THE LYSOSOMAL NATURE OF HORMONALLY INDUCED ENZYMES IN WHEAT ALEURONE CELLS

by

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Thesis submitted for the

Degree of Doctor of Philosophy

March, 1974

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SUMMARY

Aleurone tissue isolated with a roller mill device was examined in detail for its responsiveness to GA as measured by α-amylase production. It was found that tissue isolated in dilute lactic acid was most responsive and that this responsiveness could be increased by suitable incubation conditions. The method finally adopted yielded aleurone tissue that produced in response to GA more α-amylase than an equivalent amount of GA-treated half seeds.

The subcellular distribution of the GA-induced acid hydrolases, α-amylase, protease and ribonuclease in homogenates of wheat aleurone cells was determined. α-Amylase and protease were found to be almost entirely located in particulate fractions but ribonuclease was found mainly in final supernatant fractions. Control experiments were performed which indicated that the levels of hydrolytic enzymes found in particulate fractions represented true membrane enclosed enzymes and were not due to artefacts of particle preparation. Furthermore, as the membrane enclosed acid hydrolases exhibited distinct structure-linked latency and could only be liberated by physical or chemical means, it was concluded that GA-induced α-amylase and protease were lysosomal.

Using isopycnic density gradient centrifugation, lysosomes were concentrated in a single region of density gradients (ρ = 1.080 g/cc) well separated from most other aleurone cell organelles. Lysosomes were found to be rich in α-amylase and protease but contained little ribonuclease activity. The lysosomal band also contained a majority of the $NADH_2$-cytochrome c reductase, a marker enzyme for ER, found in the gradient. Because repeated attempts failed to separate lysosomal α-amylase from cytochrome c reductase on density gradients, it was concluded that GA-induced lysosomes in wheat aleurone cells were ER-derived. Examination of electron micrographs revealed that a purified band of lysosomes contained at least three vesicle types ranging in size from 0.1 - 0.5 μ.

The permeability of lysosomal membranes to GA-induced α-amylase was examined under a variety of conditions. Lysosomal membrane permeability was increased by low and high pH, and calcium ions, but was decreased by phosphate. GA was found to have no effect on α-amylase leakage from isolated lysosomes *in vitro*, but caused a significant increase in the permeability of lysosomal membranes *in vivo*. Evidence is presented that this GA-induced increase in lysosomal membrane permeability may be associated with an *in vivo* change in the thermal transition of lysosomal membranes.

It is suggested that the observed effects of GA on lysosomal membranes could have profound effects on the metabolism of cereal aleurone cells, and could be closely related to the hormonal trigger mechanism.

STATEMENT

I hereby declare that the thesis here presented is my own work, that it contains no material previously published, except where due reference is made in the text, and that no part of it has been submitted for any other degree.

(Robert Alan Gibson)

ACKNOWLEDGEMENTS

The author wishes to thank Professor L.G. Paleg and Dr. C.F. Jenner for their guidance and advice during the course of this work. Help and advice from other members of the Department of Plant Physiology are also gratefully acknowledged.

The author also wishes to thank Dr. R.I.B. Francki and Dr. J.W. Randles of the Department of Plant Pathology for their generous loan of equipment, and Dr. W.B. Wallace of the Department of Agricultural Biochemistry for many helpful discussions. The preparation of photographs by Mr. B. Palk and assistance in the electron microscope work by Mr. R. Miles are also gratefully acknowledged.

Sincere thanks are also due to Carol Gibson who prepared the figures and whose patient understanding helped make this thesis possible.

Financial support was provided by a Barley Improvement Trust Fund Scholarship, a Commonwealth Post-Graduate Studentship, and a C.S.I.R.O. Post-Graduate Studentship which are gratefully acknowledged.

PREFACE

A portion of the subject matter of this thesis (Section II) has been published under the title "Lysosomal nature of hormonally induced enzymes in wheat aleurone cells" by R.A. Gibson and L.G. Paleg, Biochem. J. (1972) 128 : 367-375.

LIST OF ABBREVIATIONS

ABA	Abscisic acid
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cc	Cubic centimetre
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid (di-sodium salt)
g	Gram(s)
g	Gravity
GA	Gibberellin A_3
hr	Hour(s)
IAA	Indole acetic acid
M	Molar
mM	Millimolar
ml	Millilitre
N	Normal
nm	Nanometer
$NADH_2$	Nicotinamide adenine diphosphate
RNA	Ribonucleic acid
m-RNA	Messenger ribonucleic acid
t-RNA	Transfer ribonucleic acid

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INTRODUCTION

The aleurone layer of cells of cereal grains, derived from the same triploid nucleus as the endosperm tissue it surrounds, has long been recognised as being important in the germination process. As early as the 19th century Haberlandt (1890) suggested that this tissue secreted enzymes that hydrolysed the storage compounds of the endosperm. This finding was disputed by Brown and Morris (1890), verified by Brown and Escombe (1898), questioned by Bruschi (1908), Mann (1915) and Bennion (1924) and reverified by Gruss (1928) and Schander (1934).

Although the early workers established that the secretion of hydrolytic enzymes by aleurone cells was somehow controlled by the embryo (Haberlandt, 1890; Brown and Escombe, 1898), it was not until 1960 that the gibberellins were identified as the compounds triggering the secretory response in aleurone cells (Paleg, 1960; Yomo, 1958, 1960a, b, c, d). Since that time evidence has continued to accumulate that gibberellins are endogenous hormones of seeds (Jones *et al.*, 1963). MacLeod and Palmer (1966) identified gibberellic acid (GA) in one day old barley seedlings and Yomo and Iinuma (1966) detected gibberellin-like substances in isolated barley embryos. This finding was confirmed by Radley (1967) who also demonstrated that gibberellin-

like substances were released from the scutellum early in the germination process although MacLeod and Palmer (1966) presented evidence that the site of gibberellin production was the nodal region of the embryo. Finally Cohen and Paleg (1967) established that the amount of gibberellin-like substances secreted by a barley embryo is sufficient to account for the mobilization of the endosperm *in vivo*.

Recently another site of synthesis of the hormone has been proposed. Kessler and Kaplan (1972) reported finding increased levels of gibberellin-like compounds in extracts of embryo-less half seeds of barley that had been treated with 5 mM-cyclic adenosine monophosphate (cyclic-AMP). Because inhibitors of DNA and RNA synthesis inhibited the synthesis of the gibberellins, these workers concluded that cyclic-AMP acts at the level of the gene causing the ultimate synthesis of GA and induction of hydrolytic enzymes in aleurone cells. Although the work indicates that seed tissues other than the embryo may be capable of synthesizing gibberellins, the involvement of cyclic-AMP plant metabolism and the actual site of synthesis of the gibberellins (endosperm or aleurone cells) remains to be demonstrated.

Although GA is the most potent substitute for the embryo, many other compounds are reported to be nearly as effective. The molecular structures of these compounds are highly diverse, encompassing such substances as the sesquiterpenoids, helminthosporal and helminthosporic acid (Briggs, 1966; Okuda *et al.*, 1967), the

nucleotides ADP and cyclic-AMP (Duffus and Duffus, 1969; Kessler, 1969; Galsky and Lippencott, 1969; Nickells *et al.*, 1971; Earle and Galsky, 1971), kinetin (Boothby and Wright, 1962), several barbiturates, most notably phenobarbital (White, 1970), amino acids such as glutamate and aspartate (Galsky and Lippencott, 1969), gibberellin precursors kaurene (K.C. Jones, 1968) and mevalonic acid (van der Groen-Petridis *et al.*, 1968), and other compounds such as sclerin (Ogawa, 1966), caffeine (Alvarez, 1969) and phaseolic acid (Redeman *et al.*, 1968). Although this list is large, none of the compounds listed induce levels of α-amylase equal to an optimal concentration of GA and few of them are known to be present in cereal seeds.

There is little doubt that the normal target tissue for the GA produced by the embryo is the aleurone layer of cells. The cells of the starchy endosperm appear to be metabolically inert in that they do not respire and do not produce α-amylase in response to applied GA when the aleurone layer is removed (Paleg, 1964). Conversely, isolated aleurone cells respire at a rate comparable with rates observed for other plant tissues (Paleg, 1964; Rowsell and Ali Khan, 1966) and produce and secrete hydrolytic enzymes in response to GA at a rate comparable to intact embryoless half-seeds (Chrispeels and Varner, 1967b). The cells of the testa-pericarp which surround the mature seed appear to be devoid of cell contents and are considered

to be dead (Esau, 1953; Evers, 1970).

The value of aleurone tissue as a system for the study of the biochemical basis of GA action was recognised (Paleg, 1965; Varner *et al.*, 1965) and it has been intensively employed for many years. The attractiveness of the aleurone layer as a biochemical tool stems from several unique features of this tissue: a) gibberellins are the natural trigger of enzyme production *in vivo*; b) its response is confined to one hormone class; c) the target tissue consists of only one cell type; d) the cells of the target tissue are differentiated, non-meristematic and non-photosynthetic; e) the tissue can be isolated relatively free of other tissues and remains responsive to the hormone; and f) the isolated tissue responds in a specific and measurable manner to the addition of the hormone independent of the addition of substrate (Jones, 1973).

One of the easiest measurable responses of aleurone tissue to GA is the synthesis and secretion of α-amylase and because of this fact, α-amylase is widely used as a reference enzyme when studying the biochemical events following GA application. However, apart from α-amylase other acid hydrolases are secreted from this tissue in response to GA including proteases (Yomo, 1961; Briggs, 1963; Jacobsen and Varner, 1967; Sundblom and Mikola, 1972), ribonucleases (Chrispeels and Varner, 1967; Bennett and Chrispeels, 1971),

β-glucanases (MacLeod and Millar, 1962; Pollard, 1969; Taiz and Jones, 1970; Jones, 1971), hexosidases (Pollard, 1969) and nucleosidases (Shuster and Gifford, 1962).

The synthesis and release of hydrolytic enzymes following GA application to aleurone layers generally occurs after a characteristic lag period of 6-8 hr in barley (MacLeod *et al.*, 1964b; Chrispeels and Varner, 1967a) and 4-6 hr in wheat (Collins *et al.*, 1972). MacLeod *et al.* (1964b) indicated that endo-β-glucanase was the first enzyme to be initiated in GA-treated barley half seed, followed by α-amylase and later by proteinase. Jacobsen and Varner (1967) on the other hand, found that α-amylase and proteinase were initiated simultaneously and had similar release curves. Chrispeels and Varner (1967b) reported that ribonuclease was initiated later than α-amylase and was not released until about 24 hr after the addition of the hormone.

As the enzymes produced are all hydrolases and because of the initiatory action of GA, it was earlier suggested that the enzymes may be either proenzymes (Paleg, 1960) or pre-formed enzymes located in membrane bound lysosomes (MacLeod and Millar, 1962). These suggestions were untenable when they failed to find a particle that could be stimulated *in vitro* by GA to release the hydrolases (MacLeod

and Millar, 1962). When hydrolytic enzyme synthesis was found to be inhibited by anaerobiosis (Briggs, 1963; Varner, 1964; MacLeod, 1963), and protein synthesis inhibitors (Briggs, 1963; Paleg, 1964; Varner and Shidlovsky, 1963) it was suggested that at least some of these enzymes may arise by *de novo* synthesis. Incorporation studies using ^{14}C-algal protein hydrolysates (Briggs, 1963) and l-phenylalanine-^{14}C (Varner and Ram Chandra, 1964) indicated that much of the protein secreted from GA-treated aleurone tissue arose by protein synthesis. Purification of α-amylase from tissue treated with GA 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 induced enzymes (Varner and Ram Chandra, 1964).

As labelled amino acids were diluted by hydrolysis products of endogenous protein it was not certain that all of the α-amylase produced was synthesized *de novo*. Filner and Varner (1967) claim to have settled the question by using an elegant density labelling technique. The α-amylase produced in tissue treated with GA, ^{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 and $H_2{}^{18}O$ was mixed with a trace (not enough to be measured enzymatically) of

the ^{14}C-α-amylase and subjected to isopycnic centrifugation in a cesium chloride density gradient. The position of the ^{18}O-α-amylase was located by measuring enzyme activity while the marker ^{16}O-α-amylase was located by measuring radioactivity. It was shown that the band of α-amylase activity corresponding to the ^{18}O-enzyme was displaced towards a region of higher density. Filner and Varner concluded that virtually all of the enzyme was synthesized *de novo*. Using identical methods, Jacobsen and Varner (1967) concluded that protease was also synthesized *de novo* in response to GA.

Filner and Varner (1967) were careful to point out that the $H_2{}^{18}O$ could be incorporated into the carbohydrate moiety rather than the amino acid portion, if α-amylase would be shown to be a glycoprotein. Although they could detect no carbohydrate in the α-amylase region of the density gradient they pointed out that if as little as 3% of the molecule was carbohydrate, incorporation of $H_2{}^{18}O$ into this portion of the α-amylase molecule could account for the density shift observed. The recent report by Mitchell (1972) that more than 3% of highly purified barley α-amylase consisted of covalently bonded carbohydrate is therefore pertinent. Although all of the carbohydrate moiety of the α-amylase molecule would have had to be labelled with ^{18}O to accound for the density shift by Filner and Varner (1967), the report nevertheless raises doubts. Further, the detailed examination of the isozymes of barley α-amylase carried out by Jacobsen *et al.* (1970) suggests that two major types of α-amylase, one stable and the other unstable, are

synthesized in response to GA. As the unstable form constitutes the major portion of total α-amylase, the apparent shift in density reported by Filner and Varner (1967) could be accounted for by a shift towards a lighter density by the radioactive marker enzyme caused by an inactivation of the unstable isozyme. Although such a suggestion may appear rather tenuous, evidence based on the density labelling technique is not unequivocal. Using this technique, Bennett and Chrispeels (1972) for example claim to have shown that ribonuclease and β-1,3-glucanase are also produced by *de novo* synthesis despite reports that the latter enzyme is pre-formed (Taiz and Jones, 1970; Jones, 1971).

In spite of these objections, the weight of evidence indicates that a majority of α-amylase and protease molecules are synthesized *de novo* in GA-treated aleurone cells. The question that therefore arises is how does GA control this protein synthesis? Until about 1960, the most prevalent view for explaining the action of hormones was in terms of a direct hormone interaction resulting in the modification of enzymic activity (Tepperman and Tepperman, 1960). However, the realization that such a mechanism could not possibly explain the multiplicity of some hormone responses, prompted Karlson (1963) to propose an explanation of hormone action that has generated great interest. Based on the simple Jacob and Monod (1961) model

for microbial enzyme induction and on the ecdysone-induced chromosome puffing in dipteran salivary glands (Clever and Karlson, 1960), Karlson suggested that hormones act as inducers, which, by combining with appropriate repressors, control m-RNA synthesis and thus regulate enzyme synthesis.

Before discussing the evidence in favor of such a mechanism it is well worth considering some of the important general features of hormone action.

1. Most hormones exhibit multiple actions, some hormones regulating growth or development as well as a variety of metabolic functions in adult tissues. This multiplicity raises the question, as yet unanswered, whether the hormone has a single site of action leading to the several actions, or many sites, one for each of the major effects. In other words, is the site of action the same in aleurone tissue, where GA induces hydrolytic enzyme secretion, as in oat stem segments where GA induces a dramatic growth response (Adams *et al.*, 1973)?

2. There is a high degree of specificity in response to hormones, which may arise from the presence of an appropriate receptor molecule. Therefore whether a response is evoked or not may depend on the receptiveness of the target tissue as much as on the specific

nature of the hormone provoking it. The difference in response of endosperm and aleurone cells to GA is a good example.

3. The same tissue may be dependent on, or responsive to, more than one hormone. The responsiveness of aleurone tissue to GA as well as ethylene (Jones, 1967) and abscisic acid (Chrispeels and Varner, 1967b) are cited as examples.

4. Most growth and development hormones are active at extremely low levels; at high doses they may be toxic or promote other effects. There is also a characteristic lag period with each hormone before its response is provoked and therefore only biochemical responses falling within this lag period may be of any causal significance.

Thus any mechanism of hormone action must take into account all of the above features of the hormone's response (Tata, 1968).

A major implication of the gene de-repression hypothesis of hormone action is that in response to the hormone a specific m-RNA will be synthesized for each of the proteins induced by GA. Workers have attempted to demonstrate this by either specifically inhibiting RNA synthesis without inhibiting protein synthesis, or by attempting to isolate a GA-induced RNA fraction. Both approaches have yielded

equivocal results.

Two kinds of inhibitors have been used to examine GA effects on RNA synthesis. Compounds such as actinomycin-D and ethidium bromide interact with DNA altering its structure and blocking RNA polymerase activity (Cerami *et al.*, 1967). Structural analogues of purine and pyrimidine bases have also been used which either compete with the natural bases for incorporation into new RNA resulting in the formation of altered protein molecules (Mahler and Cordes, 1966), or act as antimetabolites preventing the conversion of bases to their nucleotides (Ross, 1964). Such structural analogues include 8-azaadenine, 8-azaguanine, 6-azaguanine, 6-methylpurine, 6-bromouracil, 2-thiouracil, 5-fluorouracil, thiocytosine and 5-azacytidine.

Experiments carried out by Chrispeels and Varner (1967a, b) indicate that when actinomycin-D (100 µg/ml) was added at the same time as GA to aleurone layers, α-amylase synthesis was reduced by 58%, but if added at the end of the lag period (8 hr after GA) inhibition was only 10%, even though ^{14}C-uridine incorporation was more than 66% inhibited at the same time. In a more detailed study, Goodwin and Carr (1972) found that actinomycin-D was most effective in a short interval late in the lag phase but after this time it was without effect despite continuing to inhibit incorporation of ^{14}C-uridine.

The effects of purine and pyrimidine analogues on GA-induced

α-amylase synthesis are entirely different. When 6-methylpurine (0.1 mM) was added to aleurone layers at the same time as GA, α-amylase synthesis was inhibited by 90% and when added 8 hr after hormone addition it continued to have effect (45% inhibition). Removal of GA after initiation of α-amylase synthesis and secretion was found to stop further increases in enzyme production as did adding 6-methylpurine (5 mM) at the time that GA was restored to the tissue (Chrispeels and Varner, 1967a, b). Other inhibitors found to act in the same way were abscisic acid (ABA), 8-azaguanine, 5-azacytidine and rifampicin (Chrispeels and Varner, 1967a, b; Goodwin and Carr, 1972). The conclusions drawn from such experiments were that actinomycin-D blocks the formation of a stable RNA species, or an RNA species that is rapidly translated to give a stable protein, while nucleotide analogues and rifampicin (an RNA synthesis inhibitor) act by inhibiting the formation of another unstable species of RNA which is continuously required for enzyme synthesis. Inhibition of α-amylase synthesis by mid-course removal of GA has been interpreted as meaning that GA is continually required for the synthesis of the unstable RNA species (Chrispeels and Varner, 1967a, b; Goodwin and Carr, 1972).

Such conclusions can only validly be made if in fact the effects of the inhibitors are truly specific, but such is not the case. For example, 6-methylpurine (0.5 mM) has been observed to inhibit

respiration in sugar cane stem tissue (Gayler and Glaziou, 1968) and actinomycin-D elicits many effects other than on RNA synthesis. Actinomycin-D at low concentrations has been shown to inhibit glycolysis and respiration (Laszlo *et al.*, 1966), phospholipid synthesis in chick embryo (Pastan and Friedman, 1968) and barley aleurone tissue (Koehler *et al.*, 1972), and has effects on protein synthesis quite independent of its action on RNA synthesis (Honig and Rabinovitz, 1964; Revel *et al.*, 1964). In addition, actinomycin-D has been reported to inhibit RNA transcription in reovirus which has no DNA (Gomatos *et al.*, 1964) and to cause the accelerated breakdown of pre-existing RNA (Stewart and Farber, 1968).

Although the results of experiments carried out with inhibitors are inconclusive, there is nevertheless the suggestion that RNA synthesis may be involved. If so, it should be possible to detect an increase in either the total RNA or an increase in a specific RNA in response to the hormone prior to the synthesis of the induced enzymes.

Naylor (1966) reported that GA enhanced the incorporation of ^{3}H-cytidine into the RNA of the aleurone cells of wild oats, but there was no difference in the incorporation rates of ^{14}C-uridine into the RNA fraction of control and GA-treated barley aleurone cells

(Ram Chandra *et al.*, 1967). Furthermore, although Ram Chandra and Varner (1965) reported that GA enhanced the incorporation of ^{14}C-precursors into the RNA of imbibed half-seeds, this effect did not precede the initiation of new protein synthesis and a similar effect could not be shown by other workers (Laidman, 1973).

The difficulties involved in detecting an effect on RNA species in aleurone tissue prompted Varner and Johri (1968) to examine the effect of GA on the RNA synthesis of isolated dwarf pea nuclei. When GA was included in all isolation and incubation media, RNA synthesis was found to increase by 60-100% over controls and the polymer produced appeared to show slightly different nearest neighbour and chromatographic properties than species of RNA synthesized in control nuclei. Although GA is also reported to stimulate incorporation of 2-^{14}C-uracil and ^{32}P into RNA of coconut milk nuclei (Roychoudry and Sen, 1965), it is difficult to assess the importance of these results in relation to aleurone cells.

A more detailed examination of the effects of GA on aleurone tissue RNA synthesis was carried out by Chandra and Duynstee (1970). These workers reported obtaining an 80-88% increase in incorporation of ^{32}P into soluble RNA within 90 min of GA application but the effect was no longer detectable after a further 2.5 hr. Examination of uridine incorporation showed only small differences in the various RNA

fractions between hormone-treated and control samples but this was greatest in a ribosome fraction. As incorporation into both the rapidly labelled soluble RNA and the ribosomal RNA were optimal at concentrations of GA (10^{-12} M) well below the optimal concentration required for α-amylase synthesis (10^{-6} M), and in view of the conflicting results obtained with inhibitors of RNA synthesis (Chrispeels and Varner, 1967b) it is difficult to accept that increased RNA synthesis is the primary mechanism of action of GA.

More recently Zwar and Jacobsen (1972) re-examined the effects of GA on RNA synthesis in barley aleurone tissue. Using acrylamide gel electrophoresis to separate RNA species these workers demonstrated that although only a small (0-10%) increase in incorporation of ^{14}C-uridine into ribosomal RNA was detected, a 300% increase in a fraction of RNA sedimenting between 5S and 14S was measurable 16 hr after GA treatment. The effect was not observed after 4 hr but was just detectable after 8 hr of hormone application. Zwar and Jacobsen (1972) concluded that the GA-stimulated RNA fraction could represent a newly synthesized m-RNA species because of a direct correlation between ^{14}C-uridine incorporation into 5-14S RNA and α-amylase production. The effects of the following treatments on the two parameters were also similar: a) lag period; b) application of actinomycin-D; c) 5-fluorouracil treatment; and d) abscisic acid

treatment. Although the RNA species apparently induced by GA represented less than 1% of the total RNA of the cell, and the timing aspects suggest a coincidental rather than a causal relationship, this work probably represents the best evidence to date for the possible involvement of RNA synthesis in the GA response. However, until more definitive evidence (DNA hybridization, template activity, etc.) is presented about this species of RNA, we must consider the results to be inconclusive.

If protein synthesis can proceed without previous RNA synthesis then it is reasonable to assume that m-RNA, t-RNA and the required ribosomes are already present in the cell in a stable form and that it is enzyme synthesis rather than nucleic acid synthesis which is activated by addition of the hormone. In other words, the level of control may be at the translation stage rather than at the level of transcription. The question of whether there is a stable messenger in plants therefore arises, and if so, why does the protein synthesis mechanism not begin at imbibition?

The early investigations of Marcus and Feely (1964) indicated that a stable m-RNA was present in wheat and cotten seed embryos. Microsomal preparations from embryos of these seeds were only able to incorporate ^{14}C-amino acids into protein if the embryos had first been imbibed, despite the absence of RNA synthesis during the early

stage of imbibition. Subsequent observations of a transition of monosomes to polysomes during imbibition suggested that the protein synthesis machinery was inactive because of spatial separation of m-RNA from the ribosomes (Marcus and Feely, 1965; Marcus *et al.*, 1966). The conclusion that a factor or factors required for this process, were produced in embryo cells during imbibition arose when it was found that it was possible to activate ribosomes from unimbibed seeds by preincubation with homogenates of imbibed seed (Marcus and Feely, 1966). Although Dure and Waters (1965) confirmed that there was a stable m-RNA in cotton seed they found no shift from monosomes to polysomes during germination (Waters and Dure, 1966).

Chen *et al.* (1968) confirmed that a stable m-RNA is present in wheat embryos when they found that although ^{32}P incorporation into RNA was almost undetectable for the first 24 hr of germination, protein synthesis began within 30 min of imbibition. The extracted RNA from both dry and imbibed seed showed high template activity with ribosomes from *E. coli*. In addition, hybridization experiments with DNA in which RNA from dry embryos was allowed to compete with that from embryos germinated for 24 and 48 hr showed that the m-RNA was identical in all three kinds of embryo.

Thus,it is clear that stable forms of m-RNA exist in cereal

seeds and consequently RNA synthesis is not necessarily a pre-requisite for new enzyme synthesis. Although these findings add credence to the hypothesis that a stable m-RNA may be present in aleurone tissue it is possible that such a system would not be susceptible to hormonal control. In other words, if such a stable m-RNA were found to exist in aleurone cells, could we reasonably expect GA to affect protein synthesis at the level of translation?

In a detailed study, Chen and Osborne (1970) found that total protein synthesis in germinating wheat embryos was stimulated by applications of GA and that this stimulation was also observed in cell free systems of GA-treated embryos. Direct addition of GA to a cell-free system obtained from untreated embryos was, however, slightly inhibitory. ABA inhibited protein synthesis both *in vivo* and *in vitro*. The failure of GA to stimulate protein synthesis when directly added to cell-free systems suggested to the authors that a cofactor(s) was required for GA function and that it may possibly be located in the membrane fraction as this fraction was not included in their cell-free incubations.

Chen and Osborne (1970) further found that neither GA nor ABA affected the pattern of monosome and polysome distribution, nor was this pattern altered during the first 12 hr of germination, rather, increased template activity of all fractions of the ribosomes accompanied germination or treatment with GA. Hybridization

experiments with DNA showed that no new m-RNA or r-RNA was associated with the active ribosomes, indicating that the hormone regulated the use of m-RNA already stored in the embryo rather than controlling m-RNA synthesis. The authors concluded that GA acted at the level of translation by controlling the expression of preformed messages.

It is possible that the factor that determines the period between imbibition and the start of protein synthesis in wheat embryo cells is GA. Whether the process involves conversion of monosomes to polysomes as reported by Marcus and Feely (1965), or an activation of pre-existing ribosomes (Chen and Osborne, 1970), is not clear. However, the finding that GA can exert an influence over protein synthesis in the embryo in the absence of m-RNA synthesis establishes that a mechanism for hormonal control involving extra-nuclear events is reasonable.

The question of whether a similar mechanism is operating in aleurone cells has recently been investigated. Within 3-4 hr of application of GA to barley aleurone cells, Evins (1971) detected changes in the ribosome population. By 12-15 hr after hormone application the total ribosome population increased 2.5-fold while the percentage of ribosomes existing as polysomes increased to a maximum of 76% over control values.

Evins (1971) also demonstrated that the number of active ribosomes (ribosomes capable of synthesizing nascent polypeptides, measured by the formation of acid-insoluble ^{3}H-peptidylpuromycin) doubled 12 hr after hormone treatment but that the proportion of total ribosomes that were active was not affected. The rate of protein synthesis as measured by incorporation of ^{14}C-amino acids into acid insoluble material showed a doubling within 8 hr of hormone treatment and nascent polypeptides released from polysomes from GA-treated tissue by puromycin showed a high tryptophan : tyrosine ratio which is characteristic of GA-induced proteins.

Continuing their studies, Evins and Varner (1972) found that mid-course removal of GA caused a decline in both α-amylase synthesis and polysome formation and the readdition of the hormone reinitiated the processes. Actinomycin-D at concentrations that inhibited α-amylase synthesis also prevented the conversion of monosomes to polysomes, as well as increases in total ribosomes again pointing to the non-specific nature of this inhibitor. 5-fluorouracil (5-FU), which is reported to inhibit r-RNA and t-RNA synthesis but not m-RNA synthesis (Key, 1969), reduced the total ribosome population in GA-treated aleurone cells but not the percentage in the form of polysomes. α-Amylase synthesis was also unaffected by 5-FU.

Although there are certain correlations between hydrolytic enzyme synthesis and polysome formation, many aspects of the work are

confusing. Zwar and Jacobsen (1972), for example, found almost no increase in r-RNA synthesis in GA-treated aleurone cells. Examination of the density gradient profiles presented by Evins (1971) reveals much higher base lines in the polysome profiles of GA-treated tissue than in profiles of control tissue which may explain the apparent increase in total ribosomes. Further, if GA acts by increasing the percentage of polysomes, thereby activating them for protein synthesis, why is there no concomitant change in the proportion of active ribosomes? In fact, some of the data presented by Evins and Varner (1972) (their Figure 3) indicates that GA does not cause an increase in total ribosomes but does appear to change the percentage of polysomes. However, the failure of these workers to inactivate ribonuclease activity in their cell homogenates, which has been shown by Davies and Larkins (1973) to be essential for reliable polysome isolation, places even this result in doubt. The case for polysome formation as a possible mechanism of GA action remains to be proven.

Recent experiments by Carlson (1972) support the hypothesis that GA exerts its effect in aleurone cells by controlling post-transcriptional events. By examining the thermolability of α-amylase molecules produced in the presence and absence of 5-FU Carlson showed that two subpopulations of the enzyme could be distinguished. Carlson (1972) reasoned that if translational control of enzyme production was operative in aleurone cells, then treatment of tissue

with 5-FU either before or during GA treatment should result in the production of α-amylase with increasing thermolability. On the other hand, if transcriptional controls were operative, treatment with 5-FU prior to GA application should give rise to thermostable molecules, while treatment with 5-FU during GA treatment should result in the production of thermolabile α-amylase molecules. Since two populations of the enzyme were found when 5-FU was applied before and during GA treatment, Carlson (1972) deduced that control was probably at the post-transcriptional level.

Because RNA synthesis inhibitors markedly depress GA-induced α-amylase synthesis it has been assumed by many workers (Jones, 1973) that the 6-8 hr lag phase may be the time required for transcription of m-RNA and its ultimate translation to α-amylase. However, β-galactosidase induction in bacterial systems requires only 15 min (Jacob and Monod, 1961) and hormone-induced increases in protein synthesis in wheat embryos, where a stable messenger exists, begins after 30 min, which period includes the 15 min required for imbibition (Chen and Osborne, 1970). Perhaps the remaining time in the lag period is required for the conversion of monosomes to polysomes as suggested by Evins and Varner (1972). However, this same process in wheat embryos is at least 50% complete 40 min after the start of imbibition (Weeks and Marcus, 1971). Although up to 30 min may be

accounted for as the time required for GA to enter the cell (Goodwin and Carr, 1972), it seems likely that in addition to gene activation and polysome aggregation other processes are involved in the lag phase following GA treatment. The recent evidence presented by Carlson (1972), strongly suggesting that α-amylase m-RNA may be present in aleurone cells prior to GA treatment, indicates too that if only polysome formation is involved the lag period should be no longer than that found in wheat embryos.

In fact, the timing aspect is the least satisfactory part of all the work dealing with the hormonal induction of RNA synthesis. No evidence has yet been obtained which indicates a causal rather than a coincidental effect. Since protein synthesis in aleurone cells is likely to be subject to control mechanisms also operating in other tissues, it is difficult to believe that the necessary short term alteration in RNA synthesis would have continued to escape detection if GA was acting directly on this area of metabolism.

Detailed examination of the events occurring after addition of hormones in animal systems has revealed that the sequence of events is far more complicated than simple induction of m-RNA synthesis and subsequent translation, or association of ribosomes with pre-existing m-RNA (Tata, 1968). Studies with enzyme secreting tissues (Palade,1966; Siekevitz and Palade, 1966) or non-secretory tissue (Andrews and Tata, 1968) have shown that the synthesis of endoplasmic reticulum

(ER) and subsequent attachment of ribosomes is as important in regulating protein synthesis as RNA metabolism itself. Such studies have prompted Tata (1970) to propose that in animal tissues at least, synthesis of ER and its association with ribosomes (RER) occurs whenever the cell is stimulated to produce large amounts of protein. In order to account for the proliferation of RER and the qualitative changes in protein synthesis with hormone-induced shifts in the pattern of development, Tata (1970) has suggested that there is a topographical segregation of ribosomes on the ER, each population differently pre-coded according to the environmental stimulus. Such a segregation in cells that adapt rapidly to external stimuli (regulating growth, development, detoxification, for example) would provide for the preferential synthesis of proteins involved in the adaptive response with the minimum perturbation of synthesis of those proteins required for normal metabolism (Tata, 1970). Thus, in terms of the GA-induced response of aleurone cells, Tata's hypothesis would suggest that synthesis of ER and/or ribosome binding to form RER would precede translational manifestations of the hormonal effect.

The involvement of ER in the GA response in aleurone tissue has long been inferred from cytological observations. Paleg and Hyde (1964) examined aleurone cells throughout the course of imbibition and

germination. The main organelles visible in unimbibed cells were large aleurone grains (1 μ diameter), containing dense phytin globoids and protein bodies, which were completely surrounded by spherosomes or lipid-containing bodies about 0.2 μ in diameter. GA treatment resulted in the dissolution of aleurone grains and spherosomes and was accompanied by proliferation of aleurone membranes to form profiles resembling endoplasmic reticulum. These observations led the authors to think that there may be a relationship between aleurone grain membranes and the site of amylase synthesis.

Jones (1969a) reported that imbibition resulted in swelling of aleurone grains which was further increased by application of GA and the effect reached a maximum at about 10 hr after hormone treatment. An increase in RER was also observed during this period. Continued incubation in GA resulted in further proliferation of ER (10-12 hr), distention of the ER cisternae (12-16 hr of GA) and proliferation of vesicles from the ER and golgi (14-22 hr). Accompanying these changes was a reduction in the size of aleurone grains, a decrease in the number of spherosomes and an increase in the number of plastids and microbodies (Jones, 1969b). Based on the fact that α-amylase secretion was active during this period, Jones concluded that α-amylase was synthesized on the RER and secreted via ER-derived vesicles. Examination of cells incubated for longer periods with GA revealed that between 24-36 hr after hormone addition the contents of aleurone

grains had been digested and the empty organelles had fused to form a large central vacuole (Jones and Price, 1970). The proliferation of golgi vesicles during this period, which also corresponded to the period of active ribonuclease secretion, suggested to the authors that this enzyme was secreted via these vesicles.

More recently Vigil and Ruddat (1973) reported development of RER in hormone-treated aleurone cells in response to incubation with GA. Following the assembly of stacked RER, vesiculation was observed mainly in the basal portions of the cell, resulting in polar distribution of RER vesicles. It was postulated by the authors that these vesicles were involved in protein secretion because smooth vesicles, derived from the RER, apparently became oppressed to the plasma membrane. Actinomycin-D appeared to cause massive disarray of the RER. Similar observations have been made by Phillips and Paleg (1972).

Although there appears to be almost unanimous agreement that GA causes proliferation of RER in aleurone cells, a word of caution is perhaps necessary. Imbibed aleurone cells appear to contain little else but aleurone grains and numerous associated spherosomes when viewed under the electron microscope. As GA causes a reduction in the number of spherosomes and aleurone grains it is possible that the above workers may merely have a clearer view of pre-existing RER.

To distinguish between the two possibilities Evins and Varner (1971) took advantage of the fact that membranes are composed largely of phospholipids. They found that after a 4 hr lag period, GA-treated aleurone layers exhibited an enhanced capacity to incorporate ^{14}C-choline into a partially purified microsomal fraction. The GA enhancement varied from 3 to 8 times the value for control tissue after 10 hr of treatment. Although it was established that most of the radioactivity was located in the lipid (chloroform soluble) fraction of the microsomal pellet, no attempt was made to measure the total phospholipid content. Despite this, Evins and Varner (1971) concluded that GA affected ER synthesis.

In a more detailed work Koehler and Varner (1973) studied the effect of GA on the incorporation of ^{32}P into various phospholipid fractions. Although GA increased the total incorporation of ^{32}P, the authors found that the hormone did not affect either the total phospholipid-phosphorus content of the aleurone cells, or the rate of ^{14}C-acetate incorporation into the phospholipid fraction. Thus GA enhances the turnover rate of phospholipids rather than synthesis *de novo*.

In an attempt to determine whether GA changed the composition of the pre-existing membrane, Koehler and Varner (1973) also examined individual phospholipids. Phospholipid extracts from control and GA-treated tissue were chromatographed two-dimensionally, the individual

phospholipids characterized and their total radioactivity determined. On a percentage basis, GA treatment slightly enhanced the labelling of phosphatidyl-choline, -ethanolamine and -inositol while it reduced the percentage of phosphatidyl-glycerol and phosphatidic acid being labelled. On a total radioactivity basis, GA enhanced the labelling of all these phospholipids with the exception of phosphatidic acid. Fatty acid and sterol composition were not assayed. Thus, although GA treatment causes slight preferential synthesis of some phospholipids, its major effect is to stimulate overall phospholipid turnover.

There is, however, some doubt that the GA-stimulated phospholipid labelling actually represents incorporation into ER. Although Evins and Varner (1971) claimed to have found the highest specific radioactivity in the microsomal fraction, data for total counts and total protein in the various subcellular fractions were not given. On the other hand, Koehler and Varner (1973) using similar methods found that most of the label (68%) in ^{32}P treated aleurone cells was located in the 4,000 *g* pellet, a trace in the mitochondrial pellet and only 19% in the post-mitochondrial supernatant (ER, golgi, non-incorporated ^{32}P, etc.). GA had no effect on this distribution of radioactivity. Thus the claim by Evins and Varner (1971) of GA-enhanced ^{14}C-choline incorporation into ER is open to

doubt.

Another approach to examining the effect of GA on proliferation of ER was taken by Johnson and Kende (1971) who examined enzymes involved in the synthesis of lecithin (phosphatidyl-choline):

1. Choline + ATP $\xrightarrow[\text{kinase}]{\text{choline}}$ P-choline + ADP

2. P-choline + CTP $\xrightarrow[\text{transferase}]{\text{phosphorylcholine-cytidyl}}$ CDP-choline + PP_i

3. CDP-choline + 1,2 diglyceride $\xrightarrow[\text{transferase}]{\text{phosphorylcholine-glyceride}}$ lecithin + CMP

The three enzymes were detected in barley aleurone cells, choline kinase being a soluble enzyme and the two transferases being particulate. After only 2 hr of GA treatment the activities of both transferases were 150% of control and by 8 hr the enhancement rose to 300 to 400% of control levels. Inhibitors such as ABA, cyclohexamide and actinomycin-D added at concentrations that inhibit α-amylase synthesis, polyribosome formation and phospholipid labelling also reduced the GA enhancement of transferase activities. However, treatment with 0.8 M mannitol, which inhibits α-amylase synthesis (Jones, 1969c) and ^{32}P incorporation into phospholipids (Koehler

et al., 1972), had little effect on GA-stimulated transferase activity. A re-examination of this effect was carried out by Ben-Tal and Varner (1973) and they concluded that GA-dependent increases in transferase activity were due to activation rather than synthesis.

The site of phospholipid synthesis is undoubtedly located predominantly in the ER. This is certainly the case in most animal tissues which are under hormonal control (Tata, 1970). Recent studies on phospholipid synthesis in the endosperm of castor bean seeds indicate that this is also true in plant systems (Kagawa *et al.*, 1972). Lord and co-workers (1972) have shown that production of lecithin from CDP-choline occurs in a discrete fraction which could be isolated on sucrose density gradients. This fraction has been shown to be derived from the ER (Lord *et al.*, 1973). These workers have also shown that the enzymes phosphatidic acid phosphatase, CDP-diglyceride : inositol transferase and phosphatidyl-ethanolamine : L-serine phosphatidyl transferase are also strictly associated with ER (Moore *et al.*, 1973). Synthesis of phosphatidyl-glycerol occurs in both the ER and mitochondria (Moore *et al.*, 1973). Thus the membranes of the ER have a unique role in the synthesis of three of the major phospholipids found in the membranes of the cell.

Another effect consistent with the idea that GA might control phospholipid biosynthesis occurs even earlier than measurable increases in the activities of the transferases. Collins *et al.* (1972b) reported that GA stimulates the turnover rate of CTP, to a greater extent than other nucleotides, between 15 and 90 min after treatment of wheat aleurone tissue. Since CTP plays a fundamental role in the phospholipid synthesizing pathways, this result may reflect a primary action of GA at the level of phospholipid metabolism.

It seems clear that, of the studies considered, a major measurable effect of GA on barley aleurone layers is a dramatic increase in the turnover rates of phospholipids. What then is the significance of this finding? One explanation is that GA is stimulating a structural and functional reorganization of the membrane system of the aleurone cell. Cited as support of this hypothesis is the report by Johnson and Chrispeels (1973) that inhibition of a membrane-associated enzyme was responsible for a GA-induced reduction in the ability of aleurone cells to incorporate ^{14}C-arabinose into cell walls. These workers also demonstrated that GA caused a stimulation of sucrose release from aleurone layers within 2-3 hr of treatment, which was attributed to altered membrane permeability (Chrispeels *et al.*, 1973). Although both these processes have been presented by the authors as evidence for reorganization of pre-existing membrane via phospholipid

synthesis, it should be pointed out that GA-stimulated sucrose release precedes GA-induced phospholipid synthesis by 2-4 hr. Furthermore, Eastwood and Laidman (1971) have reported that GA caused an increase in leakage of phosphate, potassium and magnesium ions within 2-3 hr of treatment even in the presence of cyclohexamide which inhibits phospholipid synthesis (Koehler and Varner, 1973). Eastwood and Laidman (1971) have interpreted this effect as being due to a loosening of aleurone cell membranes perhaps by a direct hormone-membrane interaction, and a consequent increase in permeability.

An explanation of hormone action in terms of alteration of membrane permeability has been repeatedly proposed, but because of the difficulty of accounting for control of synthesis of specific proteins by this mechanism, most workers continue to concentrate on metabolic effects (Wood and Paleg, 1972). Is there any evidence that a hormone-membrane interaction is capable of exerting control of cell metabolism? Such an effect is certainly well established in animal systems where interaction of adrenalin with the plasma membrane alters the activation energy of the membrane associated adenyl cyclase resulting in increased levels of cyclic-AMP (Kreiner *et al.*, 1973). This intracellular transmitter (cyclic-AMP) affects rate control in a wide variety of metabolic pathways by activation or

inhibition of a number of pacemaker enzymes (Sutherland *et al.*, 1968). Although several investigators have attempted to implicate cyclic-AMP in the GA response in aleurone cells (Duffus and Duffus, 1969; Kessler, 1969; Galsky and Lippencott, 1969; Earle and Galsky, 1971) the role of this compound in plants has yet to be elucidated.

Although the site of action of auxin (IAA) is thought to be the plasmalemma (Morre, 1972) and the rapid effects of auxin on growth are known to occur without the necessity for synthesis of new macromolecules (Nissl and Zenk, 1969), RNA and protein synthesis are required to sustain the response. The apparent paradox of a rapid growth response due to an interaction of auxin with the plasma membrane and a delayed auxin-induced increase in RNA synthesis would appear to be resolved by the recent work of Hardin *et al.* (1972). These workers demonstrated that a factor, which is specifically released by auxin from isolated soy bean plasma membranes, enhances the activity of a purified soybean RNA polymerase. Thus, there appears to exist in plants a mechanism whereby a single interaction of a hormone with a membrane may result in a level of transcriptional control of protein synthesis.

The relatively recent concentration on membrane phenomena as potential sites of hormone action, raises anew an aspect of cellular function which had previously been considered in relation to the

aleurone layer and subsequently rejected. Lysosomes are vesicles composed of a membrane enclosing hydrolytic enzymes. Any alteration in the structure and/or function of the lysosomal membrane (as, for example, suggested by Szego *et al.*, 1971, or de Duve and Wattiaux, 1966) would have profound effects on many aspects of metabolic activity. From the standpoint of the enzymes involved in the activation of aleurone cells by GA, an explanation involving lysosomes seemed appropriate to MacLeod and Millar (1962). (At that time the lysosomal concept orientated thinking towards preformed, though inactive enzymes, the release and activation of which was in some way effected by the hormone.) This suggestion was retracted by MacLeod *et al*, (1964) when they found different enzymes developing at different times and failed to isolate a particle that could be stimulated *in vitro* by GA to release the hydrolases.

About this time the lysosomal concept was dramatically altered by de Duve and Wattiaux (1966) and Novikoff *et al*, (1964) who postulated that lysosomal enzymes were synthesized on the ribosomes of the RER and either directly budded off the ER or transported to the golgi apparatus for packaging, and that the release of the lysosomal enzymes may act as a stimulus for the synthesis of further lysosomes.

In secretory cells of animal tissue, proteins that are destined to be secreted are usually synthesized and packaged on RER

while intracellular proteins are made on free polysomes (Redman, 1969; Siekevitz and Palade, 1966; Ganoza and Williams, 1969). The synthesis and secretion of casein from mammary gland explants, for example, is characterized by an increase in RER and secretory vesicles within 12-24 hr of hormone treatment (Mills and Topper, 1970). Since similar observations have been made in GA-treated barley aleurone cells (Vigil and Ruddat, 1973) it is reasonable to suggest that GA-induced proteins could be secreted in a similar manner.

During germination, the cells of the cereal aleurone layer produce and secrete almost all of the hydrolytic enzymes involved in the complete degradation of the starchy endosperm, a process requiring up to 6-7 days. In spite of this, the aleurone cells retain their integrity until the endosperm has been entirely digested. Unless the acid hydrolases produced by the aleurone cells are separated from the rest of the cellular constituents by some sort of membrane, how do the aleurone cells escape autolysis? In animal cells, self-autolysis is prevented by the lysosomal membrane separating the hydrolytic enzymes from potential substrates (de Duve and Wattiaux, 1966). Could GA-induced hydrolytic enzymes in cereal seeds also be lysosomal?

The findings of Szego *et al.* (1971) that the properties of the

lysosomal membrane alter dramatically shortly (15 min) after administration of steroid hormones *in vivo*, is particularly significant. Steroids and gibberellins share many similar properties: both classes of compounds have isoprenoid structures, a common biosynthetic pathway, similarity in structural diversity (through insertion or deletion of double bonds, radicals and subsidiary ring structures), and control comparable physiological functions. It is therefore possible that gibberellins and steroids could also share a common hormonal mechanism.

