 8 hours (Tables 34 and 35). This trend was manifested irrespective of the pre-incubation temperatures and the phospholipid composition of the aleurone tissue of the tall selection after 20 hours of pre-incubation was remarkably similar to phospholipid composition of the aleurone tissue of the dwarf selection after the dwarf tissue had been pre-incubated at 5°C for 20 hours (Tables 15, 34 and 35). Once again, LPC was the only phospholipid found not to be affected at all by imbibition or any of the pre-incubation conditions (Tables 14, 15, 34 and 35).

4.1.1 Effects of imbibition, pre-incubation time and temperature on the acyl content and composition of phospholipids.

Tables 36, 37 and 38 present the fatty acid content of the phospholipids of the aleurone tissue from dry, as well as pre-incubated, de-embryonated seed of the tall selection. Essentially, the effects of imbibition and pre-incubation time and temperature on the acyl component of phospholipids mirrored the effect that these factors had on the total or individual phospholipid levels (Fig. 26, Tables 34 - 38).

Table 36 presents the fatty acid profiles of phospholipids in aleurone tissue dry seed of the tall selection and a comparison of these fatty acid profiles with those after 12 hours of imbibition (controls presented in Tables 37 and 38) gives the effect of imbibition on the acyl content of the phospholipids. Significant increases in some of the fatty acid components of PI, PG, PC and PE were detected as a result of imbibition, resulting in similar increases in the levels of these phospholipids (Tables 34, 35). Imbibition had no effect whatsoever on any of the fatty acids of LPC.

Table 34. Phospholipid composition of aleurone tissue of CAP x MD (rht3, tall) de-embryonated seed pre-incubated at 30°C for various lengths of time.

Experimental Treatment	Phospholipid (µg lipid/20 layers)				
	PI	PG	PC	LPC	PE
Dry Seed	32**	25*	239**	41	76*
	(7.8)[a]	(6.1)	(57.9)	(9.9)	(18.3)
12 hrs imbibition (Control)	94	44	349	40	94
	(15.1)	(7.1)	(56.2)	(6.5)	(15.1)
Pre-incubation time (hrs)					
8	141**	52	414**	44	110*
	(18.5)	(6.9)	(54.4)	(5.8)	(14.4)
16	129*	43	406**	44	118*
	(17.4)	(5.9)	(54.8)	(6.0)	(15.9)
20	140**	42	404**	43	110*
	(18.9)	(5.7)	(54.6)	(5.9)	(14.9)

* Significantly different from control at P = 0.05, ** P = 0.01

[a] Figures in parenthesis represent % composition of the phospholipids.

Table 35. Phospholipid composition of aleurone tissue of CAP x MD (rht3, tall) de-embryonated seed pre-incubated at 5°C for various lengths of time.

Experimental Treatment	Phospholipid (µg lipid/20 layers)				
	PI	PG	PC	LPC	PE
Dry Seed	32**	25*	239**	41	76*
	(7.8)[a]	(6.1)	(57.9)	(9.9)	(18.3)
12 hrs imbibition (Control)	94	44	349	40	94
	(15.1)	(7.1)	(56.2)	(6.5)	(15.1)
Pre-incubation time (hrs)					
8	136**	46	408**	44	111*
	(18.3)	(6.1)	(54.8)	(5.9)	(14.9)
16	132*	44	419**	42	112*
	(17.7)	(5.9)	(55.9)	(5.6)	(14.9)
20	135**	48	409**	46	111*
	(18.0)	(6.3)	(54.7)	(6.2)	(14.8)

* Significantly different from control at P = 0.05, ** P = 0.01

[a] Figures in parenthesis represent % composition of the phospholipids.

Table 36. Fatty acid profiles of phospholipids of aleurone tissue of CAP x MD (rht3, tall) dry seed. Control tissue was imbibed for 12 hrs at 30°C (0 hr pre-incubation).

Phospholipid	Fatty acid (µg lipid/20 layers)				
	16:0	18:0	18:1	18:2	18:3
PI	13**	1**	1	15**	1
PG	9*	1	1	13*	1
PC	96**	8	7*	119*	8*
LPC	9	2	3	27	1
PE	28*	1	4	40*	2

* Significantly different from control at P = 0.05, ** P = 0.01

Eight hours of pre-incubation at either 30°C or 5°C had a further stimulatory effect on some of the fatty acids of PI, PC and PE resulting in the increases represented in Fig. 26 and Tables 37 and 38. Pre-incubation for periods longer than 8 hours, regardless of the temperature, had no effect on the fatty acids of any of the phospholipids (Tables 37 and 38).

Tables 39, 40 and 41 provide information regarding the effect of imbibition or pre-incubation treatments on the percentage composition of fatty acids of component phospholipids of the aleurone tissue. Obviously, neither imbibition nor the pre-incubation time or temperature had any influence on the percentage fatty acid composition of any of the phospholipids of the aleurone tissue of the tall selection. This was in complete contrast to the findings for the dwarf selection (Results 3.1.1).

4.2 Sterol conversions in the aleurone tissue during imbibition of de-embryonated seed of the tall selection.

The sterol composition of the aleurone tissue of the de-embryonated seed of the tall selection, imbibed for 12 hours at 30°C and pre-incubated at 5°C or 30°C for various lengths of time (0 - 20 hours) was also monitored to ascertain the affects of these factors on the sterols. The four sterols detected were sitosterol, campesterol, stigmasterol and cholesterol and they were found in both derivative and free forms. The derivative forms were comprised of acylated steryl esters and steryl glycosides with the former being more abundant.

Table 37. Fatty acid profiles of phospholipids of aleurone tissue of CAP x MD (rht3, tall) de-embryonated seed pre-incubated at 30°C for various lengths of time. Control tissue was imbibed for 12 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	Fatty acid (µg lipid/20 layers) 16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	39	7	3	43	2
	8	61**	6	6	65*	4
	16	56**	8	3	59*	3
	20	62**	9	2	64*	2
PG	0 (Control)	19	2	3	17	3
	8	19	4	2	24	2
	16	18	2	3	15	4
	20	15	3	4	18	2

Table 37 (continued)

PC	0 (Control)	136	10	18	172	13
	8	150*	16	19	219**	11
	16	161**	12	16	206**	11
	20	160**	14	15	205*	10
LPC	0 (Control)	11	2	2	23	3
	8	12	3	2	25	1
	16	13	4	4	22	1
	20	12	3	3	20	4
PE	0 (Control)	35	2	3	50	4
	8	32	3*	8*	61*	5
	16	36	6*	6	65*	4
	20	33	5*	8	57*	7

* Significantly different from control at P = 0.05, ** P = 0.01

Table 38. Fatty acid profiles of phospholipids of aleurone tissue of CAP x MD (rht3, tall) de-embryonated seed pre-incubated at 5°C for various lengths of time. Control tissue was imbibed for 12 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	Fatty acid (µg lipid/20 layers) 16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	39	7	3	43	2
	8	59**	5	4	61*	6*
	16	58**	3	5	62*	4
	20	59**	8	4	61*	3
PG	0 (Control)	19	2	3	17	3
	8	15	3	5	19	3
	16	16	2	5	16	5
	20	19	4	3	19	4

Table 38 (continued)

PC	0 (Control)	136	10	18	172	13
	8	146	16	18	219**	10
	16	165**	14	17	213**	11
	20	166**	16	14	206*	9
LPC	0 (Control)	11	2	2	23	3
	8	11	5	5	22	1
	16	12	3	1	24	1
	20	11	5	4	22	5
PE	0 (Control)	35	2	3	50	4
	8	39	5*	8*	52	9*
	16	33	6*	8*	60*	6
	20	33	4*	6*	61*	5

* Significantly different from control at P = 0.05, ** P = 0.01

Table 39. Effect of pre-incubation time and temperature on the fatty acid percentage composition of various phospholipids of aleurone tissue of CAP x MD (rht3, tall) de-embryonated seed.

Treatment	Phospholipid[a]				
	PI	PG	PC	LPC	PE
12 hrs imbibition (1)[b]	7.66	6.21	7.36	8.33	9.21
Pre-incubation time (hrs)	8.59	1.59	0.18	4.90	1.42
Pre-incubation temp (1)	3.69	1.91	1.96	2.95	5.01
Pre-incubation time x temp (3)	3.25	5.68	3.66	5.31	3.07

[a] numerical values represent the Pillai's test-statistic obtained from a multivariate analysis of variance of the fatty acid composition of individual phospholipids.

[b] values in parenthesis represent the degree of freedom of the treatment.

Table 40. Fatty acid percentage composition of phospholipids of aleurone tissue of CAP x MD (rht3, tall) de-embryonated seed pre-incubated at 5°C for various lengths of time. Control tissue was imbibed for 12 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	% Fatty acid 16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	41.5	7.0	3.3	45.9	2.3
	8	43.2	3.8	3.1	45.3	4.6
	16	43.8	2.6	3.9	46.7	3.0
	20	44.0	5.9	2.6	45.2	2.3
PG	0 (Control)	42.9	4.1	7.4	38.6	7.0
	8	33.0	7.4	10.7	40.8	8.1
	16	36.1	5.3	10.3	37.2	11.1
	20	40.3	7.4	5.3	39.2	7.8

Table 40 (continued)

LPC	0 (Control)	27.0	4.5	3.9	57.5	7.1
	8	26.2	10.7	11.1	49.2	2.8
	16	28.8	7.4	2.4	58.1	3.3
	20	23.9	9.8	8.5	48.1	9.7
PC	0 (Control)	38.8	3.0	5.1	49.4	3.7
	8	35.7	3.9	4.4	53.6	2.4
	16	39.5	3.2	4.0	50.7	2.6
	20	40.5	3.8	3.3	50.3	2.1
PE	0 (Control)	37.2	1.8	3.3	53.7	4.0
	8	34.9	4.1	6.9	46.4	7.7
	16	29.5	5.1	6.7	53.6	5.1
	20	30.2	3.9	5.8	55.3	4.8

Table 41. Fatty acid percentage composition of phospholipids of aleurone tissue of CAP x MD (rht3, tall) de-embryonated seed pre-incubated at 30°C for various lengths of time. Control tissue was imbibed for 12 hrs at 30°C.

Phospholipid	Pre-incubation time (hrs)	% Fatty acid 16:0	18:0	18:1	18:2	18:3
PI	0 (Control)	41.5	7.0	3.3	45.9	2.3
	8	43.3	4.0	4.3	45.7	2.7
	16	43.7	6.1	2.3	45.6	2.3
	20	44.4	6.5	1.7	45.7	1.7
PG	0 (Control)	42.9	4.1	7.4	38.6	7.0
	8	36.0	8.3	4.5	46.6	4.6
	16	42.1	5.0	7.6	35.1	10.2
	20	35.7	7.7	8.4	42.2	6.0

Table 41 (continued)

LPC	0 (Control)	27.0	4.5	3.9	57.5	7.1
	8	28.2	7.8	5.1	55.8	3.1
	16	29.5	8.3	10.0	49.0	3.2
	20	28.2	6.5	8.5	47.0	9.8
PC	0 (Control)	38.8	3.0	5.1	49.4	3.7
	8	36.1	3.8	4.5	53.0	2.6
	16	39.6	2.9	3.9	50.8	2.8
	20	39.6	3.5	3.7	50.8	2.4
PE	0 (Control)	37.2	1.8	3.3	53.7	4.0
	8	29.4	3.1	6.9	55.9	4.7
	16	30.6	4.9	5.0	55.7	3.8
	20	30.4	4.3	7.5	52.0	5.8

Fig. 27 clearly shows that in aleurone tissue from the dry seed of the tall selection, most of the sterols were in the derivative form and, during the 12 hour imbibition (0 hours pre-incubation), most of them were converted to the free form. This gave rise to an equivalent complement of free sterols in the 12 hour imbibed aleurone tissue. Further pre-incubation at 5°C or 30°C, for any lengths of time examined, had no effect on the levels of either free or esterified and glycosidic sterols. In general, the behaviour of the sterols was quite similar to that found in the case of the dwarf selection (Figs. 25, 27).

Tables 42, 43 and 44 present profiles of individual sterols, in the glycosidic, esterified and free forms, during imbibition and pre-incubation. The individual sterols follow the trend represented for their totals in Fig. 27. Aleurone tissue from dry seed of the tall selection was more abundant in both steryl glycosides and steryl esters. In both cases, the esterified and glycosidic forms of sitosterol was most prevalent followed by campesterol, cholesterol and stigmasterol (Tables 42 and 43). As a result of 12 hours imbibition both steryl glycosides and steryl esters were reduced drastically, probably as a result of hydrolysis . Further pre-incubations had no influence on their levels (Tables 42 and 43).

In the case of free sterols, of the four types detected, the most abundant was sitosterol followed by campesterol (Table 44). The free sterols were the least abundant but there was a dramatic rise in their levels after 12 hours of imbibition, coinciding with the disappearance of the esterified and glycosidic sterols (Tables 42, 43 and 44). Further pre-incubation at all the conditions examined had no effect whatsoever on the levels of the free sterols (Table 44).

Figure 27: Effect of imbibition and pre-incubation temperature and time on the total sterol content of aleurone tissue of CAP x MD (rht3, tall) selection.

○ and ● derivative sterols (SG and SE)
□ and ■ free sterols
[mean values not significantly different for 5°C or 30°C pre-incubation temperature]