The effects of steroids reported by Szego *et al.* (1971) could only be elicited *in vivo* and these workers postulated that a carrier protein or intermediate was involved. However, de Duve *et al.* (1962) reported steroid hormone-induced leakage of hydrolytic enzymes from isolated lysosomes *in vitro*. Such studies prompted Bangham *et al.* (1965) to investigate the effects of steroid hormones on artificial membrane systems (liposomes). The results suggested that steroids interact with the liposomes to produce changes in their permeability (which resembled steroid-induced changes in natural membranes) by provoking a structural rearrangement of lipid layers. It is in the context of this kind of mechanism that the report by Wood and Paleg (1972) of GA increasing the permeability of liposomes composed of plant lipids is pertinent here.

Although the effects of GA on aleurone cells have obviously received a great deal of attention, most of the proposals for a mechanism of action of this hormone have not been experimentally verified. The evidence for the involvement of specific RNA synthesis rests almost entirely on results obtained with metabolic inhibitors (Chrispeels and Varner, 1967a, b) the effects of which are far from specific (Honig and Rabinovitz, 1965; Gayler and Glaziou, 1968; Koehler *et al.*, 1972). Although Zwar and Jacobsen (1972) do appear to have obtained a fraction of RNA which is synthesized about the same time as α-amylase, the effect may be coincidental rather than causal. Until a fraction of GA-induced RNA can be isolated which is specifically formed in response to the hormone prior to the formation of α-amylase, and the fraction can be shown to have high template activity, the conclusions of Carlson (1972), that a long-lived m-RNA for hydrolytic enzymes is present in the dry seed, must be accepted.

The possibility of post-transcriptional control of protein synthesis in GA-treated aleurone has also been investigated. Although a mechanism involving hormonal activation of ribosomes (Chen and Osborne, 1970) and/or conversion of monosomes to polysomes (Marcus and Feely, 1965) may be applicable to wheat embryo cells, the evidence for such a mechanism in aleurone cells (Evins, 1971; Evins and Varner, 1972) is ambiguous. Furthermore, the failure by other workers to

verify increases in r-RNA in response to GA (Zwar and Jacobsen, 1972) places the results in doubt.

Hormone-induced increases in phospholipid synthesis appear to be dramatic and verifiable (Koehler and Varner, 1973; Johnson and Kende, 1971) although claims of increased ER synthesis (Evins and Varner, 1971) can be explained in terms of phospholipid turnover (Koehler and Varner, 1973). The effects of GA on phospholipid synthesis have prompted some workers to propose that GA-induced enzymes may be only synthesized on newly made ER (Johnson and Kende, 1971). Evidence for such a mechanism is entirely lacking although cytological observations of ER proliferation have been correlated with the induction of α-amylase (Jones, 1969b; Vigil and Ruddat, 1973).

The obvious involvement of phospholipid synthesis and the nature of the enzymes induced by GA (acid hydrolases) are perhaps best explained in terms of lysosomes. In animal cells, acid hydrolases are lysosomal and thus the enclosed enzymes are prevented from randomly attacking macromolecules necessary for metabolism (de Duve and Wattiaux, 1966). Because the lysosomal membrane must be made at the same rate as the acid hydrolases they contain, phospholipid synthesis is as necessary to lysosome production as protein synthesis.

Numerous attempts have been made to identify lysosomes in plant tissue (Matile, 1969). Organelles ranging from vacuoles (Matile, 1968) to aleurone grains (Yatsu and Jacks, 1968) have been equated with animal lysosomes, but with only limited success. And yet, the many features of the lysosome concept, particularly with respect to the GA-induced response of cereal aleurone layers seem pertinent, and thus worthy of serious investigation. Thus, it is to the question of whether the acid hydrolases, produced in the aleurone layer in response to GA, are lysosomal or not, that this work is primarily directed. The latter part of the thesis also explores the relationship between GA and the vesicle membranes.

SECTION I

THE ISOLATION OF ALEURONE LAYERS AND THE CHARACTERIZATION OF THE GA RESPONSE

This section deals with the isolation methods, incubation conditions and characterization of the GA response of aleurone layers from wheat half-seeds. For the most part, the level of α-amylase induced by GA as compared with the amount of enzyme produced by similarly treated half-seeds was used as a test for aleurone tissue responsiveness.

1. The Aleurone Layer

Immediately adjacent to the seed coat of the wheat seed is a layer of differentiated cells, called the aleurone layer, formed from the outer layer of the endosperm (Evers, 1970). In the wheat grain the aleurone consists of a single layer of cells which is in contrast to barley where there are three layers of aleurone cells. It is these cells that are the target tissue for GA and secrete hydrolytic enzymes which break down the components of the endosperm tissue.

Isolation of the aleurone layer entails removal of the embryo

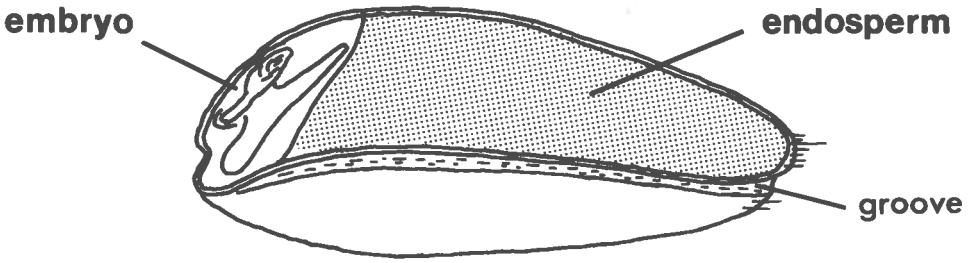
half of the seed and squeezing out the endosperm from the remaining half of the grain. Thus, the tissue that will be referred to constantly during this thesis as "aleurone layers" actually consists of the outer pericarp, the testa or seed coat and the adjacent layer of aleurone cells (Figure I.1). As the only metabolically active cells in this tissue are the aleurone cells, the presence of pericarp and seed coat cells may be considered physiologically irrelevant. The term "half-seed" refers to wheat grains which have had their embryo half removed.

2. Isolation of Aleurone Layers

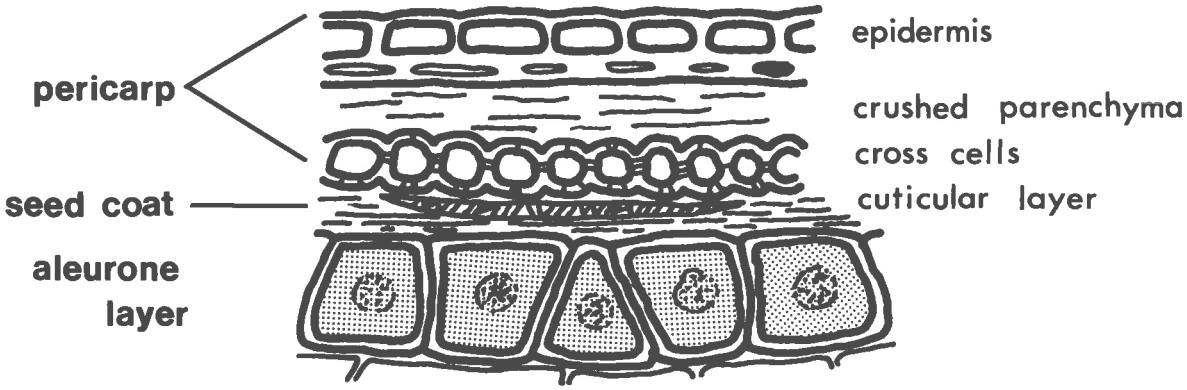
The most commonly used method of isolating aleurone layers is that of hand peeling half-seeds which have been incubated under moist, sterile conditions. This method is time consuming, necessitates much handling of the tissue which increases the likelihood of microbial contamination and yields tissue highly contaminated with starch. A more rapid method was developed by Phillips (Phillips and Paleg, 1972) using a roller mill device which has been used subsequently by other workers (Collins *et al.*, 1972b). The roller mill consists of a large screw top jar (diameter 12 cm) and an inner polythene bottle filled with fine dry sand (diameter 7 cm, weight 900 g). The glass jar is rotated by means of a set

Figure I.1

A longitudinal section through the groove of *Triticum* (wheat) seed (A) showing the cellular layers associated with aleurone layers (B). From Esau (1953).


embryo
endosperm
groove

A


pericarp
seed coat
aleurone layer
epidermis
crushed parenchyma
cross cells
cuticular layer

B

of motor driven rollers at a speed of 50 revolutions per min. Moist, sterilized wheat half-seeds are introduced into the glass jar with liquid and the rolling motion of the jar results in a flow of liquid that carries the half-seeds between the polythene bottle and the jar, resulting in the soft starchy endosperm being squeezed out of the seed coat. The starch and cell debris are then rinsed away leaving clean aleurone layers.

The rolling procedure developed by Phillips using water as the liquid was used in initial experiments and a detailed description of it has been published (Phillips and Paleg, 1972). Although this method was an improvement over existing methods it was found in preliminary experiments to give tissue with a low response to GA (see Section I.4). The relatively low solubility of endosperm protein in water necessitated prolonged rolling (36 min) which resulted in torn and damaged aleurone layers. Work by Dr. Peter Nicholls of Waite Institute indicated that the use of a weak solution of lactic acid increased the solubility of endosperm proteins (gliadin, glutenin) and thus rolling times could be reduced. A rolling method using 5 mM lactic acid as the liquid was developed and unless otherwise stated, was the method used for preparing tissue in all experiments:

1) A total of 15 g of half-seed were prepared by cutting the embryo end from the seed with a single edged razor blade.

2) The half-seeds were sterilized by soaking for 7 min in a solution of commercial bleach (4% W/V available chlorine) diluted with an equal volume of water. The sterilizing solution was decanted and the half-seeds washed ten times with 100 ml aliquots of sterile distilled water.

3) The half-seeds were then transferred asceptically to a large sterile petri dish (18 cm diameter) containing a layer of plastic gauze (2 mm mesh) and 10 ml of sterile distilled water. The petri dish was then placed in an incubator at 30°C for 26 hr.

4) All subsequent operations were carried out in an Oliphant laminar flow sterile chamber with all equipment and solutions sterilized by autoclaving for 20 min at 15 psi.

5) Imbibed half-seeds were transferred to the roller mill with 80 ml of 5 mM lactic acid (30°C) and rolled for 3 min.

6) The resulting slurry was filtered through a wire sieve (2 mm mesh) and the tissue returned to the mill with 70 ml of 5 mM lactic acid. The tissue was rolled for a further 2 min.

7) At the end of this time the polythene bottle was removed, a further 80 ml of lactic acid added, and the tissue shaken vigorously for 30 sec. The slurry was then filtered through the wire mesh as before.

8) The tissue was returned to the mill for a further 1 min roll in lactic acid and the tissue shaken and filtered as before.

9) Step 8 was repeated.

10) The tissue was washed on the metal sieve with copious amounts of distilled water and allowed to drain between many layers of tissue paper for 10 min.

11) The aleurone tissue was weighed and 1 g samples transferred to incubation flasks for preincubation in 5 ml of 2×10^{-2} M calcium nitrate for 12 hrs at $30^{\circ}C$ (recovery period) before the addition of test solutions. The preincubation period was found necessary for the tissue to recover from the rolling procedure (Collins *et al.*, 1972a, b).

This method gave clean responsive tissue which was virtually intact and showed little sign of damage.

3. Measurement of Enzyme Activity

The enzymes cited below from both the ambient solution (liquid in which the tissue was incubated) and the tissue, were assayed. The enzyme activity in the tissue was assayed after grinding in the cold in 15 ml of 5 mM calcium acetate solution in an Ultra-Turrax

model TP/180 at maximum speed for 1 min and centrifuging off the cell debris in a bench top centrifuge at 3,000 g for 10 min.

α-Amylase. This enzyme was assayed by a modification of the method of Shuster and Gifford (1962). Prior to assay, all solutions were heated at 70°C for 20 min 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 boiling for 3 min a 0.1% (W/V) solution of amylose (BDH Chemical Co.) in 5 mM calcium acetate with 50 mM KH_2PO_4, pH 4.6. The solution was filtered through two layers of Whatman No. 1 filter paper immediately before use and the concentration adjusted so that the absorbance reading of a solution diluted 1:1 with water and reacted with IKI solution was 1.3 at 620 nm. As enzyme activity is dependent on substrate concentration it is important to ensure that the substrate concentration is the same for each experiment. Substrate dilution was carried out with a 5 mM calcium acetate/50 mM KH_2PO_4 solution.

IKI stock solution contained 0.6 g iodine and 6.0 g potassium iodide made up to 100 ml with distilled water. For use in the enzyme assay, 2.5 ml of the stock solution was made up to 100 ml with distilled water.

As α-amylase was used as a marker enzyme for all experiments reported here, there follows a detailed description of the assay. Firstly each enzyme solution to be tested was diluted with 5 mM calcium acetate/50 mM KH_2PO_4 solution so that the reaction was about 50% complete in 6-8 min 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 to balance zero absorbance on the spectrophotometer. The substrate and the diluted enzyme solution were heated to 30°C and 5 ml of each were mixed and the time noted (time zero). At two minute intervals after time zero up to 10 min, 1 ml samples were taken and placed in other tubes containing 1 ml of diluted IKI solution. The sample tubes were then diluted with 4 ml of distilled water and the O.D. of each sample determined in a Unicam SP350 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 from the spectrophotometer 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 and by any dilution factors gave total enzyme units.

Acid Protease. This assay, based on the liberation of ninhydrin positive products from the wheat storage protein gliadin, was used as described by Jacobsen and Varner (1967). An aliquot of the enzyme mixture to be assayed (generally 5 ml) was made up to 25 ml by slowly stirring in ice cold redistilled ethanol (final concentration 80% (V/V) ethanol). The mixture was allowed to stand for 5 min in ice before being centrifuged at 20,000 g for 10 min at 5°C. The resulting supernatant was discarded and the pellet resuspended in 2 ml of 1 mM acetate buffer, pH 4.8, and made 10 mM with respect to 2-mercaptoethanol (BDH Chemical Co.). The resulting enzyme mixture was mixed with 4 ml of substrate solution and incubated for 60 min at 30°C. The substrate solution consisted of a saturated solution of gliadin (Sigma Chemical Co.) in 50 mM acetate buffer, pH 4.8. Samples (0.6 ml) were taken at 0, 30 and 60 min and reacted with 2 ml of ninhydrin reagent prepared according to Moore and Stein (1948). The samples were heated in a boiling water bath for 20 min, allowed to cool and diluted with 5 ml of a 1:1 (V/V) solution of propanol : water. The optical density of the samples were recorded in a Unicam SP350 spectrophotometer set at 580 nm.

Enzyme activity is expressed in units where one unit is defined as a change of 1 O.D. unit per hour per sample.

Acid Ribonuclease. The hydrolysis of RNA was determined by the method of Wilson (1963) using yeast RNA (BDH Chemical Co.) purified by the method of Cantoni and Davies (1966) as substrate. The reaction mixture contained 2 ml enzyme, 1.6 ml substrate and 0.4 ml 1 M KCl. The enzyme used was that obtained from the ambient or tissue without further purification. The substrate contained 1% (W/V) RNA in 0.2 M tris-acetate buffer, pH 5.0. The reaction was carried out at 37°C for 60 min. Samples (1 ml) were taken at 0, 30 and 60 min and pipetted into centrifuge tubes containing 1 ml of a mixture of 2.5%(W/V) trichloroacetic acid and 0.25% (W/V) uranyl acetate. If kept cold, the samples could be kept for long periods at this stage. The mixture was centrifuged at 3,000 g for 10 min and the resulting supernatant poured off and diluted to 10 ml with distilled water. The optical density of each sample was then determined on a Unicam SP800 spectrophotometer set at 260 nm. Enzyme activity is expressed in units where 1 unit represents 0.1 E_{260} generated in 1 hr at 37°C and pH 5.0. When ribonuclease activity was being assayed care was taken to avoid contamination by autoclaving all glassware before use and by wearing rubber gloves during all operations.

4. Response of Isolated Aleurone Tissue to GA

4.1 Viability of Seed

All work reported in this thesis was performed on aleurone layers from wheat seed var. OLYMPIC, as this variety was earlier shown to be most responsive to applications of GA (Phillips, 1968). Collins of this laboratory (Collins, 1970) used stocks of Olympic from the 1967 crop and it was considered advisable to use the newly arrived 1969 crop seed. To compare the response of the new seed to GA with that of the old, the following experiment was performed.

Half-seed from both the 1967 crop (old seed) and the 1969 crop (new seed) were prepared, sterilized and imbibed for 24 hrs at 30°C. Duplicate 1 g samples of half-seeds were placed in 9 cm petri dishes containing a single layer of plastic screen (2 mm mesh) and 5 ml of test solution. Test solutions were either water or GA solution (10 µg/ml). The half-seeds were incubated for 24 hr at 30°C and then both the ambient and tissue were assayed for α-amylase activity. The results are shown in Table I.1.

The 1969 crop seed appeared to respond in an identical manner to the older seed and so the new seed was chosen for all subsequent experiments.

4.2 Incubation Conditions

The method of incubating aleurone tissue used by both Phillips

Table I.1

Production of α-amylase by half-seeds of wheat (var. Olympic). Comparison of 1967 and 1969 crop seed. Incubation medium: water or GA (10 µg/ml). Temperature: 30°C. Time: 24 hr. Figures given for α-amylase activity represent the average value of two samples.

	α-Amylase (SIC units/g wet weight)		
	Ambient	Tissue	Total
New seed			
Water	0	0	0
GA	23.8	50.0	73.8
Old seed			
Water	0	0.2	0.2
GA	22.6	50.5	73.1

(1968) and Collins (1970) was to place the tissue over plastic gauze (2 mm mesh) in a petri dish containing the incubation medium and place the petri dishes in an incubator. Neither of the above workers used Ca^{++} in the incubation medium although this ion had been reported to improve yields of α-amylase (Chrispeels and Varner, 1967b). To test the effect of Ca^{++} ions and various incubation conditions aleurone tissue was isolated by the water method of Phillips (Phillips and Paleg, 1972) and 0.5 g samples placed in containers with 5 ml water. The tissue was allowed to recover from the rolling process for 4 hr at 30°C before the addition of the test solutions. Three incubation conditions were used: 9 cm petri dishes with plastic mesh (Phillips, 1968; Collins, 1970), 125 ml erlenmeyer flasks placed in the same incubator, and 125 ml erlenmeyer flasks shaken on a water bath adjusted to vibrate at 50 oscillations per minute. All treatments were incubated for 24 hr at 30°C after application of the test solutions. The results are listed in Table I.2.

Several important points should be noted about the results (Table I.2). Firstly, the levels of α-amylase obtained with GA treated aleurone tissue were greatest when the tissue was incubated in shaking 125 ml flasks, indicating that aeration may be a limiting factor in other incubation conditions. Secondly, although the level

Table I.2

Production of α-amylase by wheat aleurone tissue in various conditions. Temperature: 30°C. Time: 24 hr. Incubation medium: water, Ca^{++} (20 mM calcium nitrate), or GA (10 µg/ml) as indicated.

Incubation Container	Test Solution	α-Amylase (SIC units/g fresh weight) Ambient	Tissue	Total
Petri dish	H_2O	0	4	4
	Ca^{++}	0	6	6
	GA	50	50	100
	GA + Ca^{++}	70	45	115
Flask, still	H_2O	0	7	7
	Ca^{++}	5	10	15
	GA	80	46	126
	GA + Ca^{++}	100	50	150
Flask, shaking (experiment I)	H_2O	0	3	3
	Ca^{++}	0	8	8
	GA	52	44	96
	GA + Ca^{++}	150	54	204
Flask, shaking (experiment II)	H_2O	0	0	0
	Ca^{++}	0	3	3
	GA	88	100	188
	GA + Ca^{++}	288	100	388
Half-seed	GA + Ca^{++}	400	400	800

of α-amylase in the tissue is about the same with or without calcium, α-amylase levels are higher in the ambient when calcium is present, indicating that calcium may be stabilizing the secreted enzyme or promoting secretion. Calcium had little effect on the yields of α-amylase in control tissue. Lastly, and perhaps most importantly, the highest levels of α-amylase detected in isolated aleurone tissue (GA + Ca^{++}, expt. II) only represented 50% of the enzyme made by an equivalent amount of half-seed. Such a difference was commonly observed when tissue was isolated by the water method of Phillips (1968) and further variation of incubation methods failed to improve the percentage yield (cf. Section 4.6).

All subsequent experiments were performed in 125 ml flasks containing 20 mM calcium nitrate and were incubated in a shaking water bath at 50 oscillations per min.

4.3 Effect of GA Concentration

All experiments reported thus far used GA at a concentration of 10 µg/ml as this was the optimal concentration for α-amylase production reported by Phillips (1968) and Collins (1970) in aleurone tissue isolated by the water method. To see whether the tissue was responding in a similar manner here it was thought advisable to examine the relationship between the concentration of GA and the

production of α-amylase. Aleurone tissue was isolated by the water method (Phillips, 1968) and allowed to recover in water for 4 hr at 30°C. The water was poured off the 0.5 g samples of aleurone tissue and various amounts of GA added to the standard incubation medium of 5 ml of 20 mM calcium nitrate to give final GA concentrations of 0.1, 1.0, 10 and 100 µg/ml. The tissue was incubated in stoppered 125 ml flasks in a shaking water bath at 30°C for 24 hr and α-amylase assayed in both ambient and tissue.

The results are shown in Table I.3 and Figure I.2, and clearly demonstrate that the highest levels of α-amylase are obtained with 10 µg/ml GA with this particular tissue. It should also be noted that the levels of α-amylase vary most widely in the ambient with GA concentrations. A GA concentration of 10 µg/ml was chosen for subsequent experiments.

4.4 Induction of α-Amylase, Protease and Ribonuclease

At various times after the addition of GA to isolated aleurone tissue, namely 0, 9, 12, 18, 24 and 36 hr, samples were removed from the water bath for the assay of these enzymes. Both the ambient and the tissue extract were made up to a volume of 15 ml and from this 5 ml was made available for the assay of each of the three enzymes. α-Amylase, protease and ribonuclease were all assayed as

Figure I.2

Effect of various concentrations of GA on the production of α-amylase in isolated aleurone tissue. Temperature: 30°C. Time: 24 hr. Incubation medium: 5 ml of 20 mM calcium nitrate plus GA at the concentration indicated. α-Amylase: SIC units/g fresh weight.

○ Tissue

● Ambient

□ Total

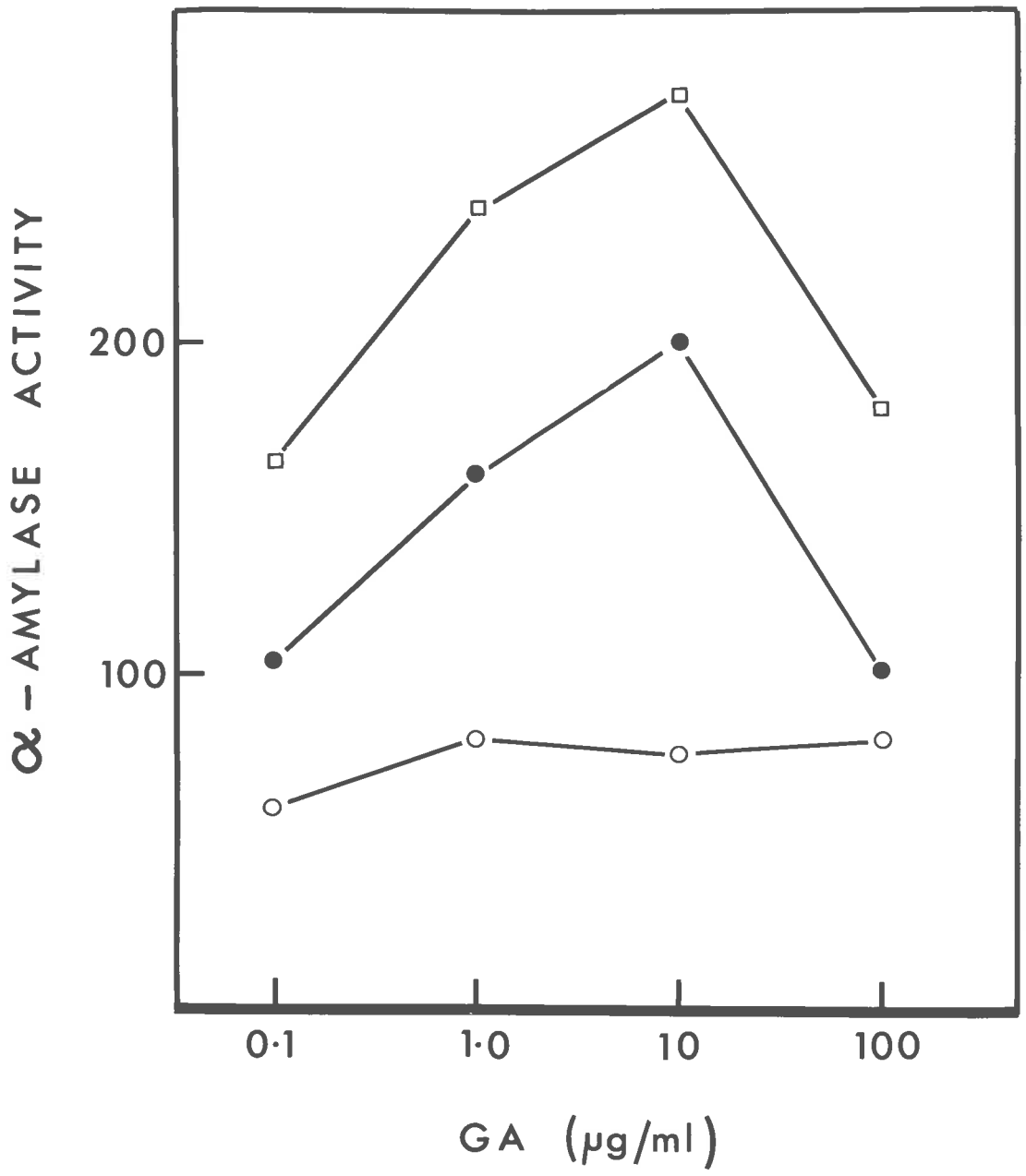

α – AMYLASE ACTIVITY
200
100
0·1
1·0
10
100
GA (µg/ml)

Table I.3

Effect of various concentrations of GA on the production of α-amylase in isolated aleurone tissue. Temperature: 30°C. Time: 24 hr. Incubation medium: 5 ml of 20 mM calcium nitrate plus GA at the concentration indicated.

GA concentration (µg/ml)	α-Amylase (SIC units/g fresh weight)		
	Ambient	Tissue	Total
0	0	1	1
0.1	104	60	164
1.0	160	80	240
10	200	75	275
100	100	80	180

described in Section I.3. The results are presented in Table I.4 for both ambient and tissue while for simplicity, only the total activities are plotted in Figure I.3.

Although no attempt was made to determine precisely the lag period for hydrolytic enzyme induction in this experiment, it is obvious that increases in both α-amylase and protease are detectable in the tissue by 9 hr after GA treatment. Increases in total ribonuclease activity are not measurable until 18-24 hr after hormone application (Table I.4). At the start of the experiment considerable ribonuclease is associated with the tissue which appears to leak into the incubation medium without apparent synthesis occurring. In view of this and because ribonuclease activity reaches such high levels in the absence of GA by 36 hr, it is difficult to ascertain from the data presented whether ribonuclease has a longer lag period than α-amylase in this tissue. As large amounts of all three acid hydrolases were present in the tissue at 24 hr after application of GA, this incubation period was chosen for all future experiments.

4.5 Effect of Actinomycin-D

As the ultimate aim of this work was to determine whether α-amylase is membrane bound in GA treated aleurone tissue, any treatment that increased the amount of α-amylase retained by the

Figure I.3

Time course of production of α-amylase, protease and ribonuclease in isolated aleurone tissue. Incubation medium: 20 mM calcium nitrate plus or minus GA (10 µg/ml). Temperature: 30°C. Enzyme activity expressed as units per gram fresh weight as defined in Section I.3.

○ α-Amylase

▲ Protease

■ Ribonuclease

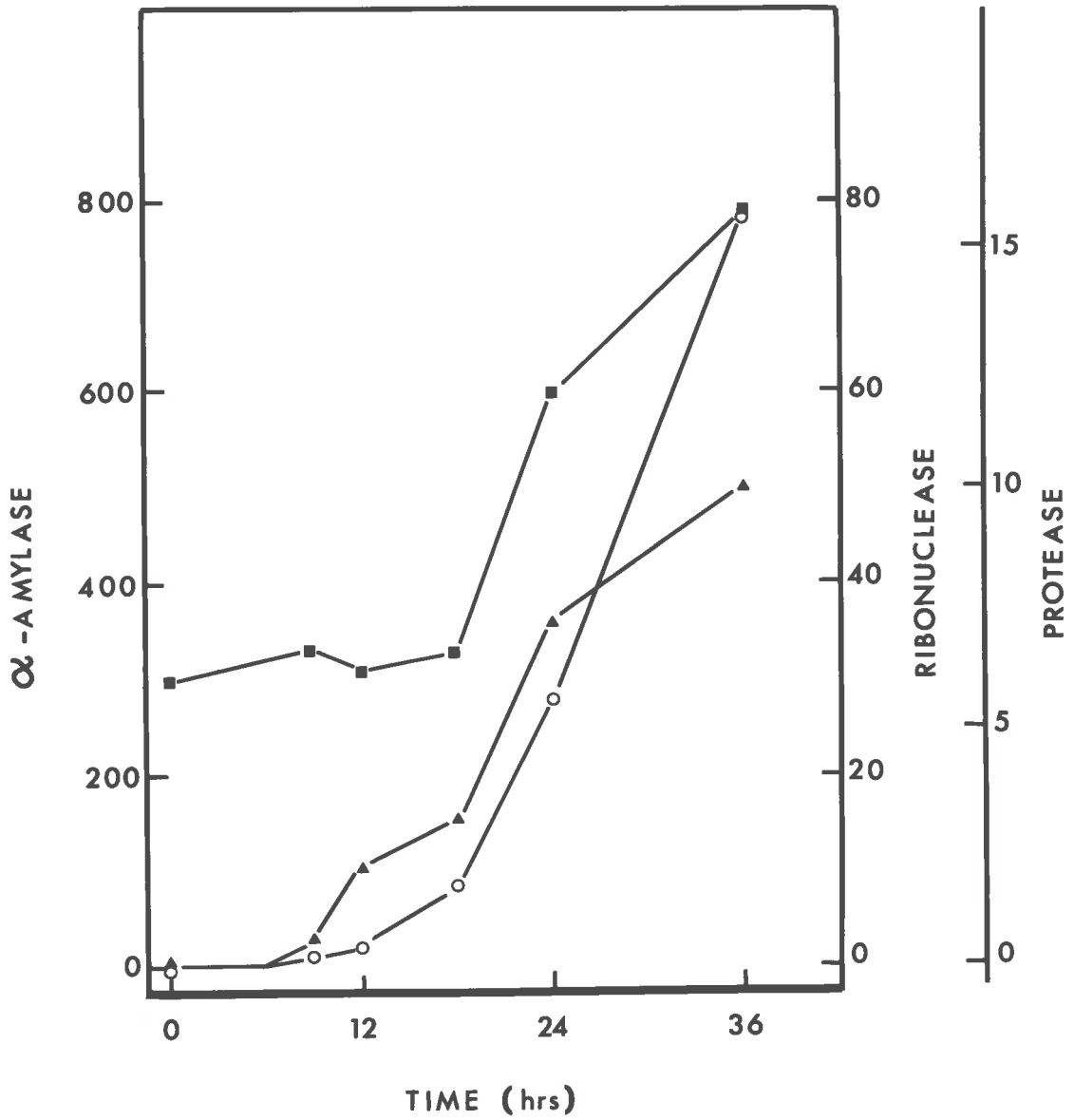

800
600
400
200
0
α -AMYLASE
80
60
40
20
0
RIBONUCLEASE
15
10
5
0
PROTEASE
0
12
24
36
TIME (hrs)

Table I.4

Time course of production of α-amylase, protease and ribonuclease in isolated aleurone tissue. Incubation medium: 20 mM calcium nitrate plus or minus GA (10 µg/ml). Temperature: 30°C. Enzyme activity expressed as units per gram fresh weight as defined in Section I.3.

Incubation Time (hr)	GA	α-Amylase			Protease			Ribonuclease		
		Ambient	Tissue	Total	Ambient	Tissue	Total	Ambient	Tissue	Total
0		0	0	0	0	0	0	0	30	30
9	+	2	7	9	0	0.6	0.6	17	16	33
12	+	8	12	20	1.2	1.2	2.4	15	16	31
18	+	33	30	63	2.0	1.2	3.2	18	15	33
24	+	198	84	282	4.0	2.3	7.3	35	25	60
24	-	1	2	3	0.2	0.3	0.5	17	18	35
36	+	585	198	783	6.3	3.3	9.6	52	26	78
36	-	15	7	22	0.5	0.5	1.0	21	22	43

tissue and thus increased the chances of detecting particulate α-amylase was desirable. Hence the report by Chrispeels and Varner (1967b) that low concentrations (25 µg/ml) of actinomycin-D (an inhibitor of RNA transcription) caused more α-amylase to be accumulated in barley tissue, was investigated.

Isolated and preincubated aleurone tissue was treated with GA (10 µg/ml) together with actinomycin-D at 0, 5, 10, 25 and 50 µg/ml for 24 hr at 30°C. α-Amylase was assayed in the tissue and the ambient solution and the results are presented in Table I.5. Actinomycin-D inhibited both the total synthesis of α-amylase and the amounts detected in both fractions. Although it is clear that the amount of enzyme found in the ambient was affected most, no net accumulation of α-amylase could be detected in the tissue.

4.6 Re-examination of the Rolling Procedure

It was pointed out earlier that although aleurone tissue isolated by the water method (Phillips, 1968) gave responsive tissue, the amounts of α-amylase induced by GA never exceeded about 50% of those produced by an equivalent weight of half-seeds. Therefore the question whether the lactic acid procedure (Section 2) yielded more responsive tissue was examined.

Aleurone tissue was isolated by both the old water method and the new lactic acid method and both batches of tissue were allowed to

Table I.5

Inhibition of α-amylase synthesis in isolated aleurone tissue by actinomycin-D. Aleurone tissue was treated at time zero with GA (10 µg/ml) and various concentrations of actinomycin-D. Incubation medium: 20 mM calcium nitrate. Time: 24 hr. Temperature: 30°C. Figures in parenthesis indicate inhibition as a percentage of control values.

Actinomycin-D (µg/ml)	α-Amylase (SIC units/g fresh weight)		
	Ambient	Tissue	Total
0	240	136	376
5	152 (27)	112 (18)	264
10	152 (27)	112 (18)	264
25	18 (93)	50 (63)	68
50	5 (98)	5 (96)	10

recover for 16 hr at 30°C. GA was added to give a final concentration of 10 µg/ml and the tissue incubated for a further 24 hr. The resulting ambient and tissue fractions were assayed for α-amylase activity and the results are shown in Table I.6. It should be noted that the data are expressed as SIC units/g dry weight of original half-seed because the dry weight of the seed was found to vary less than the fresh weight of aleurone tissue. This practise was followed in subsequent experiments.

Although aleurone tissue isolated by the water method yielded only 54% of the amount produced by half-seeds, the tissue isolated in lactic acid on the other hand gave almost as much (90%) α-amylase in response to GA as the equivalent weight of half-seed (Table I.6). No α-amylase could be detected in the tissue or the ambient solution of layers isolated in lactic acid and treated in solutions lacking the hormone. The lactic acid method was adopted as a standard procedure for aleurone isolation.

4.7 Effect of Glucose, Mannitol and Leucine

As pointed out earlier, the ultimate aim of the present investigation was to obtain aleurone tissue rich in α-amylase and to determine where in the cell the enzyme was located. Thus it was reasoned that any treatment that increased the amount of α-amylase

Table I.6

Comparison of α-amylase production by wheat aleurone isolated by two different rolling techniques. Both tissues preincubated 16 hr in 20 mM calcium nitrate. Tissue incubated for 24 hr after addition of GA (10 µg/ml) at 30°C.

	α-Amylase (SIC units/g fresh weight)			Total units/g dry weight	%
	Ambient	Tissue	Total		
Half seed					
-GA	0	0	0		
+GA	470	330	800	800	100
Aleurone (water method)					
-GA	3	3	6		
+GA	415	204	614	430	54
Aleurone (lactic acid method)					
-GA	0	0	0		
+GA	600	330	930	712	89

accumulating in the tissue could facilitate the study. So the reports by Chrispeels and Varner (1967b) that amino acids increased α-amylase production and by Phillips (1968) that 10 mM glucose caused accumulation of α-amylase in aleurone tissue were further investigated.

Aleurone tissue was isolated by the lactic acid procedure and was allowed to recover from rolling for 16 hr in calcium nitrate at 30°C. In contrast to the usual procedure of simply adding a concentrated GA solution, the preincubation medium was poured off and replaced with 10 mM solutions of glucose, mannitol or leucine together with calcium nitrate, plus or minus GA at a final concentration of 10 µg/ml. The tissue was incubated for a further 24 hr and α-amylase activity assayed.

The results of this experiment were rather surprising (Table I.7). Only when glucose or mannitol were included in the incubation medium did the tissue respond to GA. Since no treatment was included where the preincubation medium was retained, conclusions are difficult. However, the data suggest that a factor necessary for the tissue to respond to the hormone was poured off with the preincubation medium and this factor could be replaced by glucose or mannitol but not leucine. As it is obviously an advantage to culture the tissue in solutions of known and controllable composition, instead of the preincubation medium of unknown and probably variable composition

Table I.7

Effect of glucose, mannitol or leucine on production of α-amylase in aleurone tissue isolated by the lactic acid procedure. Preincubation period: 16 hr. Incubation: 24 hr. Incubation medium 20 mM calcium nitrate. Other additions as indicated: glucose, mannitol and leucine all at 10 mM, GA 10 µg/ml. Temperature: 30°C.

		α-Amylase (SIC units/g fresh weight)		
Addition	GA	Ambient	Tissue	Total
None	-	0	1	1
	+	25	15	40
Glucose	-	6	10	16
	+	300	212	512
Mannitol	-	0	3	3
	+	600	188	788
Leucine	-	15	19	34
	+	9	6	15

the extent to which glucose or mannitol substitute for the factors in the preincubation medium was investigated.

In the next experiment leucine was not included and the effects of glucose and mannitol added either directly to the preincubation medium or by themselves in solution were compared. The results are shown in Table I.8.

The results of this experiment clearly show that changing the preincubation medium results in almost a complete loss of α-amylase activity despite the presence of the hormone, but this loss of activity is more than restored by the presence of glucose or mannitol. Of the two sugars, glucose was the most effective, resulting in yields of α-amylase in excess of 90% of the half-seed value whether added directly or by changing the solution. Changing the solutions resulted in slightly higher yields (99%) than the direct addition method (92%, Table I.8).

Because of the importance of having extremely high yielding tissue before any thorough investigation into the subcellular distribution of acid hydrolases in aleurone cells was commenced, the experiment was repeated with minor modifications. As well as testing the effect of glucose during incubation with the hormone, the sugar was also tested in the preincubation medium. Because of the apparent superiority of glucose in enhancing the GA induced production of

Table I.8

Effect of glucose or mannitol by either direct addition (ADD) or by changing the pre-incubation medium (CHANGE) on production of GA-induced α-amylase in isolated aleurone. Conditions are the same as given for Table I.7. Glucose and mannitol both at 10 mM, GA 10 µg/ml.

	Addition	Method of Addition	GA	α-Amylase (SIC units/g fresh weight) Ambient	Tissue	Total	Total units/g dry weight	%
Half-seed	None	ADD	-	2	23	25		
	"	ADD	+	340	500	840	840	100
Aleurone	"	ADD	-	0	0	0		
	"	ADD	+	600	180	780	530	64
	"	CHANGE	-	0	0	0		
	"	CHANGE	+	7	25	32	21	3
	Glucose	ADD	-	0	3	3		
		ADD	+	890	270	1160	770	92
		CHANGE	-	0	0	0		
		CHANGE	+	1000	265	1265	830	99
	Mannitol	ADD	-	0	0	0		
		ADD	+	650	230	880	580	69
		CHANGE	-	0	0	0		
		CHANGE	+	1070	288	1158	760	90

α-amylase (Table I.8), mannitol was not included.

The results listed in Table I.9 confirm the earlier ones (Tables I.7 and I.8) which showed that α-amylase production by GA is almost negated when the preincubation medium is changed unless glucose is included in the new incubation medium. These results (Table I.9) also indicate that glucose in the preincubation medium is without effect on the subsequent response of the tissue to GA, for only when glucose was included in the incubation medium were high yields of α-amylase obtained in response to the hormone.

Glucose in both the preincubation medium and the incubation medium gave the greatest quantities of enzyme with yields of α-amylase in excess of 150%. However, as the possibility of bacterial or fungal infection was undoubtedly increased, and because the inclusion of glucose in the incubation medium only, gave an α-amylase production in excess of 100% of an equivalent weight of half-seed, it was decided to leave glucose out of the preincubation medium.

From the results of the foregoing experiments a standard method of isolation and incubation was developed giving aleurone tissue highly responsive to GA. The tissue was isolated in the roller mill using lactic acid as the liquid, allowed to recover from rolling during a 12 hr preincubation in 20 mM calcium nitrate and finally incubated

Table I.9

Effect of glucose added directly (ADD) or by changing the preincubation medium (CHANGE) in both preincubation and incubation media, on the production of α-amylase. Conditions and concentrations are the same as stated for Table I.8.

	Addition to preincubation medium	Addition to incubation medium	Methods of addition	GA	α-Amylase Ambient	α-Amylase Tissue	α-Amylase Total	Total units/ g dry weight	%
Half-seed	None	None	ADD	-	0	0	0		
	"	"	ADD	+	470	330	800	800	100
Aleurone	None	None	ADD	-	0	0	0		
	"	"	ADD	+	600	330	930	712	89
	"	"	CHANGE	-	0	3	3		
	"	"	CHANGE	+	26	24	50	39	5
	"	Glucose	CHANGE	-	0	0	0		
	"	"	CHANGE	+	882	388	1270	980	123
	Glucose	None	CHANGE	-	0	0	0		
	"	"	CHANGE	+	24	35	59	45	6
	"	Glucose	CHANGE	-	0	0	0		
	"	"	CHANGE	+	1305	390	1695	1300	163

Figure I.4

Effect of GA concentration on production of α-amylase in isolated aleurone tissue. Incubation medium: 20 mM calcium nitrate, 10 mM glucose and GA at the concentration indicated. Temperature: 30°C. Time: 24 hr. α-Amylase: SIC units/g fresh weight.

□ Tissue

▼ Ambient

▽ Total

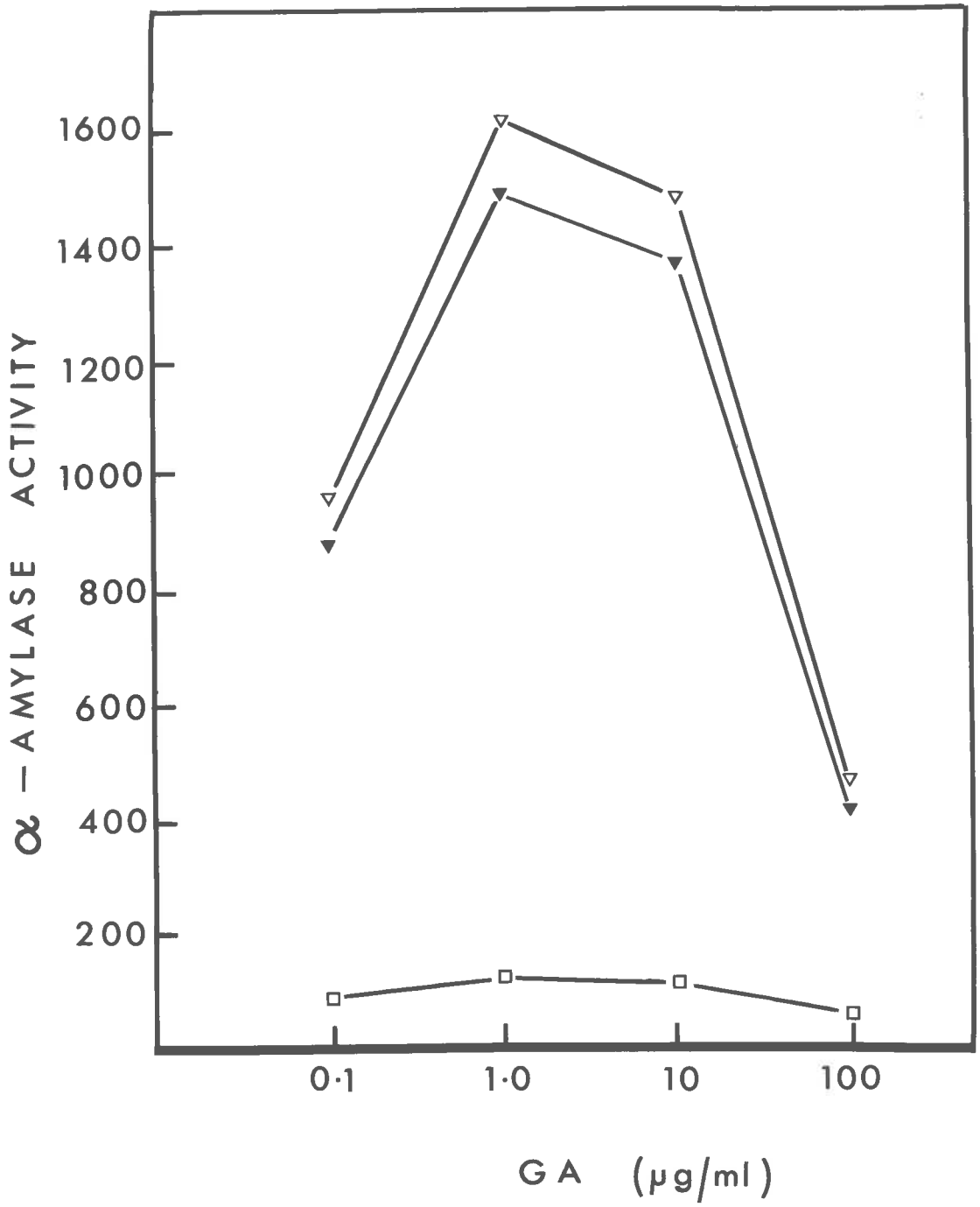

1600
1400
1200
1000
800
600
400
200
α – AMYLASE ACTIVITY
0·1
1·0
10
100
GA (µg/ml)

Figure I.5

Determination of lag period of α-amylase production in isolated aleurone tissue. Incubation medium: 20 mM calcium nitrate, 10 mM glucose, plus or minus GA (1 µg/ml). Temperature: 30°C. α-Amylase: SIC units/g fresh weight.

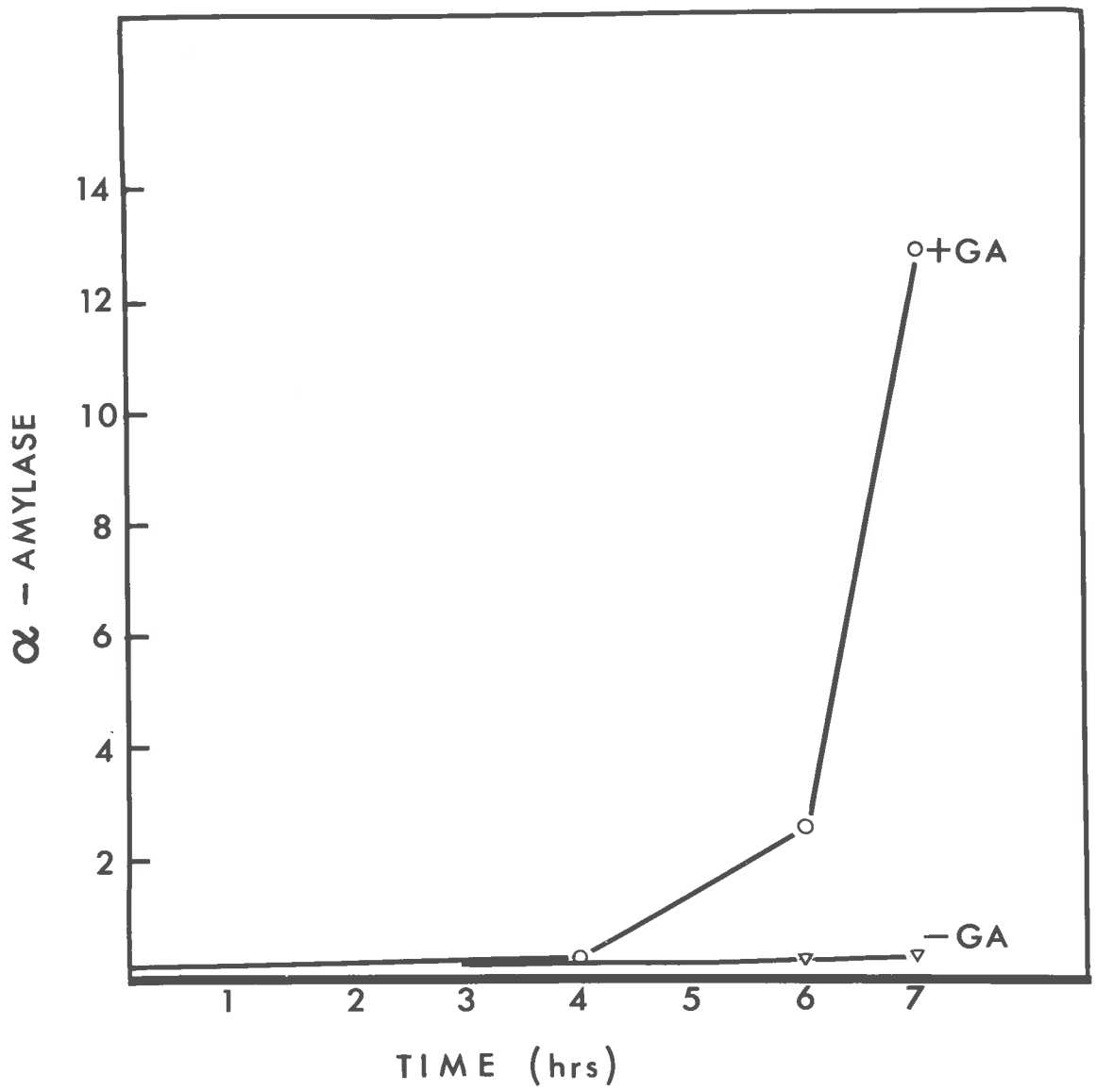

α - AMYLASE
14
12
10
8
6
4
2
+GA
−GA
1
2
3
4
5
6
7
TIME (hrs)

Table I.10

Increase in α-amylase production at various times after the addition of GA (1 µg/ml). Temperature: 30°C.

	Time (hr)	α-Amylase (SIC units/g fresh weight) Ambient	Tissue	Total
-GA	0	0	0	0
	4	0	0	0
	6	0	0.1	0.1
	7	0	0.2	0.2
+GA	0	0	0	0
	4	0	0.2	0.2
	6	0	2.5	2.5
	7	2.8	10.0	12.8

for 24 hr in calcium nitrate and 10 mM glucose with or without GA.

Using this combination of methods, aleurone tissue treated with a range of GA concentrations responded in terms of α-amylase production as Figure I.4 illustrates. The maximum amount of α-amylase could be obtained with a concentration of GA as low as 1 μg/ml, so this concentration of the hormone was chosen for all future experiments.

As a further check on the responsiveness of the tissue, an attempt was made to estimate the lag time between the addition of GA and the first appearance of α-amylase. The first trace of α-amylase was detected in the tissue 4 hr after the application of GA (Table I.10 and Figure I.5) and by 7 hr α-amylase could be detected in the ambient solution. In the absence of GA however, only a trace of α-amylase could be detected in the tissue even by 7 hr. It was concluded therefore, that the lag period for α-amylase induction by GA was approximately 4 hr at 30°C in wheat aleurone cells.

5. Discussion

The bulk of a cereal seed such as wheat, both by weight and volume, is made up of endosperm (Figure I.1). It is this tissue that contains the majority of the storage carbohydrates and proteins

of the seed. Starch represents 75% of the total dry weight of the grain while protein represents a further 14% (Jennings and Morton, 1963). Thus, from an economic viewpoint, the endosperm is the most important portion of the mature grain. The physiological significance of the endosperm however, lies in the fact that it is the source of nutrients for the embryo in the interval between maturation of the grain and the establishment of the new seedling.

Most of the reserves in the endosperm are stored in the form of macromolecules and at any given time are not necessarily available to the embryo. The enzymes hydrolysing these reserves are not present in the endosperm but are secreted by the single layer of aleurone cells which surround it (Paleg, 1960) and the synthesis and secretion of these hydrolytic enzymes is under the control of the plant hormone, GA. In one of the most elegant systems known in the plant kingdom, GA is released by the recipient of the ultimate hydrolysis products, the embryo. It has also been suggested that the soluble products of endosperm digestion can exert osmotic control of hydrolytic enzyme secretion thus extending the total control of the rate of food mobilization in the germinating seed (Jones and Armstrong, 1971).

Although the aleurone layer is apparently well separated from other tissue, there are many practical obstacles to its isolation. Firstly, the hardness of cereal seed makes it extremely difficult to

handle in the dry state. Milling separates aleurone tissue (bran) from starch grains (flour) of the endosperm and from the embryo (wheat germ). Unfortunately bran has generally been found to be a poor source of physiologically active aleurone cells, due probably to the great heat generated during milling.

Soaking the seeds in water softens them, but unless the embryo is removed germination procedes and the aleurone cells begin enzyme secretion before the cells can be isolated. Hence it has become standard practice to remove the embryo half of the seed before imbibition (Paleg, 1960; Chrispeels and Varner, 1967). The actual period and conditions of this imbibition period are critical and vary from 2 hr in water (Eastwood and Laidman, 1971) to 3 days on moist sand (Varner, 1964). Also, to avoid microbial contamination, the seeds must be surface sterilized and all operations performed under asceptic conditions. The period of 26 hr imbibition at 30^{o}C was found by Phillips (1968) to give maximal softness to the endosperm and maximally responsive aleurone tissue for this variety of wheat (Olympic).

The separation of the aleurone layer from the enclosed endosperm presents other difficulties. Peeling by hand (Varner, 1964; Jones, 1968) may be manageable in laboratories where a large staff is available, but for workers where assistance is not available or where

large amounts of tissue are required, this method is too time-consuming. To date, the roller mill method developed by Phillips and Paleg (1972) is the only practical alternative to hand peeling.

The physiological responsiveness of the isolated aleurone tissue is, of course, the prime test of the effectiveness of the method of isolation. However, even this criterion is somewhat dubious since the isolated aleurone layer is free of at least some of the control mechanisms operative in both the half seed and the intact grain systems. In the latter two systems for example, enzyme production may be subject to osmotic feed-back regulation by the hydrolysis products formed during endosperm digestion (Jones and Armstrong, 1971). However, practical considerations and an absence of suitable alternatives, dictate that GA-induced α-amylase production in embryo-less half-seed serve as at least the minimum level that should be induced in isolated aleurone layers obtained from an equivalent weight of seed and incubated for a similar time and temperature.

Yields of α-amylase in excess of 100% of an equivalent weight of half-seed were recorded when glucose was included in the incubation medium of GA-treated aleurone tissue (Table I.9). Glucose may serve as an energy source for the tissue during the lag period before degradation of phytate has begun. Although glucose stimulation of

GA-induced α-amylase production has been observed in barley aleurone (Chrispeels and Varner, 1967b), there is no satisfactory explanation of its action.

The presence of calcium in the incubation medium appears to be beneficial for high yields of α-amylase (Table I.2) from GA-treated tissue. This is perhaps not surprising in view of the manifold roles of calcium in this system. Calcium is required for enzymic activity of the α-amylase protein (Kneen *et al.*, 1943; Ohlsson, 1930) in spite of the fact that the majority of this ion is only loosely bound by malt and wheat α-amylases and may be removed by dialysis even in the absence of chelating agents (Schwimmer and Balls, 1949). In addition, Varner and Mense (1972) have shown, by using a flow through cell device and measuring continuously the secretion rate of α-amylase from GA-treated barley aleurone layers, that if no calcium or similar divalent cation such as magnesium was present, the secretion of α-amylase does not take place. Finally, the addition of calcium to presumably fully calcium saturated α-amylase, protects the enzyme against both heat and proteolytic inactivation (Mitchell, 1972). Although it is impossible to delineate the specific role played by the calcium ions added to the incubation medium, this work confirms again the desirability of their presence.

As the ultimate aim of this study was to determine the

subcellular location of GA-induced acid hydrolases it was necessary to establish how long after hormone application wheat aleurone tissue attained a suitably high level of the three hydrolases chosen for study, namely α-amylase, protease and ribonuclease. The sequence of production of the three enzymes was difficult to determine from the data obtained (Table I.4). Although increases in both α-amylase and protease are easily detectable after 9 hr of GA treatment, ribonuclease does not appear to increase until 18-24 hr. However, the high levels of ribonuclease in control tissue make it difficult to differentiate between the development of the three enzymes. In barley, synthesis and secretion of α-amylase and protease occur after a 6-8 hr lag period; apparent synthesis of ribonuclease begins immediately after GA application although its secretion is delayed until 24 hr (Chrispeels and Varner, 1967a). In this regard, the 4 hr lag period observed for α-amylase induction by GA (Figure I.5) is among the shortest reported (Jacobsen and Varner, 1967; Collins *et al.*, 1972b).

These results and those described above, indicated that wheat aleurone tissue isolated and treated as described was highly responsive to low levels of GA application. Furthermore, because as much α-amylase was produced as by half-seeds and the lag time was short, and in view of the ease of isolating large amounts of aleurone, it was considered that both the tissue and the methods of isolation were ideally suited to the proposed investigation.

SECTION II

DIFFERENTIAL CENTRIFUGATION OF HOMOGENATES OF GA-TREATED ALEURONE TISSUE

This Section is concerned primarily with the subcellular distribution of the GA-induced acid hydrolases, α-amylase, protease and ribonuclease, in homogenates of wheat aleurone cells. Conditions of homogenization, centrifugation and enzyme analysis are described in detail. Control experiments are described which suggest that levels of acid hydrolases found in particulate fractions represent true membrane-enclosed enzymes and are not due to artefacts of particle preparation. A major portion of this work has been reported under the heading "Lysomal Nature of Hormonally Induced Enzymes in Wheat Aleurone Cells" by R.A. Gibson and L.G. Paleg (Biochem. J. (1972) *128*, 367-375).

1. Preparation of Aleurone

All aleurone tissue used in experiments cited in this Section was isolated by the lactic acid method described in Section I.2. Unless otherwise stated the tissue was incubated for 24 hr in the calcium-glucose incubation medium (± GA) following a 16 hr recovery

period in calcium nitrate (Section I.4). At the end of the incubation period the incubation medium was decanted and the tissue washed with 10 ml of calcium acetate solution (5 mM). The calcium acetate was decanted and the combined liquid fraction (ambient) was set aside under refrigeration for later enzyme analysis. The tissue was then shaken vigorously with three 100 ml aliquots of distilled water and the washings discarded. Finally the tissue was drained over a plastic screen (1 mm mesh) and transferred to a 4°C cold room where subsequent operations were carried out.

2. Homogenisation

Two methods of grinding aleurone tissue were employed.

(a) Ultra Turrax Apparatus. Samples of treated aleurone tissue (1 g) were placed in 40 ml glass centrifuge tubes with 10 ml of grinding medium, and the tissue was allowed to settle to the base of the tube. The grinding probe of the Ultra Turrax (Model TP 18/2) was lowered carefully to the base of the tube ensuring that no air was trapped in the probe. The power was turned on, and the thyrister control gradually turned up until sufficient power was delivered to the Ultra Turrax to just set the revolving portion of the probe in motion. The action of the probe was such that broken tissue was constantly being ejected into the grinding medium, while new

unbroken tissue was simultaneously drawn up for grinding. After 30 sec grinding, with movement of the probe to ensure complete homogenization of all tissue, the power was turned off and the resulting homogenate was filtered through two double layers of cheesecloth into clean centrifuge tubes.

(b) Mortar and Pestle. Samples of treated aleurone (1 g) were placed in a pre-chilled mortar (6 cm diameter) with 4 ml of grinding medium, but without sand. The tissue was ground by rotating the pestle with a steady motion for 60 sec with frequent checks to see that all the tissue was being homogenized. The sides of the mortar were then washed with 6 ml of grinding medium and the homogenate filtered through two double layers of cheesecloth into clean centrifuge tubes.

A variety of grinding media were tested but unless otherwise stated the medium used was based on one developed by Breidenbach and Beevers (1967): 0.4 M sucrose, 0.05 M Tris-HCl (pH 7.0), 0.1% bovine serum albumin (BSA), 0.01 M KCl, 1 mM EDTA and 0.1 mM $MgCl_2$.

3. Centrifugation of Aleurone Homogenates

Low speed centrifugations (1,000 *g*, 20,000 *g*) were carried out in an MSE Mk 1 model refrigerated centrifuge using an 8 x 50 ml rotor

and plastic centrifuge tubes. High speeds (60,000 *g*, 105,000 *g*) were attained on a Beckman model L refrigerated ultracentrifuge using a 50 Ti rotor and 13.5 ml polyallomer tubes. All centrifugations were carried out at 0-5°C. Unless otherwise stated centrifugation times were 10 min at 1,000 *g*; 20 min at 20,000 *g*; 30 min at 60,000 *g* and 90 min at 105,000 *g*.

Where more than 1 g of treated aleurone was required for analysis, both the initial homogenates and the subsequent supernatants from each successive centrifugation were mixed thoroughly prior to the next centrifugation step. Immediately after centrifugation, the resulting pellet was resuspended in the required buffer and stored on ice until enzyme assays could be performed. Pellets were suspended in 5 ml of 5 mM calcium acetate for α-amylase assay, or 2 ml of 1 mM potassium acetate buffer, pH 4.8, containing 20 mM 2-mercaptoethanol for the protease assay, or 2 ml of 50 mM potassium acetate buffer, pH 5.0, for the ribonuclease assay. All pellets were thoroughly ground in a close fitting glass homogenizer in 0.1% Triton X-100 before assay.

4. Measurement of Enzyme Activity

α-Amylase. After inactivation of β-amylase activity by heating at 70°C for 20 min (Paleg, 1960), enzyme activity was determined by the method described in detail in Section I.3. When

EDTA was present care was taken to add excess calcium acetate before inactivating the β-amylase. All results are expressed in starch-iodine colour (SIC) units as described by Briggs (1967).

Acid Ribonuclease. The depolymerization of yeast RNA was determined by the method of Wilson (1963), described earlier (Section I.3). One unit of enzyme activity represents 0.1 E_{260} generated in 1 hr at 37°C and pH 5.0.

Acid Protease. The hydrolysis of the wheat storage protein, gliadin, was assayed at 30°C and pH 4.8 by the method of Jacobsen and Varner (1967). One unit of enzyme activity represents 1.0 E_{580} generated under the conditions cited in Section I.3.

Boiled enzyme controls were included in all enzyme assays.

Protein was measured by the method of Lowry *et al.* (1951).

4.1 Latency of α-Amylase

The latency of α-amylase was established by two methods. The first involved centrifugation, the latent enzyme being that proportion of a suspension of a 60,000 *g* pellet preparation that was sedimented (by a further centrifugation) after incubation under various conditions. The second method, used commonly with animal lysosomes

(Wattiaux and de Duve, 1956), involved assaying enzyme activity with and without a surface-active agent such as Triton X-100, after incubation of lysosomes under similar conditions. Equal volumes of a suspension of a 60,000 *g* pellet were assayed for α-amylase activity in substrate solutions containing 0.4 M sucrose. One sample, without additive, was taken as the measure of free or available α-amylase and another, containing 0.1% Triton X-100, was assayed as a measure of total α-amylase activity. Both samples were preincubated at 30°C for 30 min before assay. Latent α-amylase activity was calculated by subtracting the value obtained for free α-amylase activity from the value obtained for total α-amylase activity:

i.e. latent enzyme = (activity with Triton X-100) - (activity without Triton X-100).

5. Differential Centrifugation of Aleurone Homogenate and the Distribution of Enzyme Activity

5.1 Choice of Grinding Medium

Work carried out in this laboratory by Phillips (1968) on fractions obtained by differential centrifugation of wheat aleurone homogenates indicated that a portion (15%) of the total tissue

α-amylase could be sedimented. Phillips used an Ultra Turrax to homogenize the aleurone in grinding medium described by Honda *et al.* (1966) which was developed for the isolation of intact chloroplasts. As this grinding medium may not have been the best choice for the isolation of particles containing hydrolytic enzymes, a search was initiated for a grinding medium giving a higher, yet consistent percentage of precipitable α-amylase. A total of 7 different grinding media were tested. Their composition and the distribution of α-amylase resulting from their use is summarized in Table II.1.

As already pointed out, there are two criteria for a suitable grinding medium for the detection of particulate enzymes. Firstly, the medium should give as high a percentage as possible of the enzyme in question in the particulate fractions, and secondly the total amount of enzyme recovered from all fractions should be high; that is, there should be no component of the medium which inhibits the activity of the enzyme being assayed.

With the exception of grinding medium No. 1 (glycerol) all the media tested were aqueous in nature, and with the exception of this medium all resulted in approximately 50% of the total α-amylase being found in the particulate fractions. However in many of these media the total amounts of α-amylase recovered were low. Thus in medium No. 5 only 19.2 units were recovered compared with 60 units recovered using medium No. 4. Because medium No. 4

Table II.1

Effect of grinding medium on distribution of α-amylase in cell fractions obtained from aleurone tissue treated with GA (10 µg/ml) for 24 hr. Tissue was ground for 30 sec (Ultra Turrax apparatus) and the resulting homogenate subjected to differential centrifugation as described in the text. Composition of media: (1) glycerol (Yatsu and Jacks, 1968); (2) 0,25 M sucrose, 2.5% ficoll, 5% dextran, 0.01% bovine serum albumin, 0.025 M Tris-HCl (Honda *et al.*, 1966); (3) 20% sucrose, 1 mM EDTA, 0.1% polyvinylpyrrolidone, 0.1 M Tris-HCl (Balz, 1966); (4) 0.4 M sucrose, 0.05 M Tris-HCl, 0.01 M dithiothreitol, 0,1% bovine serum albumin, 10 mM KCl, 1 mM EDTA, 0.1 mM $MgCl_2$ (Breidenbach and Beevers, 1967; (5) 0.35 M mannitol, 0.35 M sucrose, 10 mM K_2HPO_4, 0.1% bovine serum albumin, 1.0 mM EDTA, 2.0 mM $Na_2S_2O_5$ (Stokes *et al.*, 1968); (6) 0.5 M sorbitol, 0.05 M Tris-HCl, 1.0 mM EDTA (Matile, 1968); (7) 0.4 M mannitol, 0.01 M Tris-HCl (Spichiger, 1969). All media were at pH 7.0

	Distribution of α-amylase (%)						
Grinding medium	1	2	3	4	5	6	7
Cell fraction							
1,000	12,7	48,8	38,6	11,9*	6,2	3,7	3,5
20,000	16.4	2.4	7,4	18.8*	10.4	23.4	25.9
105,000	0	2.4	3.1	14,1*	39.1	24.4	15.7
Supernatant	70.9	46.4	50,9	55.2*	44.3	48.5	54.9
Total	100	100	100	100	100	100	100
	(13,4 units)	(37.9 units)	(32.4 units)	(60.1 units)	(19.2 units)	(42.7 units)	(40.1 units)

* Values represent the means of two experiments.

(Breidenbach and Beevers, 1967) gave both high yields of α-amylase in the particulate fractions and the highest recoveries of total enzyme activity, this medium was chosen for subsequent experiments.

5.2 Tissue Homogenization

Very high shear forces are required to break the massive cell walls surrounding the aleurone cells and thus liberate the organelles and cytoplasm into the grinding medium. Unfortunately the very forces required to burst the cells often are sufficient also to damage the liberated organelles. To minimise this effect a variety of homogenization techniques were tested, again with the intent of finding a system that yielded the maximum proportion of α-amylase activity in the particulate fraction along with the maximum extraction of the enzyme from the tissue.

Aleurone tissue was homogenized in grinding medium of Breidenbach and Beevers (1967) by the methods outlined in Table II.2. The results indicate that regardless of the method of homogenization chosen, the percentage of α-amylase found in the particulate fractions was always between 45-50%. However, the amount of α-amylase extracted from the tissue varied markedly with as little as 18 units extracted by the razor blade method, and as much as 60 units obtained using the Ultra Turrax apparatus for 30 sec. Grinding the tissue with a mortar and pestle also gave excellent results with 50% of the total α-amylase in the

Table II.2

The effect of grinding technique on the distribution of α-amylase in fractions obtained by differential centrifugation of aleurone cell homogenates. All tissue treated with GA (10 µg/ml) for 24 hr at 30°C. The grinding medium used in all cases was that of Breidenbach and Beevers (1967). All other conditions were as described in the text.

	Ultra Turrax								Mortar & Pestle		Razor Blade	
	10 sec grind		20 sec grind		30 sec grind		60 sec grind		60 sec		10 min	
	units	%	units	%	units	%	units	%	units	%	units	%
1,000 *g*	1.6	3.3	1.3	3.0	2.0	3.3	2.0	3.4	2.5	4.9	6.6	36.5
20,000 *g*	12.0	25.0	9.6	22.5	16.6	27.1	15.0	25.4	13.2	25.8	1.9	10.5
105,000 *g*	9.4	19.6	8.3	19.4	10.9	17.8	8.6	14.6	9.8	19.1	0.4	2.2
Supernatant	25.0	52.1	23.5	55.1	31.8	51.8	33.4	56.6	25.7	50.2	9.2	50.8
Total	48.0	100	42.7	100	61.3	100	59.0	100	51.2	100	18.1	100

particulate fractions and over 50 units total enzyme extracted from the tissue. Because of the similarities in the results obtained, these last two methods were both employed in subsequent experiments.

5.3 Control Experiments

If the majority of an enzyme's activity can be sedimented by centrifugal forces sufficient to sediment cell organelles, it can be erroneous to assume that the enzyme in question is normally associated with an organelle (either enclosed in it or associated with its membrane) unless control experiments are conducted to preclude possible artefacts. For example, on rupturing the cell, an enzyme which is normally cytoplasmic may bind non-specifically to membrane fractions and thus be sedimented by centrifugation. To test this possibility a series of experiments were performed.

In one experiment, α-amylase from aleurone tissue treated with GA was included in the grinding medium used to homogenize untreated aleurone tissue, to see if increased α-amylase could be found in the various pellet fractions. A 1 g sample of GA (10 µg/ml) -treated tissue was homogenized with the Ultra Turrax for 1 min at maximum speed in 7.5 ml of water. After passage through two double layers

of cheesecloth, the filtrate was centrifuged at 20,000 *g* for 30 min, and a portion (5 ml) of the resulting supernatant was mixed with an equal volume of double-strength grinding medium. This mixture was then used to grind 1.0 g of control tissue. The resulting fractions obtained after centrifugation were compared with both control tissue and GA-treated tissue, homogenized without added α-amylase.

The results of this experiment (Table II.3) indicated that there was little non-specific binding of added α-amylase to the organelles obtained in the various pellets. A majority of the enzyme was located in the final supernatant fraction. In contrast, GA-treated aleurone tissue homogenized under the same conditions (but without added α-amylase) showed the normal distribution of α-amylase, with over 45% of the enzyme in particulate fractions.

An objection could reasonably be raised that because the source of the tissue was untreated aleurone, the experiment was not valid. This may be particularly important in the light of the vast ultra-structural changes observed in GA-treated aleurone (Paleg and Hyde, 1964; Jones, 1969a, b). Therefore the experiment was repeated using GA-treated tissue instead of control tissue.

It could further be argued that the addition of α-amylase to the grinding medium would not be expected to cause an increase in

Table II.3

Distribution of α- amylase in control aleurone tissue after homogenization with added α-amylase, and subsequent differential centrifugation. The grinding medium used was that described by Breidenbach and Beevers (1967). All other conditions were as described in the text.

	α-Amylase					
	Control tissue		Control tissue + α-Amylase		GA-treated tissue	
	units	%	Units	%	units	%
Cell fraction						
1,000 *g*	0	0	1.0	1.1	2.7	2.1
20,000 *g*	1.1	25.6	1.0	1.1	41.5	31.9
105,000 *g*	0.8	18.6	0.9	1.0	14.8	11.4
Supernatant	2.4	55.8	86.0	96.8	71.0	54.6
Total	4.3	100	88.9	100	130.0	100

the measurable particulate α-amylase if there were only a limited number of binding sites on the membrane of the organelles and these were all occupied by existing cellular α-amylase. It was therefore reasoned that if an alternative binding site were provided, any soluble α-amylase in the cell would bind to that in preference to the organelles. To test this hypothesis, 100 mg of amylose (the substrate for α-amylase) was dissolved by boiling in 5 ml of water and the solution centrifuged for 30 min at 20,000 *g*. The resulting supernatant was mixed with an equal volume of double-strength grinding medium and this mixture used to homogenate 1 g of GA-treated aleurone.

Regardless of the addition of either α-amylase or its substrate, amylose, to the grinding medium, approximately 80 units of α-amylase was found in the particulate fractions of each of the three treatments (Table II.4). Although the tissue homogenised with added α-amylase showed a slightly different sedimentation pattern (more enzyme activity in the 1,000 *g* pellet and less in the 105,000 *g* pellet than control), the added α-amylase appears to be entirely associated with the supernatant fraction. The addition of amylose to the grinding medium caused little change in either the distribution or the amount of precipitable α-amylase. Both of these results were taken as indicating that the α-amylase measured in pellet fractions was a true measure of organelle-associated enzyme and was not a preparative

Table II.4

Distribution of α-amylase in GA-treated (10 µg/ml) aleurone tissue after homogenization with added α-amylase or amylose and subsequent differential centrifugation. The grinding medium used was prepared according to Breidenbach and Beevers (1967). All other conditions were as described in the text.

	α-Amylase					
	GA-treated		GA-treated + α-amylase		GA-treated + amylose	
	Units	%	Units	%	Units	%
Cell fraction						
1,000 *g*	18.6	9.7	27.5	8.7	13.7	7.5
20,000 *g*	39.1	20.4	45.5	14.3	54.6	29.8
105,000 *g*	25.3	13.2	9.1	2.8	16.6	9.2
Supernatant	109.0	56.9	236.0	74.2	98.0	53.5
Total	192.0	100	318.1	100	182.9	100

artefact.

5.4 Distribution of GA-Induced Acid Hydrolases

The experiments described thus far have all demonstrated that a significant proportion of the GA-induced α-amylase in wheat aleurone was particulate in nature and could be sedimented by differential centrifugation. The enzymes, protease and ribonuclease, also reported to arise by *de novo* synthesis (Jacobsen and Varner, 1967; Bennett and Chrispeels, 1972) were of interest, and experiments were designed to determine the subcellular distribution of these enzymes.

Three 1 g samples of GA-treated aleurone tissue were homogenized separately in 10 ml of grinding medium. The homogenates were filtered through cheesecloth and the filtrates combined. This combined homogenate was mixed thoroughly and divided into three 10 ml fractions for centrifugation. The same process was repeated for control tissue. After each centrifugation step, one tube from each treatment (± GA) was set aside for the assay of each of the three enzymes. Samples were also taken from the final supernatant fraction for enzyme analysis.

Although 46% of the α-amylase was detected in the pellet fractions, only 25% of the protease activity was found in these same fractions (Table II.5). Ribonuclease on the other hand, could not

Table II.5

Distribution of α-amylase, protease and ribonuclease in fractions obtained by differential centrifugation of homogenates of isolated aleurone tissue incubated 24 hr at 30°C with or without GA (10 µg/ml). Tissue homogenized with an Ultra Turrax in the grinding medium described by Breidenbach and Beevers (1967).

	% Distribution					
	α-Amylase		Protease		Ribonuclease	
	-GA	+GA	-GA	+GA	-GA	+GA
Cell fraction						
1,000 *g*	0	2	8	7	0	0
20,000 *g*	23	32	5	11	0	0
105,000 *g*	18	12	12	8	0	0
Supernatant	57	54	75	74	0	0
Total	100	100	100	100		
	(4 units)	(130 units)	(3 units)	(6 units)		

be detected in any of the fractions.

The lack of ribonuclease activity was thought to be due to a component of the grinding medium as enzyme activity was detected in the tissue from the same experiment when the tissue was homogenized in 5 mM calcium acetate (data not shown). A prime suspect for the cause of this inhibition of ribonuclease activity was dithiothreitol which also interfered with the α-amylase assay. This possibility was tested in the next experiment.

The results listed in Table II.6 show that dithiothreitol completely inhibited ribonuclease activity but its presence or absence did not seriously affect either the total amount of α-amylase or the percentage sedimented by centrifugation. It was therefore decided to remove this compound from the grinding medium.

Using this modified grinding medium, the distribution of α-amylase, protease and ribonuclease was examined in detail using the methods already outlined. The results obtained from three such experiments including the average values are listed in Table II.7. An average of 59% of the total α-amylase in GA-treated tissue was sedimentable and about half of this activity was located in the 20,000 *g* pellet. Protease showed a similar trend with an average of 70% of the activity present in particulate fractions. Ribonuclease, on the other hand, was primarily located in the supernatant fraction

Table II.6

Effect of dithiothreitol on the distribution and recovery of α-amylase and ribonuclease in fractions obtained by differential centrifugation of homogenates of aleurone tissue treated with GA (10 µg/ml) for 24 hr at 30°C.

Dithiothreitol	α-Amylase		Ribonuclease	
	+	-	+	-
Total enzyme units/g	130	112	0	129
% Sedimentable	45	46	0	10

Table II.7

Distribution of α-amylase, protease and ribonuclease in cell fractions obtained from aleurone tissue treated with or without GA (10 µg/ml) for 24 hr at 30°C. Tissue homogenized in grinding medium minus dithiothreitol. All other conditions described in the text.

	α-Amylase				Protease				Ribonuclease			
	-GA		+GA		-GA		+GA		-GA		+GA	
Cell Fraction	units	%	units	%	units	%	units	%	units	%	units	%
Experiment 1												
1,000 *g*	0.6	13.3	7.3	9.1	0	0	0.4	6.1	0.2	2.6	0.9	3.4
20,000 *g*	1.1	24.5	14.7	18.3	0.3	42.9	2.0	30.3	0.2	2.6	1.7	6.4
105,000 *g*	0.6	13.3	16.3	20.3	0.1	14.2	1.8	27.3	0.1	1.3	2.0	7.5
Supernatant	2.2	48.9	42.0	52.3	0.3	42.9	2.4	36.3	7.2	93.5	22.1	82.7
Total	4.5	100	80.3	100	0.7	100	6.6	100	7.7	100	26.7	100
Experiment 2												
1,000 *g*	0.8	15.7	11.1	11.3	0.3	37.5	0.9	20.5	1.0	10.2	2.9	11.4
20,000 *g*	0.8	15.7	37.0	37.5	0.1	12.5	1.4	31.8	0.2	2.0	2.2	8.6
105,000 *g*	0.4	7.8	14.5	14.7	0.1	12.5	1.4	31.8	0.1	1.0	1.8	7.0
Supernatant	3.1	60.8	36.0	36.5	0.3	37.5	0.7	15.9	8.5	86.8	18.6	73.0
Total	5.1	100	98.6	100	0.8	100	4.4	100	9.8	100	25.5	100
Experiment 3												
1,000 *g*	2.2	21.0	19.3	12.1	0.3	17.6	1.2	17.9	1.4	7.8	2.2	7.3
20,000 *g*	1.4	13.3	67.0	41.8	0.6	35.3	2.7	40.3	0.2	1.1	2.6	8.6
105,000 *g*	0.6	5.7	11.9	7.4	0.1	5.9	0.6	9.0	0.1	0.6	1.5	5.0
Supernatant	6.3	60.0	62.0	38.7	0.7	41.2	2.2	32.8	16.2	90.5	24.0	79.1
Total	10.5	100	160.2	100	1.7	100	6.7	100	17.9	100	30.3	100
Average Values												
1,000 *g*	1.2	17.9	12.6	11.1	0.2	20.0	0.8	13.6	0.9	7.6	2.0	7.2
20,000 *g*	1.1	16.4	39.6	35.0	0.3	30.0	2.0	33.9	0.2	1.7	2.2	8.0
105,000 *g*	0.5	7.5	14.2	12.6	0.1	10.0	1.3	22.0	0.1	0.8	1.8	6.5
Supernatant	3.9	58.2	46.7	41.3	0.4	40.0	1.8	30.5	10.6	89.9	21.6	78.3
Total	6.7	100	113.1	100	1.0	100	5.9	100	11.8	100	27.6	100

and only about 20% of the enzyme was detected in the pellets.

The ratio of total enzyme activity of GA-treated tissue over control tissue is also interesting (Table II.7). GA caused an 18-fold increase in α-amylase, a 6-fold increase in protease and only a 2-fold increase in ribonuclease activity. A final point to be noted is the rather variable distribution of both α-amylase and protease activities in the various pellet fractions. This aspect will be discussed in detail in Section II.6.

It was desirable, to aid purification of the α-amylase containing particles, to develop a rapid method of concentrating the particles into a single centrifugal fraction. An experiment was performed to compare the level of acid hydrolases obtained by centrifuging the post-1,000 *g* supernatant at 60,000 *g* for 30 min, instead of the usual 20,000 *g* for 20 min and 105,000 *g* for 90 min centrifugation steps.

A centrifugal force of 60,000 *g* sedimented almost all of the enzymes that were previously distributed between the 20,000 *g* and 105,000 *g* pellets (Table II.8). As in the preceding experiments, only 20% of the ribonuclease appeared in the pellets and most of this was in the 60,000 *g* pellet. The total α-amylase activity values (160 and 161 units) shown for the two GA treatments, correspond to a 78% recovery of the total α-amylase activity in the tissue before differential centrifugation, as measured by a separate analysis

Table II.8

Effect of increased centrifugal force from 20,000 *g* to 60,000 *g* on distribution of α-amylase from homogenates of aleurone tissue treated with GA for 24 hr at 30°C. All other conditions as described in the text.

	α-Amylase						Protease						Ribonuclease					
	-GA		+GA				-GA		+GA				-GA		+GA			
	units	%	units	%	units	%	units	%	units	%	units	%	units	%	units	%	units	%
1,000 *g*	2.2	21.0	19.3	12.1	19.3	12.0	0.3	18.8	1.2	17.9	1.2	17.4	1.9	10.3	2.2	7.1	2.2	7.4
20,000 *g*	1.4	13.3	67.0	41.8	-		0.6	37.5	2.7	40.3	-		0.2	1.1	2.6	8.4	-	
60,000 *g*	-		-		76.0	47.2	-		-		3.3	47.8	-		-		4.0	13.4
105,000 *g*	0.6	5.7	11.9	7.4	1.8	1.1	0.1	6.2	0.6	9.0	0.2	2.9	0.1	0.5	1.5	5.0	0.2	0.7
Supernatant	6.3	60.0	62.0	38.7	64.0	39.7	0.6	37.5	2.2	32.8	2.2	31.9	16.2	88.1	24.5	79.5	23.5	78.5
Total	10.5	100	160.2	100	161.1	100	1.6	100	6.7	100	6.9	100	18.4	100	30.8	100	29.9	100

(grinding in calcium acetate solution). Thus, the recovery of activity through the diverse operational techniques was good.

5.5 Distribution of α-Amylase and Protein

If an enzyme is particulate then one should observe not only the bulk of the enzyme activity in a single centrifugal fraction, but also an enrichment of the specific activity of the enzyme in that fraction. The assumption being that most of the particulate enzyme, but little of the soluble protein, should be sedimented by centrifugation. Conversely, one should also observe a concomitant decrease in the specific activity of the final supernatant. Therefore, the distribution of both α-amylase and protein in each of the centrifugal fractions was determined in the next experiment.

Two important features of the data cited in Table II.9 must be emphasized. Firstly, although 56% of the α-amylase was found in the 60,000 *g* pellet, this fraction only contained 23% of the total protein. Thus, differential centrifugation resulted in a 2.5-fold enrichment of α-amylase when compared with the specific activity of α-amylase found in the total homogenate. This was accompanied by a decrease in the specific activity of α-amylase in the final supernatant. Secondly, the total number of α-amylase units obtained by adding the activities found in the various

Table II.9

Distribution of α-amylase and protein in cell fractions obtained from homogenates of aleurone tissue treated with GA (10 µg/ml) for 24 hr at 30°C.

	α-Amylase Units	%	Protein mg	%	Specific activity (units/mg protein)
Cell fraction					
1,000 *g*	8.3	11.4	2.0	25.0	4.2
60,000 *g*	41.0	56.0	1.8	22.5	22.8
Supernatant	23.8	32.6	4.2	52.5	5.7
Total	73.1	100	8.0	100	
Original homogenate	76.0		8.2		9.3

centrifugal fractions agrees closely with the total number of α-amylase units found in the original cell homogenate. This is important evidence that the activity found in the pellet fractions represents an equivalent amount of enzyme and is not due to a small amount of enzyme activated by being removed from a cytoplasmic inhibitor.

5.6 Effect of GA Concentration

The level of α-amylase induced in aleurone tissue is dependent on the concentration of GA applied (Figure I.5). It was therefore of interest to determine whether GA had any effect on the distribution of α-amylase in various fractions obtained by differential centrifugation.

The data listed in Table II.10 indicates that although the hormone affected the total amount of α-amylase in the tissue it had little effect on either the distribution of the enzyme or the percentage of α-amylase sedimented. An average of 53% of the total enzyme was found in particulate fractions and 90% of this was located in the 60,000 *g* pellets. The optimum concentration of GA for α-amylase production in the tissue appeared to be 1.0 µg/ml.

5.7 Effect of Calcium

Because of the high levels of calcium used in the incubation medium (20 mM), it was important to see what effect this cation had on

Table II.10

Effect of GA concentration on the distribution of α-amylase in subcellular fractions obtained from aleurone tissue incubated for 24 hr at 30°C.

	α-Amylase							
	0.1		1.0		10		100	
	Units	%	Units	%	Units	%	Units	%
Cell fraction								
1,000 *g*	2.6	3.1	5.3	4.4	5.8	5.0	4.8	8.6
60,000 *g*	40.0	47.5	55.5	46.5	58.8	51.3	23.3	41.6
Supernatant	41.7	49.4	58.8	49.1	50.0	43.7	27.8	49.8
Total	84.3	100	119.6	100	114.6	100	55.9	100

the subcellular distribution of α-amylase in GA-treated tissue.

Calcium (5 mM) caused a dramatic shift in the distribution of α-amylase when it was included in the grinding medium (Table II.11), but had almost no affect on the total amount of enzyme that was sedimented. The enzyme that normally appeared in the 60,000 *g* pellet was located in the 1,000 *g* pellet when calcium was present. Apparently, calcium at the concentration used, was sufficient to cause aggregation of all organelles which resulted in the α-amylase-containing particles sedimenting at lower centrifugal forces than normal. The phenomenon of calcium aggregation of organelles has also been observed in animal tissue (Kamath and Narayan, 1971).

5.8 Effect of Chelating Agents

The calcium-induced change in the distribution of α-amylase containing particles was thought to explain the different percentages of hydrolytic enzymes observed in the various particulate fractions. in earlier experiments. It was reasoned, therefore, that if increased amounts of tissue were homogenised in the same volume of grinding medium, an increased amount of calcium (mainly in the cell walls) would also be introduced. Thus the distribution of α-amylase in the various fractions would be altered. Conversely, if the concentration of compounds capable of chelating calcium was increased, the effect should

Table II.11

Effect of calcium on the subcellular distribution of α-amylase in homogenates of aleurone tissue treated for 24 hr with GA (1 µg/ml) at 30°C. Tissue homogenized in either normal grinding medium or grinding medium containing 5 mM calcium nitrate and without EDTA.

	α-Amylase			
	Normal grinding medium		Grinding medium + calcium	
	Units	%	Units	%
Cell fraction				
1,000 *g*	2.0	1.8	58.9	56.2
60,000 *g*	67.5	61.4	0	0
Supernatant	40.5	36.8	46.1	43.8
Total	110.0	100	105.0	100

be reversed. These possibilities were tested in the next experiment.

The effect of increasing the ratio of tissue to grinding medium was most notable when medium I was used (Table II.12). The increased percentage of α-amylase found in the 1,000 *g* pellet as a result of grinding 3 g of tissue instead of the usual 1 g, parallels the effect of adding calcium to the grinding medium (Table II.11). These data tend to support the view that calcium in the tissue can affect the distribution of the subcellular particles containing acid hydrolases.

This proposal gains further support from the distribution of α-amylase obtained when medium II (containing a higher level of EDTA) and medium III were used (Table II.12). In these two cases, the bulk of the particulate α-amylase was present in the microsomal (70,000 *g*) pellet rather than in the mitochondrial (10,000 *g*) pellet as occurred when medium I was used. Increasing the amount of tissue homogenised had little effect on this distribution.

Although their effects on the distribution of α-amylase in tissue homogenates were similar, EDTA and phosphate probably act in different ways. EDTA is known to be an excellent chelator of divalent cations and probably neutralizes the effect of calcium by absorbing the charge on the ion. Phosphate, on the other hand, reacts with calcium to form insoluble calcium phosphate, thus removing the cation

Table II.12

Effect of aleurone tissue concentration and chelating agents on the subcellular distribution of α-amylase. Homogenates prepared from aleurone tissue treated with GA (1 μg/ml) for 24 hr at 30°C. Composition of grinding media: (I), 0.4 M sucrose, 0.05 M Tris-HCl (pH 7.0), 0.1% BSA, 0.01 M KCl, 1 mM EDTA and 0.1 mM $MgCl_2$; (II), same as I with EDTA at 10 mM; (III), same as I but with 0.05 M KH_2PO_4 (pH 7.0) substituting for Tris-HCl. α-Amylase: SIC units/g fresh weight.

	% α-Amylase*					
	Medium I (+ 1 mM EDTA)		Medium II (+ 10 mM EDTA)		Medium III (+ KH_2PO_4)	
Weight of tissue	1 g	3 g	1 g	3 g	1 g	3 g
Cell fraction						
1,000 *g*	16.7	68.0	5.2	10.8	15.8	11.0
10,000 *g*	29.2	3.9	18.0	21.1	18.8	19.0
20,000 *g*	0	0	10.5	12.0	4.9	14.6
70,000 *g*	17.1	0	36.3	29.7	38.4	39.8
Supernatant	37.0	28.1	30.0	26.4	22.2	15.8
Total	100	100	100	100	100	100
Total units	(64.8)	(237.9)	(70.2)	(201.5)	(83.1)	(211.2)

* Tissue treated with 0.1 N-HCl before grinding (Section 5.9).

from the grinding medium.

5.9 Effect of Acid Treatment

If an enzyme is to be considered truly particulate rather than existing free in the cytoplasm of the cell, provided that homogenisation techniques are sufficiently gentle to retain the integrity of a majority of the organelles, then most of the enzyme should be found in the pellet fractions after differential centrifugation rather than in the final supernatant. In most of the experiments cited thus far, only about 50% of the total α-amylase found in tissue homogenates was located in the pellet fractions. The work of Varner and Mense (1972) indicated that a significant proportion of the α-amylase secreted from GA-treated aleurone cells is retained for a period in the cell walls. They further indicated that this enzyme could be inactivated by soaking the tissue in dilute acid without affecting the cells themselves. As any enzyme associated with cell walls would be expected to be released into the grinding medium during homogenisation, it was decided to investigate the possibility of increasing the percentage of particulate α-amylase by acid treatment of the tissue prior to homogenization. In the next experiment, one portion of GA-treated aleurone tissue was washed with water in the usual way while another portion of tissue was washed in dilute acid. The acid washing entailed soaking the tissue in 0.1 N HCl (10 ml) for 15 min, washing with 50 ml of 0.05 M Tris-HCl (pH 7.0) for 2 min to neutralize the acid, and finally rinsing the

tissue with excess distilled water. Both samples of tissue were homogenized with 10 ml of the grinding medium normally used (medium I, Table II.12).

The data in Table II.13 clearly demonstrate that much of the α-amylase usually found in the final supernatant fraction was derived from outside the cell, presumably trapped in the cell walls. This is indicated by the fact that the amount of α-amylase found in the 60,000 *g* pellet was unaffected by acid washing while the same treatment caused a reduction in the amount of the enzyme detected in the final supernatant. It was therefore concluded, that the majority of α-amylase in GA-treated aleurone cells (> 80%) occurs within organelles.

5.10 Latency of Particulate α-Amylase

Early experiments indicated that particulate α-amylase was structurally latent, disruptive treatments being required to release the enzyme so that it could act on the substrate. The data listed in Table II.14 show the percentage of α-amylase released from 60,000 *g* pellet preparations in response to various disruptive treatments.

At 5°C only 20% of the α-amylase leaked into the supernatant during the 30 min incubation period. The value was increased by the physical stresses imposed by ten-fold dilution, grinding in an Ultra

Table II.13

Effect of washing GA-treated aleurone with dilute acid before homogenization. Conditions described in the text.

	α-Amylase			
	Water-washed tissue		Acid-washed tissue	
	Units	%	Units	%
Cell fraction				
1,000 *g*	10.5	9.0	2.6	4.3
60,000 *g*	47.6	40.8	47.6	78.2
Supernatant	58.8	50.2	10.7	17.5
Total	116.9	100	60.9	100

Table II.14

Effect of various treatments on the latency of α-amylase in 60,000 *g* pellet preparations. Pellets were resuspended in 1 ml of the following medium: 0.4 M sucrose, 0.1 M Tris-HCl (pH 7.0), 5 mM calcium nitrate and 0.1% bovine serum albumin. The resuspended organelles were incubated for 30 min at the temperature indicated and recentrifuged at 60,000 *g* for 30 min. Original 60,000 *g* pellet obtained from aleurone tissue treated with GA (10 µg/ml) for 24 hr at 30°C.