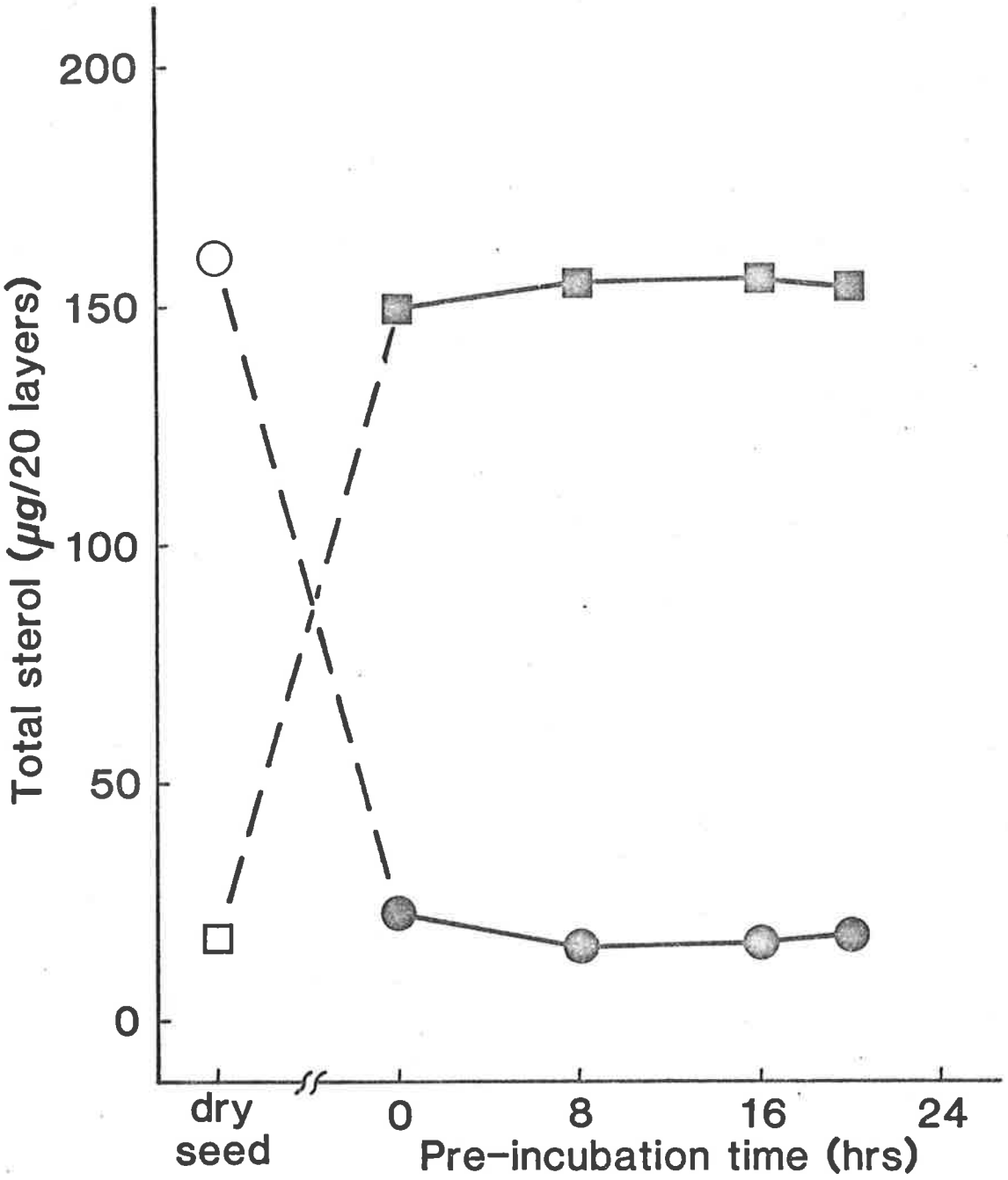

200
150
100
50
0
Total sterol (μg/20 layers)
dry seed
0
8
16
24
Pre-incubation time (hrs)

Table 42. Steryl glycoside composition of aleurone tissue of CAP x MD (rht3, tall) de-embryonated seed pre-incubated at 30°C or 5°C for various lengths of time.

Experimental Treatment	Sterol (μg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	4.2*	13.0**	4.1**	35.1**
12 hrs imbibition (Control)	1.3	2.2	0.8	3.7
Pre-incubation time (hrs) at 30°C				
8	0.5	2.6	0.3	2.2
16	0.4	2.0	0.6	2.3
20	0.3	1.5	0.8	2.8
Pre-incubation time (hrs) at 5°C				
8	0.2	1.2	1.0	2.6
16	0.2	1.6	0.8	2.6
20	0.4	1.6	1.0	3.1

* Significantly different from control at P = 0.05, ** P = 0.01

Table 43. Steryl ester composition of aleurone tissue of CAP x MD (rht3, tall) de-embryonated seed pre-incubated at 30°C or 5°C for various lengths of time.

Experimental Treatment	Sterol (µg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	8.2**	25.6**	6.3*	63.6**
12 hrs imbibition (Control)	2.1	4.2	1.4	6.7
Pre-incubation time (hrs) at 30°C				
8	0.7	3.2	0.6	6.0
16	0.6	3.2	1.0	7.1
20	1.0	4.1	1.5	5.8
Pre-incubation time (hrs) at 5°C				
8	0.6	3.0	1.0	6.3
16	0.5	4.7	1.0	4.3
20	0.5	4.6	1.0	6.3

* Significantly different from control at P = 0.05, ** P = 0.01

4.3 Discussion

The main advantages of examining the behaviour of the phospholipids in the aleurone tissue of the tall selection, under similar conditions of imbibition and pre-incubation to that used with the dwarf, is that it yields useful comparisons with respect to the effect of low temperature on the two genotypes. This is emphasized by the facts that both genotypes were the product of the same initial cross, were grown and harvested under similar environmental conditions, and were isogenic with respect to the Rht3 gene.

Two important contrasts emerged from the comparison of the phospholipid behaviour of the aleurone tissue of the tall selection with that of the dwarf selection. Firstly, only the imbibition of water was required to initiate the synthesis of all the major phospholipids of the aleurone tissue of the tall selection. This was quite similar to earlier findings (Varty and Laidman, 1976 ; Mirbahar and Laidman, 1982). Secondly, pre-incubation temperature had no effect on either the phospholipids or their fatty acids of the tissue of the tall selection. These two differences, when considered with the absence of any Rht (Rht3 in this case) allele in the tall selection, underlines the possibility that the presence of the Rht gene might be responsible for the observed aberrations in phospholipid metabolism of both Kite and the dwarf selection aleurone tissues. Further comparison of the phospholipid composition of the aleurone tissue of the tall selection (pre-incubated for 20 hours at 5^{o} or $30^{o}C$) with that of a 20 hour - $5^{o}C$ pre-incubated aleurone tissue of the dwarf selection shows them to be remarkably similar Tables 15, 34 and 35). It is pertinent to point out that under the above mentioned conditions of pre-incubation, the aleurone tissue of the two lines displayed similar GA_3 sensitivities (Results 1.2.2).

Table 44. Free sterol composition of aleurone tissue of CAP x MD (rht3, tall) de-embryonated seed pre-incubated at 30°C or 5°C for various lengths of time.

Experimental Treatment	Sterol (µg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	0.8**	3.5**	0.8**	12.3**
12 hrs imbibition (Control)	10.0	35.6	8.3	95.6
Pre-incubation time (hrs) at 30°C				
8	10.4	38.6	9.3	99.3
16	8.2	37.6	8.3	100.1
20	9.2	35.6	9.2	96.3
Pre-incubation time (hrs) at 5°C				
8	9.5	40.2	7.8	96.3
16	11.2	39.8	9.3	98.6
20	7.2	38.3	8.6	97.2

* Significantly different from control at P = 0.05, ** P = 0.01

Thus, the low temperature treatment is not only able to equalize the GA_3-sensitivities of the two tissues (Results 1.2.2) but also their phospholipid compositions. This underlines the likelihood that, at least in the case of the aleurone tissue, low temperature treatment is able to circumvent or 'cure' the lesions caused by the presence of the Rht gene, with respect to both GA_3-sensitivity as well as phospholipid composition. Further, since the aleurone tissue of the tall selection attained its maximal GA_3-sensitivity during a period of 12 hours imbibition followed by 8 hours of pre-incubation (Results 1.2.2), and, as it is also during this period that all the phospholipid synthesis takes place (Tables 34 and 35), it does seem that phospholipid synthesis during imbibition/germination and GA_3-sensitivity may have a 'cause-and-effect' relationship.

With respect to the sterols, once again, as in the case of the aleurone tissue of Kite and the dwarf selection, none of the pre-incubation factors examined had any specific effect on their metabolism. Once again the dramatic nature of the disappearance of the esterified and glycosidic sterols during imbibition, and the appearance of free sterols in equivalent amounts, underlines a storage role for the derivative sterols in the mature wheat aleurone tissue.

5. Effect of various inhibitors on lipid metabolism and GA_3-sensitivity of the wheat aleurone tissue of CAP x MD (rht3, tall) selection.

In the previous sections (Results 3 and 4), it was found that, in the aleurone tissue, active phospholipid biosynthesis was linked with the development of GA_3-sensitivity. In this section, this observation has been examined by using various inhibitors which have been reported to be involved in the inhibition of phospholipid, fatty acid or sterol biosynthesis.

Of the inhibitors used in this study, Gammexane (Michell, 1975), Diclofop-methyl (Hoppe, 1980, 1981), Chlorpromazine and Trifluoperazine (Allan and Michell, 1975) are reported to be inhibitors of phospholipid biosynthesis; BASF 13-338 is reported to be an inhibitor of 18:3 biosynthesis (St. John and Christiansen, 1976 ; St. John et al., 1979) and SK&F 7997-A_3 is reported to be an inhibitor of sterol biosynthesis in both animal (Kraml et al., 1964) and plant (Douglas and Paleg, 1981a) systems.

5.1 Effect of various inhibitors on GA_3-sensitivity

De-embryonated seed of the tall selection (CAP x MD, rht3) were used to test the effects of the inhibitors on GA_3-sensitivity. The pre-incubation period in this case was of 24 hours at 30°C and, in essence, it was equivalent to the 12 hours of imbibition + 8 hours of pre-incubation period studied previously (Results 1.2.2). This decision to combine the two periods, imbibition and pre-incubation, into one was

made on the basis of the fact that at the end of the 8 hours of pre-incubation the CAP x MD(rht3, tall) tissue displayed maximal GA_3-sensitivity (Results 1.2.2) and any inhibitory effects of the inhibitors would presumably be easily picked up.