Treatment	α-Amylase (% of total)	
	Supernatant (free)	Pellet (latent)
5°C		
Control	20	80
Dilution (x10)	42	58
Grinding	82	18
Freezing (four times)	55	45
Triton X-100	91	9
Sodium deoxycholate	98	2
30°C		
Control	63	37
Phospholipase A	79	21
Phospholipase C	61	39
Phospholipase D	61	39

Turrax apparatus at maximum speed for 1 min or freezing and thawing four times. Treatment with the surface-active agents Triton X-100 and sodium deoxycholate at 5°C resulted in almost complete loss of α-amylase from the pellet. At 30°C, leakage of α-amylase into the supernatant was increased to 60% indicating a temperature-dependent autolysis of the α-amylase-containing particles. Phospholipase A at 30°C slightly enhanced this leakage but phospholipase C or D appeared to be without effect.

Lysosomal enzymes from animal origin usually exhibit three important characteristics: 1) they usually have acid pH optima; 2) they are particulate and can be sedimented by centrifugation; 3) the particulate enzymes exhibit structural latency. It should be noted that the techniques used in the experiment indicated in Table II.14 were perhaps more rigorous than those usually employed with lysosomes of animal origin. In experiments with animal lysosomes, enzyme latency is usually determined as the difference in activity with and without the addition of a surface-active agent (e.g. deoxycholate, Triton X-100, etc.) to the incubation medium during enzyme assay. In the experiment just described, latency was estimated by the amount of enzyme that was sedimented during a second centrifugation at 60,000 *g*. When α-amylase-containing particles comparable with those used in the experiment reported in Table II.14 were tested for enzyme latency by the technique established for animal lysosomes,

completely comparable results were obtained (Table II.15).

Because the data in Tables II.14 and II.15 so clearly demonstrated that particulate α-amylase exhibited structural latency, the three criteria previously listed for lysosomal enzymes appeared to be satisfied. It was therefore concluded that the α-amylase-containing particles could justifiably be referred to as lysosomes. This will be done for the remainder of this thesis.

6. Discussion

During germination, the cells of the cereal aleurone layer produce and secrete almost all of the hydrolytic enzymes involved in the complete degradation of the starchy endosperm, a process requiring up to 6-7 days under optimal conditions. Despite the fact that the aleurone cells continue to synthesize and secrete these potent hydrolases until the endosperm has been entirely digested, the aleurone cells themselves retain their viability and integrity for the entire period. The question therefore arises, how do the aleurone cells escape the action of the acid hydrolases which are so effective at degrading endosperm cells and their contents?

A number of explanations can be put forward to explain this apparent paradox.

(a) The enzymes synthesized and secreted by aleurone cells may have a high degree of specificity and may not attack substrates

Table II.15

Latency of α-amylase in 60,000 *g* pellet preparations. Comparison of two methods of determining structural latency.

Incubation conditions	Method of determining latency	Fraction	α-Amylase (% of total)
5°C, 30 min	Centrifugation	Supernatant (free)	20
		Pellet (latent)	80
30°C, 30 min	Centrifugation	Supernatant (free)	59
		Pellet (latent)	41
30°C, 30 min	Triton X-100	Free	63
		Latent	37

in aleurone cells. This possibility is unlikely as hydrolases such as proteases and ribonucleases attack specific bond linkages (i.e. peptide bonds in proteins) and these would have to be absent in aleurone cell macromolecules.

(b) The hydrolases present in aleurone cells may be prevented from attacking substrates in these cells by the presence of cytoplasmic inhibitors which are not secreted by aleurone cells, thus allowing the hydrolases to function on endosperm cells once they have been secreted.

(c) The enzymes, which are destined for secretion by aleurone cells, may be made in such a way that they require a portion of their structure to be removed before they become active, and this can only occur outside the aleurone cell. Such a relationship is known in animal cells with the release of the potent protease trypsin from the inactive trypsinogen (Northrop *et al.*, 1948). Again this is an unlikely mechanism to protect aleurone cells as it would entail all hydrolases secreted behaving in this way.

(d) A mechanism by which the GA-induced acid hydrolases are made at the plasmalemma and the growing polypeptide chain fed directly through the membrane is also a possibility. By this mechanism the active hydrolases would never be in contact with the contents of the aleurone cell. Such a mechanism has been suggested

for the α-amylase-secreting micro-organism *B. amyloliquefaciens* which contains no detectable α-amylase in its cytoplasm (Grant and Coleman, 1972).

(e) The GA-induced hydrolases may be synthesized and secreted from aleurone cells via discrete membrane-bound particles, thus limiting access of the enzymes to their substrates within the aleurone cell. Such a mechanism is widely found in animal tissues (Wattiaux and de Duve, 1956), where acid hydrolases are contained in lysosomes.

To help distinguish between these possible mechanisms it was essential to know where in the aleurone cell the acid hydrolases were located. Thus, if the GA-induced enzymes were found to be free in the cytoplasm, mechanisms (a), (b) and (c) would be likely. If little or none of the acid hydrolases were found in aleurone cells then mechanism (d) would be a distinct possibility. Finally, if a significant proportion of the acid hydrolases were found in organelles within the aleurone cell then it would be probable that aleurone cells secreted GA-induced hydrolases by means of mechanism (e).

Cereal aleurone cells are surrounded by massive cell walls and comparatively high shear forces are required to rupture the cells and liberate the cell contents into the grinding medium. Ideally, a method is required that ruptures all the cells without, in the

process, rupturing all the enclosed organelles. The idea of using enzymes capable of digesting cell walls prior to homogenization (thus reducing the shear forces required) was rejected because of the long period of incubation required (4-8 hr) and the possibility that these enzyme preparations could contaminate or damage the cell homogenates. Therefore, only mechanical methods of rupturing the cells were tested (Table II.2). Both grinding in a mortar and pestle and controlled grinding in an Ultra Turrax were found to give good recoveries of particulate α-amylase from GA-treated aleurone tissue. Although the Ultra Turrax was easier to use, the mortar and pestle method was retained for two reasons: 1) it is a grinding device available to workers in all laboratories, and 2) the Ultra Turrax was not always available.

The isolation of all organelles is facilitated by the correct choice of grinding medium. Basically there are two distinct types of media available, aqueous and non-aqueous. Non-aqueous media have proved suitable for the isolation of some organelles where the distribution of water-soluble, small molecular weight molecules is being examined (Stocking, 1971). Unfortunately many of the solvents used are toxic and others readily dissolve lipid components of organelles (Mathius, 1966).

Aqueous media are often preferred by many workers on the basis that living cells are highly hydrated; this may or may not be an adequate reason. Ideally, the grinding medium chosen should closely approximate the composition of the cytoplasm of the cells being homogenized, to minimize damage to the organelles. While no thorough study of the essential requirements of grinding media for the isolation of α-amylase containing particles was carried out, a large number of different recipes were tested (Tables II.1, II.4 and II.12). The yields of α-amylase found in the various particulate fractions varied from 15-50% depending on the type of medium used. The grinding medium giving both the highest percentage of particulate α-amylase and the highest recovery of total enzyme activity was the medium designed for the isolation of glyoxysomes by Breidenbach and Beevers (1967). The medium minus dithiothreitol (Tables II.5 and II.6) was used throughout this study.

Other workers have also shown that the composition of the grinding medium can critically affect the percentage of an enzyme that is found in the particulate fraction. For example, Miflin (1970) found that the percentage of particulate nitrate reductase detected in homogenates of barley roots was affected by the concentration of sucrose used in his grinding medium. Furthermore, Parish (1971) reported that while a sucrose-based grinding medium

yielded only 30% of the total catalase activity in particulate fractions, a sorbitol-ficoll mixture increased the percentage of particulate catalase to over 70%.

While a certain amount of reassurance may be found if different methods of isolation yield essentially similar results (Tables II.1 and II.2), even these results must be interpreted with caution. The phospholipid portion of cell membranes contain highly charged head-groups and thus most organelles can act as ion-exchange particles and bind, in a non-specific manner, suitably charged protein molecules. This occurs particularly when organelles and soluble proteins are exposed to an environment vastly different from the carefully regulated intra-cellular fluid, such as occurs during homogenization (Mathius, 1966).

Several of the experiments cited in this section were designed to measure the degree of non-specific binding of α-amylase to organelle fractions. The addition of tissue α-amylase to the medium used for grinding both control and GA-treated aleurone tissue, failed to increase the level of α-amylase in the particulate fractions (Table II.3 and II.4). Furthermore, the addition of a soluble substrate (amylose) to the grinding medium did not cause more α-amylase to appear in the final supernatant fraction (Table II.4). It was therefore concluded that the α-amylase detected in the pellet fractions after differential centrifugation of aleurone cell homogenates represented enzyme

normally associated with organelles in the cell.

Detailed analysis of the pellets obtained by differential centrifugation of homogenates of aleurone layers indicated that the 20,000 *g* and 105,000 *g* pellets were particularly rich in α-amylase and protease activity (Table II.7). Enzyme activity distributed between these fractions could be concentrated in a single fraction if a centrifugal force of 60,000 *g* was imposed (Table II.8). The percentage yield obtained in these fractions (50-60%) is considered significant in view of the high shear forces necessary to break aleurone cell walls. These values can be significantly increased (to 80%) if all secreted enzyme located in the cell walls is inactivated by treatment with dilute acid prior to homogenization (Table II.13). Since all of the enzyme activity was originally derived from within GA-treated aleurone tissue, it was concluded that the GA-induced enzymes α-amylase and protease are particulate in nature in the cells of the aleurone layer.

The nature of ribonuclease is less clear, as most of this enzyme is found in the supernatant fraction. The amount of ribonuclease present in control tissue is large and the enzyme activity undergoes the smallest percentage increase in the incubation period (24 hr) employed. In this time, the percentage of enzyme in either the 20,000 *g* and 105,000 *g* fractions or the 60,000 *g* fraction showed the greatest increase (Tables II.7 and II.8). If, as

suggested by Chrispeels and Varner (1967a), ribonuclease secretion is initiated later than α-amylase and protease, it seems possible that the proportion of sedimentable ribonuclease would increase as the incubation time was extended. Indeed, other workers (Jones and Price, 1970) have suggested that after GA induction, this enzyme in barley aleurone tissue may be sedimentable.

The effects of divalent cations on membranes are diverse. While low concentrations of calcium and magnesium ions appear to be necessary for the stability of certain membranes (Mathius, 1966), high concentrations of these ions (> 0.1 mM) appear to result in the organelles of a tissue homogenate aggregating, so that severe contamination of particulate fractions occur. This effect is well documented by workers with animal tissue (Dallner and Nilson, 1966) and in fact has been made use of in developing a method of isolating ER (microsomes). The method involves treating the post-mitochondrial (20,000 *g*) supernatant with 8 mM calcium chloride and centrifuging at 1,000 *g* for 10 min instead of the usual 100,000*g* for 90 min (Kamath and Narayan, 1972; Kupfer and Levin, 1972). Aggregation of organelles by calcium was also observed in the present study (Table II.11).

Because of the problems of aggregation encountered, almost certainly caused by the presence of excess calcium ions, it is most probable that the acid hydrolase-containing particles are of a size

and density that would normally locate them in the microsomal fraction (105,000 *g*). This suggestion is borne out in part by the fact that increasing the concentration of chelating agents in the grinding medium resulted in a majority of the particulate α-amylase being detected in the microsomal fraction (Table II.12).

The organelle aggregation caused by calcium probably explains the results of Koehler and Varner (1973) who unsuccessfully attempted to measure ^{32}P incorporation into the ER of barley aleurone layers. These workers found a majority of the ^{32}P counts in the 4,000 *g* pellet rather than in the microsomal pellet and concluded that serious cross contamination of the fractions had occurred during isolation. Since Koehler and Varner did not include chelating agents in their grinding medium, calcium from the cell walls (Table II.12) almost certainly resulted in sedimentation of their organelles at centrifugal forces lower than would be expected.

Assessment of whether an enzyme is particulate or not can be complicated if cytoplasmic inhibitors are present, which can seriously affect the activity of the enzyme being assayed. For example, Tolbert *et al.* (1969) found that the activity of cytochrome c oxidase in each of the centrifugal fractions totalled more than was present in the original homogenate of sugar cane and tobacco plants. This type of activation of particulate (mitochondrial) enzymes was found to be due to a cytoplasmic inhibitor. Clearly, no such situation occurs with

particulate α-amylase in wheat aleurone tissue (Table II.9) where the sum of the various fractions equalled the amount found in the original homogenate. Furthermore, a gain in the specific activity of particulate α-amylase was due to a preferential sedimentation of α-amylase rather than other proteins from the homogenate.

The degree to which an enzyme is membrane-bound can vary with both the physiological state of the cell and the species from which the tissue is obtained. For example, the percentage of glyoxysomal marker enzymes measured in microbody fractions has been found to vary greatly with the age of the tissue (Schnarrenberger *et al.*, 1971; Gerhardt and Beevers, 1970) and with the plant species examined (Huang and Beevers, 1971). The association of acid hydrolases with lysosomes varies widely in animal tissue, particularly in response to age, disease and hormones (Dingle and Fell, 1969). However, for at least one physiological variable, GA concentration, the percentage of particulate α-amylase in wheat aleurone tissue does not vary (Table II.10).

Particulate α-amylase exhibits pronounced structural latency regardless of the method of determination (Tables II.14 and II.15). Mild treatments such as osmotic shock, and freezing and thawing failed to liberate all the enzyme and even grinding in the Ultra Turrax was not completely successful in this regard. Only treatment with surface-active agents which dissolved the membrane, such as

deoxycholate and Triton X-100, liberated all of the membrane associated α-amylase.

Numerous attempts have been made to equate various plant organelles, ranging from aleurone grains to vacuoles, with animal lysosomes (Matile, 1969). Although most of these studies have reported various amounts of hydrolytic enzymes in particulate fractions, none have demonstrated the most important criterion of lysosomal enzyme, structural latency (Beaufay and de Duve, 1959). Recently however, Hirai and Asahi (1973) have reported that particulate α-amylase in peas is tenaciously bound to membrane fractions and is probably lysosomal.

Jones (1972) failed to detect particulate α-amylase in homogenates of barley aleurone cells despite gentle homogenization and the use of an identical grinding medium (Breidenbach and Beevers, 1967). However, as many of the precautions that have been found necessary to maximise the yields of α-amylase (acid washing, disruption of pellets, etc.) were not taken, the result is not surprising. In this regard it is interesting to note that Firn (1973) has reported that by following the methods of Gibson and Paleg (1972) and employing both gel filtration as well as differential centrifugation, 30% of the total α-amylase in barley aleurone homogenates was located in particulate fractions.

A number of possibilities were cited at the beginning of this discussion that could explain how aleurone cells escape the autolytic action of the enzymes they secrete. They included (a) enzyme specificity, (b) cytoplasmic inhibitors, (c) structural inhibition, (d) synthesis on the plasmalemma, and (e) membrane-enclosed enzymes. Although only possibility (e) was seriously investigated, several points can be noted about the other suggestions. Because α-amylase and protease have been definitely established as being particulate, the other mechanisms (a, b and c) would hardly be required; however, the possibility of the existence of a cytoplasmic inhibitor (b) for α-amylase at least, has been refuted. Furthermore, since such a large proportion of the non-secreted tissue α-amylase was lysosomal, synthesis of hydrolytic enzymes at the plasmalemma (d) must be considered an unlikely mechanism.

SECTION III

ISOPYCNIC DENSITY GRADIENT SEPARATION OF ALEURONE CELL LYSOSOMES

This Section is concerned with the characterization of aleurone cell lysosomes. Using isopycnic density gradient centrifugation, lysosomes were concentrated in a single region of the gradient (ρ = 1.080 g/cc) well separated from most other aleurone cell organelles. Lysosomes were found to be rich in α-amylase and protease but not ribonuclease. The lysosomal band also contained a majority of the $NADH_2$-cytochrome c reductase, a marker enzyme for ER, found in the gradient. Repeated attempts failed to separate α-amylase from cytochrome c reductase and it was concluded that wheat aleurone lysosomes were ER derived. Examination of electron micrographs revealed that a purified band of lysosomes contained at least three vesicle types ranging in size from 0.1 - 0.5 μ.

1. Tissue

All aleurone tissue was prepared by the lactic acid method outlined in Section I. The tissue was pre-incubated for 12 hr before the addition of GA (1 μg/ml) and allowed to incubate for a further 24 hr. Both pre-incubation and incubation of the tissue (1 g fresh

weight) was carried out in 125 ml erlenmeyer flasks in a shaking water bath at 30°C, set to oscillate at 50 o.p.m.

2. Preparation of Aleurone Cell Organelles

At the end of the incubation period the incubation medium was decanted and set aside for later assay. The tissue was washed with 2 x 100 ml of distilled water, 10 ml of 10 mM EDTA solution for 5 min, and finally rinsed with 2 x 100 ml of distilled water. The tissue was then transferred to a cold room (2°C) where it was allowed to chill for 15 min before homogenization.

The chilled aleurone tissue (1 g) was homogenized in 10 ml of the medium described in Section II using either a mortar and pestle or the Ultra Turrax. The cell homogenate was filtered through 2 double layers of cheesecloth, and the filtrate centrifuged at 1,000 *g* for 10 min. The resulting supernatant was then recentrifuged at 60,000 *g* for 30 min. The resulting supernatant was decanted and set aside, and the pellet resuspended in 1 ml of a resuspension medium using a close fitting plastic piston. The piston device (1.5 cm long) was connected to a glass rod (10 cm) and was constructed to fit the round base of the centrifuge tube and to provide the parallel sides (1.0 cm long) with a 0.1 mm clearance. The composition of the resuspension medium was 0.4 M sucrose, 1 mM EDTA and 0.01 M Tris-HCl, pH 7.0. The resuspended pellets from 4 g of tissue were mixed and

then layered on the top of the density gradient.

3. Density Gradients

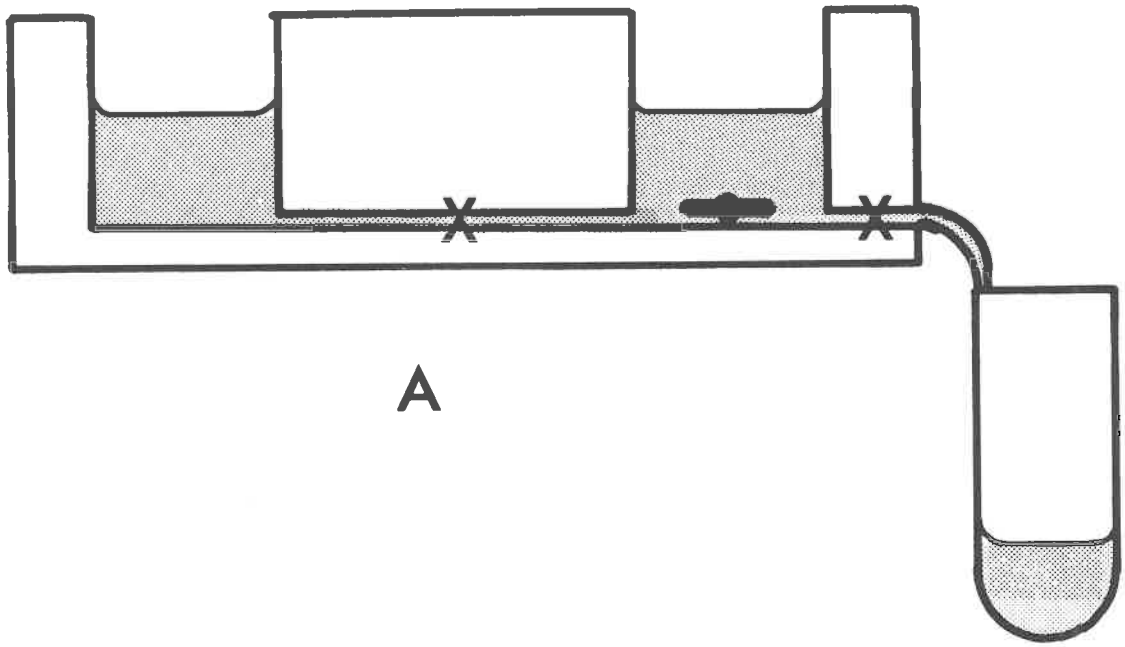
Two different devices were employed to prepare density gradients. The gravity flow device (Figure III.1A) proved to give erratic gradients, but with extreme care linear gradients were obtained provided that the flow rates were kept to a minimum and sufficient time (12 hr) was allowed for equilibration after pouring. When solutions of high viscosity (ficoll) were used, it was found to be necessary to use a peristaltic pump device (Figure III.1B).

a) Gravity Flow Device. The left chamber contained the dilute solution of sucrose while the right chamber contained an equal volume of concentrated sucrose solution. If a gradient of 20% to 60% sucrose was required, for example, the left chamber contained the 20% sucrose while 60% sucrose was placed in the right hand chamber.

To pour the gradient, both valves were opened, the stirring bar in the right chamber set in motion and the mixed sucrose solution allowed to run down the side of the centrifuge tube. Best results were obtained when the flow rate, controlled by the right valve, was kept to a minimum (1 ml/min). The speed of rotation of the stirring bar was also critical; too slow and insufficient mixing occurred,

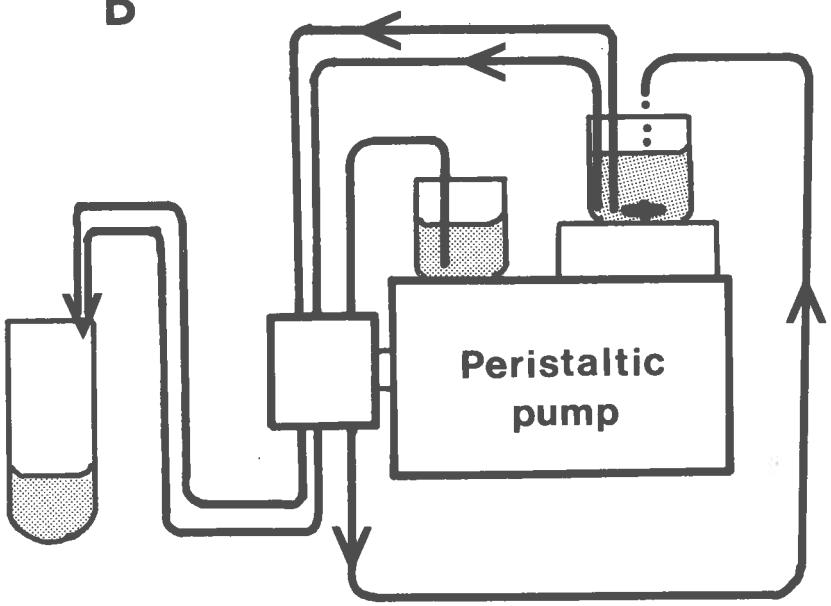
Figure III.1

Sucrose density gradients were prepared using the gravity flow device (A) while ficoll density gradients were made with the peristaltic pump device (B).



A

B



too fast and dilute sucrose solution was forced into the right chamber faster than mixed sucrose was running out.

b) Peristaltic Pump Device. To pour gradients, the tubing connected to the dilute solution on the left was filled with this solution by activating the peristaltic pump. When the first drop of dilute solution reached the right hand container holding the concentrated solution, the two remaining tubes were placed in the concentrated solution and the stirring motor activated. The mixed solution from the right hand container then passed through the two lengths of tubing to run down the side of the centrifuge tube.

Provided that all three lengths of tubing were identical, extremely linear gradients were obtained with this device. As the solutions were pumped rather than flowing under gravity, viscosity did not present a problem. Hence, this device was used for pouring all ficoll gradients.

All gradients reported in this thesis were 30 ml in volume including the load placed on the top of the gradient. Gradients were always poured into cellulose nitrate tubes made for use in the Beckman SW25.1 rotor. Exact composition of gradients and length of time in the centrifuge vary and are explained in the text.

At the end of the centrifugation period, fractions (1 ml) were collected by means of an ISCO density gradient fraction collector

set at a flow rate of 2.5 ml/min. Density gradient profiles were obtained by simultaneously recording the absorbancy of material in the gradient at 280 nm on an ISCO density recorder set at 2.5 units full scale absorbancy. The collected fractions (1 ml) were immediately set in ice and enzyme activity was measured in each fraction as soon as possible.

4. Enzyme Assays

α-Amylase (EC 3.2.1.1). When this was the only enzyme to be assayed, each fraction from the gradient was collected in tubes containing 4 ml of 10 mM calcium acetate solution. When other enzymes were also assayed a 0.2 ml sample from each of the 1 ml fractions from the gradient was taken and placed in separate tubes containing 0.8 ml of 10 mM calcium acetate solution. This dilution of the gradient fractions was generally necessary for measurement of α-amylase activity and the excess calcium provided protection to the enzyme when EDTA or phosphate was present. The diluted α-amylase samples were then made 0.1% with respect to Triton X-100 and heated for 10 min at 70°C before measurement of enzyme activity by the method detailed in Section I.

Acid Ribonuclease (EC 2.7.7.16). Samples (0.2 ml) were taken from each of the density gradient fractions and diluted with

0.8 ml of 0.1 M acetate-tris buffer (pH 5.0) and the diluted enzyme fractions made 0.1% with Triton X-100. The samples were then assayed for ribonuclease activity by the method detailed in Section II.

Acid Protease (EC 3.4.4). Samples (0.5 ml) were taken from each of the density gradient fractions, diluted with 0.5 ml of 0.1 M acetate-tris buffer, pH 4.8, and the diluted samples made 0.1% with Triton X-100. The samples were then assayed for protease activity by the method described in Section II.

Cytochrome c Oxidase (EC 1.9.3.1). This enzyme was assayed according to the method of Simon (1958). From 25-50 µl of enzyme taken directly from the gradient fraction was pipetted into a bottom corner of a 1.5 ml spectrophotometer cuvette (5 mm light path) and 5 µl of 2% digitonin was added. After 30 sec, 1.2 ml of a solution containing 0.2 M sucrose, 0.05 M KH_2PO_4 (pH 7.1) and cytochrome c (20 µM) was added to the cuvette and mixed. The cytochrome c had previously been reduced with dithionite and oxygenated to remove the excess reagent. The disappearance of reduced cytochrome c was recorded on a Unicam SP1800 continuously recording spectrophotometer at 550 nm against a reference cuvette containing reduced cytochrome c. Enzyme activity is expressed in ΔE_{550} nm/min at 25°C.

Catalase (EC 1.11.1.6). Assay was at 25°C by the method

of Maehly and Chance (1954), by measuring spectrophotometrically at 240 nm the disappearance of H_2O_2. The complete reaction mixture contained, in a final volume of 3 ml, 0.01 M KH_2PO_4 (pH 7.5), freshly prepared 9.5 x 10^{-4} M H_2O_2 and 25 to 50 µl of enzyme taken directly from the density gradient fractions. A unit of activity is defined as a change of 1 O.D. in 1 min and based on the extinction coefficient, was equal to 2.76 µmoles of H_2O_2.

Isocitratase (EC 4.1.3.1). This activity was determined by the method of Dixon and Kornberg (1959). The reaction mixture contained, in a volume of 1.2 ml, 0.05 M KH_2PO_4 (pH 6.9), 5 mM magnesium chloride, 13 mM isocitrate, 4 mM dithiothreitol, freshly prepared 20 mM phenylhydrazine and 25-50 µl of enzyme. The assay was initiated by the addition of isocitrate in the cuvette to be measured but was not included in the reference cuvette. Enzyme activity is expressed in ΔE_{324} nm/min.

$NADH_2$ Cytochrome c Reductase (EC 1.6.2.1). The reduction of cytochrome c was followed in a Unicam SP1800 recording spectrophotometer at 550 nm and 25°C. The final reaction volume was 1.2 ml and contained 25 µM $NADH_2$, 25 µM cytochrome c, 0.167 mM KCN, 50 mM KH_2PO_4 (pH 7.6) and 25-50 µl of enzyme taken directly from the gradient fractions. The reaction was initiated by adding the required

amount of $NADH_2$. This nucleotide was not included in the reference cuvette. Enzyme activity is expressed in ΔE_{550} nm/min (Wray and Filner, 1970).

Presentation of Data. Unless otherwise stated, all enzyme data are presented in the figures as arbitrary units, where one unit equals 1% of the total enzyme activity found in that particular density gradient.

5. Incorporation of ^{14}C-Lysine

Two 1 g samples of aleurone tissue that had been incubated for 23 hr with or without GA, were incubated for a further 2 hr with 1 μCi of DL-lysine-1-^{14}C monohydrochloride (specific activity 314 μCi/mg, The Radiochemical Centre Ltd., Amersham, England). The tissue was washed in copious amounts of distilled water and homogenates were prepared by the method described (Section III.2). The organelles were separated on a sucrose-ficoll gradient and thirty 1 ml fractions were obtained using an ISCO gradient fraction collector. From each fraction, 0.1 ml samples were taken and placed in glass vials containing 10 ml Bray's solution (Bray, 1960). The vials were placed in a Packard model "Tri-Carb" scintillation counter for the determination of radioactive counts.

6. Electron Microscopy

The 60,000 *g* pellets obtained from homogenates of 8 g of GA-treated aleurone tissue were resuspended in a total of 4 ml of resuspension medium and an equal volume of this organelle mixture was layered on to two sucrose-ficoll gradients (20% sucrose - 20% sucrose + 30% ficoll). The gradients were centrifuged and subsequently fractionated as described earlier. Fractions from each gradient that corresponded to visible bands were combined and fixed in 2% (v/v) glutaraldehyde in phosphate buffer (pH 7.5) for 8 hr. The samples were then centrifuged at 60,000 *g* for 30 min and the resulting pellet washed with an isotonic solution prepared in phosphate buffer (pH 7.5). The samples were centrifuged for a second time and the resulting supernatant discarded.

The pellets were resuspended in one drop of a 2% (w/v) agar solution at 45°C. The cooled agar-organelle mixture was easier to handle than loose pellets. The organelles were post-fixed in 2% (w/v) osmium tetroxide in 0.1 M phosphate buffer, pH 7.0, for 2 hr. The pellet fractions were then dehydrated in an alcohol series and embedded in Araldite. Ultrathin sections were then cut on a Si-Ro-Flex ultramicrotome, mounted on copper grids and examined, after counterstaining in lead citrate, in a Siemans electron microscope.

7. Density Gradient Separation of Organelles from Homogenates of Wheat Aleurone Cells

The principle of density gradient centrifugation is that a mixture of particles is applied as a thin layer to the top of a liquid column of graded density which is then centrifuged. The particles move into the column at a speed proportional to their sedimentation rate and eventually form discrete zones, which can be recovered. Sedimentation can be continued until each group of particles has reached a position in the gradient where the density of the surrounding fluid equals the buoyant density of the particles. Their movement then becomes infinitely slow and isopycnic separation has been achieved.

Ideally, if the composition of the gradient is within the required range of densities and centrifugation is carried out until isopycnic separation is achieved, bands will appear in the gradient which correspond to different organelles. Furthermore, if the marker enzyme is located in one of these organelles then a majority of the enzyme activity should correspond to one of the bands. Therefore, the aim of the project was to obtain a band on a density gradient that was pure biochemically (free of enzymes known to be located in organelles not associated with secretion, e.g. mitochondria) and pure morphologically (only one type of organelle visible when examined in the electron microscope).

7.1 Enzyme Analysis of Density Gradients

The composition of a density gradient is as important as the composition of the grinding medium if good separation of organelles is to be achieved. As yet, there exists no gradient recipe that is suitable for the separation of all organelles.

Preliminary gradients were prepared that not only would provide a suitable range of densities to separate most known organelles, but would also prevent as much loss of the marker enzyme (α-amylase) activity as possible. The 60,000 *g* pellets obtained from 4 g of GA-treated aleurone tissue were resuspended, as described, in 5 ml of a medium containing 0.4 M sucrose, 0.01 M Tris-HCl (pH 7.0), 0.1% BSA and 5 mM calcium nitrate (included to protect α-amylase). The resulting organelle mixture was carefully layered on the top of a 25 ml column of sucrose grading from 30 to 60% sucrose. In addition, the gradient contained 0.01 M Tris-HCl (pH 7.0), 0.1% BSA and 5 mM calcium nitrate. The gradient was centrifuged for 4 hr at 23,000 rpm in a Beckman SW25.1 rotor at 5°C. At the end of the centrifugation period, 1 ml fractions were collected as already described, and the α-amylase activity determined in each fraction.

Although many gradients were run on many different occasions the results were always similar to those shown in Figure III.2. Apart from a band at the 0.4 M sucrose : 30% sucrose interface and

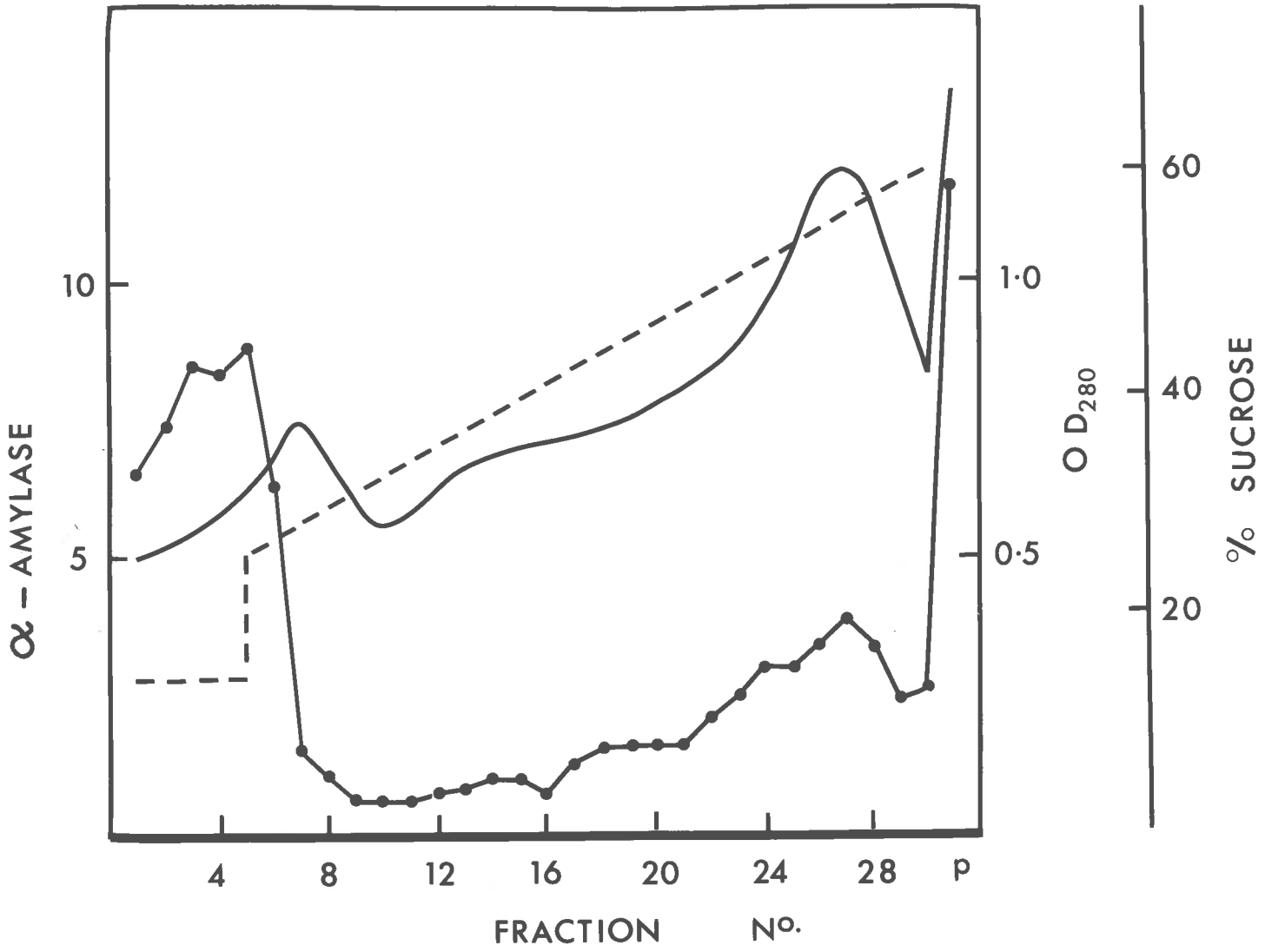
Figure III.2

Distribution of α-amylase on a 30-60% sucrose density gradient containing 5 mM calcium nitrate. The gradient solutions also contained 0.01 M Tris-HCl (pH 7.0) and 0.1% BSA. The gradient was centrifuged for 4 hr at 23,000 rpm in a Beckman SW25.1 rotor. α-Amylase activity is expressed in arbitrary units where 1 unit equals 1% of the total enzyme detected on the gradient. Tissue: GA (10 µg/ml) treated aleurone.

α-Amylase

O.D. 280 nm

% Sucrose


α – AMYLASE
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% SUCROSE
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12
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FRACTION Nº.

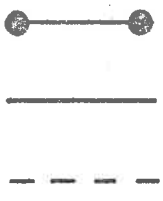
a slightly larger band at the base of the gradient, most of the particles were smeared along the length of the gradient or were located in the pellet at the base of the tube. The α-amylase activity showed a similar trend; there was a peak of activity near the top of the gradient but it did not appear to correspond to any particular band. The remainder of the enzyme activity seemed to be associated with membrane wherever it was located.

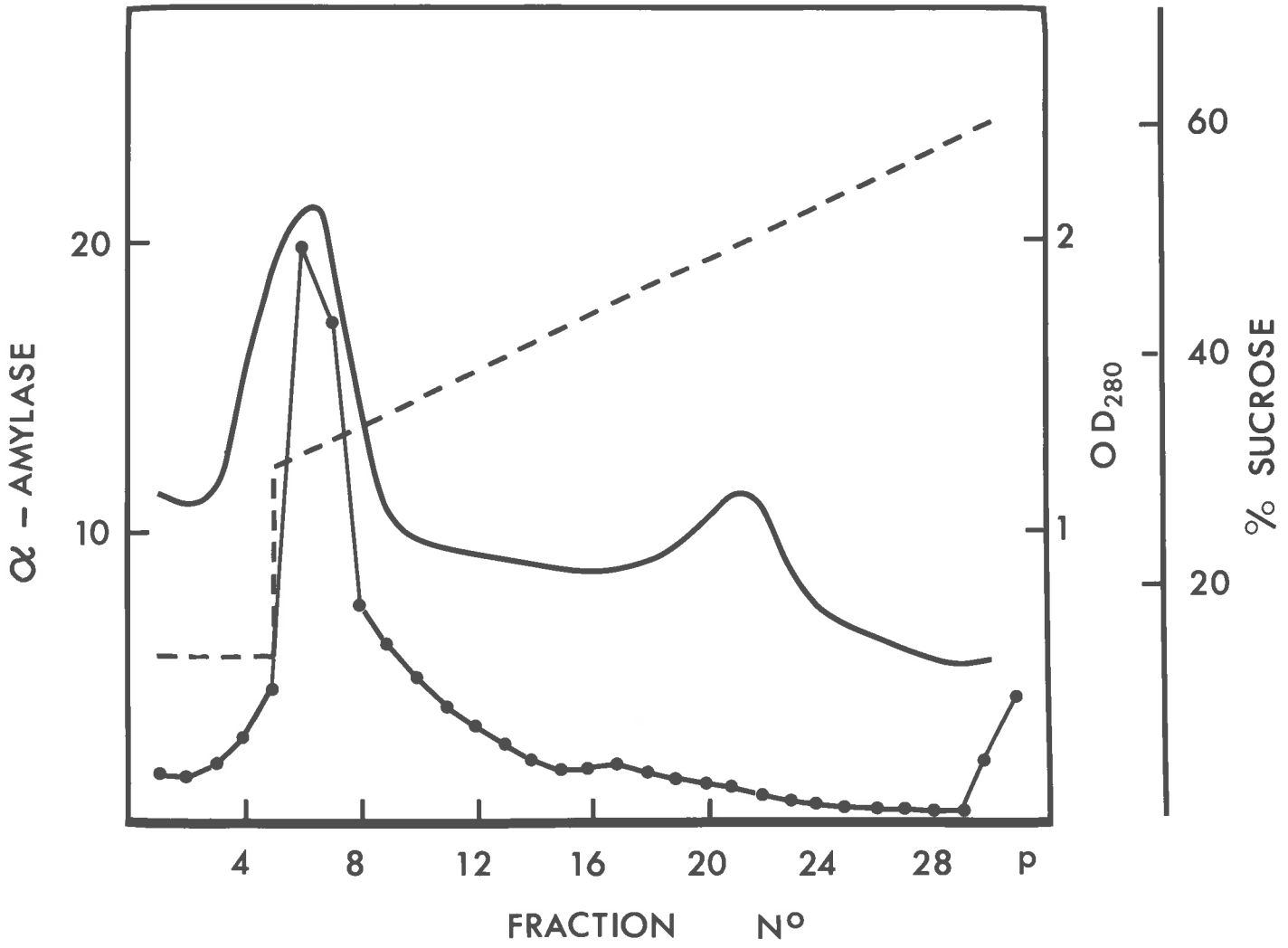
It was not until an experiment was carried out to determine the effect of calcium in the grinding medium (Section II) that it was appreciated that this cation was causing aggregation of organelles and could not be included in the gradients. As a result, 60,000 *g* pellets obtained from the homogenates of 4 g of GA-treated aleurone tissue were resuspended in a medium similar to that described for the previous experiment but without calcium nitrate. The suspension of organelles was then layered on the top of a 30-60% sucrose gradient prepared (minus calcium) as described for the previous experiment.

The results (Figure III.3) showed a vast improvement in resolution over gradients previously obtained. A total of 56% of the α-amylase found in the gradient was concentrated in fractions 6-10 where there was also a large peak of absorbance at 280 nm. A second peak of organelles was concentrated at about 50% sucrose but was almost devoid of α-amylase activity. Although this result was encouraging, it was felt that only two bands of organelles were

Figure III.3

Distribution of α-amylase on a 30-60% sucrose density gradient minus calcium. The gradient solutions also contained 0.01 M Tris-HCl (pH 7.0) and 0.1% BSA. Centrifugation conditions are the same as for Figure III.2. α-Amylase activity is expressed in arbitrary units where 1 unit equals 1% of the total enzyme detected on the gradient. Tissue: GA (10 µg/ml) treated aleurone.




α – AMYLASE
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40
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hardly enough to account for all the organelles present in aleurone cells.

The large amount of BSA included in each gradient was not desirable if accurate protein determinations were to be made on the gradient fractions. To determine whether this additive was necessary a further experiment was conducted. Organelles from GA-treated aleurone were isolated and resuspended in a 0.4 M sucrose solution. The resuspended organelles were then separated on a 30-60% sucrose gradient which contained no other additives.

The results (Figure III.4) illustrated that BSA had little beneficial effect on organelle separation and so this compound was excluded from all subsequent gradients. The distribution of α-amylase in the sucrose-only gradient was similar to that observed in the previous experiment (Figure III.3) with over 50% of the total enzyme activity located in a band of organelles near the load - 30% sucrose interface (Figure III.4). Two other bands were clearly visible, one corresponding to a sucrose concentration of 50% and the other banding at about 55% sucrose. Neither band contained appreciable amounts of α-amylase.

A disturbing feature of this and previous gradients was that a majority of the organelles appeared to be blocked at the load - 30% sucrose interface and were prevented from entering the gradient. To investigate whether by decreasing the initial sucrose concentration

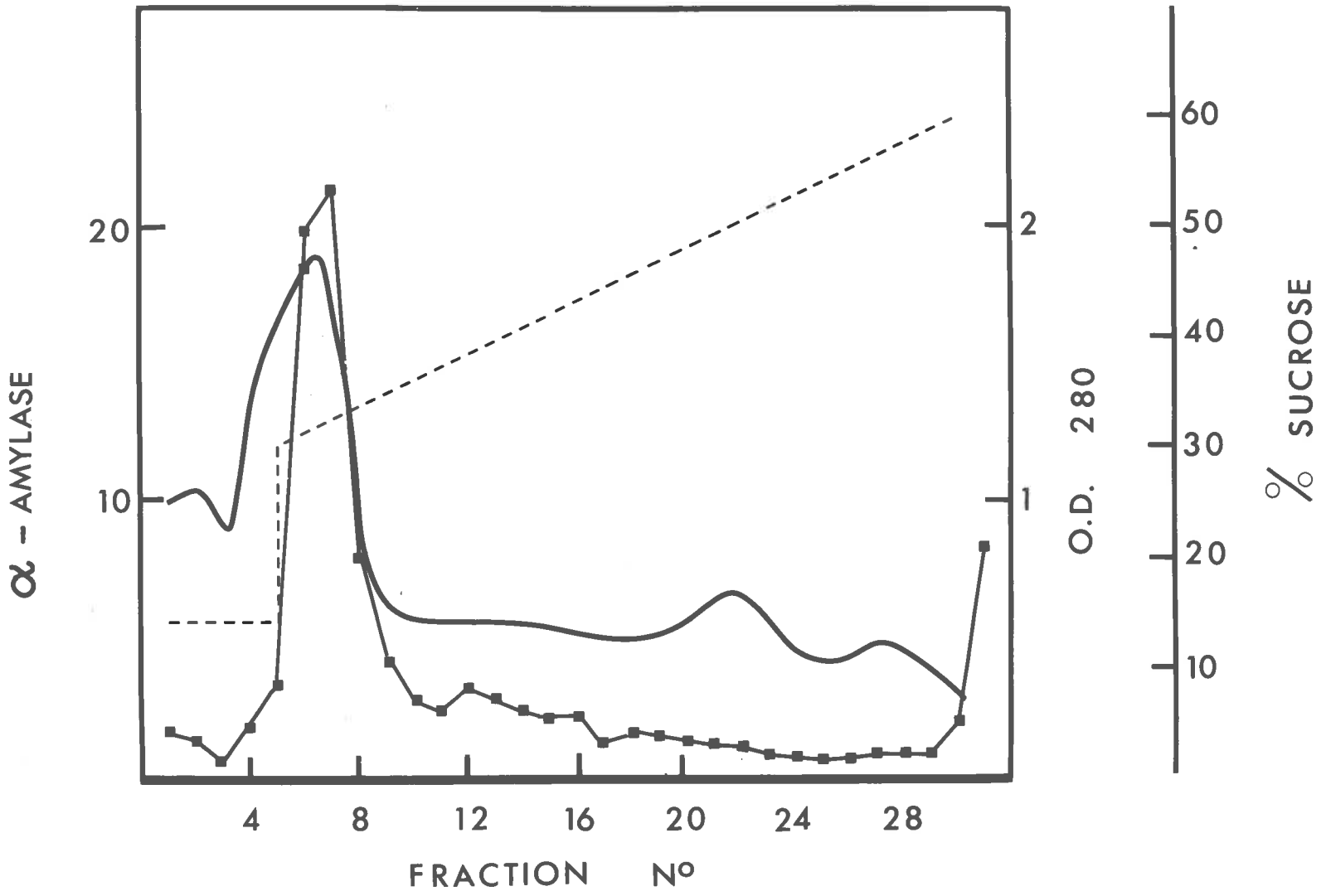
Figure III.4

Distribution of α-amylase on 30-60% sucrose density gradient minus BSA and Tris. Centrifugation time: 4 hr. Speed: 23,000 rpm. Temperature: 5°C. Rotor: SW25.1. α-Amylase activity expressed in arbitrary units. Tissue: GA (10 µg/ml) treated aleurone.