Various concentrations of SK&F 7997-A_3, BASF 13-338, Chlorpromazine and Trifluoperazine were made up in 5 ml of distilled water. In the case of both Chlorpromazine and Trifluoperazine, the pH was adjusted to 5.3 before carrying out the pre-incubations or incubations. Surface sterilized de-embryonated seed of the tall selection were pre-incubated with the stipulated concentrations of the inhibitors for 24 hours at 30°C. At the end of this period the de-embryonated seed were thoroughly washed and transferred to 5 ml of $Ca(NO_3)_2$ containing 0.1 µg/ml GA_3. After a further incubation of 24 hours at 30°C, the α-amylase activity in the ambient solution and tissue were assayed to determine the effect of the inhibitors on the GA_3-sensitivity during pre-incubation. In the case of the other two inhibitors, Gammexane and Diclofop-methyl, all procedures were exaclty as detailed above except that the inhibitors were delivered in 20 µl of acetone. The controls for these inhibitors also contained 20 µl of acetone.

When the effect of the inhibitors during the incubation period was to be studied, the de-embryonated seed were pre-incubated for 24 hours at 30°C before exposing them to the stipulated concentrations of the inhibitors and 0.1 µg/ml GA_3 for a further 24 hours. Alpha-amylase produced at the end of this period was measured to determine the effect of the inhibitors on the GA_3-sensitivity during incubation.

Tables 45, 46 and 47 provide information regarding the effects of BASF 13-338, Chlorpromazine and Trifluoperazine on the GA_3-sensitivity

Table 45

Effect of BASF 13-338 included in the pre-incubation or incubation periods, on α-amylase production by CAP x MD (rht3, tall) de-embryonated seed. Pre-incubation was carried out for 24 hours at 30°C ± inhibitor followed by 24 hour incubation with 0.1 µg/ml GA_3 and ± inhibitor.

Treatment	Inhibitor concentration (µg/ml)	α-amylase SIC per halfseed
Inhibitor included during pre-incubation		
	0 (control)	25.68
	50	23.74 n.s.
	100	24.18 n.s.
	200	22.75 n.s.
	400	24.62 n.s.
Inhibitor included during incubation		
	0 (control)	26.35
	50	24.72 n.s.
	100	23.85 n.s.
	200	25.32 n.s.
	400	24.68 n.s.

n.s. Treatment not significantly different from control at P = 0.05.

Table 46

Effect of Chlorpromazine included in the pre-incubation or incubation periods, on α-amylase production by CAP x MD (rht3, tall) de-embryonated seed. Pre-incubation was carried out for 24 hours at 30°C ± inhibitor followed by 24 hour incubation with 0.1 µg/ml GA_3 and ± inhibitor.

Treatment	Inhibitor concentration (mM)	α-amylase SIC per halfseed
Inhibitor included during pre-incubation		
	0 (control)	25.65
	0.01	26.84 n.s.
	0.10	27.48 n.s.
	1.0	23.24 n.s.
	2.0	25.41 n.s.
Inhibitor included during incubation		
	0 (control)	27.45
	0.01	24.95 n.s.
	0.10	25.91 n.s.
	1.0	26.84 n.s.
	2.0	28.32 n.s.

n.s. Treatment not significantly different from control at P = 0.05.

Table 47

Effect of Trifluoperazine included in the pre-incubation or incubation periods, on α-amylase production by CAP x MD (rht3, tall) de-embryonated seed. Pre-incubation was carried out for 24 hours at 30°C ± inhibitor followed by 24 hour incubation with 0.1 µg/ml GA_3 and ± inhibitor.

Treatment	Inhibitor concentration (mM)	α-amylase SIC per halfseed
Inhibitor included during pre-incubation		
	0 (control)	24.63
	0.01	26.21 n.s.
	0.10	25.32 n.s.
	1.0	27.23 n.s.
	2.0	25.85 n.s.
Inhibitor included during incubation		
	0 (control)	26.32
	0.01	24.84 n.s.
	0.1	25.75 n.s.
	1.0	26.38 n.s.
	2.0	27.10 n.s.

n.s. Treatment not significantly different from control at P = 0.05.

of the aleurone tissue from the de-embryonated seed of the tall selection. All concentrations of BASF 13-338, included either during pre-incubation or incubation periods had no effect on the GA_3-sensitivity of the aleurone tissue of the tall selection. This was shown by the comparable amounts of α-amylase produced by both the control and treated tissues (Table 45). Similar conclusions can be drawn for both Chlorpromazine (Table 46) and Trifluoperazine (Table 47).

Fig. 28 gives the effect of various concentrations (0.25 - 5 mg/ml) of SK&F 7997-A_3 on the GA_3-sensitivity of the aleurone tissue from the de-embryonated seed of the tall selection. While the presence of the inhibitor during the pre-incubation period was able to curtail the α-amylase response, the inhibitor had no effect whatsoever on the response during the incubation period. This inhibitory effect was concentration dependent and, at a concentration of 5 mg/ml, SK&F 7997-A_3 was able to cut down the GA_3-response of the tissue to only 3% of the control.

Figs. 29 and 30 give the effect of Gammexane and Diclofop-Methyl on the GA_3-sensitivity of the aleurone tissue from the de-embryonated seed of the tall selection. Essentially, the nature of the effects of the inhibitors on the GA_3-sensitivity, in the case of Gammexane and Diclofop-Methyl, was the same as that observed with SK&F 7997-A_3 (Figs. 28, 29, 30). One difference in the case of Diclofop-Methyl (1 mM) was the slight inhibition of the α-amylase response when the inhibitor was present in the incubation period (Fig. 30).

Figure 28: Effect of SK&F-7997-A_3 included in the pre-incubation or incubation periods, on α-amylase production by CAP x MD (rht3, tall) de-embryonated seed.

Pre-incubation was carried out for 24 hrs at 30°C ± the stipulated dose of the inhibitor. Incubation was carried out for 24 hrs at 30°C with 0.1 µg/ml GA_3 ± the stipulated dose of the inhibitor.