α-Amylase

O.D. 280 nm

% Sucrose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FRACTION Nº

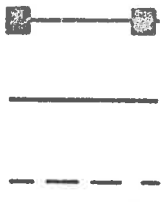
of the gradient it was possible to move the α-amylase-containing particles away from contaminating organelles, a similar experiment to the previous one was carried out. In this experiment, however, the 25 ml gradient was made linear between 20% and 60% sucrose. The results are illustrated in Figure III.5.

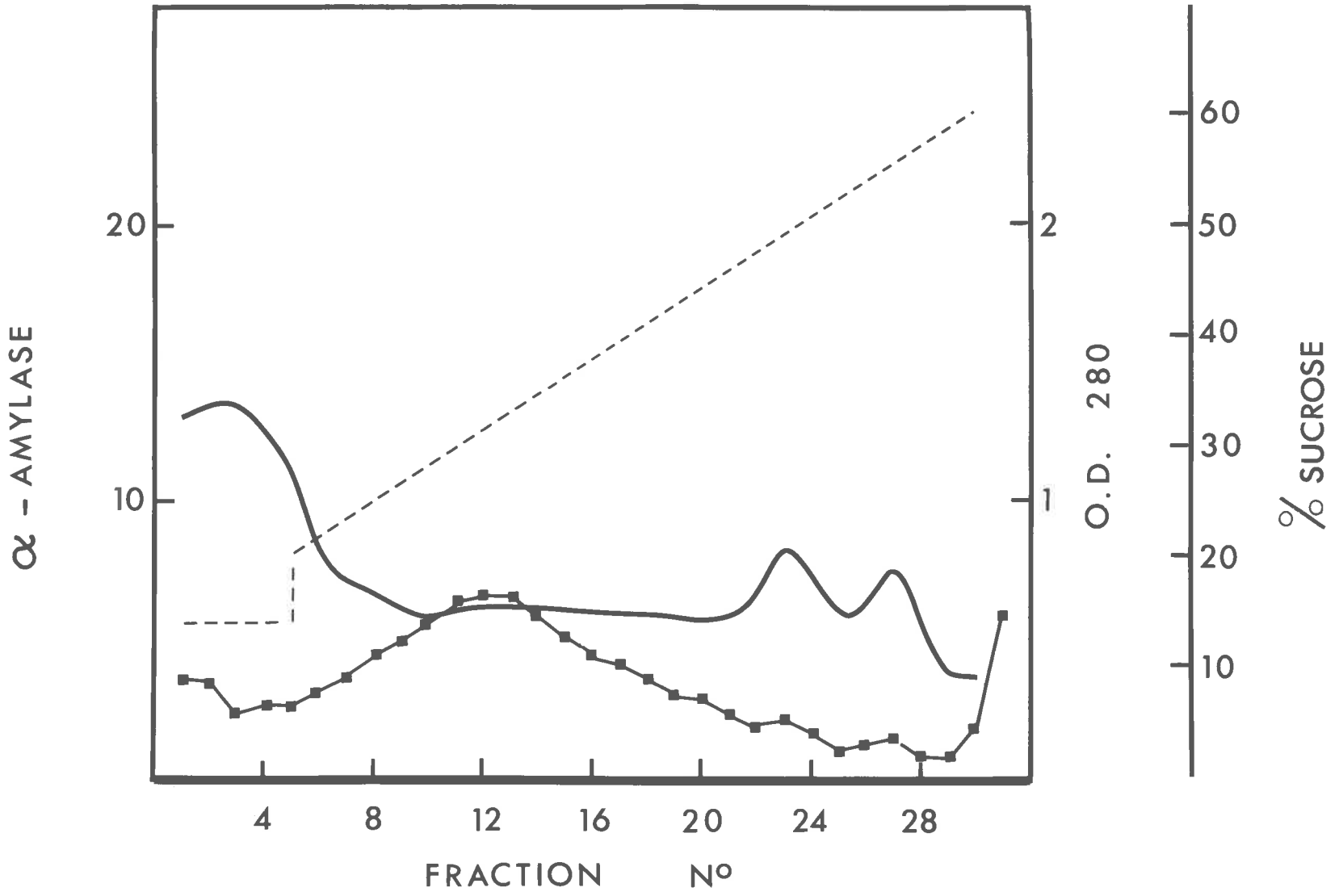
The major band, that in previous gradients had appeared at the top, was now spread in a broad band in the middle of the gradient. Unfortunately, no separation of α-amylase activity from this broad band was achieved. The two bands that were observed at the base of the gradient in the previous experiment, were still present although they now appeared to contain some α-amylase activity. Because of the extreme broadness of the α-amylase peak it was decided that 20-60% sucrose gradients were not useful for the purification of lysosomes.

An alternative method of preparing the gradients was tried. A total of 4 g of GA-treated aleurone tissue was homogenized and the post-1,000 *g* supernatant centrifuged in an SW25.1 rotor over a 3 ml cushion of 60% sucrose. After centrifuging for 1 hr at 25,000 rpm, the supernatant was drawn off and a 30-60% sucrose gradient was poured under the layer of organelles that had formed at the surface of the sucrose cushion. The idea behind such a technique was to isolate particles that were not damaged by the resuspension method

Figure III.5

Distribution of α-amylase on a 20-60% sucrose density gradient. Sucrose solutions prepared in water without other additives. Centrifugation conditions are the same as cited for the previous figure. α-Amylase activity expressed in arbitrary units. Tissue: GA (10 µg/ml) treated aleurone.

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then in use. The resulting gradient was then subjected to centrifugation and subsequent enzyme analysis in the usual way.

The results of this experiment (Figure III.6) were also very disappointing. Although the gradient was resolved into three major bands, they were very diffuse and consequently merged into one another. Furthermore, the banding did not appear to be specific since the level of α-amylase found in the various fractions almost exactly corresponded to the amount of particulate matter (as measured by O.D. 280). It was therefore concluded that pursuance of this technique would be fruitless.

Despite the apparently excessively large peak observed at the load - 30% sucrose interface in previous experiments (Figures III.3 and III.4), it was possible that this band was relatively free of contaminating organelles and that repeated attempts to purify it further were unnecessary. It was therefore decided to assay a typical 30-60% sucrose gradient for both α-amylase and cytochrome c oxidase, a marker enzyme for mitochondria. The results are illustrated in Figure III.7.

The gradient profile resembled those observed in earlier experiments, with the majority of the α-amylase activity located in the band at the top of the gradient. However, this band also contained a large proportion of the total cytochrome c oxidase activity which indicated that it was contaminated with mitochondria.

Figure III.6

Distribution of α-amylase on a 30-60% sucrose density gradient. Gradient poured under the band formed at the homogenate-60% sucrose interface after centrifugation for 1 hr at 25,000 rpm. Further details of the experiment are described in the text. Gradient centrifugation conditions are the same as described for Figure III.4. α-Amylase activity expressed in arbitrary units. Tissue: GA (10 µg/ml) treated aleurone.

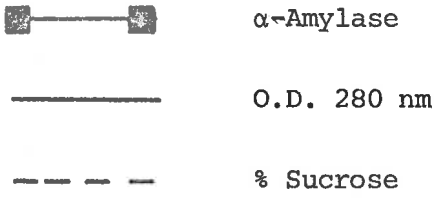
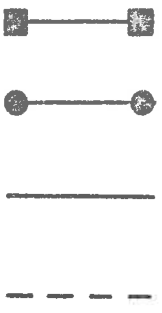
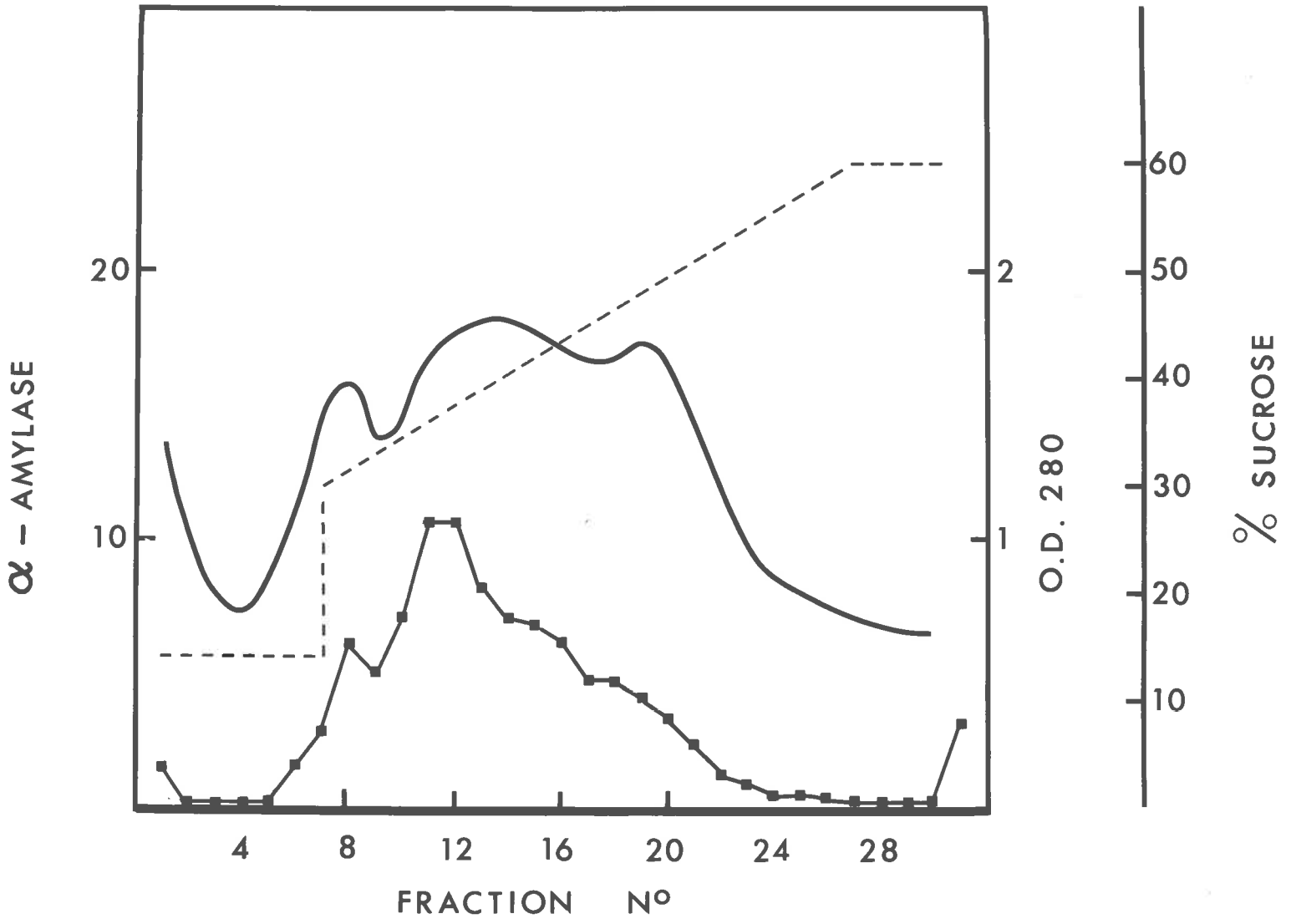


Figure III.7

Distribution of α-amylase and cytochrome c oxidase on a 30-60% sucrose density gradient. Sucrose solutions prepared in 0.01 M Tris-HCl (pH 7.0). Centrifugation conditions as described in Figure III.4. Both α-amylase and cytochrome c oxidase activities expressed in arbitrary units where 1 unit equals 1% of the total activity detected on the gradient.
Tissue: GA (10 µg/ml) treated aleurone.




α – AMYLASE
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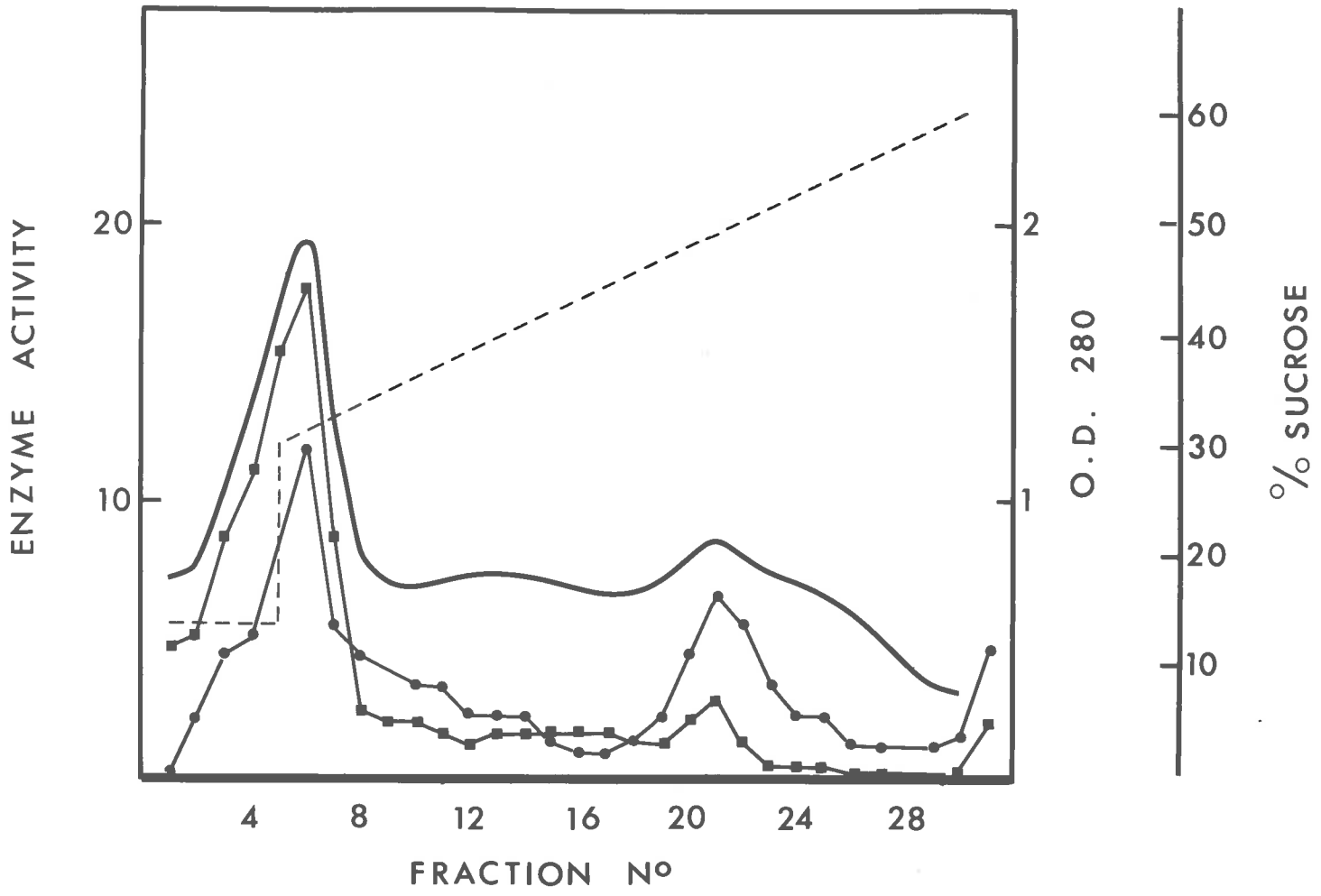
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It was concluded that the band at the load - 30% sucrose interface required further purification.

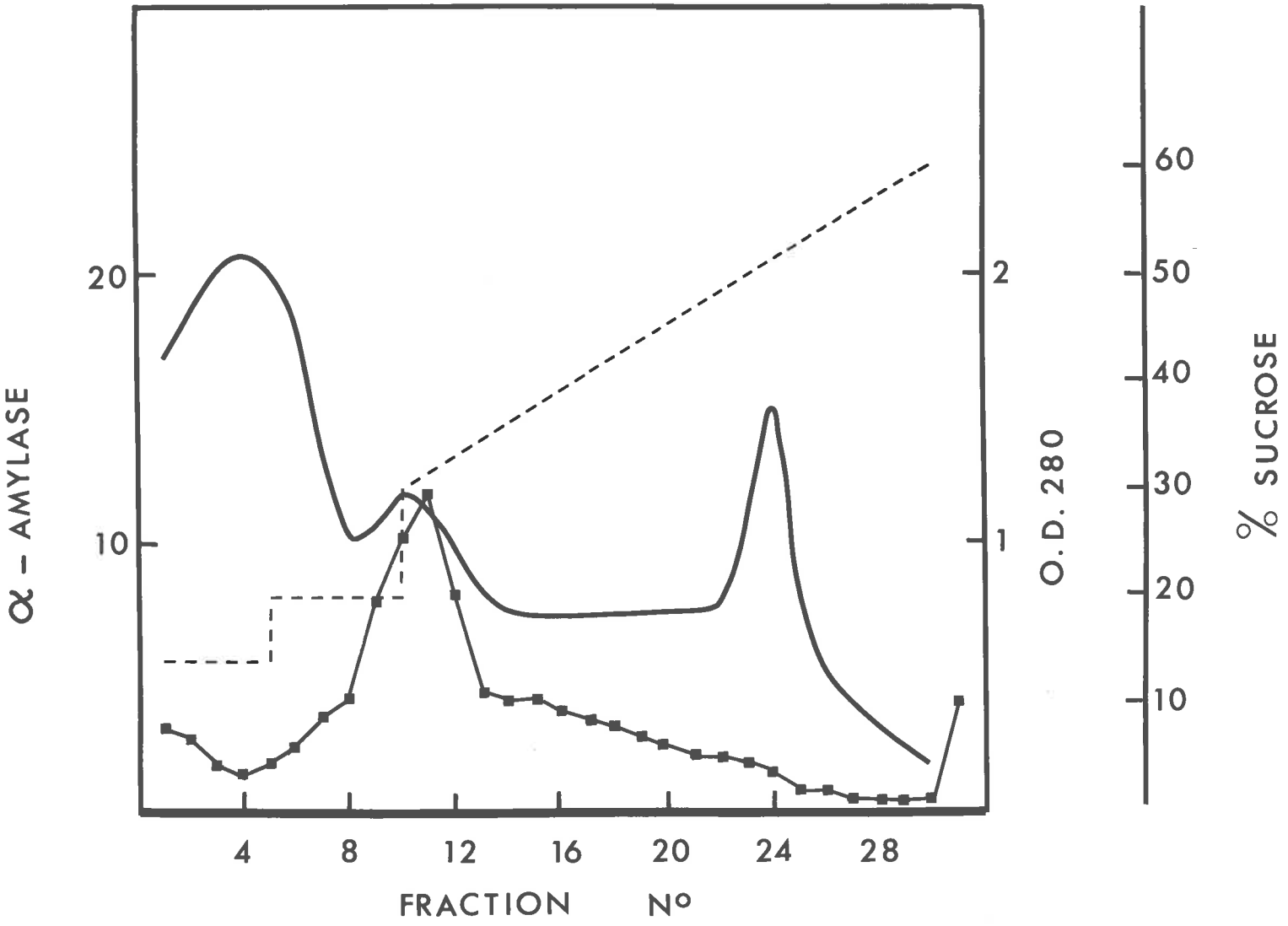
From the results obtained thus far it seemed obvious that the sudden increase in density from 0.4 M sucrose to 30% sucrose was too great a discontinuity to allow the organelles to enter the gradient properly. Although an increase from 0.4 M sucrose to 20% sucrose enabled the α-amylase containing particles to enter the gradient, the 20-60% sucrose gradient resulted in the formation of too broad a band to be of use for further purification (Figure III.5).

It was hoped, therefore, that by partially combining the two gradients, an improved separation could be obtained. A 20 ml column was prepared grading from 30% to 60% sucrose and over this was layered 5 ml of 20% sucrose. Over this was placed 5 ml of resuspended organelles from GA treated tissue and the gradient centrifuged and analysed.

The results of this gradient were most encouraging (Figure III.8). A large peak of particulate matter was located at the load - 20% sucrose interface but this band contained little α-amylase. A second smaller band was located at the 20-30% sucrose interface which contained over 40% of the total α-amylase on the gradient. A third large band was centered at the 50% sucrose position of the gradient but it was largely devoid of enzyme activity. Although there was some smearing of α-amylase in the centre of the gradient, it

Figure III.8

Distribution of α-amylase on a 20 : 30-60% sucrose density gradient. Sucrose solutions prepared in 0.01 M Tris-HCl (pH 7.0). Centrifugation time: 4 hr. Speed: 23,000 rpm. Rotor: SW25.1. Temperature: 5°C. α-Amylase activity expressed in arbitrary units. Tissue: GA (1 µg/ml) treated aleurone.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8
12
16
20
24
28
FRACTION Nº

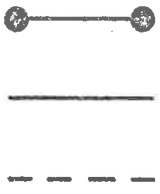
appeared that a significant purification of the α-amylase band had been achieved.

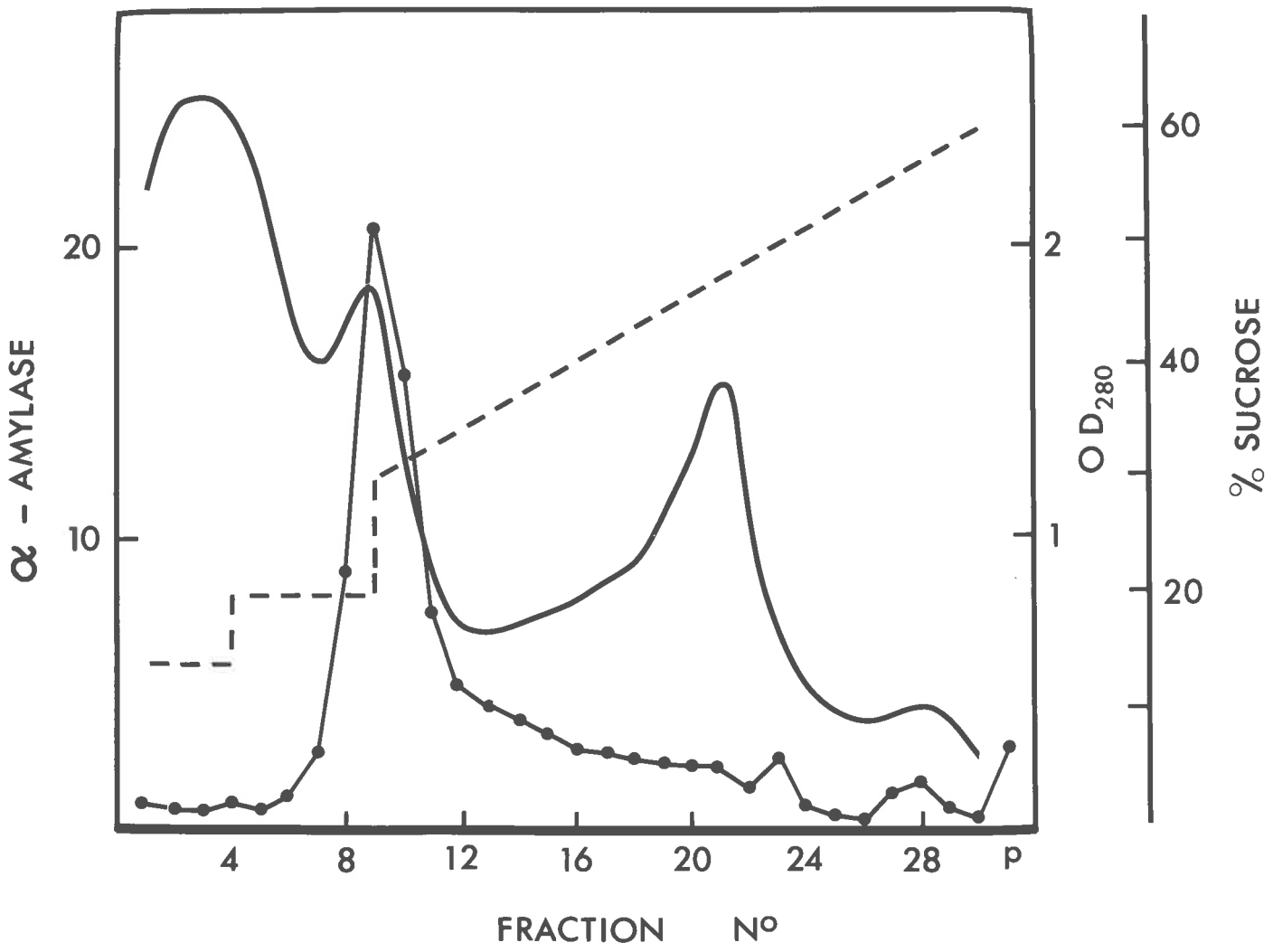
Unfortunately, subsequent experiments failed to confirm this result. This was thought to be due to the use of a new batch of sucrose which was thought to contain excessive calcium or divalent cations. It was therefore decided that if consistent gradients were to be regularly attained, a chelating agent such as EDTA would have to be employed despite the deleterious effect this compound has on α-amylase. Accordingly, gradients were prepared exactly as described for the previous experiment with the exception that all sucrose solutions were prepared in 0.01 M Tris buffer (pH 7.0) containing 1 mM EDTA. Furthermore, the load was made only 4 ml instead of 5 ml in an attempt to narrow the band width along the gradient.

The results of this experiment (Figure III.9) were almost identical to those illustrated in Figure III.8. Only three bands were visible, one at the load - 20% sucrose interface, another at the 20%-30% sucrose interface and another at a position corresponding to about 50% sucrose (Figure III.9). The major peak of α-amylase activity was associated with the second band although there was still significant spreading of the enzyme activity down the length of the gradient.

Figure III.9

Distribution of α-amylase on a 20 : 30-60% sucrose density gradient containing 1 mM EDTA. Sucrose solutions prepared in 1 mM EDTA and 0.01 M Tris-HCl (pH 7.0). Centrifugation conditions as described for Figure III.8. α-Amylase activity expressed in arbitrary units. Tissue: aleurone that had been treated with GA (1 µg/ml) for 24 hr.




α - AMYLASE
20
10
O D280
2
1
% SUCROSE
60
40
20
4
8
12
16
20
24
28
P
FRACTION Nº

Although gradients as just described gave highly repeatable results from week to week, no amount of manipulation with rotor speed, centrifugation time or slope of the density gradients improved the separation of particulate matter from the α-amylase peak or increased the number of bands observed. Since Breidenbach and Beevers (1967) had been able to separate proplastids, mitochondria and glyoxysomes (microbodies) from castor bean endosperm on essentially identical density gradients, my results were puzzling especially since these organelles were reported to also occur in cereal aleurone cells (Jones, 1969b).

There was the possibility that many of the organelles were taking up sucrose or losing water as they moved down the gradient, thus altering their buoyant density. It was therefore decided to use ficoll (MW 400,000), which has a much lower osmotic potential than sucrose, as a solute. Ficoll proved to be extremely difficult to work with as it is difficult to dissolve and very viscous at concentrations higher than about 5%. However, with the aid of the peristaltic pump device illustrated in Figure III.1B, workable gradients were regularly prepared. The standard gradient consisted of a column of liquid 22 ml in volume grading in a linear fashion from 20% sucrose to 20% sucrose + 30% ficoll. Over this was layered 6 ml of 20% sucrose and on top of this was placed a 2 ml load of

resuspended organelles. Conditions of centrifugation and enzyme assay were the same as stated for sucrose density gradients. All gradients contained 0.01 M Tris buffer (pH 7.0) and 1 mM EDTA.

The results of the first ficoll gradient attempted are illustrated in Figure III.10. There was a significant improvement in resolution over the earlier sucrose gradients in that many more bands were visible, particularly at the base of the gradient. Band 1 corresponded to the band similarly located in the sucrose gradients and was devoid of α-amylase activity. Bands 2 and 3 contained a majority of the α-amylase activity and almost certainly corresponded to the single band observed at the 20%-30% sucrose interface in the sucrose gradients. The resolution of the single band into two bands was due to a slight discontinuity in the ficoll gradient. Band 4 appeared as a diffuse shoulder of band 3 and contained significant amounts of α-amylase. Bands 5, 6 and 7 were located in the densest region of the gradient and were devoid of α-amylase activity. This region of the gradient did not always resolve itself into 3 bands; generally only 2 bands were present in this region.

Although the resolution achieved on ficoll gradients was less than ideal, it was the best that could be achieved in my hands. It was decided to proceed with the ficoll gradients and characterize biochemically the bands observed.

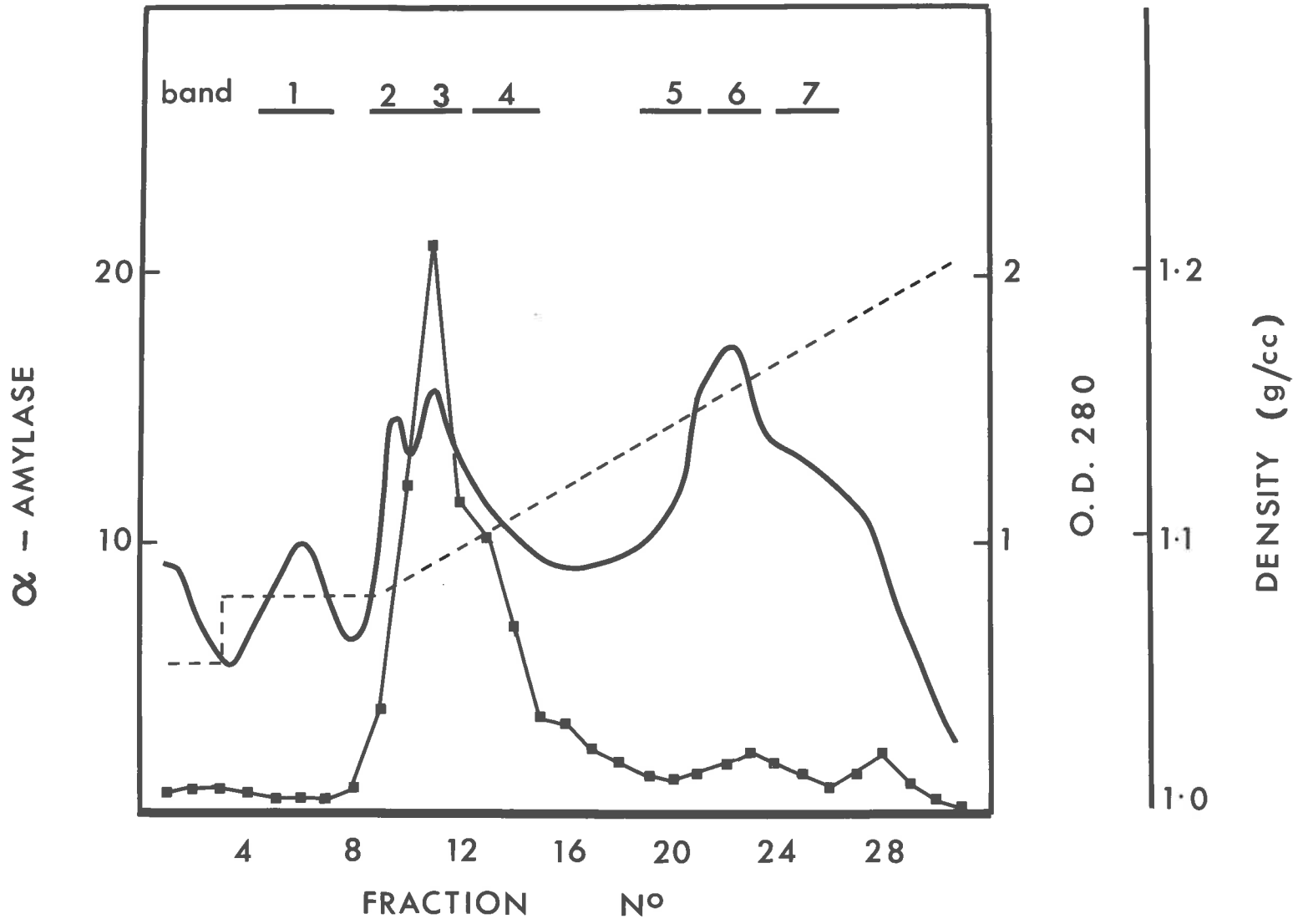
Figure III.10

Distribution of α-amylase on a 20% sucrose - 20% sucrose + 30% ficoll density gradient. Sucrose and ficoll solutions prepared in 1 mM EDTA and 0.01 M Tris-HCl (pH 7.0). Centrifugation conditions as described for Figure III.8. Load: 2 ml. α-Amylase activity expressed as arbitrary units. Organelles obtained from aleurone tissue treated with GA (1 µg/ml) for 24 hr at 30°C.

α-Amylase

O.D. 280 nm

Density (g/cc)


band 1 2 3 4 5 6 7
20
10
2
1
α – AMYLASE
O.D. 280
1·2
1·1
1·0
DENSITY (g/cc)
4 8 12 16 20 24 28
FRACTION Nº

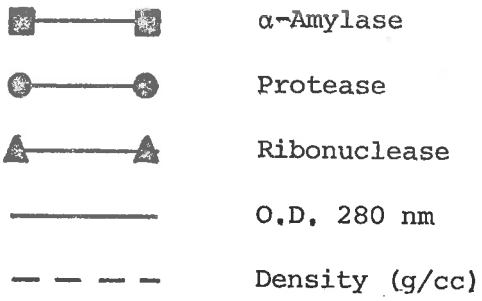
Earlier experiments (Section II) had indicated that while α-amylase and protease were particulate enzymes the exact nature of ribonuclease was in doubt. It was therefore important to establish the distribution of these three hydrolytic enzymes in the various fractions obtained by density gradient centrifugation. This was done in the next experiment.

Most of the ribonuclease activity was associated with the supernatant (top) of the gradient although a significant proportion was found down the length of the gradient (Figure III.11). Because no peak of ribonuclease activity was found associated with any particular band, it was concluded that a majority of this enzyme was cytoplasmic. Acid protease on the other hand showed an identical distribution pattern to that of α-amylase, with a majority of the enzyme associated in the region of bands 2 and 3. It seemed likely therefore, that these two enzymes were either associated with the same particle or in different particles of the same density which the gradient was unable to resolve.

It was important to attempt to identify the various bands observed in the density gradients biochemically by assaying for enzymes known to be associated with particular organelles. The marker enzymes chosen were $NADH_2$-cytochrome c reductase (ER), cytochrome c oxidase (mitochondria), and catalase and isocitratase

Figure III.11

Distribution of α-amylase, protease and ribonuclease on a sucrose : ficoll density gradient prepared as described for the previous figure. Sucrose and ficoll solutions prepared in 1 mM EDTA and 0.01 M Tris-HCl (pH 7.0). Enzymes assayed by the methods described in the text. Enzyme activities of all three enzymes expressed as arbitrary units where 1 unit equals 1% of the total activity detected on the gradient. Load: 2 ml. Centrifugation conditions as described for Figure III.8. Tissue: GA (1 µg/ml)-treated aleurone.



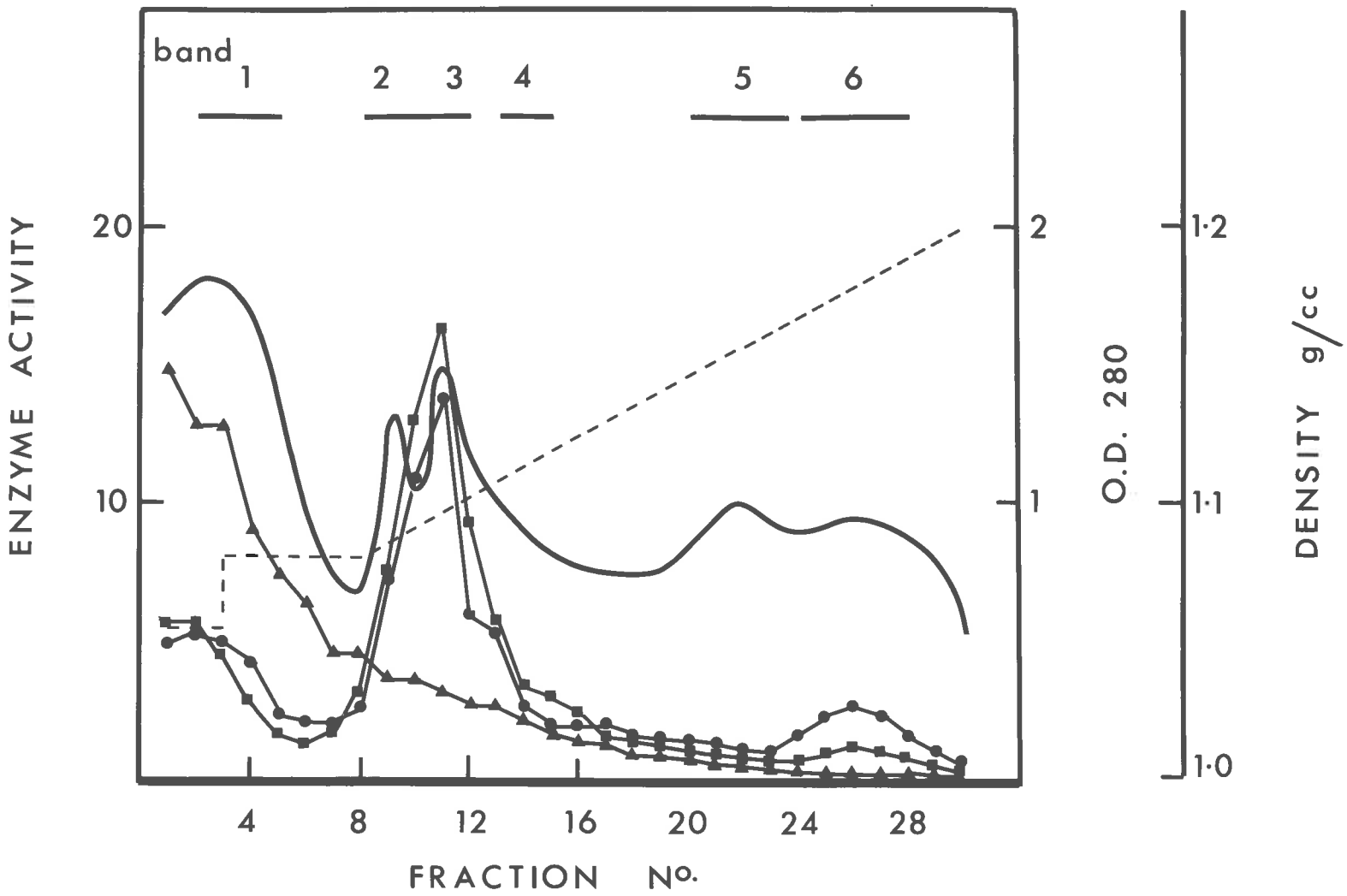
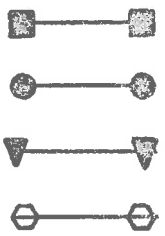

band
1
2
3
4
5
6
ENZYME ACTIVITY
20
10
2
1
O.D. 280
1·2
1·1
1·0
DENSITY g/cc
4
8
12
16
20
24
28
FRACTION No.

Figure III.12

Distribution of various organelle marker enzymes on a sucrose : ficoll density gradient prepared as described in Figure III.10. Sucrose and ficoll solutions prepared in 1 mM EDTA and 0.01 M Tris buffer (pH 7.0). Enzymes assayed by the methods described in the text. Centrifugation conditions as described in Figure III.8. Load: 2 ml. Activities of all enzymes expressed as arbitrary units. Tissue: Aleurone treated with GA (1 µg/ml) for 24 hr at 30°C.



α-Amylase

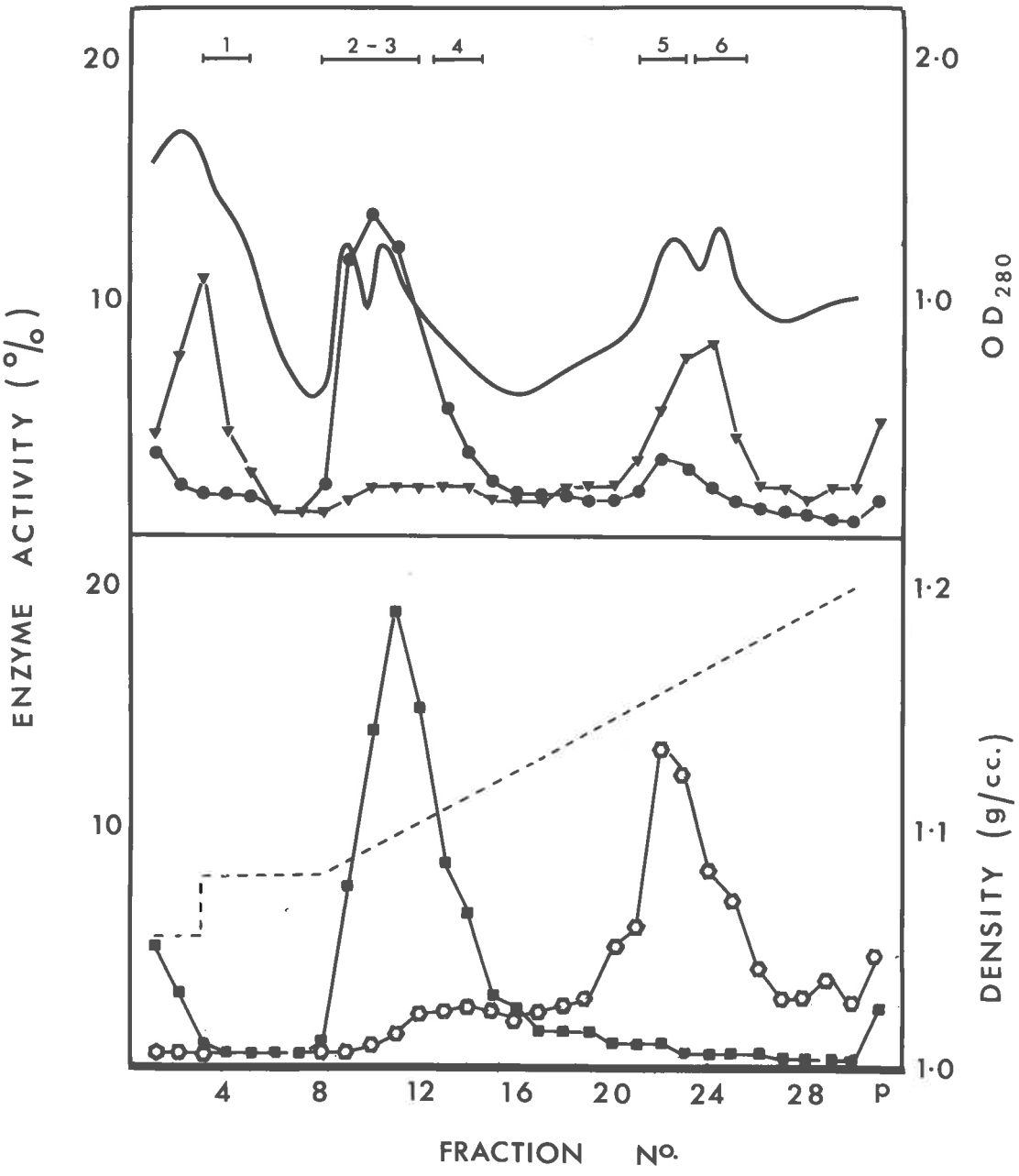
$NADH_2$-cytochrome c reductase

Catalase

Cytochrome c oxidase

O.D. 280 nm

Density (g/cc)


1
2 - 3
4
5
6
20
10
2·0
1·0
OD280
ENZYME ACTIVITY (%)
20
10
1·2
1·1
1·0
DENSITY (g/cc.)
4
8
12
16
20
24
28
P
FRACTION No.

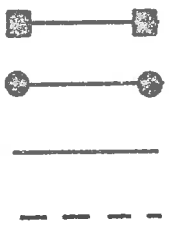
(glyoxysomes). The loading and preparation of the gradients were exactly as described for the previous experiment and the results are illustrated in Figure III.12.

Cytochrome c oxidase activity was almost entirely located in band 5 although a certain amount was associated with band 6. Catalase activity on the other hand, was equally distributed between band 1 and bands 5 and 6. Once again most of the α-amylase activity was found in bands 2, 3 and 4 which also appeared to contain a majority of the cytochrome c reductase activity. Although it was pleasing to note that the α-amylase peak contained only one other enzyme, the resolution of bands 5 and 6 was poor. Isocitratase was only found in trace amounts and the data were not reliable enough to be reported.

The results suggested that the α-amylase-containing particles were either ER vesicles or particles that were of a similar density. In an attempt to separate α-amylase and cytochrome c reductase activities, 60,000 *g* pellets obtained from 8 g of GA-treated aleurone were resuspended in 4 ml of medium and 2 ml were layered on each of two gradients. The first gradient was linear from 20% sucrose to 20% sucrose + 15% ficoll (Figure III.13), while the second gradient was discontinuous with concentrations above 20% being obtained by additions of ficoll (Figure III.14). Both gradients were centrifuged

Figure III.13

Distribution of α-amylase and $NADH_2$-cytochrome c reductase on a 20% sucrose - 20% sucrose + 15% ficoll linear density gradient. Sucrose and ficoll solutions prepared in 1 mM EDTA and 0.01 M Tris buffer (pH 7.0). Centrifugation time: 4 hr. Speed: 25,000 rpm. Temperature: 5°C. Rotor: SW25.1. Enzyme activity expressed as arbitrary units where 1 unit equals 1% of the total activity detected on the gradient. Tissue: GA-treated (1 µg/ml) aleurone.