● SK&F-7997-A_3 included during incubation

■ SK&F-7997-A_3 included during pre-incubation

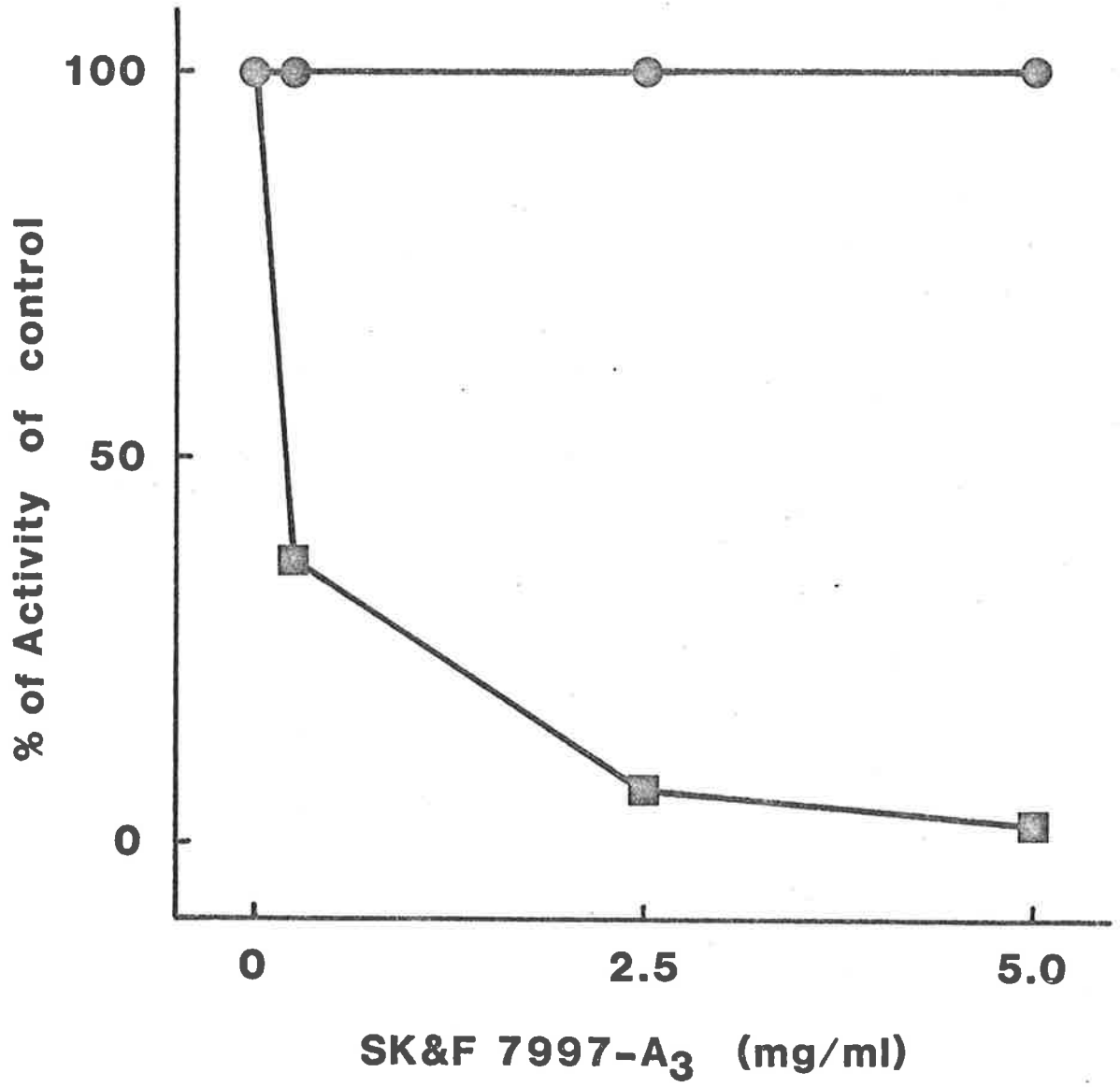

100
50
0
% of Activity of control
0
2.5
5.0
SK&F 7997-A3 (mg/ml)

Figure 29: Effect of Gammexane included in the pre-incubation or incubation periods, on the α-amylase production by CAP x MD (rht3, tall) de-embryonated seed.

Pre-incubation was carried out for 24 hrs at 30°C ± the stipulated dose of the inhibitor. Incubation was carried out for 24 hrs at 30°C with 0.1 µg/ml GA_3 ± the stipulated dose of the inhibitor.

● Gammexane included during incubation

■ Gammexane included during pre-incubation

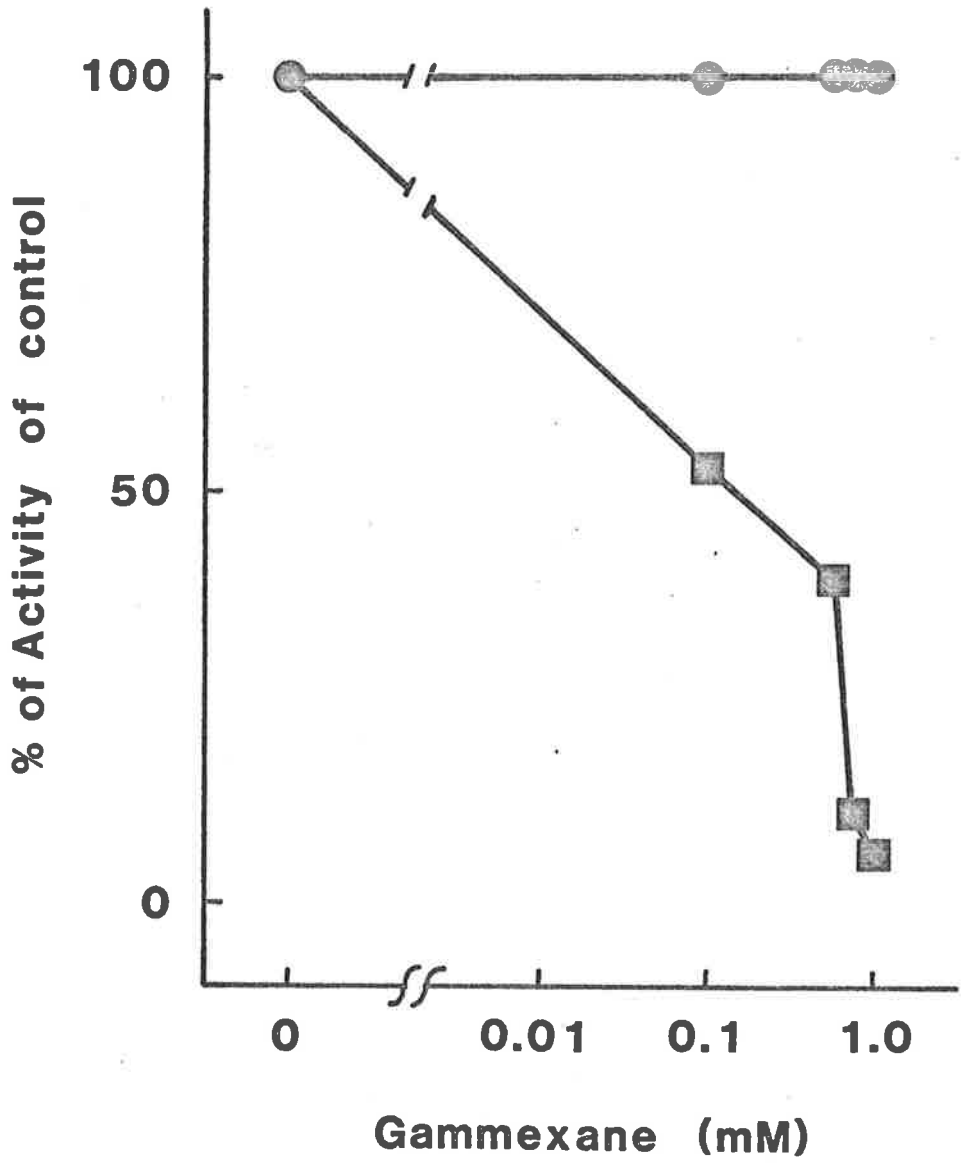
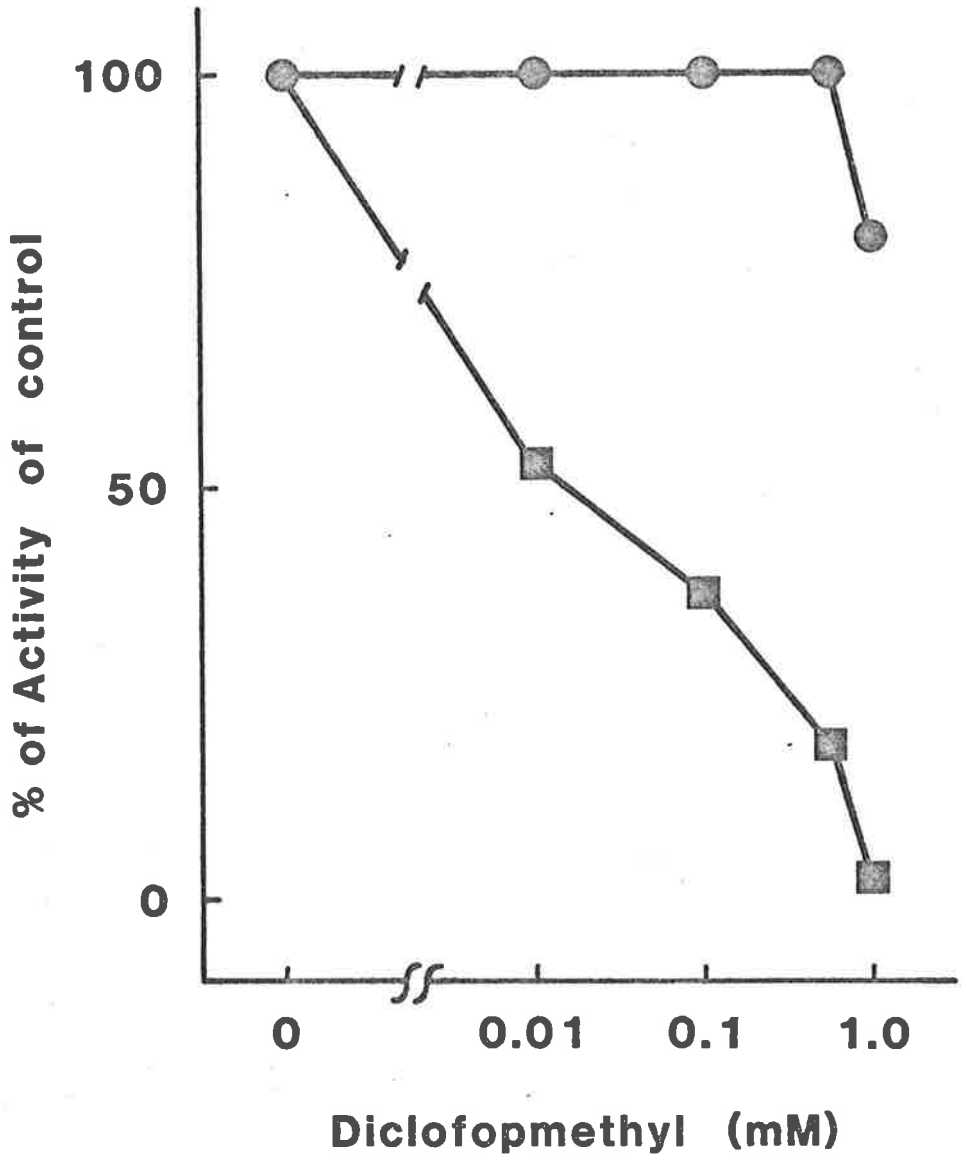