α-Amylase

$NADH_2$-cytochrome c reductase

O.D. 280 nm

Density (g/cc)

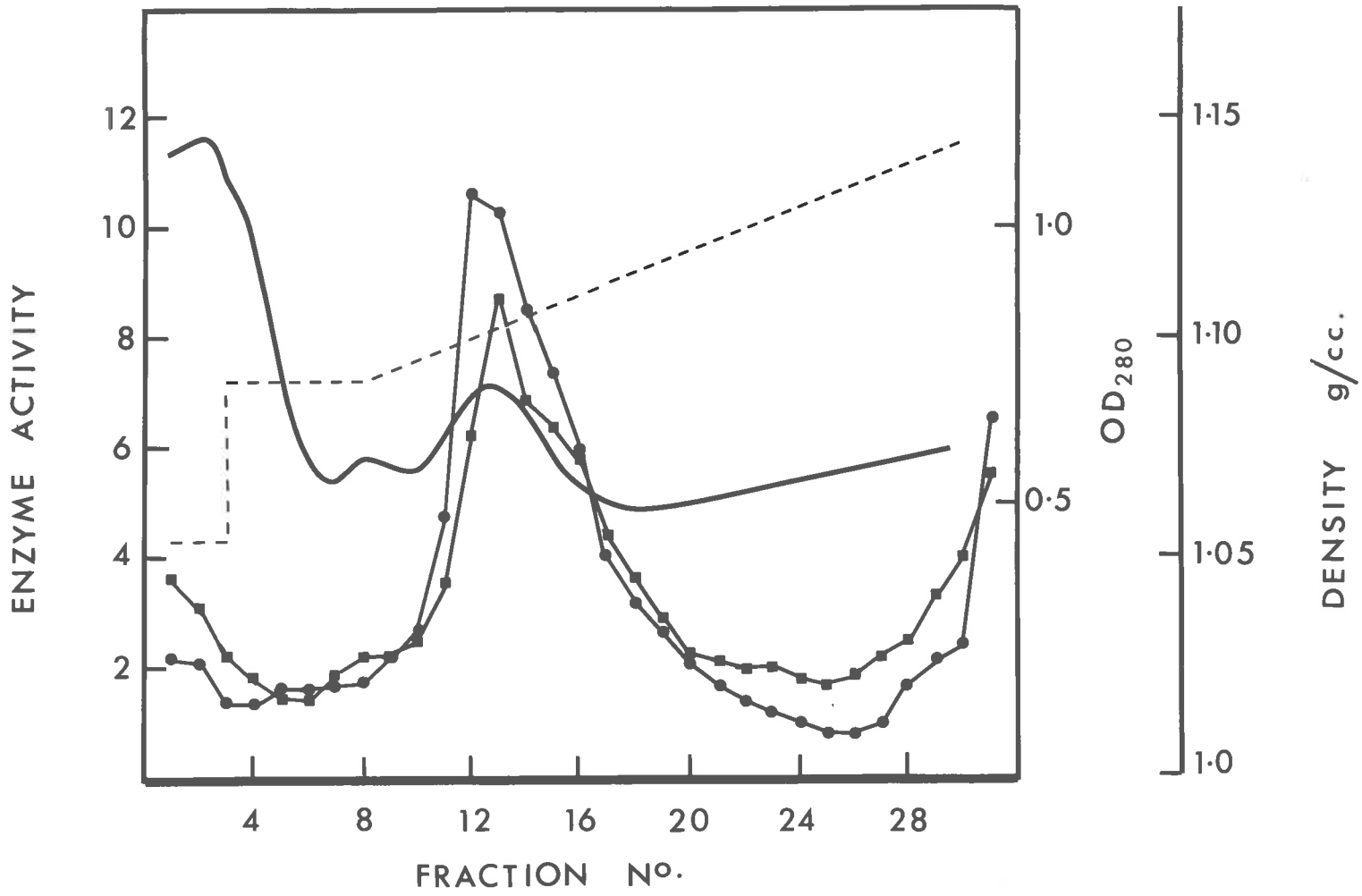
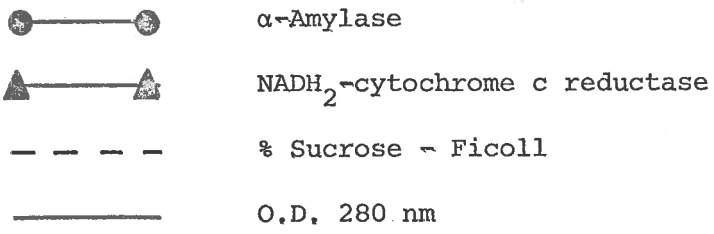
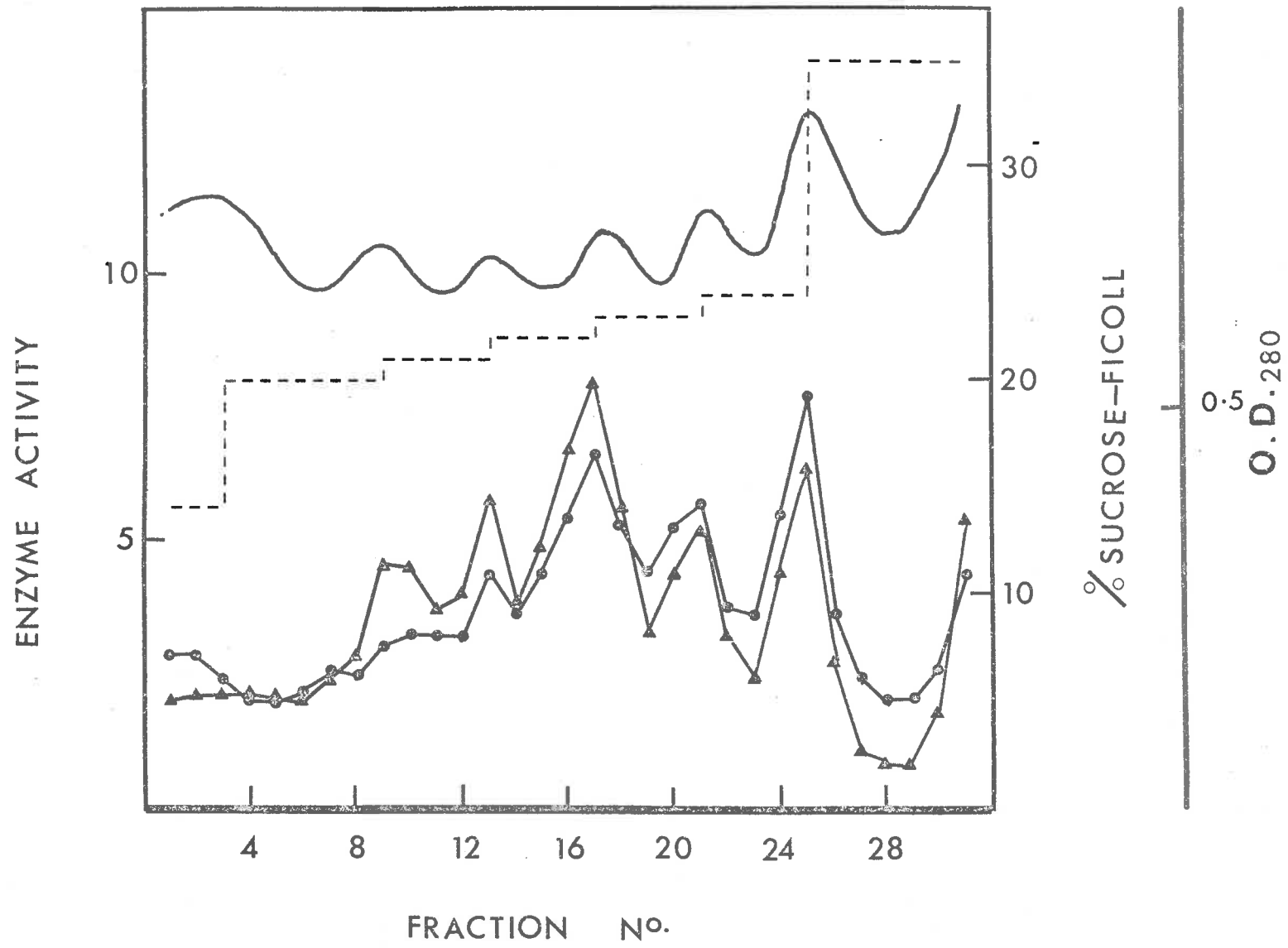

ENZYME ACTIVITY
12
10
8
6
4
2
4
8
12
16
20
24
28
FRACTION Nº.
1·0
0·5
OD280
1·15
1·10
1·05
1·0
DENSITY g/cc.

Figure III.14

Distribution of α-amylase and $NADH_2$-cytochrome c reductase on a sucrose : ficoll discontinuous density gradient. Solutions of sucrose and ficoll prepared in 1 mM EDTA and 0.01 M Tris-HCl (pH 7.0). All solutions above 20% were achieved by additions of ficoll to 20% sucrose solutions. Centrifugation conditions as described for, Figure III.13. Enzyme activity expressed as arbitrary units. Load: 2 ml. Tissue: GA-treated (1 µg/ml) aleurone.




ENZYME ACTIVITY
10
5
30
20
10
% SUCROSE–FICOLL
0·5
O. D. 280
4
8
12
16
20
24
28
FRACTION Nº.

for 4 hr at 25,000 rpm.

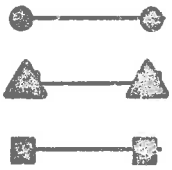
The peak of α-amylase activity in the continuous gradient corresponded to band 3 which was also the region of cytochrome c reductase activity. This region corresponded to a density of 1.080 g/cc (20% sucrose + 3% ficoll). Bands of membranous material were visible at every interface of the discontinuous gradient and both enzymes were associated with each of the bands. The major peak of both α-amylase and cytochrome c reductase appeared to be at the 22-24% interface. No clear separation of the two enzymes was achieved in either gradient.

Although cytochrome c reductase had been reported to be associated with the ER (Moore *et al.*, 1973), it was considered to be important to establish the identity of this region of the gradient by other means. Because the ER is a site of protein synthesis it was reasoned that the apparent ER region of the gradient should incorporate ^{14}C-amino acids. Accordingly, two 1 g samples of aleurone tissue that had been incubated for 23 hr with or without GA, were incubated for a further 2 hr with 1 μCi ^{14}C-lysine, and the tissue homogenates subjected to density gradient centrifugation.

The results of the experiment are presented in Figures III.15 and III.16 and several interesting points can be noted. Although there was a large peak of radioactivity in the ER region of the control gradient (Figure III.15), this peak was very much reduced in the gradient containing organelles from GA-treated tissue

Figure III.15

Incorporation of ^{14}C-lysine into organelles isolated from control aleurone tissue and separated on a sucrose : ficoll density gradient prepared as described in Figure III.10. Sucrose and ficoll solutions prepared in 1 mM EDTA and 0.01 M Tris buffer (pH 7.0). Radioactivity and enzyme activities determined as described in the text. Centrifugation time: 4 hr. Speed: 23,000 rpm. Rotor: SW25.1. Temperature: 5°C. Enzyme activities expressed in arbitrary units.



α-Amylase

$NADH_2$-cytochrome c reductase

Radioactivity (cpm X 10^3)

O.D. 280 nm

% Sucrose - Ficoll

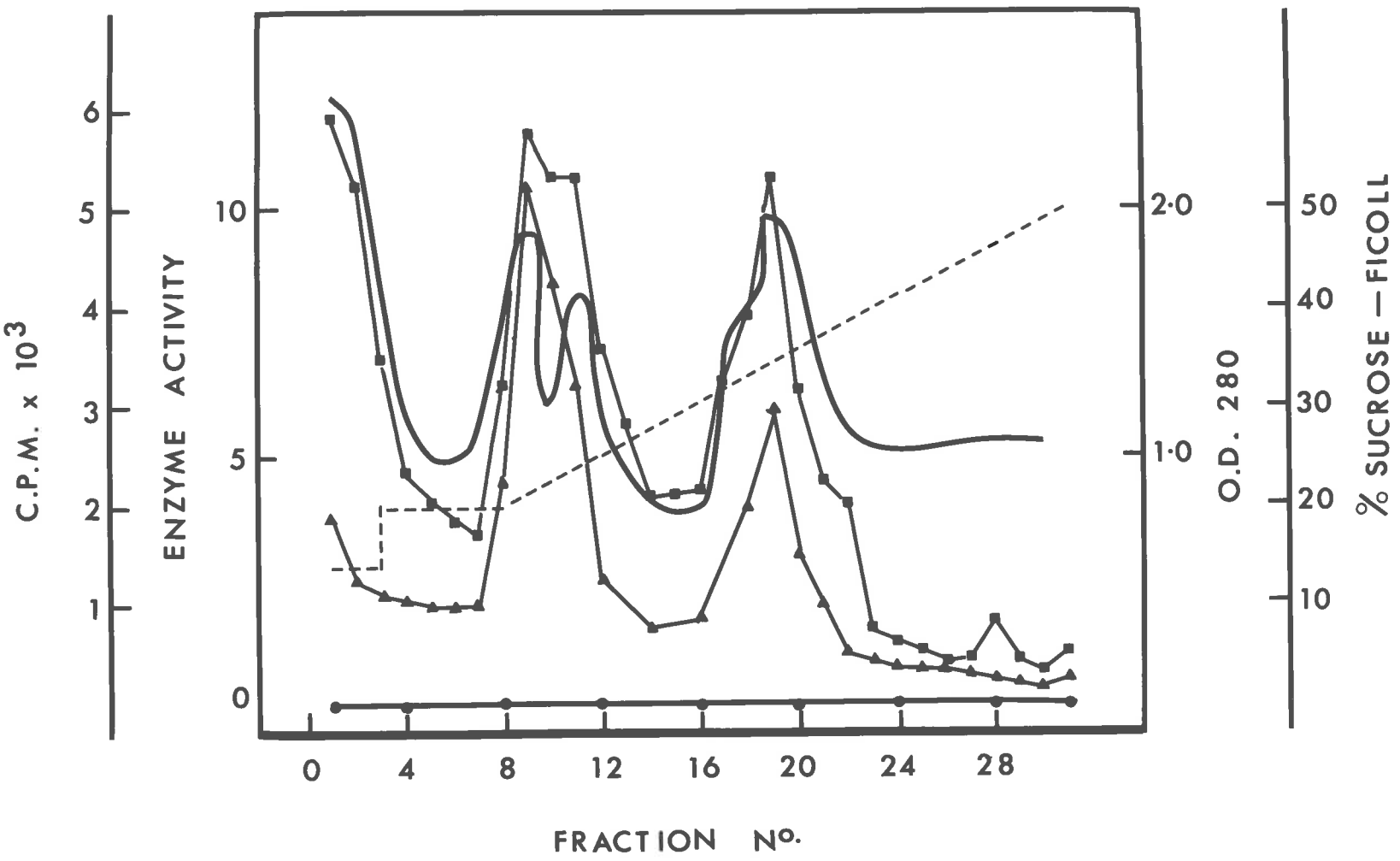
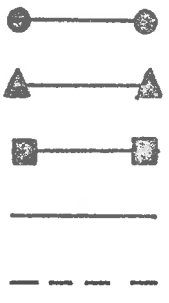

C.P.M. x 10^3
6
5
4
3
2
1
ENZYME ACTIVITY
10
5
0
2·0
1·0
O.D. 280
50
40
30
20
10
% SUCROSE — FICOLL
0
4
8
12
16
20
24
28
FRACTION Nº.

Figure III.16

Incorporation of ^{14}C-lysine into organelles isolated from GA (1 µg/ml) treated tissue and separated on a sucrose : ficoll gradient prepared as described in Figure III.10. All other conditions as described for Figure III.15.



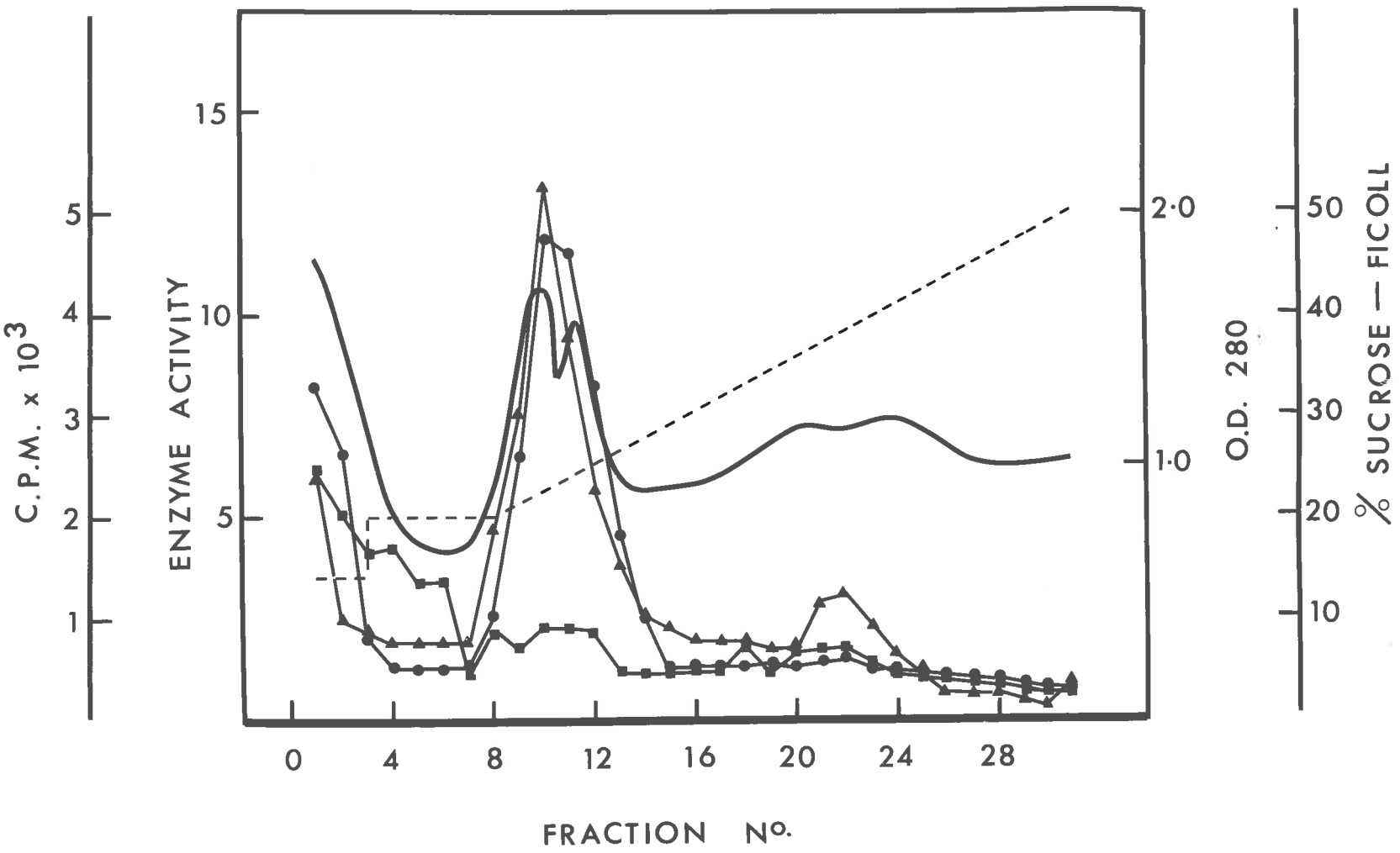
α-Amylase

$NADH_2$-cytochrome c reductase

Radioactivity (cpm X 10^3)

O.D. 280 nm

% Sucrose - Ficoll


C.P.M. x 10^3
ENZYME ACTIVITY
O.D. 280
% SUCROSE — FICOLL
FRACTION Nº.
0
4
8
12
16
20
24
28
1
2
3
4
5
10
15
1·0
2·0
10
20
30
40
50

(Figure III.16). The apparently reduced level of ^{14}C-lysine incorporation in the GA-treated tissue was almost certainly due to isotope dilution and proteolysis in this tissue (Varner *et al.*, 1965). A second major peak of radioactivity was also observed in control-tissue gradients in the mitochondria-glyoxysome region which was not observed in GA-treated-tissue gradients. The reason for this peak of radioactivity is not understood.

α-Amylase was not detected on the control-tissue gradient (Figure III.15) but was present as a single band on the gradient obtained with organelles from GA-treated tissue (Figure III.16). Furthermore, the α-amylase peak corresponded exactly to the peak obtained for cytochrome c reductase. These data represent important evidence that the two enzymes are separate proteins and not simply sub-units of each other as has been reported for nitrate reductase and cytochrome c reductase (Wray and Filner, 1970).

Finally, and perhaps most importantly, the amount of cytochrome c reductase measured in the ER region of both gradients was identical (6.5 units). Thus GA does not appear to cause an increase in ER as measured by the marker enzyme for this fraction of the cell contents.

7.2 Cytological Observations

It was considered important to obtain electron micrographs of the various bands observed in the density gradients of GA-treated

aleurone cell homogenates. A gradient prepared in exactly the same manner as described in previous experiments was assayed for α-amylase activity and the results are illustrated in Figure III.17. Samples taken from each of the six bands observed as well as the pellet were fixed in 2% glutaraldehyde and prepared for viewing in the electron microscope by the methods outlined earlier.

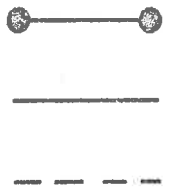
Band 1 contained many ruptured aleurone grains as well as many small vesicles which appeared to be similar in size (0.1 - 0.6 μ) to vesicles contained in aleurone grains (1 - 2 μ) (Figure III.18). The fraction was far from homogeneous with a variety of membranous structures visible including an occasional mitochondrion. The band corresponded to a buoyant density of about 1.060 g/cc.

Vesicles of many sizes were also visible in sections obtained from band 2 (Figure III.19). Although an occasional aleurone grain was visible, vesicles (0.1 - 0.6 μ) were the main constituent, some of which appeared to be slightly electron dense (0.1 - 0.2 μ) and others that seemed to be constructed of two separate membranes (0.2 - 0.4 μ). Occasional small mitochondria were also visible. The band was rich in α-amylase activity (Figure III.17) and had a buoyant density of 1.070 g/cc.

The other α-amylase-containing region (band 3) was also rich in a variety of vesicles (Figure III.20). Aleurone grains were also occasionally seen but double-membrane vesicles (0.2 - 0.4 μ) and

Figure III.17

Distribution of α-amylase on a sucrose : ficoll density gradient (20% sucrose - 20% sucrose + 30% ficoll). Organelles in bands indicated were prepared for electron microscopy as described in the text. Sucrose and ficoll solutions prepared in 1 mM EDTA and 0.01 M Tris-HCl (pH 7.0). Centrifugation conditions as described in Figure III.15. α-Amylase activity expressed in arbitrary units. Tissue: aleurone treated with GA (1 µg/ml) for 24 hr at 30°C.



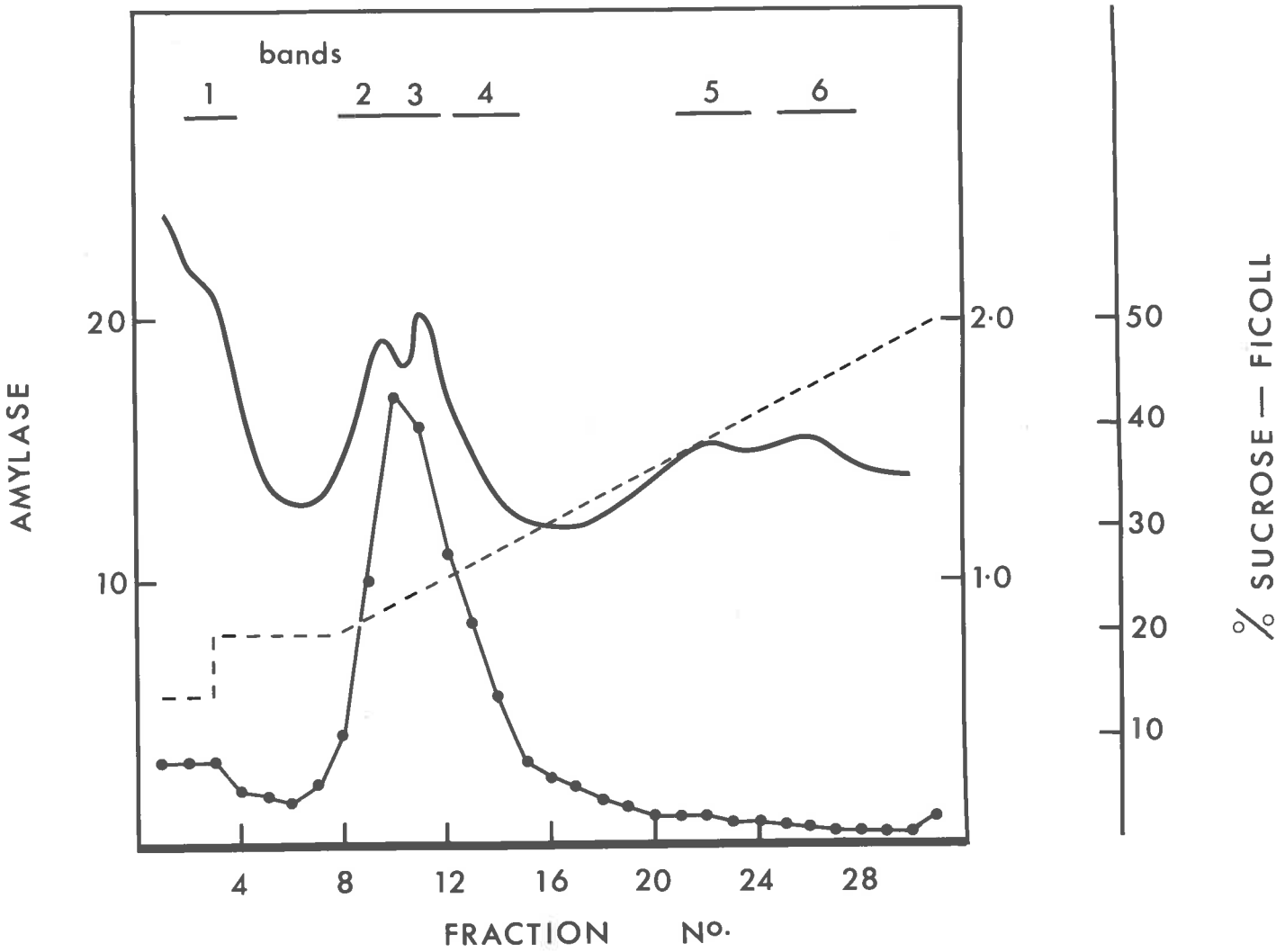

bands
1
2 3
4
5
6
AMYLASE
20
10
2·0
1·0
50
40
30
20
10
% SUCROSE — FICOLL
4
8
12
16
20
24
28
FRACTION Nº.

Figure III.18

Electron micrograph of organelles in band 1 of density gradient illustrated in Figure III.17. Many aleurone grains are visible as well as many small vesicles similar in size (0.1 - 0.6μ) to vesicles contained in aleurone grains. Magnification: 32,000x.

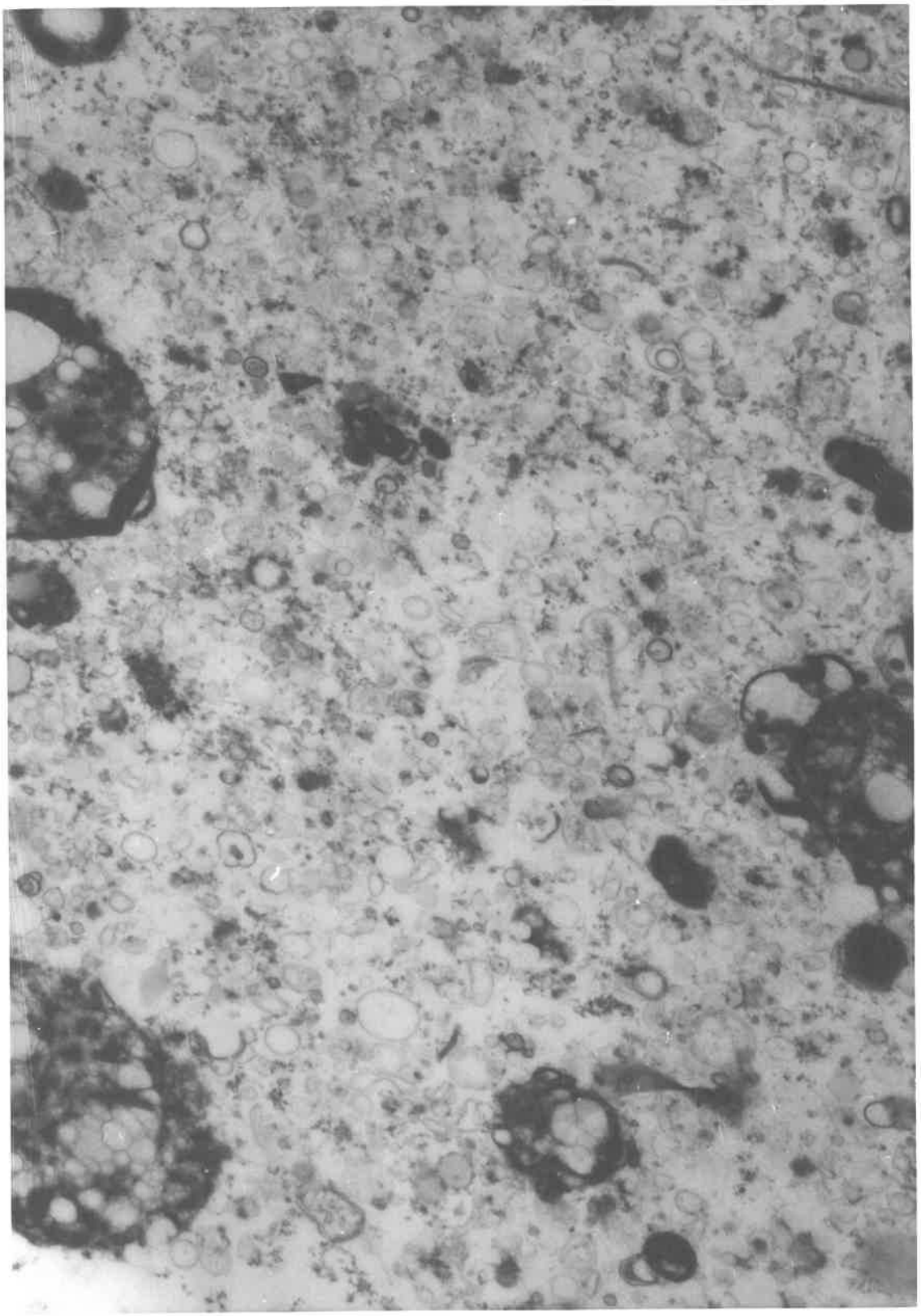


Figure III.19

Electron micrograph of organelles in band 2 of density gradient of organelles obtained from GA (1 µg/ml) treated aleurone tissue illustrated in Figure III.17. Many small (0.1 - 0.6µ) vesicles visible and an occasional aleurone grain or small mitochondrion. Magnification: 32,000x.

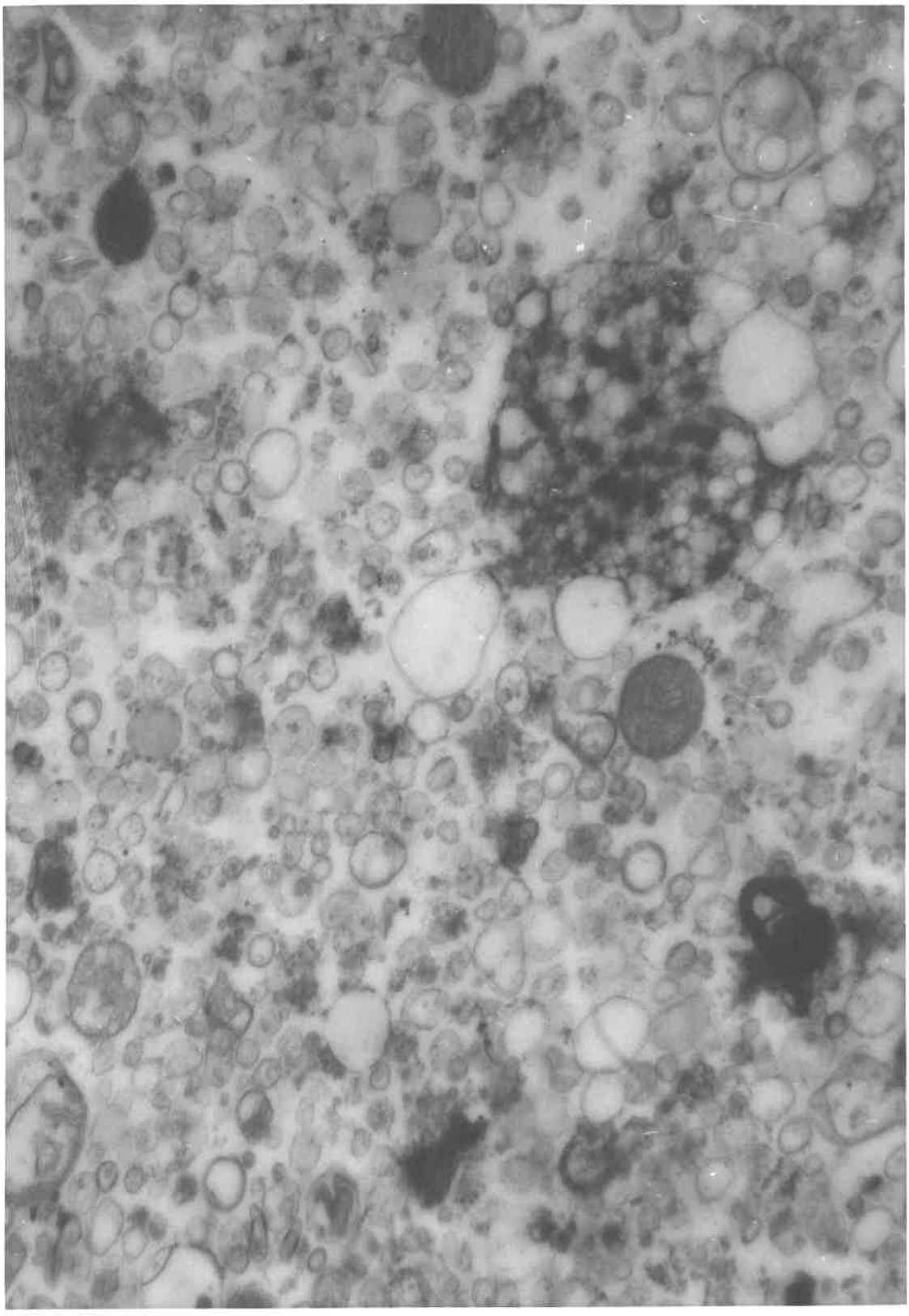
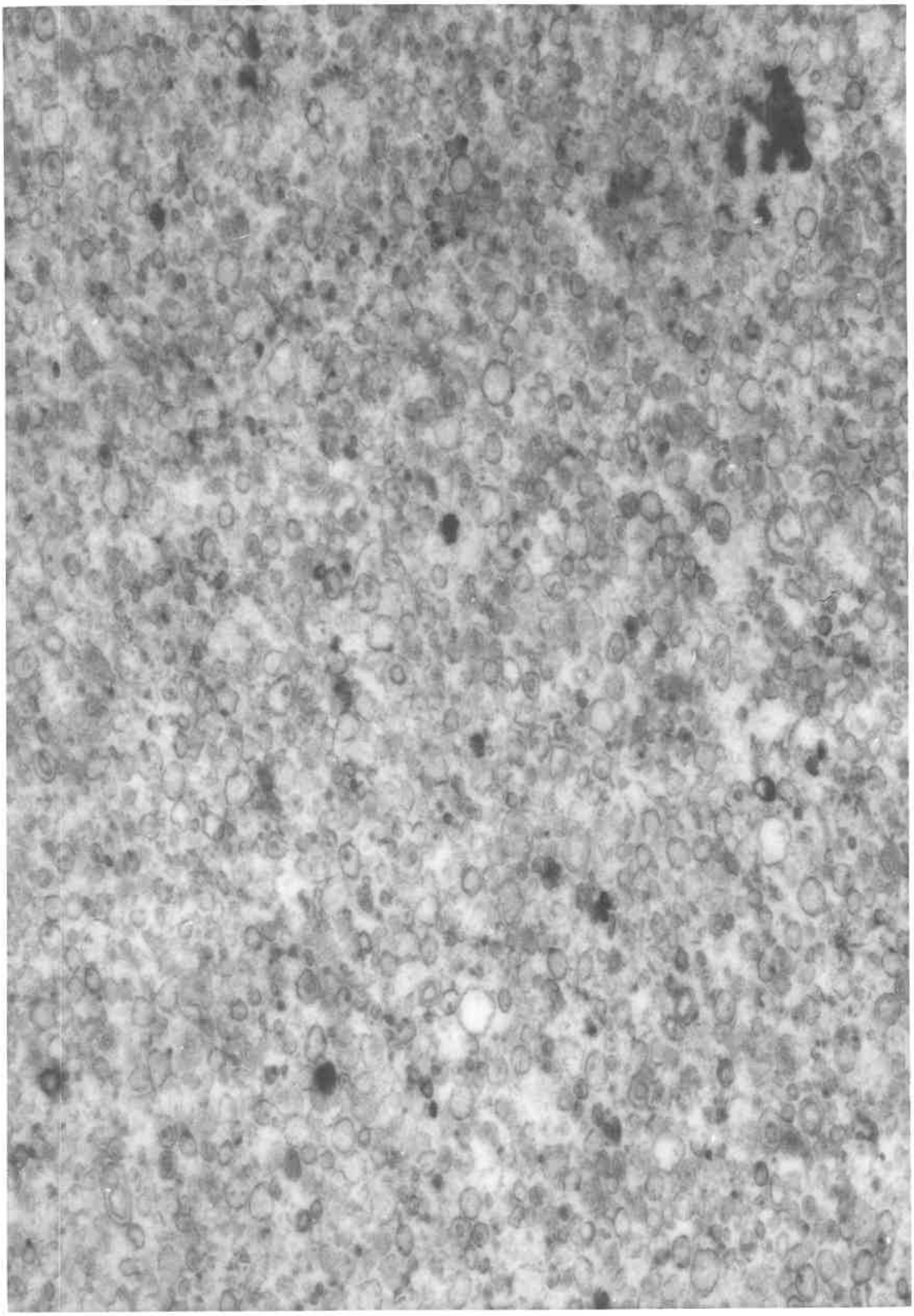


Figure III.20

Electron micrograph of organelles in band 3 of density gradient illustrated in Figure III.17. Organelles obtained from GA (1 µg/ml) treated aleurone tissue. Many vesicles are visible, some of which contain double membranes (0.2 - 0.4µ) and others (0.1 - 0.2µ) which are slightly electron dense. Magnification: 32,000x.



slightly electron dense vesicles (0.1 - 0.2 μ) were the main constituent. Also present were the occasional long membranous structures resembling a portion of ER. The band had a buoyant density of 1.080.

Band 4 (Figure III.21), which was actually a shoulder of band 3, and also contained some α-amylase activity, contained similar organelles to those observed in band 3. The band was rich in both double-membrane vesicles (0.2 - 0.4 μ) and small, slightly electron dense, vesicles (0.1 - 0.2 μ). The band had an apparent buoyant density of 1.090.

Band 5 (Figure III.22) was the most homogeneous fraction obtained. Intact mitochondria were almost the only organelles visible (0.5 - 0.75 μ) and had a buoyant density of 1.150 g/cc.

The organelles in band 6 (Figure III.23) were difficult to identify. Although some disruptive mitochondria were clearly visible, some smaller bodies (0.2 - 0.4 μ) which were electron dense and vaguely reminiscent of microbodies, were also seen. The fraction was also rich in various vesicles and broken pieces of membrane and had a density of 1.170 g/cc.

The pellet at the base of the gradient was small but in some experiments small amounts of α-amylase had been detected and so was of interest. Apart from a few scattered vesicles the fraction

Figure III.21

Electron micrograph of organelles in band 4 of density gradient illustrated in Figure III.17. This band appears rich in both double-membrane vesicles (0.2 - 0.4μ) and electron-dense vesicles (0.1 - 0.2μ). Magnification: 32,000x.

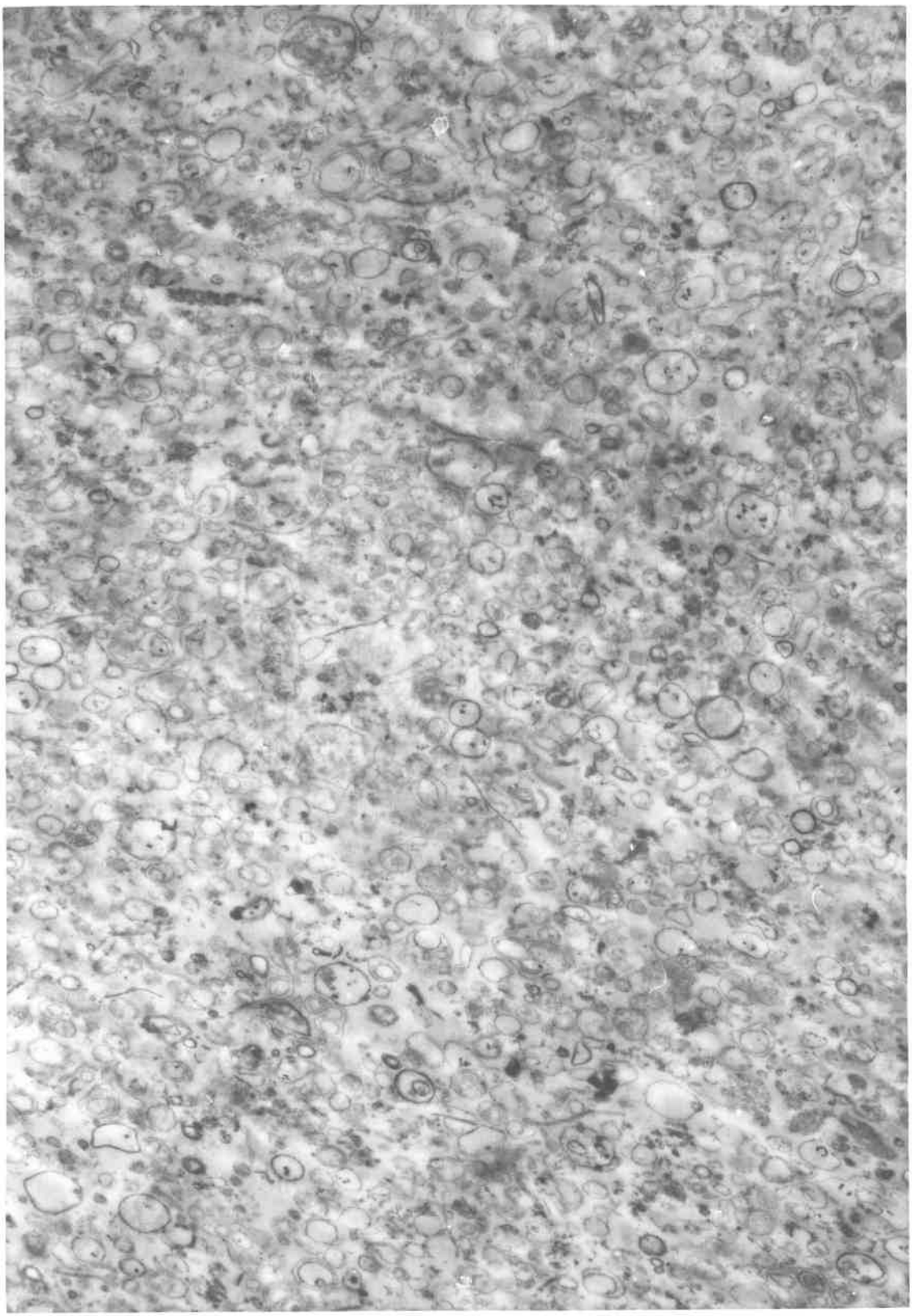


Figure III.22

Electron micrograph of organelles in band 5 of density gradient of organelles obtained from GA (1 µg/ml) treated aleurone tissue illustrated in Figure III.17. Mitochondria (0.5 - 0.75µ) and an occasional vesicle are the only organelles visible. Magnification: 80,000x.