100
50
0
% of Activity of control
0
0.01
0.1
1.0
Gammexane (mM)

Figure 30: Effect of Diclofop-Methyl included in the pre-incubation or incubation periods, on the α-amylase production by CAP x MD (rht3, tall) de-embryonated seed.

Pre-incubation was carried out for 24 hrs at 30°C ± the stipulated dose of the inhibitor. Incubation was carried out for 24 hrs at 30°C with 0.1 μ g/ml GA_3 ± the stipulated dose of the inhibitor.

● Diclofop-Methyl included during incubation

▒ Diclofop-Methyl included during pre-incubation


100
50
0
% of Activity of control
0
0.01
0.1
1.0
Diclofopmethyl (mM)

5.2 Effect of various inhibitors on the lipid metabolism

Once again, the two periods of 12 hours of imbibition and 8 hours of pre-incubation, employed in earlier experiments (Results 4), were combined into a 24 hours pre-incubation period. This decsion to combine the two periods, imbibition and 8 hours of pre-incubation, into one was made on the basis of the fact that during these periods the CAP x MD (rht3, tall) aleurone tissue displayed active lipid biosynthesis and any inhibitory effects of the inhibitors would presumably be easily picked up. De-embryonated seed of the tall selection were pre-incubated for 24 hours at 30^{o}C with the various concentrations of the inhibitors and, at the end of this period, the phospholipids were extracted and analysed. The three inhibitors Gammexane, Diclofop-Methyl and SK&F 7997-A_3 were selected on basis of the results presented earlier (Results 5.1) where these three inhibitors, when present during the pre-incubation period, were shown to inhibit the α-amylase response. Only the concentration of the inhibitor having the maximum inhibitory effect on the α-amylase response was selected for these experiments (Figures 28, 29 and 30).

Table 48 shows the effect that 1 mM Gammexane, 1 mM Diclofop-Methyl and 2.5 mg/ml SK&F 7997-A_3 had on the constituent phospholipids of the aleurone tissue of the tall selection. Once again, only the imbibition of water was required to initate the synthesis of PI, PG, PC and PE, and the levels of these phospholipids in the control tissue was quite comparable to that found earlier (Tables 34, 35 and 48). While the presence of Diclofop-Methyl and Gammexane had no effect on the levels of any phospholipid, 2.5 mg/ml SK&F 7997-A_3 had a strong inhibitory effect on the levels of PI, PC and PE. It is of interest that these were the three phospholipids whose levels were significantly increased by low

Table 48. Effect of various inhibitors included in the pre-incubation period on the phospholipids of CAP x MD (rht, tall) aleurone tissue . Pre-incubation of de-embryonated seed was carried out for 24 hrs at 30°C with various inhibitors and phospholipids were analysed at the end of this period.
The control had 20 µl acetone/5 ml of the pre-incubation medium.

Treatment	Phospholipid (µg lipid/20 layers)				
	PI	PG	PC	LPC	PE
Dry Seed	40**	23*	239**	44	82**
24 hrs pre-incubation at 30°C (control)	146	46	438	49	114
24 hrs pre-incubation with 1 mM Gammexane	133	43	419	46	119
24 hrs pre-incubation with 1 mM Diclofop-Methyl	138	52	422	49	120
24 hrs pre-incubation with 2.5 mg/ml SK & F-7997-A_3	47**	37	266**	48	87**

* Significantly different from Control at P = 0.05, ** P = 0.01

temperature pre-incubation of the de-embryonated seed of the dwarf selection (Tables 14 and 15).

Table 49 shows the lack of effect of the inhibitors on the free fatty acids of the aleurone tissue from the de-embryonated seed of the tall selection. The free fatty acids were analysed after a pre-incubation with the inhibitors of 24 hours at 30^{o}C. Neither the 24 hours pre-incubation nor the presence of any of the inhibitors had any effect on the free fatty acids of the aleurone tissue.

Tables 50 and 51 show the effects that a 24 hour pre-incubation with the inhibitors had on the derivative (steryl esters and steryl glycosides) and free sterols of the aleurone tissue from the de-embryonated seed of the tall selection. The levels of derivative sterols detected in aleurone layers from dry seed were quite high and comparable with those found earlier. A 24 hour pre-incubation/imbibition period dramatically decreased their levels, a situation also found earlier (Tables 42, 43 and 50). None of the inhibitors had any effect on the levels of derivative sterols of the aleurone tissue from the de-embryonated seed of the tall selection (Table 50). A 24 hour pre-incubation brought about large increases in the levels of free sterols of the aleurone tissue of the tall selection (Table 51). This was again akin to the earlier findings (Table 44). Once again none of the inhibitors had any effect on the levels of any of the free sterols of the aleurone tissue (Table 51).

5.3 Discussion

De-embryonated seed of the tall selection were used in this study as they were found to have both high GA_3-sensitivity and active phospholipid biosynthesis.

Table 49. Effect of various inhibitors included in the pre-incubation period on the free fatty acids of the aleurone tissue of CAP x MD (Rht, tall) seed.
Pre-incubation was carried out for 24 hrs at 30°C with various inhibitors and free fatty acids were analysed at the end of this period.
The control had 20 µ l acetone/5 ml of the pre-incubation medium.

Treatment	Free Fatty Acid (µ g lipid/20 layers)					Total free fatty acid (µg lipid/20layers)
	16.0	18.0	18.1	18.2	18.3	
Dry Seed	28	6	27	46	4	112
24 hrs pre-incubation at 30°C (control)	33	7	27	51	5	122
24 hrs pre-incubation with 1 mM gammexane	27	5	21	54	4	110
24 hrs pre-incubation with 1 mM Diclofop-Methyl	29	9	25	47	6	117
24 hrs pre-incubation with 2.5 mg/ml SK & F-7997-A_3	30	7	27	43	6	114

Table 50. Effect of various inhibitors included in the pre-incubation period on the derivative sterols (steryl glycosides and esters) of aleurone tissue of CAP x MD (rht3, tall) seed. Pre-incubation was carried out for 24 hours at 30°C with various inhibitors and the derivative sterols were analysed at the end of this period. The control had 20 µl acetone/5ml of the pre-incubation medium.