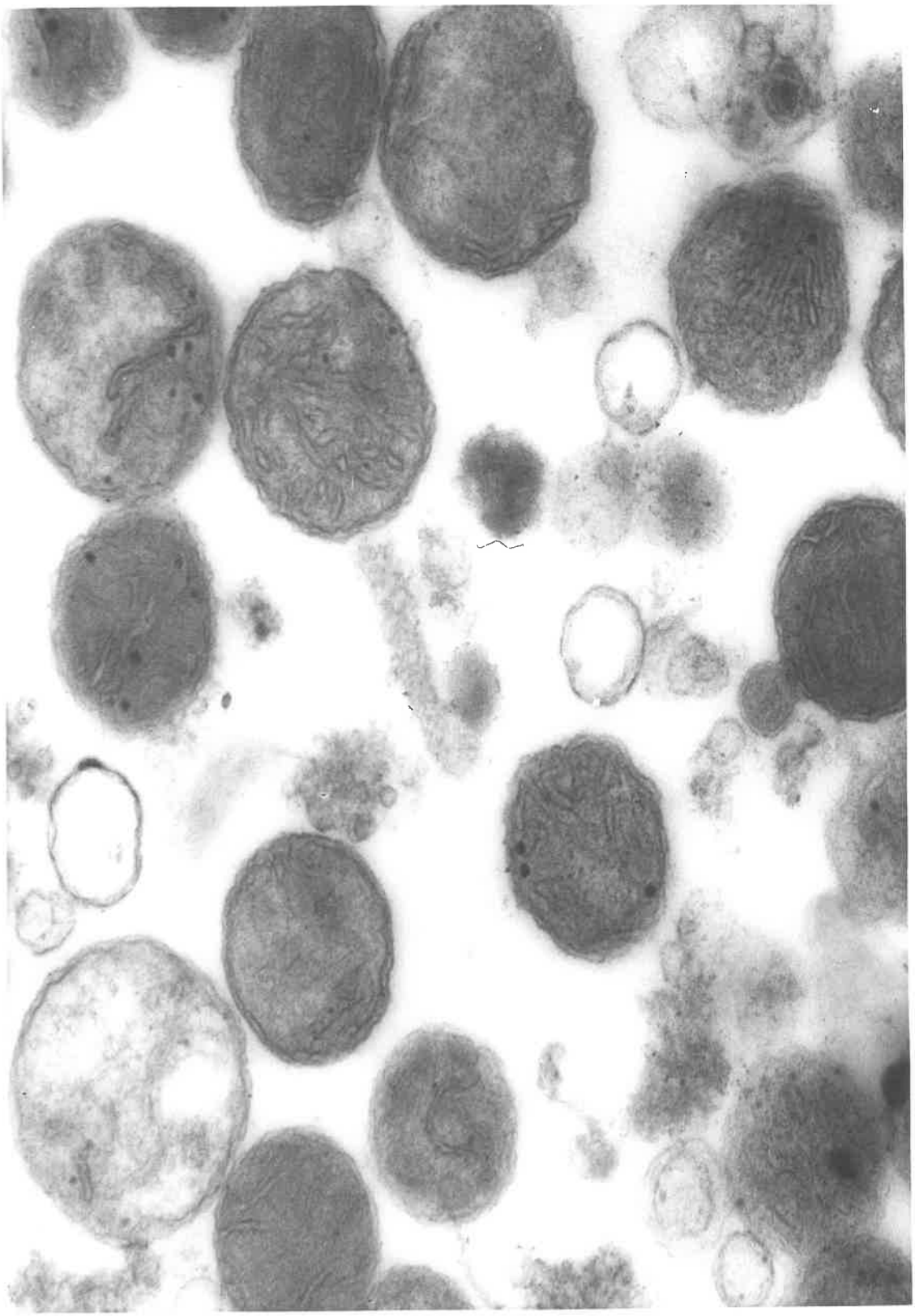
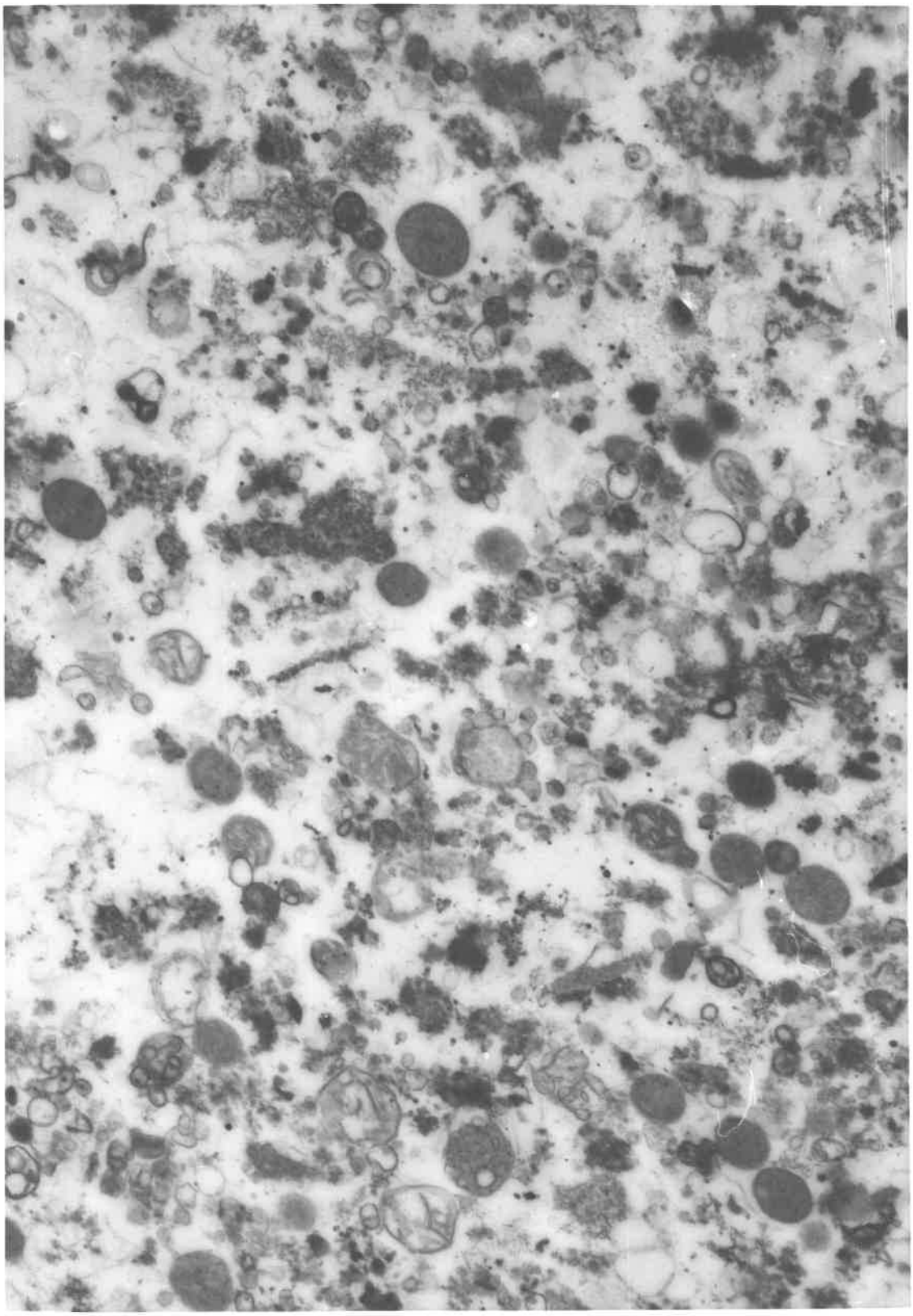


Figure III.23

Electron micrograph of organelles in band 6 of density gradient illustrated in Figure III.17. Ruptured mitochondria and small (0.2 - 0.4μ) electron dense bodies as well as a variety of vesicles are visible. Magnification: 32,000x.



appeared to be rich in only small pieces of cell wall (Figure III.24).

It was obvious that the fractions obtained on density gradients were rather heterogeneous even though biochemical analysis had indicated a fair degree of marker enzyme purity (Figure III.12). An attempt was therefore made to further purify bands 2, 3 and 4 so that the α-amylase-containing particles (lysosomes) could be visually identified more precisely.

A gradient was prepared identical to the one shown in Figure III.17 and bands 2, 3 and 4 were collected and layered on a new gradient. The new gradient was linear between 20% sucrose and 20% sucrose + 15% ficoll, and contained a 20% sucrose overlay. The gradient was centrifuged for 4 hr at 25,000 rpm and was assayed for both α-amylase and cytochrome c reductase activity. No separation of these two enzymes was achieved (Figure III.25). The band corresponding to the α-amylase peak was then prepared for electron microscopy.

The upper portion of this band (region 1, Figure III.25) was rich in three types of organelles; large vesicles (0.5 μ) often containing small electron dense bodies, vesicles of smaller (0.2 - 0.4 μ), irregular size made up of double membranes, and smaller slightly electron dense vesicles (0.1 - 0.2 μ) (Figure III.26). An examination of these three types of vesicles at a higher

Figure III.24

Electron micrograph of organelles in the pellet at the base of the gradient illustrated in Figure III.17. Small pieces of cell wall and some vesicles are visible. Magnification: 32,000x.

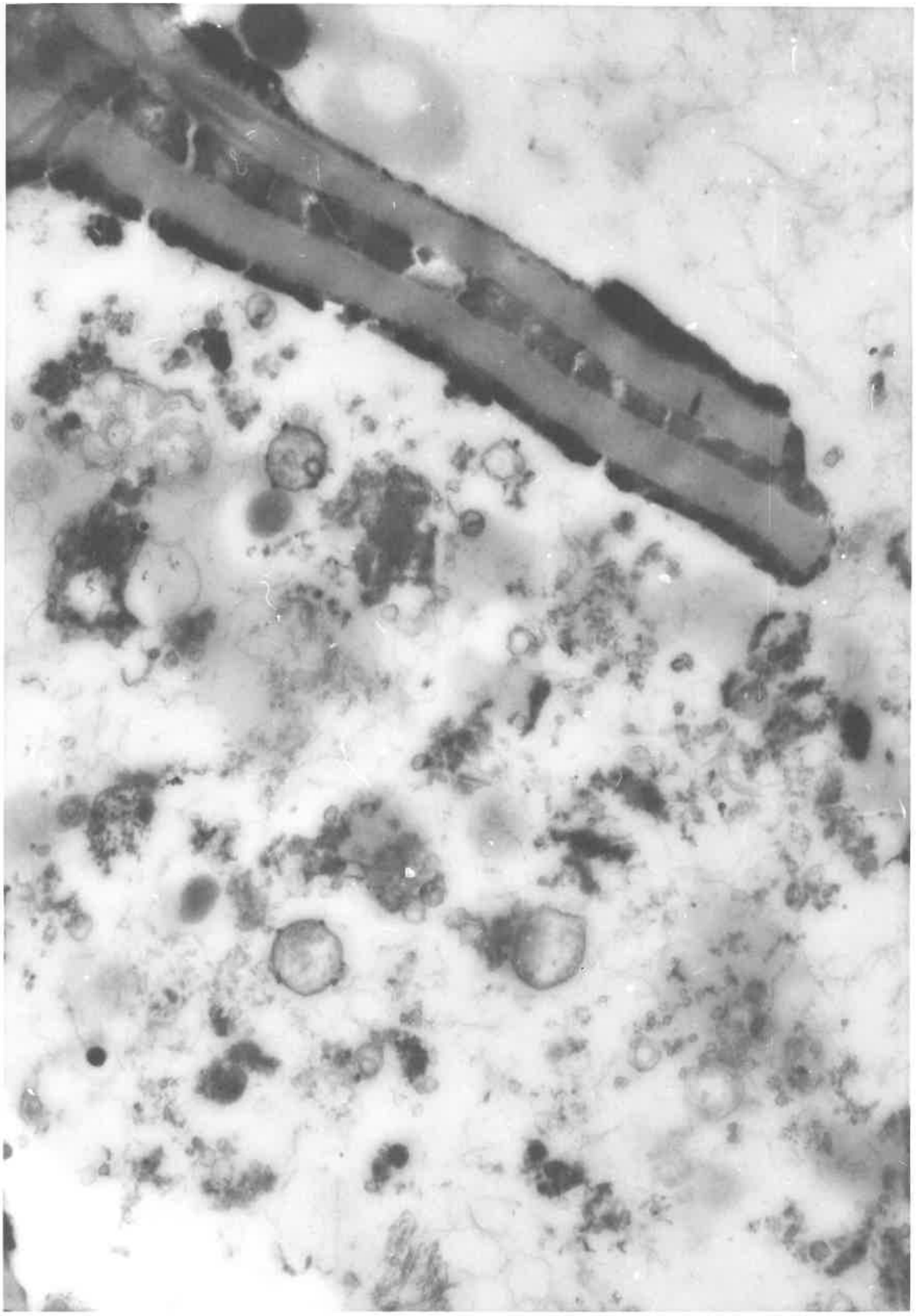
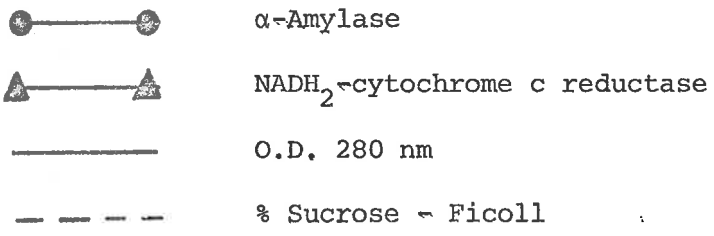


Figure III.25

Purification of lysosome rich peak obtained from a density gradient similar to that shown in Figure III.17 (including bands 2, 3 and 4) by a second centrifugation on a 20% sucrose - 20% sucrose + 15% ficoll density gradient. All solutions contained 1 mM EDTA and 0.01 M Tris-HCl (pH 7.0). Centrifugation time: 4 hr. Speed: 25,000 rpm. Temperature: 5°C. Rotor: SW25.1. α-Amylase and cytochrome c reductase assayed as described in the text and expressed as arbitrary units. Tissue: Aleurone treated with GA (1 µg/ml) for 24 hr at 30°C.



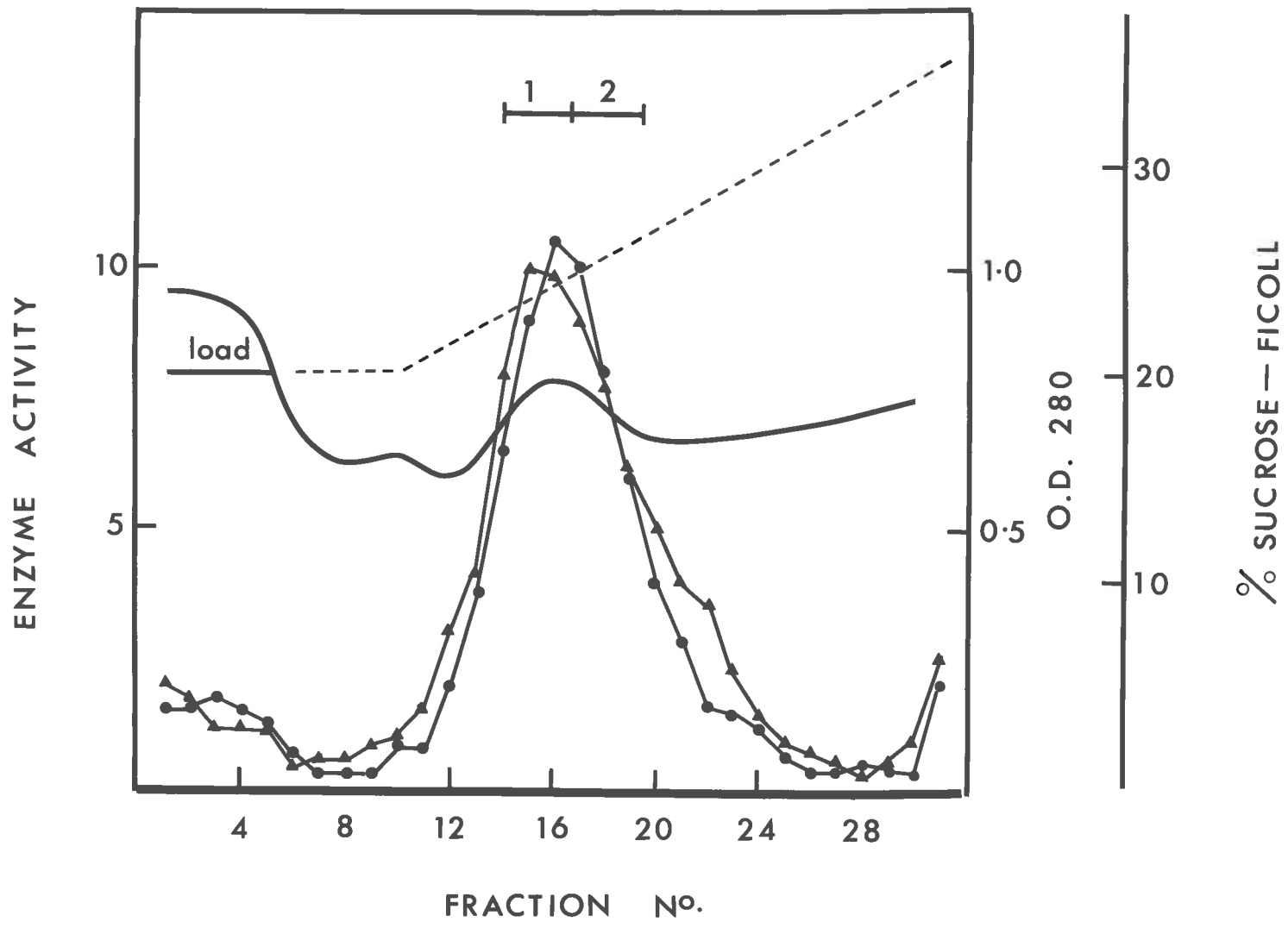

1
2
load
10
5
1·0
0·5
30
20
10
4
8
12
16
20
24
28
ENZYME ACTIVITY
O.D. 280
% SUCROSE — FICOLL
FRACTION No.

Figure III.26

Electron micrographs of organelles in region 1 of lysosome band of density gradient illustrated in Figure III.25. Large vesicles (0.5μ), small electron dense vesicles (0.1 - 0.2μ) and double-membraned vesicles (0.2 - 0.4μ) are visible. Magnification: 80,000x.

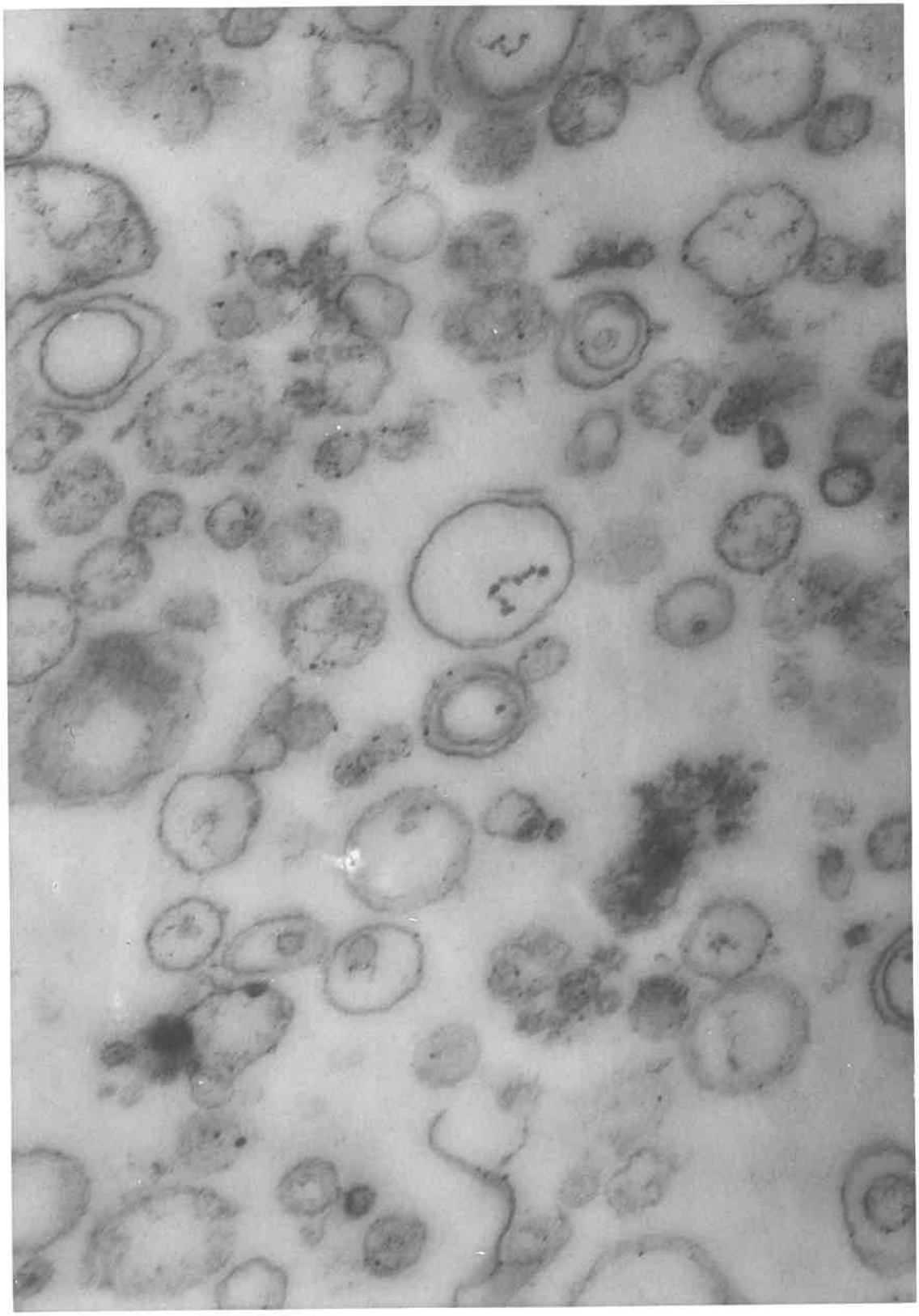
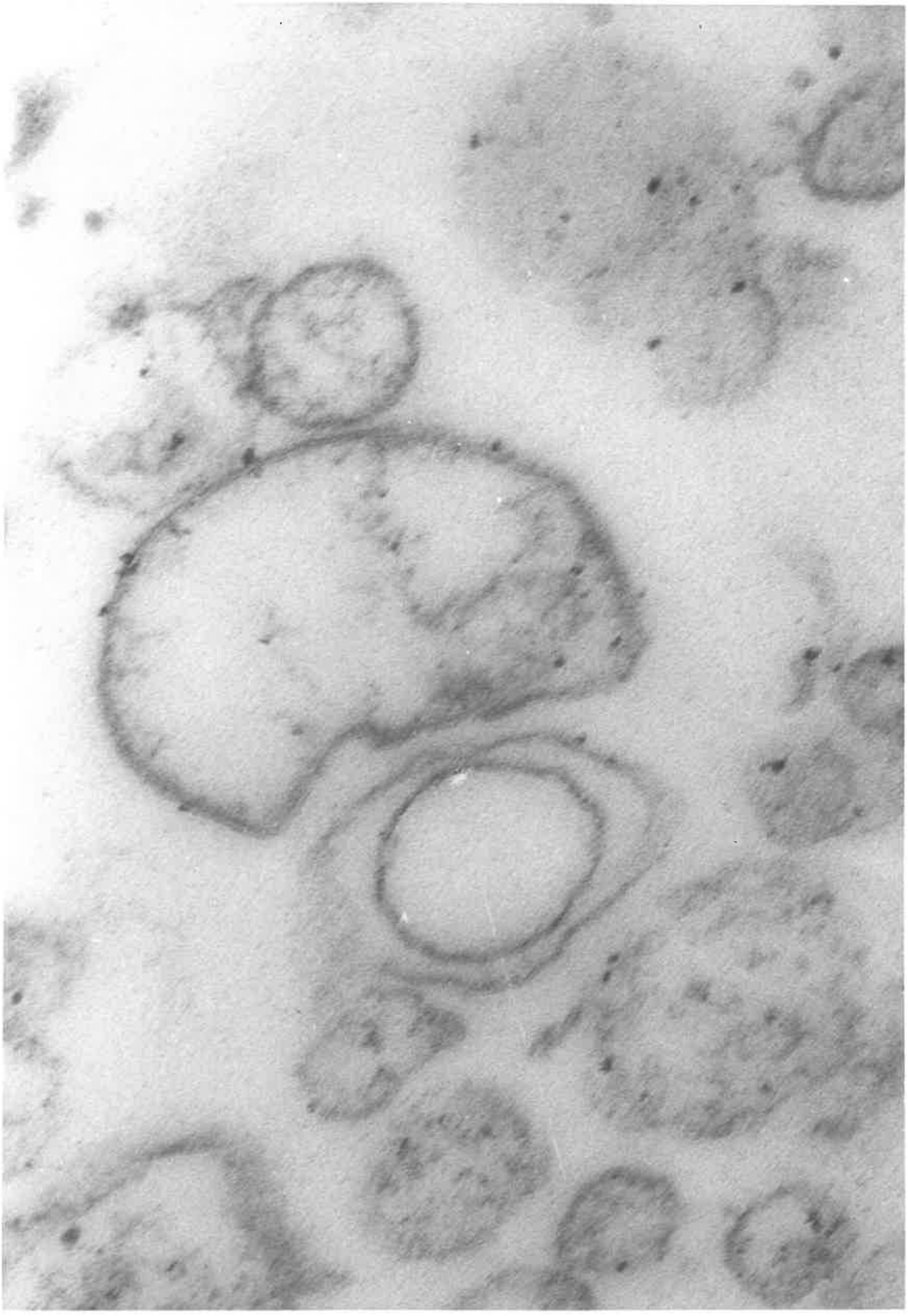


Figure III.27

Enlargement of a region of the electron micrograph illustrated in the previous figure (Figure III.26). Unit membranes are clearly visible in all three classes of vesicles. Magnification: 160,000x.



magnification (Figure III,27) showed that all were contained by unit membranes. Furthermore, the double-membrane structures were clearly constructed of two separate unit-membranes.

The lower portion of this band (region 2, Figure III.25) contained similar organelles as well as the long ER-like vesicles (Figure III.28) mentioned earlier.

It was obvious that considerable purification, though not a single homogeneous preparation of lysosomes, had been achieved.

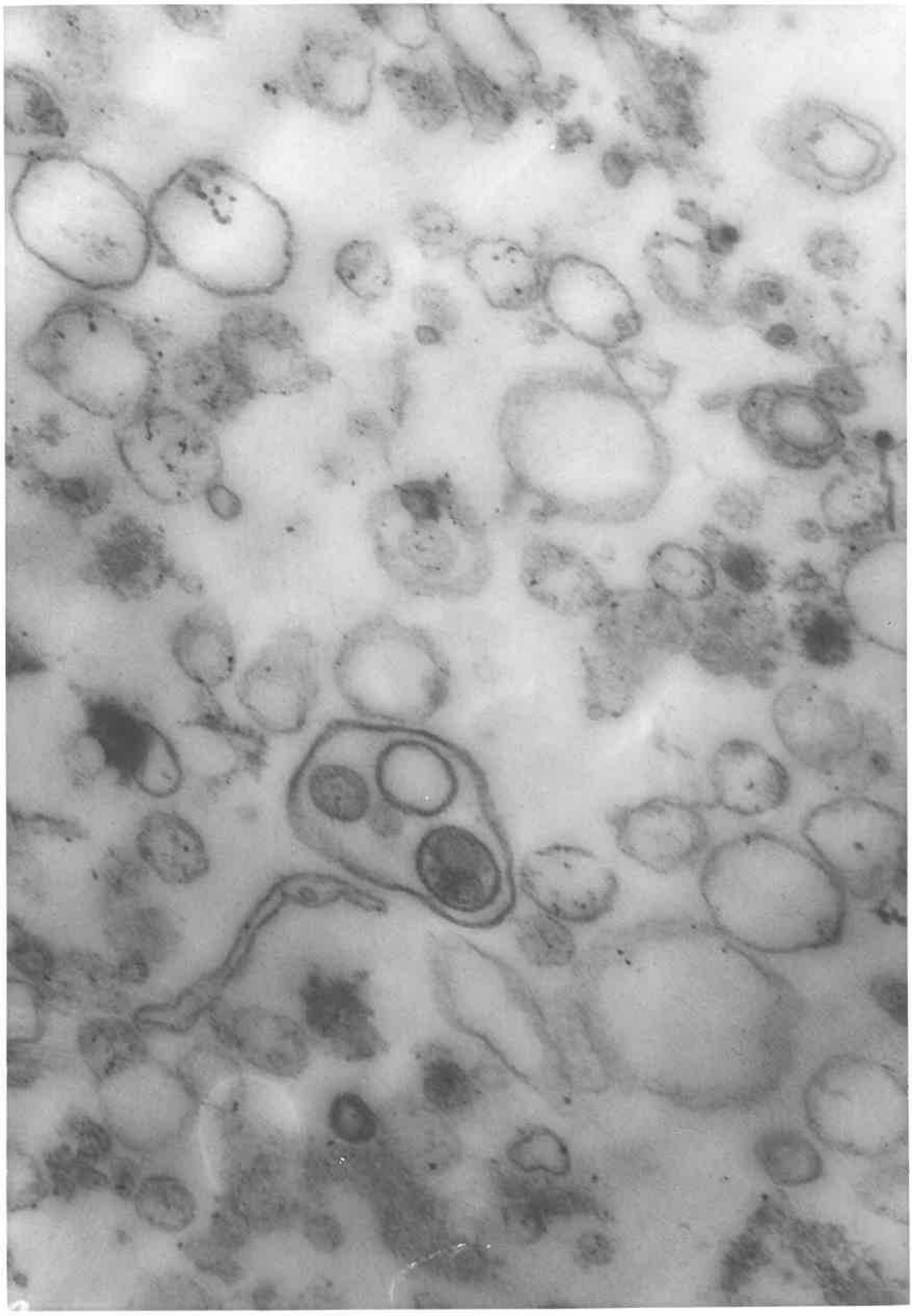
8. Discussion

It was pointed out in the previous section that although differential centrifugation was an adequate technique for determining whether an enzyme was particulate or not, the resolution was poor. For this reason, discussion as to the nature of aleurone cell lysosomes was minimal. For the same reason, isopycnic linear gradient centrifugation was adopted to examine in detail the origin and location of GA-induced acid hydrolases within the cell.

Considering that isopycnic centrifugation is a well established technique for organelle isolation (Tolbert, 1973), the degree of difficulty experienced in obtaining satisfactory separation of lysosomes from other organelles was surprising. Although the poor results obtained in preliminary experiments (Figure III.2) could be attributed to the presence of calcium (Figure III.3), the indifferent

Figure III.28

Electron micrograph of organelles in region 2 of lysosome band of density gradient illustrated in Figure III.25. Similar vesicles to those observed in Figure III.26 are visible as well as long, ER-like vesicles. Magnification: 80,000x.



resolution of organelles on sucrose density gradients even in the presence of EDTA (Figure III.9) was disappointing. Similar difficulties have been reported by Parish (1971) but the reasons are far from clear.

Although the separation of organelles was greatly improved when ficoll was used as the solute (Figure III.10) the results were again, not completely satisfactory. Pitt and Galpin (1973) also reported poor separation of lysosomes from potato shoots on sucrose density gradients and were forced to use ficoll gradients. However, the use of ficoll is difficult due to the viscosity of even dilute solutions although the use of the peristaltic pump device (Figure III.1) made preparation of the gradients easier. Furthermore, ficoll was found to seriously interfere with reagents used to measure protein and thus protein assays were impossible. The effect of ficoll on other organelles was not examined but it should be pointed out that Tolbert (1972) has reported serious losses of microbody marker enzymes when ficoll was used as the solute. So although ficoll-sucrose density gradients yielded the best separation of organelles, there is little doubt that better results are achievable.

Analysis of the sucrose-ficoll linear density gradient adopted (Figure III.12) revealed that excellent separation of lysosomes from other major organelles had been achieved. Cytochrome c oxidase,

a marker enzyme for mitochondria was located in band 5 and to a lesser extent, band 6. The conclusion that band 5 contained almost pure mitochondria was supported by the electron micrographs of this fraction (Figure III.22). The apparent buoyant density of mitochondria in ficoll gradient is 1.150 g/cc which is lower than the density (1.20 g/cc) normally reported for mitochondria (Rocha and Ting, 1970; Breidenbach and Beevers, 1967). This is almost certainly due to the use of ficoll which does not penetrate readily into organelles and whose viscosity slows the movement of organelles in the gradient (Pitt and Galpin, 1973).

The distribution of the microbody (peroxisome, glyoxysome) marker enzyme is puzzling. Catalase was found associated both with band 1 and band 6 (Figure III.12). As microbodies are reported to have a density of 1.25 g/cc (Breidenbach and Beevers, 1967), which would normally locate them immediately below mitochondria, the catalase activity at the top of the gradient (ρ = 1.06) is difficult to explain. Tolbert (1969) and Parish (1971) have reported similar observations. The failure to detect appreciable levels of the glyoxysomal marker enzyme, isocitratase, could be due to the removal of dithiothreitol from the gradients. This enzyme was detected in the original homogenates in high levels (data not shown). Although

microbodies may be visible in electron micrographs of band 6 (Figure III.23) they do not appear to be visible in band 1 (Figure III.18). Whether catalase is associated with the aleurone grains and derived vesicles which predominate in band 1 is difficult to determine from the available data. The activity in band 1 may simply represent soluble catalase. Because of the microbody-like organelles in band 6 and the coincidence of catalase in this region of the gradient, the electron dense bodies present (Figure III.23) have tentatively been called microbodies.

Bands 2 and 3 are probably more properly considered as one band. The enzyme activity profile and the electron micrographs indicate identity of components and the small discontinuity in the gradient (Figure III.17) would account for the formation of two peaks. This region of the gradient was found to be rich in both α-amylase and the ER marker enzyme, cytochrome c reductase. Attempts to displace these two enzymes by using discontinuous (Figure III.14) or shallow continuous (Figure III.13) gradients were unsuccessful. This does not indicate that the two enzymes are different forms of each other as cytochrome c reductase activity was still present in gradients of control tissue homogenates in which α-amylase could not be detected (Figure III.15). Rather, the data have been interpreted to mean that the density of lysosomes and ER are so close as to

preclude separation by this method and, perhaps, that the lysosomes are derived from the ER.

The lysosome region of the density gradient not only is rich in α-amylase but also contains most of the protease activity (Figure III.11). Ribonuclease on the other hand, must be considered to be either a soluble enzyme or, if lysosomal, very permeable with respect to the lysosomal membrane. Reports of ribonuclease activity associated with plant lysosomes (Matile, 1968; Pitt and Galpin, 1973) must be considered in the light of the work of Matsushita and Ibuki (1960) who showed that most of the particulate ribonuclease in peas was associated with ribosomes attached to membranes. Furthermore Hirai and Asahi (1973) have shown that ribonuclease associated with pea microsomes was only loosely attached to the membranes. It is also possible, as mentioned earlier, that 24 hr hormone-treated tissue does not have a sufficiently high percentage of newly formed ribonuclease to adequately identify it as lysosomal.

Wheat aleurone lysosomes appear to be associated with the ER as measured by cytochrome c reductase activity (Figures III.12 and III.13) and by incorporation studies with ^{14}C-lysine (Figure III.15). Thus, the site of synthesis of GA-induced α-amylase and protease is almost certainly the ER although direct proof of this proposal was not obtained in this study. However, because the particulate α-amylase was associated with the ER region of all gradients examined,

it is difficult to believe that the ER is not associated with α-amylase synthesis.

Measurement of amounts of ER in GA-treated and control tissue, by assaying cytochrome c reductase in the ER region of gradients (Figure III.15), failed to indicate a significant difference between treatments. Thus, reports by Jones (1969a, b) and Vigil and Ruddat (1973) of massive proliferation of RER in response to GA treatment in barley aleurone cells must be questioned. While it is conceivable that increases in ER could occur in the absence of a concomitant increase in ER-associated cytochrome c reductase, this is not considered probable. Since cytochrome c reductase has been definitely shown to be associated with the ER in animals (Goldstone *et al.*, 1973) and plants (Moore *et al.*, 1973) it is considered that this enzyme is probably a good measure of ER level when assayed from the correct region of a density gradient. It is concluded, therefore, that the cytological observations of apparent ER proliferation (Jones, 1969a, b; Vigil and Ruddat, 1973) may have been actually an unmasking of pre-existing ER as the aleurone cells expand greatly during imbibition and the aleurone grains and spherosomes reduce in number in response to GA (Jones, 1969a, b).

Although animal lysosomal acid hydrolases are generally thought to be synthesized on the ribosomes of the RER and transported

to the golgi for packaging into primary lysosomes (de Duve and Wattiaux, 1966) it is only recently that firm experimental evidence has been obtained for the involvement of RER in lysosomal enzyme synthesis (Goldstone *et al.*, 1973). However, the ER has long been implicated in the synthesis of plant lysosomal enzymes and membranes (Matile, 1969) by both cytological and biochemical observations.

An examination of particulate acid hydrolases in corn roots by Matile (1968) indicated that two distinct classes of lysosomes were present. Light lysosomes (ρ = 1.06 g/cc) were correlated with vacuoles while heavy lysosomes which had an apparent buoyant density of about 1.11 g/cc were thought to have been derived from the ER. The heavy lysosomal membranes showed a striking similarity to ER membranes when examined by the freeze-etch process and the lysosomes were rich in cytochrome c reductase activity.

Hydrolytic enzymes have also been detected in spherosomes of tobacco (Spichiger, 1969) and corn (Semadeni, 1967) seedlings and these organelles have thus been implicated as lysosomes. Semadeni (1967) isolated three populations of lysosomes, two of which he determined were spherosomes while the other (ρ = 1.070 g/cc) appeared to be ER-derived vesicles. The latter fraction also contained α-amylase activity. Morphological evidence in favour of a homology between ER and spherosome membranes has been presented by Frey-Wyssling

et al. (1963).

More recently Pitt and Galpin (1973) examined the distribution of several acid hydrolases of homogenates of potato shoots using ficoll density gradients. These workers found two populations of lysosomes corresponding to densities of 1.10 and 1.07 g/cc. Both fractions were rich in RNA and were probably derived from the ER. Electron micrographs of the fractions revealed the presence of a heterogeneous collection of vesicles, many of which contained double membranes similar to those observed in Figure III.26 in the present study. Furthermore, the density of aleurone lysosomes observed in this study (1.080 g/cc) is in close agreement with the density calculated for potato lysosomes by Pitt and Galpin (1973).

Finally, Hirai and Asahi (1973) critically re-examined the reports of several populations of lysosomes in plant roots (Matile, 1968). These workers (Hirai and Asahi, 1973) demonstrated that although density gradient experiments indicated at least two lysosome populations (as measured by acid hydrolases), neither population could be separated from cytochrome c reductase activity. It was therefore concluded that root lysosomes at least are all ER-derived and the separate populations obtained were probably due to preparative artefacts.

Attempts at purification of lysosomes were only partially successful in that the purest fraction obtained (Figure III.26) contained at least three different vesicles. It was earlier reasoned that if lysosomes were budded off the ER there should be two populations of hydrolase-containing vesicles in cell homogenates; one group arising as a result of the grinding procedure which is known to cause the formation of vesicles from the sheets of ER (Mathius, 1966), and a second group, naturally derived by budding off the ER as observed by Vigil and Ruddat (1973). Although these two populations of vesicles must be present in aleurone cell homogenates the techniques used failed to resolve them and they may, in fact, be unresolvable.

The double-membrane vesicles frequently observed in purified lysosome preparations (Figures III.26 and III.28) probably represent vesicles formed by the grinding procedure. Such structures have been demonstrated by other workers to be derived directly from the ER (Hirai and Asahi, 1973; Pitt and Galpin, 1973). The smaller, slightly electron dense particles also seen in purified fractions (Figure III.26) may be naturally derived lysosomes although no data are available on this point.

As EDTA was found to be essential in both the grinding

medium and the density gradients (Figure III.9) to obtain reliable organelle separation, an essential structure to visibly distinguish between naturally-occurring and preparative ER vesicles was removed, ribosomes. Vigil and Ruddat (1973) reported that ER-derived vesicles found in the vicinity of the plasmalemma were free of ribosomes. As EDTA is known to cause dissociation of ribosomes from ER membranes (James *et al.*, 1969), and separation of RER from ribosome-free ER is routinely achieved (Bloemendal *et al.*, 1967), a separation of the two populations of vesicles is theoretically possible if organelle isolation could be achieved in the absence of EDTA. This may not be possible with aleurone tissue and α-amylase as the marker enzyme.

It is clear, from a functional standpoint at least, that α-amylase and protease qualify as lysosomal enzymes. They are acid hydrolases almost certainly synthesized and packaged on the ER (Figures III.9 and III.15) and they exhibit structural latency (Section II). That these enzymes are secreted and are also under hormonal control makes them additionally interesting.

Although lysosomes have been identified in most animal tissues (Dingle and Fell, 1969), very few lysosomal enzymes from these tissues are actively secreted from the cells. However, the few cases that have been reported do appear to be under hormonal control. Secretion of protease by bone cells, for example, is

controlled by parathyroid hormone and the secretion of a similar protease from thyroid cells is stimulated by thyroid stimulating hormone (Dingle, 1969). Although Balz (1966) has reported a measure of control of synthesis of the lysosomal enzymes ribonuclease, protease and esterase in senescing tobacco leaves by kinetin, these enzymes are not secreted. Thus, the GA-induced synthesis and secretion of lysosomal enzymes by cereal aleurone cells appears to be unique.

SECTION IV

LEAKAGE OF α-AMYLASE FROM WHEAT ALEURONE LYSOSOMES

This Section is concerned with the permeability of lysosomal membranes to GA-induced α-amylase. Lysosomal membrane permeability was increased by low and high pH, and calcium but was decreased by phosphate. GA was found to have no effect on α-amylase leakage from isolated lysosomes *in vitro*, but caused a significant increase in the permeability of lysosomal membranes *in vivo*. Lysosomes from tissue treated with high levels of GA (100, 1 µg/ml) released more α-amylase at elevated temperatures than lysosomes from tissue treated with lower (0.1, 0.01 µg/ml) hormone levels.

i. Lysosome Isolation

Unless otherwise stated, the words lysosomes or lysosome preparation, are used to describe the α-amylase-containing particles in a 60,000 *g* pellet obtained by the methods described in Section II. All lysosome preparations were obtained from aleurone tissue isolated by the lactic acid procedure, allowed to recover from rolling for 16 hr at 30°C in 5 mM calcium nitrate, and incubated for 24 hr at 30°C in a solution containing 10 mM glucose, 5 mM calcium nitrate

plus or minus GA at a final concentration of 1 µg/ml.

The tissue was washed exhaustively in distilled water and homogenized in a medium containing 0.4 M sucrose, 0.05 M Tris-HCl (pH 7.0), 0.1% BSA, 10 mM KCl, 0.1 mM $MgCl_2$ and 1 mM EDTA. The tissue : grinding medium ratio was always 1 : 10 (g/ml). When phosphate grinding medium was employed the composition of the medium was the same except that 0.05 M KH_2PO_4 was substituted for Tris-HCl as the buffer.

After grinding the tissue in either an Ultra Turrax or mortar and pestle the homogenate was filtered through 2 layers of cheesecloth. The filtrates from identical tissue (e.g. control or 1 µg/ml GA treated) were combined and thoroughly mixed before 10 ml aliquots were removed for centrifugation at 1,000 *g* for 10 min at 0-2°C. The subsequent supernatants were decanted and mixed as before, prior to the removal of 10 ml aliquots for centrifugation at 60,000 *g* for 30 min at 0-2°C. The resulting supernatant was discarded and the pellets resuspended in 1 ml of medium with the aid of a plastic piston device (Section III) having 0,1 mm clearance. Unless otherwise stated the resuspension medium contained 0.4 M sucrose and 0.05 M Tris-HCl (pH 7.0). The pellets were resuspended by maintaining a twisting-pumping action with the piston device until a creamy, homogeneous suspension was obtained (no more than 30 sec). Any

additions were made at this stage in a volume of 0.1 ml. When additions of GA were required, the hormone was included in samples of resuspension medium at the concentration required.

2. Leakage of α-Amylase

During resuspension of the 60,000 *g* pellets, the samples were kept on ice until all operations were completed. The resuspended lysosomes, still in polyallomer centrifuge tubes, were transferred to circulating water baths, pre-set to the required temperature, for 30 min. The tubes were then removed from the water baths, made up to 10 ml with ice cold 0.4 M sucrose and centrifuged at 60,000 *g* for a second time. The resulting supernatant was poured into glass test tubes and calcium acetate added to give a final concentration of 5 mM with respect to calcium. When phosphate was used as the buffer, sufficient (twice the molarity of phosphate) calcium was added to overcome its tendency to precipitate the calcium present. Just prior to the α-amylase assay, and after heating for 20 min at 70°C, the supernatant fractions were adjusted to pH 5.0 and made up to a known volume with distilled water.

The pellets obtained from the second centrifugation were treated for 5 min with 1 ml of 0.1% Triton X-100 during which time they were stirred with a glass rod. After adding 4 ml of 5 mM calcium acetate to each tube, the contents were individually transferred

to a Kontes glass homogenizer (7 ml volume), and the membranous material thoroughly ground by hand. After transferring the homogenized pellet suspension to a glass test tube, the original centrifuge tube and the glass homogenizer were washed thoroughly with 5 ml of 5 mM calcium acetate. The washings were added to the glass test tube and the combined sample (10 ml) heated for 20 min at 70°C and assayed for α-amylase activity.

The amount of α-amylase detected in the supernatant fraction was calculated as a percentage of the total α-amylase for that treatment (i.e. supernatant + pellet = total). Since the α-amylase in the supernatant represents enzyme not enclosed by membranes, it is referred to as "% free" enzyme.

Because incubations under various conditions were potentially capable of inactivating α-amylase, a control sample was included in each experiment. The control sample was resuspended in 5 mM calcium acetate and set aside for later assay. The total α-amylase detected in the incubated samples was calculated as a percentage of the total enzyme measured in the control sample and is referred to as "% recovered".

3. Enzyme Assays

α-Amylase. The method used to assay this enzyme has been described in detail in Section I.

Cytochrome c reductase was assayed according to the method of Wray and Filner (1970) by following the reduction of cytochrome c in the presence of $NADH_2$ at 25°C. Enzyme activity is expressed in ΔE_{550}/min.

4. Spin-label Studies

Phase changes in the lipid components of lysosomal membranes were inferred from changes in the effects of temperature on the motion of the spin-labelled analogue of methyl stearate (M12NS) as determined by electron spin resonance (esr) spectroscopy. Preparations of isolated lysosomes obtained from a 12,000 - 60,000 *g* pellet were infused with spin label, and the spectra were recorded with a Varian E9 spectrometer. The instrument was fitted with a temperature control unit which maintained the temperature of the sample (0.2 ml) at ± 0.1°C. The motion of the spin label (T_o) was calculated as described by Raison *et al.* (1971).

5. Effect of GA on Wheat Aleurone Lysosomes

It has been clearly demonstrated in the previous sections of this thesis that the GA-induced hydrolases, α-amylase and protease, are lysosomal. Although little information is available about plant lysosomes, animal lysosomal enzymes have been investigated for a number of years (see Dingle and Fell, 1969) and the mechanism of

action of steroid hormones has received particular attention. The ultimate manifestations of steroid hormones are manifold, but one of the earliest measurable events is the rapid (2-30 min) change in the permeability of lysosomal membranes (Szego, 1972a, b; Szego and Seeler, 1973).

It was of interest to determine whether GA, which shares many of the properties of animal steroid hormones, affected the permeability of cereal aleurone lysosomes. In other words, does GA share a common mechanism of action with steroid hormones as has been suggested by the work of Wood and Paleg (1972, 1974)?

An established procedure for investigating the effects of drugs on animal lysosomes is to measure the rate of leakage of a hydrolytic enzyme from isolated lysosomes at various temperatures (Dingle and Fell, 1969). Any compound that results in more free enzyme than control values, after incubation for known times, is assumed to act by increasing the permeability of the lysosomal membrane thus allowing more enzyme to leak out into the incubation medium.

Accordingly an experiment was carried out to determine if GA had any effect on isolated lysosomes obtained from aleurone cells.

Although GA at the highest concentration (1,000 µg/ml) appeared to result in less enzyme leaking from the lysosomes into the

surrounding medium (Table IV.1 and Figure IV.1), there were several disturbing features of the experiment. The amount of α-amylase in the final supernatant after incubation at 5°C (free enzyme) was higher than that observed in previous experiments and, generally, the control values showed little effect of temperature. Equally disturbing, the percentage of α-amylase recovered in each treatment varied from 87 to 112%. Furthermore, measurement of the supernatant fractions obtained after the final centrifugation showed that the highest GA concentration (1,000 µg/ml) had caused a decrease in the pH from 7.0 to 6.1. Consequently the apparent effect of high concentrations of GA on lysosomal leakage could possibly be accounted for by the pH change. It was concluded that an examination of the effects of incubation conditions was essential before any more attempts were made to determine possible GA effects on lysosomes.

5.1 Effect of Incubation Conditions on Stability of Lysosomes

The composition of the medium used to resuspend the 60,000 *g* pellet was examined to see if all the constituents were necessary for stable lysosomes. The results of this experiment are listed in Table IV.2.

Calcium, which had been included in the resuspension medium to stabilize the α-amylase molecules, appeared to cause an increase in leakage of the enzyme from the lysosomes. The other constituents

Table IV.1

Effect of GA on lysosomes isolated from GA (10 µg/ml) treated aleurone cells. The 60,000 *g* pellet preparations were resuspended in 1 ml of the medium described for Figure IV.1, plus or minus GA at the concentrations shown and incubated for 30 min at the temperature indicated. The resulting percentage free enzyme was determined by the centrifugation method described.