Treatment	Steryl glycosides + Steryl esters (µ g lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	14.6**	34.3**	11.3**	100.4**
24 hrs pre-incubation at 30°C (control)	5.6	6.8	4.2	11.5
24 hrs pre-incubation with 1 mM Gammexane	3.2	5.2	3.4	9.3
24 hrs pre-incubation with 1 mM Diclofop-Methyl	4.2	4.3	2.3	10.8
24 hrs pre-incubation with 2.5 mg/ml SK & F-7997-A_3	5.2	5.1	3.8	9.6

** Significantly different from Control at P = 0.01

Effects of SK&F 7997-A_3 on the α-amylase response, as well as on phospholipid biosynthesis, quite clearly support the earlier findings in this work. The ability of SK&F 7997-A_3 to inhibit the α-amylase response could be ascribed to its ability to inhibit the biosynthesis of PI, PC and PE (Fig. 28 and Table 48), especially since PI, PC and PE were found to be highly correlated with the α-amylase response (Results 3.1.2) and, also, as their synthesis was found to be occurring during the 24 hour imbibition/pre-incubation period in the aleurone tissue of the tall selection (Results 4.1 and Table 48). This would also explain why the inhibitor had no effect on the α-amylase response when present only during the incubation period (Fig. 28); the de-embryonated seed used in this case had undergone a 24 hour pre-incubation and thus, the aleurone tissue would already have its normal content of these phospholipids (Table 48). These results indicate that, in addition to being an inhibitor of sterol biosynthesis, SK&F 7997-A_3 may also be a phospholipid biosynthesis inhibitor. This is an interesting, important and unexpected finding.

The fact that SK&F 7997-A_3 had no effect on sterol metabolism (Tables 50 and 51) supports the earlier findings of this work. It was concluded earlier (Results 2.2, 3.1.3 and 4.2) that the free sterols appearing at the end of the imbibition period were the result of mobilization of the steryl esters and glycosides. This suggests that little sterol synthesis was occurring and, since SK&F 7997-A_3 is an inhibitor of sterol biosynthesis, it would be expected to have no effect on the free sterol levels found at the end of the 24 hour pre-incubation period (Table 51).

Table 51. Effect of various inhibitors included in the pre-incubation period on the free sterols of the aleurone tissue of CAP x MD (rht3, tall) seed. Pre-incubation of de-embryonated seed was carried out for 24 hours at 30°C with various inhibitors and the free sterols were analysed at the end of this period.
The control had 20 µ lacetone/5ml of the pre-incubation medium.

Treatment	Free sterols (µg lipid/20 layers)			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Dry Seed	1.2**	3.6**	1.5**	13.8**
24 hrs pre-incubation at 30°C (control)	9.6	31.4	9.4	96.4
24 hrs pre-incubation with 1 mM Gammexane	10.5	32.5	10.8	94.5
24 hrs pre-incubation with 1 mM Diclofop-Methyl	8.8	34.7	9.2	95.5
24 hrs pre-incubation with 2.5 mg/ml SK & F-7997-A_3	9.8	38.4	11.2	97.4

** .Significantly different from Control at P = 0.01

The fact that neither 24 hours pre-incubation nor any of the inhibitors examined had any effect on the levels of free fatty acids found in the aleurone tissue of the tall selection (Table 49), would indicate either that the free fatty acids as such are not involved in the GA_3-response of the tissue, or that their concentrations were not sufficiently altered to interfere with the response of the tissue.

The effects of Gammexane and Diclofop-Methyl on the α-amylase response and phospholipid metabolism seem to be more difficult to interpret, especially since they are known to be inhibitors of phospholipid synthesis. However, it is possible that the lack of any inhibitory effect of Gammexane and Diclofop-Methyl on the phospholipid synthesis of the wheat aleurone might be due to an intrinsic property of the tissue itself. The ability of the inhibitors (Gammexane and Diclofop-Methyl) to inhibit the α-amylase response without affecting the phospholipids might indicate that, in addition to active phospholipid biosynthesis, there is an additional factor or factors which is involved in the development of GA_3-sensitivity during the pre-incubation period. Obviously, while SK&F 7997-A_3 influences phospholipid biosynthesis, both Gammexane and Diclofop-Methyl might be acting on this other factor/s.

Another possibility is that both Gammexane and Diclofop-Methyl interact with a certain component/s of the aleurone tissue, and 'change' its property/ies. Obviously, such a 'change' would not only have to be the cause of the observed inhibition of the α-amylase response but also be a time-dependent process to explain why the inhibitors had no effect on the α-amylase response when present exclusively during the incubation period. Changes in membrane permeability seem to be one such candidate as both Diclofop-Methyl and Gammexane have been shown to perturb ionic

contents of tissues. A requirement for Ca^{++} ions has been shown for the synthesis and secretion of α-amylase in isolated cereal aleurone layers. In addition, both Gammexane and Diclofop-Methyl have been shown to increase the leakage of amino compounds and inorganic ions in plants and, more importantly, this effect took place in a time-dependent manner. (Hoppe, 1980 ; Schefszuik and Simonis, 1979 ; Chrispeels and Varner, 1967 ; Goodwin and Carr, 1972b). A period of 24 hours pre-treatment, at least in the case of Diclofop-Methyl, was required to obtain a significant leakage of the amino compounds (Hoppe, 1980). Perhaps the use of shorter pre-incubation periods or longer incubation periods, with the inhibitors, would have clarified the situation further.

Overall, the experiments with the inhibitors once again underline the arguments discussed in the Introduction, (2.2.2), regarding the difficulties in interpreting their results. Lack of specificity of action of the inhibitors is the main difficulty and is always going to be a problem. Hence, in the light of our incomplete knowledge of the effects of the inhibitors, any conclusions drawn from the experiments with SK&F 7997-A_3, Gammexane and Diclofop-Methyl must be regarded with caution.

The effects of BASF 13-338, Chlopromazine and Trifluoperazine on the lipid biosynthesis of the aleurone tissue from the de-embryonated seed of the tall selection, were not examined in this study due to their lack of effect on the GA_3-sensitivity. Obviously, further experimentation involving the effects of these inhibitors on the lipids of the aleurone tissue is needed, before drawing any conclusions

regarding their lack of effect on the GA_3-response. The lack of any effect of Trifluoperazine on the GA_3-sensitivity found in this work does not agree with work carried out earlier by Elliot et al. (1983) in which Trifluoperazine was shown to curtail the α-amylase response of the barley aleurone layers.

IV. GENERAL DISCUSSION

The main aim of this work was to examine the possibility of a membrane-based site of GA_3 action in the wheat aleurone system. The low temperature-induced increases in GA_3-sensitivity and phospholipid augmentation in the wheat aleurone system, when considered in unison, clearly underline this possibility.

The low GA_3-insensitivity detected in the aleurone tissue/de-embryonated seed of Rht2/Rht3-containing varieties, unless exposed to low temperature (Results 1.1 and 1.2), is probably due to the complete lack of synthesis of certain phospholipids during imbibition. This suggestion is supported by the generally accepted view that the imbibition of water is the principal event responsible for initiating the synthesis of phospholipids in wheat aleurone tissue, and that this synthesis of phospholipids, and its associated development of membranes, are probably pre-requisites for the GA_3 responsiveness of the tissue (Varty and Laidman, 1976 ; Laidman, 1982). More support for the above stated hypothesis comes from the fact that, in the tall selection, active synthesis of all the phospholipids (except LPC) during imbibition is also associated with high GA_3-sensitivity (Results 4.1). Results from the experiment with inhibitors also support, albiet with certain qualifications, the concept of a link between phospholipid augmentation and subsequent development of GA_3-sensitivity (Results 5).

It has been suggested, that the GA_3-insensitivity in wheat aleurone tissue containing the Rht3 gene, may be related to a lack of receptor molecules of GA_3 (Ho *et al.*, 1981 ; Gale and Marshall, 1975). Due to the probability that the low temperature-induced increase in GA_3-

sensitivity is not a result of changes in the relative amounts of isozyme produced by α-amylase structural genes, or the starch liquefaction capacity of the enzymes, or the lag-time of α-amylase production, or alterations in the cellular metabolism of the aleurone tissue (except phospholipid synthesis) (Discussion 1.3), it seems likely that the low temperature treatment which "cures" or circumvents the genetic lesion manifest in the aleurone tissue of fully matured seed containing the Rht3 gene is a result of an increase of effective hormone receptor sites.

Strong evidence for this statement comes from the experiments of other research workers with isogenic lines (Rht3/rht3). Flintham and Gale (1982) found that the introduction of the Rht3 gene caused a 77% reduction in α-amylase produced by the dwarf selection when compared to the tall selection. The fact that a 20 hour pre-incubation at $5^{o}C$ is able to bring the maximal α-amylase responses of the dwarf and tall selections to par, indicates the full restoration of the α-amylase response, affected probably, by the low temperature-induced increase in GA_3-receptor sites (Results 1.2.2). According to this line of argument, the tall selection would have the full/normal complement of GA_3-receptor sites, and, thus, pre-incubation temperature would have little or no effect on the GA_3 response of the tall selection. This was indeed what was found (Results 1.2.2).

Given that the already stated similarities of the low temperature-induced increase in GA_3-sensitivity of aleurone tissue/de-embryonated seed of Rht1, Rht2 and Rht3 mutants, and the fact that low temperature-induction of GA_3-sensitivity seems to be operative in aleurone tissue of only varieties having at least one of the three Rht alleles (Results 1),

it is probable, therefore, that the low temperature treatment effect which "cures" or circumvents the genetic lesions manifest in the Rht1 and Rht2 genotypes, is the same as that effective in the Rht3-containing genotype i.e. an increase in GA_3 receptor sites.

Invoking a 'low temperature-induced increase in GA_3-receptor sites' hypothesis also explains why the magnitude of the low temperature-induced increase in GA_3-sensitivity is GA_3-concentration dependent in all the experiments. At the lower GA_3 concentration (0.01 µg/ml), GA_3 itself would become a limiting factor, unable to saturate the increased number of GA_3-receptor sites produced as a result of the low temperature treatment (Results 1.2.1 and 1.2.2 Ho et al., 1981). Furthermore, the results from all experiments indicate that the low temperature-induced increase in GA_3-receptor sites is not an 'all-or-none' process but a quantitative, temporal one, since a 20 hour pre-incubation at $5^{o}C$ always produced a significantly greater amount of α-amylase than a 16 hour pre-incubation at the same temperature (Results 1.2.1 and 1.2.2). This would imply the co-existance of both activated and non-activated cells in the same aleurone tissue, a situation very similar to the one suggested earlier (Gibson and Paleg, 1982).

If the overall effect of low temperature on Rht3-containing varieties is an increase in hormone receptor sites, then clearly, the simplest interpretation of the extremely close relationships between phospholipid composition and hormone sensitivity implicates and underlies the possibility that the GA_3-receptor sites are membrane-based (Wood and Paleg, 1972; 1974). Phospholipid composition is of major importance in the control of the properties of membranes and membrane-based enzymes (McMurchie et al., 1983 ; King and Spector, 1978 ;

Livingstone and Schacter, 1980 ; Buckland et al., 1981). This is further highlighted by the fact that the phospholipid composition, both relative and absolute, of the aleurone tissues with low GA_3-sensitivity, is drastically different than that of the highly sensitive tissue (Results 2.1, 3.1 and 4.1).

As most of the phospholipid synthesized during the periods studied in this work are likely to be incorporated into membranes (Varty and Laidman, 1976), different phospholipid compositions may be expected to react with GA_3 to greater or lesser extents, and even in different ways (Pauls et al., 1982 ; Wood et al., 1974). The structure and function of different membranes may be differentially modulated by GA_3, producing differential effects on the physical parameters of the different lipid membranes and on the membrane-based enzymes (Livingstone and Schacter, 1980 ; McMurchie et al., 1983). One of the important membrane properties influenced by changed membrane composition is permeability (Wood and Paleg, 1974). This presents a potential control mechanism of the movement of regulatory compounds, ions and diffusion-limited substrates all of which could influence the subsequent response of the tissue (Goodwin and Carr, 1972b ; Chrispeels and Varner, 1967).

Evidence indicating membranes as the primary targets for hormones has been growing over the years (Pauls et al., 1982 ; Jusaitis et al., 1982 ; Gibson and Paleg, 1977). More recent evidence to support this hypothesis comes from the data of Jones and Paleg (1984a,b) and Jones et al. (1984). These workers have been able to demonstrate complex formation between IAA and phospholipid membrane components in aqueous media. One of the important features of such hormone-lipid complex formation is that IAA binds to both the hydrocarbon and head group

regions of amphiphiles containing the trimethyl ammonium group. This is further highlighted by the results with GA_3, inasmuch as both the acyl (hydrocarbon) and the polar head groups of phospholipids are strongly correlated with the GA_3-sensitivity.

Most of the metabolic changes induced by cold temperatures are associated with lipids and thus, it was not surprising to find the changes described in Sections 2, 3 and 4. However, lipids are not the only cellular components to respond to low temperatures, and it is possible that other metabolites may also change. Of course, the possible importance of changes in other areas of metabolism will depend heavily on their temporal relationships with changes in GA_3-sensitivity and their correlations with hormonal responsiveness.

Overall, the results obtained in this work support the concept of a membrane-based site of GA_3 action in the wheat aleurone layer. The exact composition (or range of compositions), and the precise site of the receptor membrane is still unknown, but the approach and the tissue will continue to prove useful in the solution of the problem.

One very interesting aspect which emerges from this study is the influence of the Rht gene on phospholipid metabolism. The presence of either Rht2 or Rht3 results in a gross aberration of the phospholipid metabolism of the wheat aleurone tissue. The results of experiments with Kite (Rht2), with the dwarf (Rht3) and the tall (rht3) selections, all support this conclusion.

In view of the likely role of the active phospholipid synthesis in the aleurone tissue in relation to GA_3-sensitivity, the influence of the

Rht gene on phospholipid synthesis probably constitutes a mechanism by which this gene is able to impart GA_3-insensitivity to the wheat aleurone tissue. Further, the Rht3 gene can be considered to be more potent than the Rht2 gene in its ability to influence phospholipid metabolism, inasmuch as the synthesis of three phospholipids (PI, PC and PE) is curtailed by the presence of Rht3, while Rht2 is only able to influence the synthesis of two phospholipids (PC and PE). The fact that GA_3-insensitivity due to the presence of Rht2 gene may not be fully operative in the aleurone tissue as compared to its being fully operative in the case of Rht3 gene (Gale and Marshall, 1975), further highlights this observation.

Whether these explanations can also be extrapolated to the GA_3-insensitivity of aerial tissues of wheat is open to conjecture. However, it is worth bearing in mind that in a previous study with Avena stem segments, the growth response of the tissue to GA_3 was strongly correlated with, among other things, the phospholipid composition of the tissue (Jusaitis et al., 1982). Furthermore, Vincent and Suty (1980) found that both Rht1 and Rht2 genes had an effect on the quantitative lipid (phospholipid) content of six day old etiolated wheat seedlings, and suggested this as being instrumental in the decrease in the rate of subapical growth.

The similarity of the sterol metabolism in the aleurone tissues of Kite, and the dwarf and tall selections, indicates the lack of influence of the Rht gene on sterols. This indicates that the difference in physiological responses of dwarf and tall wheat tissues to GA_3 is not a result of differences in their sterol content or metabolism. This is supported by the fact that the sterol composition of dwarf and tall Pisum sativum seedlings is identical (Westerman and Roddick, 1982).

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