GA (µg/ml)	Temperature (°C)	α-Amylase % free	% recovered
0	5	41	87
	15	45	93
	30	44	100
10	5	42	94
	15	47	96
	30	51	100
100	5	42	101
	15	46	101
	30	49	101
1000	5	38	110
	15	41	103
	30	43	112

100% = 36.4 units

Table IV.2

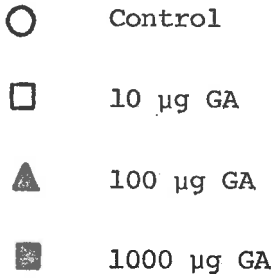
Effect of various compounds in the resuspension medium (described in Figure IV.1) on leakage of α-amylase from lysosomes. The 60,000 *g* pellet preparations were obtained from homogenates of aleurone tissue incubated for 24 hr in GA (10 µg/ml). Time of lysosome incubation: 30 min. Abbreviations: Ca^{++}, calcium; BSA, bovine serum albumin; MSH, 2-mercaptoethanol; $BrO_3^=$, potassium bromate.

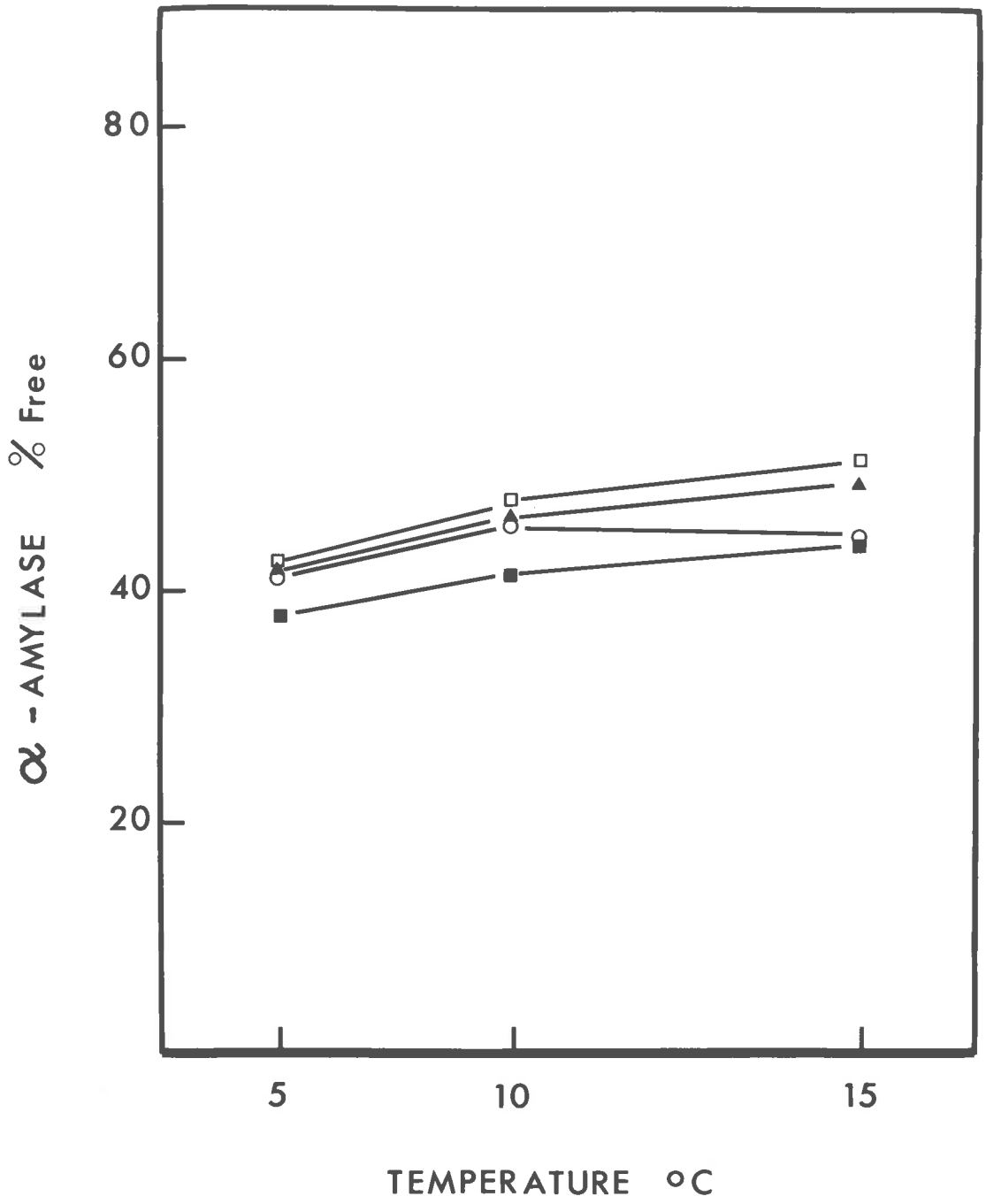
Treatment	α-Amylase % Free	α-Amylase % Recovered
5°C		
Resuspension medium	34	104
" " + Ca^{++}	25	103
" " + BSA + Ca^{++}	22	110
0.4 M sucrose	27	112
30°C		
Resuspension medium	66	100
" " + Ca^{++}	39	97
" " + BSA + Ca^{++}	32	99
0.4 M sucrose	38	97
Resuspension medium + Ca^{++}		
" " + $BrO_3^=$ (1 mg)	32	89
" " + MSH (0.01 M)	38	101
" " + Lecithin (1 mg)	37	98

100% = 35.0 units

Figure IV.1

Effect of addition of various concentrations of GA on leakage of α-amylase from lysosomes isolated from aleurone tissue treated with 10 µg/ml GA for 24 hr at 30°C. Lysosomes resuspended in a medium containing 0.01 M Tris-HCl (pH 7.0), 5 mM calcium nitrate, 0.1% BSA and 0.4 M sucrose. Lysosomes incubated for 30 min at the temperature indicated.




80
60
40
20
α - AMYLASE % Free
5
10
15
TEMPERATURE °C

of the medium had little effect on α-amylase leakage. Bovine serum albumin (BSA) was not included in subsequent media. Potassium bromate ($BrO_3^=$) which was included to prevent protease action and lecithin which was added to provide an alternative substrate for any possible phospholipases present, were also without effect. The reducing agent 2-mercaptoethanol (MSH) also did not lower the amount of α-amylase that leaked from the lysosomes at 30°C.

Because calcium has been reported to be necessary for the stability of certain membranes (Mathias, 1966) the effect of this cation was re-investigated over a range of concentrations (Table IV.3). Although calcium consistently improved the total amount of α-amylase recovered after incubation at 30°C this ion also increased the amount of free α-amylase and the effect appeared to be concentration dependent. Calcium was not included in subsequent resuspension media.

Because it had been reported that the stability of certain plant organelles was greatly affected by the concentration of the osmoticum (Miflin, 1970) and the type of sugar used as an osmoticum (Parish, 1971), these factors were investigated in the next experiment.

The results of the experiment (Table IV.4) indicated that leakage of α-amylase was inversely proportional to the concentration of sucrose but that the effect was not great. Slightly higher leakage rates were obtained when sorbitol or mannitol were used. The concentration of osmoticum had no effect on the amount of α-amylase

Table IV.3

Effect of calcium on leakage of α-amylase from lysosomes isolated from GA (10 µg/ml) treated aleurone. Conditions as described for previous Table. Temperature: 30°C. Time: 30 min.

Calcium (mM)	α-Amylase	
	% free	% recovered
0	41	95
0.1	46	96
1.0	47	98
5	52	101
10	55	102

100% = 37.8 units

Table IV.4

Effect of osmoticum on leakage of α-amylase from lysosomes isolated from GA (10 µg/ml) treated aleurone. Resuspension medium contained 0.01 M Tris-HCl (pH 7.0) and sugars at the concentration indicated. Time: 30 min. Temperature: 30°C.

Osmoticum	α-Amylase	
	% Free	% Recovered
0.2 M Sucrose	43	101
0.4 M "	41	100
0.6 M "	39	97
0.8 M "	34	101
0.4 M Sorbitol	45	101
0.4 M Mannitol	47	101

100% = 37.8 units

recovered. Because 0.4 M sucrose was used in the original homogenization medium and because this concentration did not adversely affect enzyme leakage, the molarity of sucrose in the resuspension medium was retained at 0.4.

As the leakage of hydrolytic enzymes from lysosomes of animal origin have been reported to be affected by the pH of the incubation medium (Sawant *et al.*, 1964), this parameter was investigated in lysosomes isolated from GA-treated aleurone tissue. Leakage of α-amylase was minimal between pH 6.5 - 7.0 which also was the range over which greatest recoveries of the enzyme were obtained (Figure IV.2 and Table IV.5). α-amylase appeared to be most stable at or near neutrality for at low pH values loss of total enzyme activity was greatest. Subsequent resuspension media were prepared at pH 7.0.

It was also considered essential to investigate the effect of phosphate on the leakage of α-amylase from aleurone lysosomes as this anion is found in high levels in aleurone cells and is secreted in response to GA (Eastwood and Laidman, 1971). Furthermore, potassium phosphate has been used as a buffer for many years. It was also desirable to check the effects of an alternative buffer, tris.

Although lysosomes in tris leaked about the same percentage of α-amylase into the medium as was observed in earlier experiments, leakage of the enzyme in the phosphate medium was extremely low at both temperatures (Table IV.6). Phosphate did not appear to adversely

Figure IV.2

Effect of pH on the leakage of α-amylase from lysosomes isolated from GA-treated (10 µg/ml) aleurone tissue. Lysosomes resuspended in a medium containing 0.05 M Tris-acetate buffer at the pH indicated and 0.4 M sucrose. Incubation time: 30 min. Temperature: 30°C.

● α-Amylase (% Free)

■ α-Amylase (% Recovered)

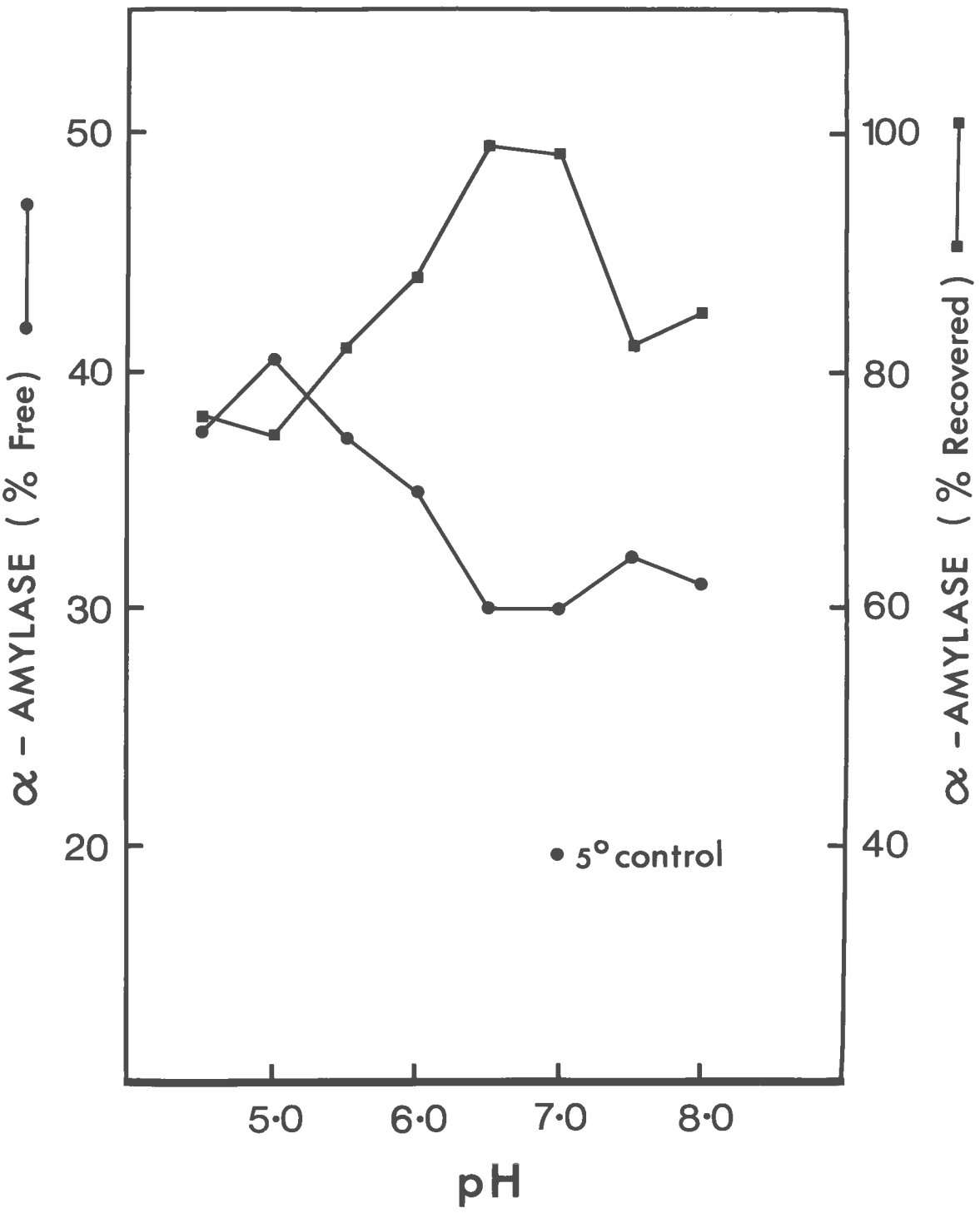

α - AMYLASE (% Free)
α - AMYLASE (% Recovered)
50
40
30
20
100
80
60
40
5·0
6·0
7·0
8·0
pH
• 5° control

Table IV.5

Effect of pH on leakage of α-amylase from lysosomes isolated from GA-treated (10 µg/ml) aleurone tissue. The resuspension medium contained 0.05 M Tris-acetate buffer at the pH indicated and 0.4 M sucrose. Incubation time: 30 min. Temperature: 30°C.

pH	α-Amylase	
	% Free	% Recovered
Control (pH 7.0, 5°C)	19.6	101
4.5	37.5	76
5.0	40.6	75
5.5	37.2	82
6.0	35.0	88
6.5	29.9	99
7.0	30.0	98
7.5	32.2	82
8.0	31.0	85

100% = 49.6 units

Table IV.6

Comparison of effects of tris and phosphate buffers on leakage of α-amylase from lysosomes isolated from GA (10 µg/ml) treated aleurone layers. The 60,000 *g* pellet preparations were resuspended in media containing tris (0.05 M) or phosphate (0.05 M) and incubated for 30 min at the indicated temperature. All media were prepared at pH 7.0.

Treatment		α-Amylase % Free	α-Amylase % Recovery
5°C	Tris	20	100
	Phosphate	5	99
30°C	Tris	39	98
	Phosphate	10	101

100% = 51.9 units

affect the amount of α-amylase recovered at either temperature. However, phosphate at the concentration used (0.05 M) caused the formation of massive amounts of precipitate (calcium phosphate) when calcium was added to the final supernatant fraction at the end of the experiment. This made the assay of α-amylase very difficult. As the amount of precipitate was lessened when the concentration of phosphate was reduced, without significantly increasing the percentage free enzyme, phosphate was used at a concentration of 0.01 M in subsequent experiments.

It was important to determine if the reduced leakage of α-amylase observed with phosphate was specific for that ion or whether other ions could substitute. The effect of phosphate was therefore compared with arsenate which is also a divalent anion with four oxygen atoms. Since arsenate has poor buffering capacity it was added in the presence of tris buffer. Also, because α-amylase secretion has been reported to be an energy-requiring process (Varner and Mense, 1972), the phosphorylated compounds ATP and ADP were also tested.

Regardless of whether phosphate was present alone or with tris or ATP the leakage of α-amylase was always about 27% compared with 40% for tris control (Table IV.7). The amount of free enzyme measured with arsenate, ATP or ADP in the presence of tris was almost the same as found for tris alone. It was concluded that arsenate, ATP and

Table IV.7

Effect of phosphate, arsenate and phosphorylated nucleotides on the leakage of α-amylase from lysosomes isolated from GA (1 µg/ml) treated aleurone layers. All media were prepared at pH 7.0. Incubation period: 30 min. Temperature: 30°C

Treatment	α-Amylase	
	% Free	% Recovered
Phosphate (0.01 M)	27	90
Tris (0.05 M)	40	100
Tris + phosphate	28	98
Tris + arsenate	36	100
Tris + ATP (1 mg)	36	91
Tris + ADP (1 mg)	40	97
Tris + phosphate + ATP	27	85

100% = 38.0 units

ADP did not affect the lysosomal membrane and that the effect of phosphate was specific.

5.2 Further Effects of GA on Lysosomes

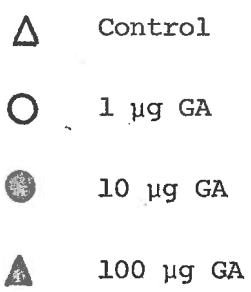
Having examined many of the factors that affected α-amylase leakage from lysosomes it was decided to re-examine the *in vitro* response to GA. It was reasoned that there may be a limited number of binding sites on the membrane, and if so, they may be fully occupied on lysosomal membranes isolated from tissue incubated in high (10 µg/ml) concentrations of GA. Accordingly lysosomes were isolated from tissue treated with lower amounts of the hormone (0.1 or 1.0 µg/ml) and subsequently incubated in the presence of GA at various concentrations. The GA solutions were adjusted to pH 7.0 prior to addition to the lysosomes.

Although the amount of free α-amylase detected in control lysosomes was encouragingly low from both 0.1 and 1.0 µg/ml GA-treated tissues, GA had little *in vitro* effect at any of the concentrations tested (Figures IV.3 and IV.4). Since the amount of α-amylase recovered from each treatment was high (Table IV.8) it was concluded that under these conditions at least, GA did not cause an increase in the permeability of the lysosomal membrane.

Analysis of variance of the data contained in Table IV.8 confirmed that there was no effect of direct addition of hormone to

Figure IV.3

Effect of addition of various concentrations of GA on leakage of α-amylase from lysosomes isolated from aleurone tissue treated with 0.1 µg/ml GA for 24 hr at 30°C. Lysosomes resuspended in a medium containing 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose, and incubated for 30 min at the temperature indicated.



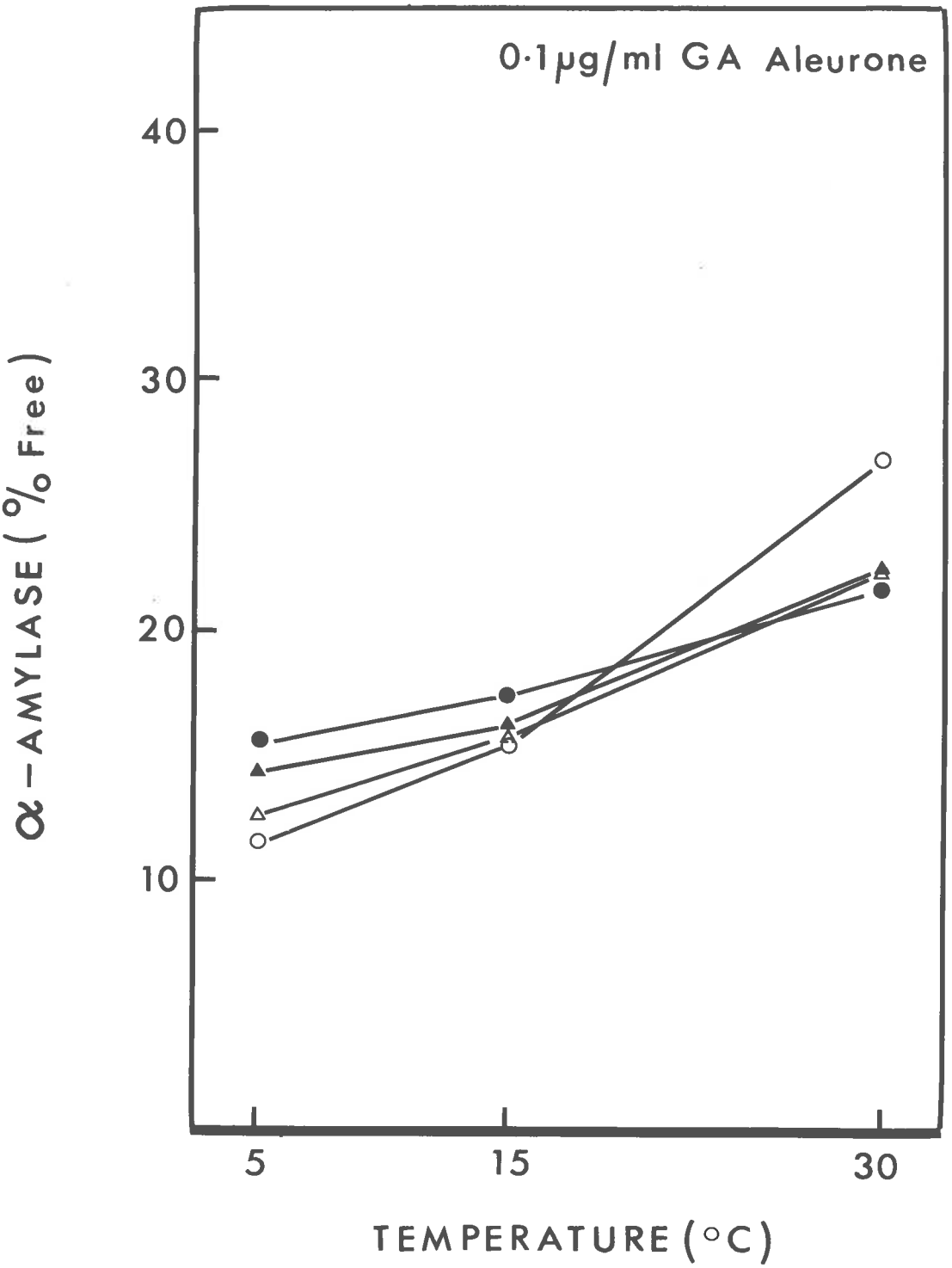

0·1µg/ml GA Aleurone
40
30
20
10
α – AMYLASE (% Free)
5
15
30
TEMPERATURE (°C)

Figure IV.4

Effect of addition of various concentrations of GA on leakage of α-amylase from lysosomes isolated from aleurone tissue treated with 1.0 µg/ml GA for 24 hr at 30°C. Lysosomes resuspended in a medium containing 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose and incubated for 30 min at the temperature indicated.

△ Control

○ 1 µg GA

10 µg GA

100 µg GA

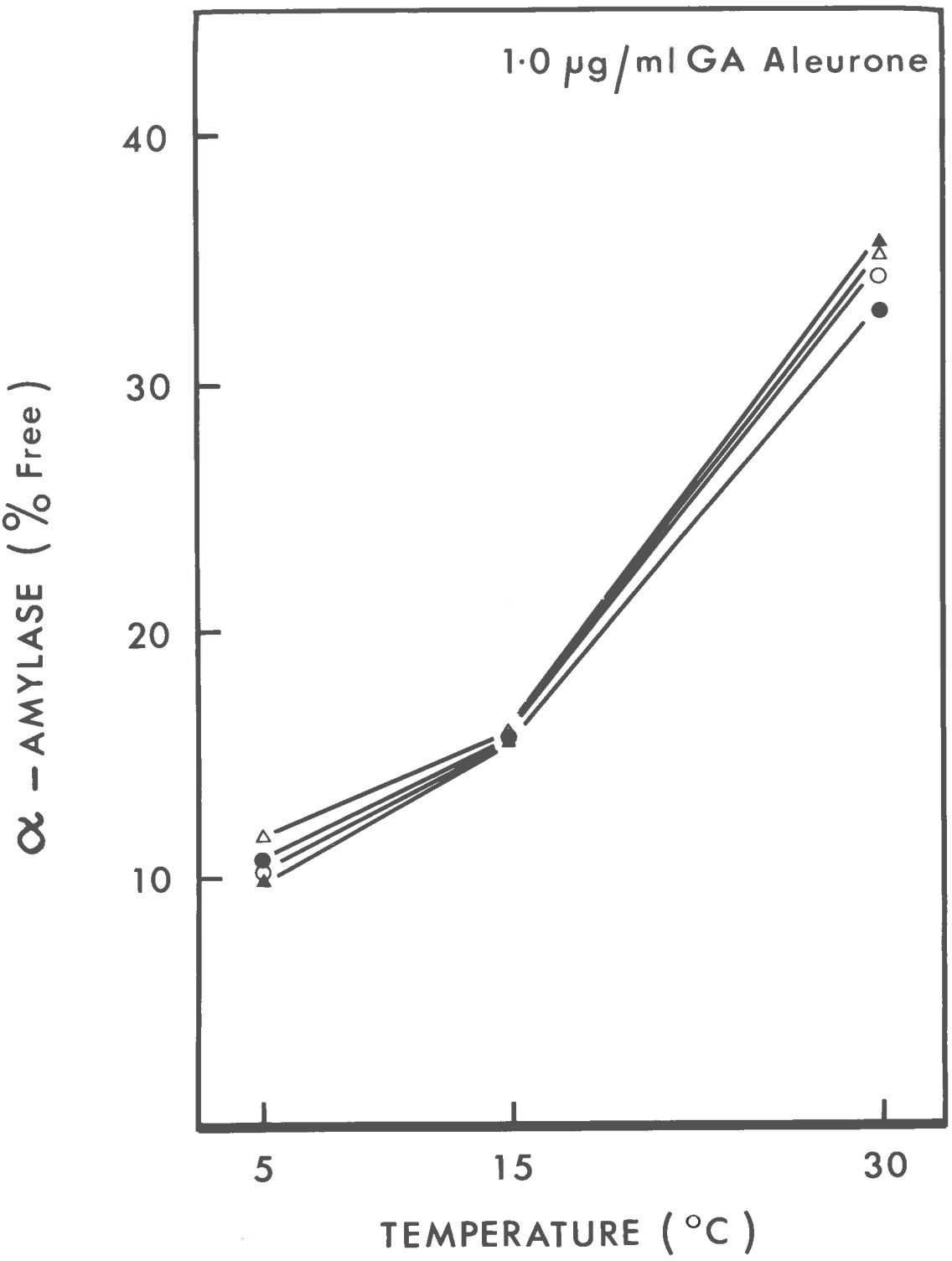

1·0 µg/ml GA Aleurone
40
30
20
10
α – AMYLASE (% Free)
5
15
30
TEMPERATURE (°C)

Table IV.8

Effect of GA on lysosomes isolated from aleurone tissue incubated for 24 hr in either 0.1 µg/ml or 1.0 µg/ml GA at 30°C. Lysosomes (60,000 *g* pellet) resuspended in 1 ml of medium containing 0.4 M sucrose, and 0.05 M Tris-HCl (pH 7.0). Lysosomes incubated for 30 min at the temperature indicated.

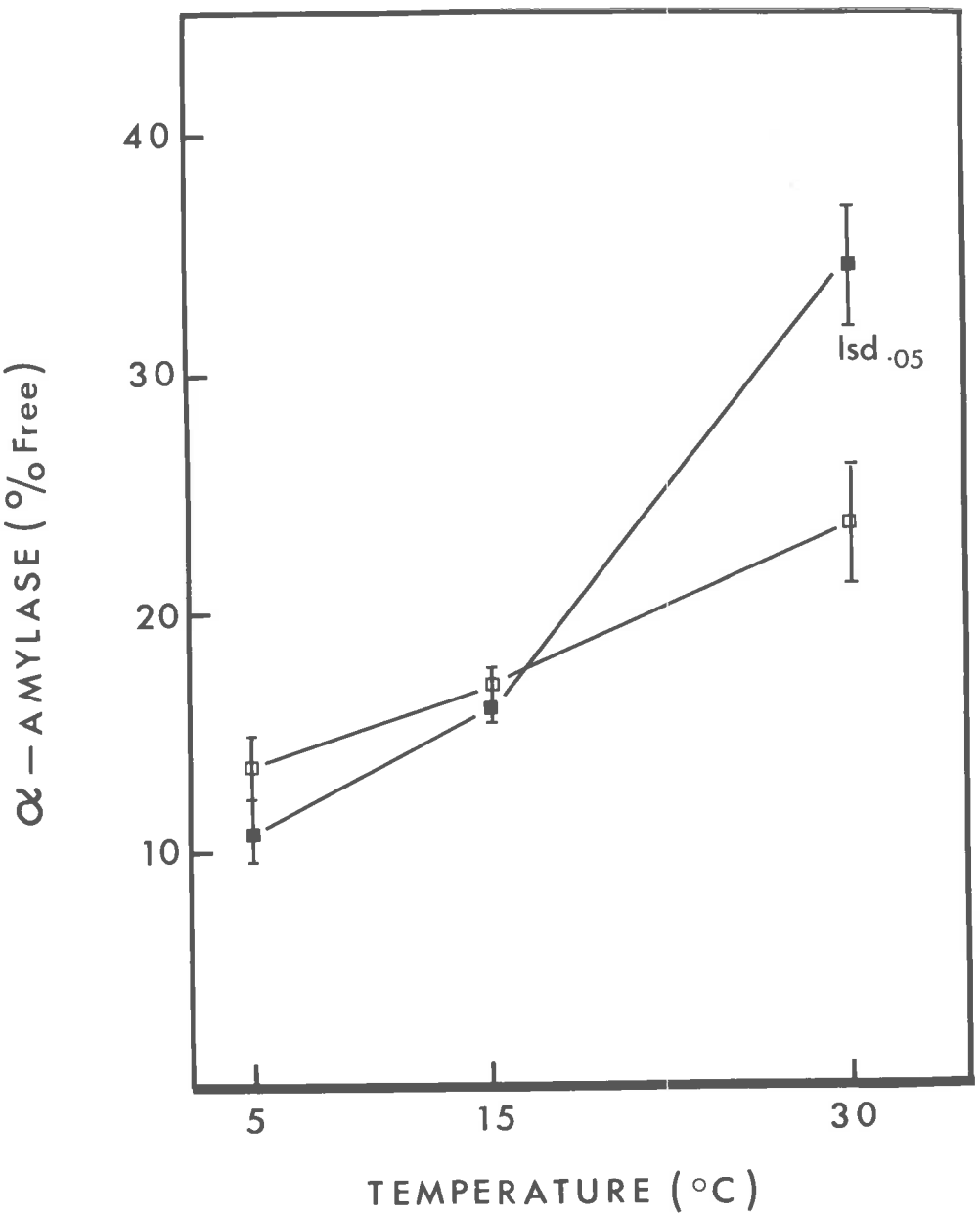
Treatment	Temperature (°C)	α-Amylase			
		0.1 µg/ml Aleurone		1.0 µg/ml Aleurone	
		% Free	% Recovered	% Free	% Recovered
Control	5	12	102	12	98
	15	16	97	16	97
	30	22	102	35	105
1 µg GA	5	11	102	10	104
	15	16	101	16	99
	30	27	97	34	97
10 µg GA	5	15	99	11	100
	15	17	101	16	102
	30	22	100	33	98
100 µg GA	5	14	103	10	100
	15	19	99	16	101
	30	23	98	35	99
		100% = 37.5 units		100% = 38.3 units	

Figure IV.5

Summary of all data contained in Figure IV.3 and IV.4 showing least significant difference ($lsd_{.05}$) at the 5% level. Lysosomes isolated from aleurone tissue treated with 1.0 or 0.1 µg/ml GA for 24 hr at 30°C.

■ 1.0 µg/ml GA-lysosomes

□ 0.1 µg/ml GA-lysosomes


40
30
20
10
α – AMYLASE (% Free)
lsd .05
5
15
30
TEMPERATURE (°C)

isolated lysosomes. However the analysis revealed that there was a significant difference between the lysosomes obtained from 0.1 µg/ml GA-treated tissue and those obtained from 1.0 µg/ml treated tissue (Figure IV.5). Thus it appeared that there was an *in vivo* effect of the hormone on the lysosomal membrane. This effect was examined in detail and will be discussed later in the report.

Continuing with the hypothesis that an *in vitro* response was difficult to demonstrate because the sites of GA attachment to the membrane were already occupied, the effect of GA on lysosomes isolated from control aleurone tissue was examined. Control aleurone tissue was incubated in water for 48 hr at 30°C to allow tissue α-amylase to build up to a measurable level, and lysosomes extracted from this tissue were then incubated in a range of temperatures with or without GA.

The results (Figure IV.6) were again disappointing. The curve of percentage free α-amylase shows a definite peak at 40°C which also corresponds to an over-recovery of total enzyme activity at this temperature, which cannot be explained. Addition of GA had no effect on the percentage of free enzyme found at any of the temperatures tested (Table IV.9).

Since lower percentages of free α-amylase were found when phosphate was used as a buffer instead of tris, it was decided to

Figure IV.6

Effect of GA (100 µg/ml) on leakage of α-amylase from lysosomes isolated from control aleurone tissue incubated for 48 hr at 30°C. Lysosomes resuspended in a medium containing 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose and incubated for 30 min at the temperature indicated.

● +GA

○ -GA

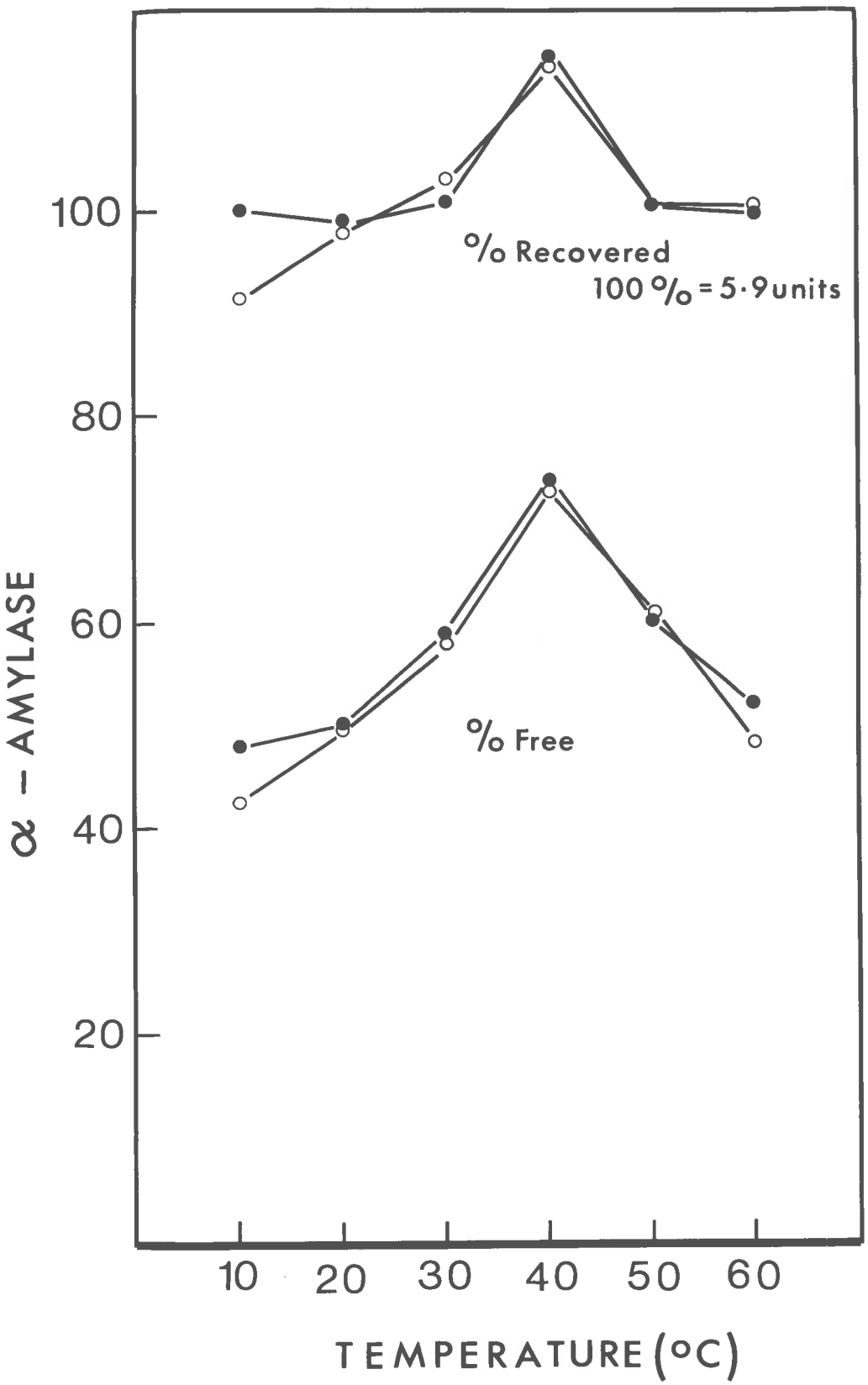

100
80
60
40
20
α - AMYLASE
% Recovered
100 % = 5·9 units
% Free
10
20
30
40
50
60
TEMPERATURE (°C)

Table IV.9

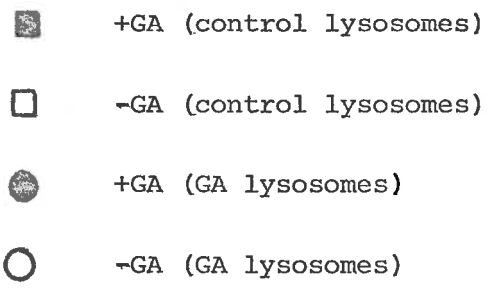
Effect of GA on aleurone lysosomes isolated from control tissue that had been incubated for 48 hr at 30°C. Isolated lysosomes incubated for 30 min at the temperature indicated, with or without GA (100 µg/ml). Resuspension medium contained 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose.

Temperature (°C)	GA	α-Amylase	
		% Free	% Recovered
10	-	48.1	92
	+	42.4	100
20	-	50.0	98
	+	50.0	98
30	-	59.0	103
	+	58.3	101
40	-	73.8	114
	+	72.2	115
50	-	60.0	101
	+	60.0	101
60	-	52.5	101
	+	48.3	100

100% = 5.9 units

Figure IV.7

Effect of GA (100 µg/ml) on leakage of α-amylase from lysosomes isolated from GA (1 µg/ml) treated, and control aleurone tissue. Lysosomes resuspended in a medium containing 0.01 M KH_2PO_4 (pH 7.0) and 0.4 M sucrose. Lysosomes incubated for 30 min at the temperature indicated.

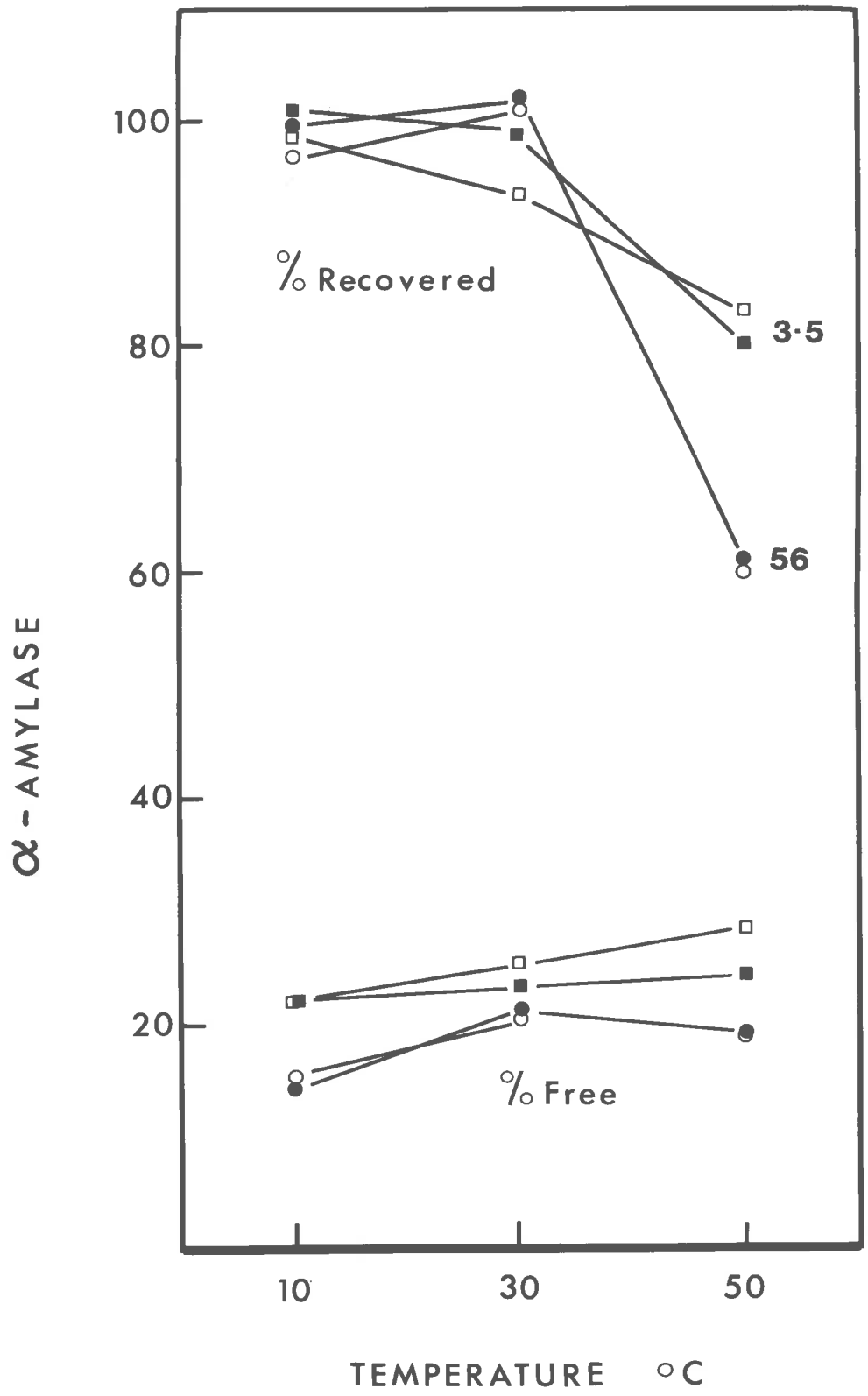


+GA (control lysosomes)

-GA (control lysosomes)

+GA (GA lysosomes)

-GA (GA lysosomes)


100
80
60
40
20
α - AMYLASE
% Recovered
3·5
56
% Free
10
30
50
TEMPERATURE °C

examine the effects of GA on lysosomes isolated from both control and GA (1 µg/ml)-treated tissue using phosphate. The results are listed in Table IV.10.

The amount of α-amylase found in the final supernatant was low at all temperatures tested from lysosomes isolated from both control and GA-treated tissue (Figure IV.7), confirming earlier observations (Table IV.6). Added GA had no effect on lysosomes from GA-treated tissue but did appear to slightly reduce α-amylase leakage from lysosomes from control tissue treated at 50°C (Figure IV.7). However the recoveries of total α-amylase from this treatment were extremely low (60-80%) making definite conclusions impossible.

The loss of total α-amylase activity at 50°C was thought to be due to heat inactivation of the enzyme caused by removal of calcium ions on the α-amylase molecule by phosphate. Since incubation with phosphate had yielded the only *in vitro* GA response recorded thus far, it was thought advisable to attempt to counter the loss of enzyme activity with additions of calcium to the incubation medium. Unfortunately additions of calcium above 1 mM caused the phosphate buffer (0.01 M) to precipitate so this was the highest concentration of calcium tested.

The losses of α-amylase in phosphate were partly overcome by 0.5 and 1.0 mM calcium, but magnesium was without effect even at 10 mM (Table IV.11). However the losses incurred at 60°C were sufficiently large even in the presence of calcium to cause this approach to be abandoned.

Table IV.10

Effect of GA on aleurone lysosomes isolated from control and GA (1 µg/ml) treated tissue using phosphate as the buffer for both the grinding medium (0.05 M) and resuspension medium (0.01 M). Isolated lysosomes incubated for 30 min with or without GA (100 µg/ml) at the temperature indicated. Both control and GA-treated tissue incubated for 24 hr at 30°C.

Treatment	GA (100 µg/ml)	α-Amylase % Free	α-Amylase % Recovered
Control tissue			
10°	-	22	99
	+	22	101
30°	-	25	94
	+	23	99
50°	-	28	83
	+	24	80
		100% = 3.5 units	
GA-treated tissue			
10°	-	15	97
	+	14	99
30°	-	20	101
	+	21	102
50°	-	19	60
	+	19	61
		100% = 56 units	

Table IV.11

Effect of divalent cations on loss of α-amylase activity by phosphate in lysosomes from GA treated (1 µg/ml) tissue. Additions of cations at the concentrations indicated. Time of incubation: 30 min.

Treatment	Temperature (°C)	α-Amylase % Free	α-Amylase % Recovered
Phosphate (0.01 M)	20°	20	100
	40°	21	90
	60°	0	0
Phosphate + Ca^{++} (0.5 mM)	20°	12	98
	40°	19	101
	60°	21	74
Phosphate + Ca^{++} (1.0 mM)	20°	11	99
	40°	19	100
	60°	22	81
Phosphate + Mg^{++} (10 mM)	20°	10	99
	40°	17	79
	60°	0	0

100% = 34.8 units

5.3 *In vivo* Effects of GA

Results obtained in an earlier experiment (Figure IV.5) indicated that although direct addition of GA had little effect on isolated lysosomes, the leakage of α-amylase was faster from lysosomes obtained from tissue incubated in 1 µg/ml GA than from those isolated from tissue treated with 0.1 µg/ml GA. Since this result indicated a possible *in vivo* hormone effect on the permeability of lysosomal membranes, the experiment was repeated using lysosomes isolated from aleurone tissue treated with sub-optimal (0.1 µg/ml), optimal (1.0 µg/ml) or supra-optimal (100 µg/ml) concentrations of GA.

The results (Figure IV.8 and Table IV.12) were essentially similar to those obtained in an earlier experiment (Figure IV.5). The percentage free enzyme was the same from lysosomes isolated from 0.1 and 1.0 µg/ml GA-treated tissue up to 20°C (Figure IV.8). Above this temperature 1 µg/ml GA-lysosomes leaked a higher percentage of α-amylase into the surrounding medium than 0.1 µg/ml GA-lysosomes. The curve for 100 µg/ml GA-lysosomes was similar to the curve obtained with 1.0 µg/ml GA-lysosomes except that the base value (0°C) was higher for the former lysosomes. Although the recovery of α-amylase was constant over the temperature range tested for 100 and 0.1 µg/ml GA-lysosomes, the recovery values were more variable for 1.0 µg/ml GA-lysosomes (Table IV.12).

The experiment was repeated in an attempt to obtain more reliable data and the results are shown in Figure IV.9. Lysosomes from 1.0 and

Figure IV.8

Effect of temperature on leakage of α-amylase from lysosomes isolated from aleurone tissue treated with various concentrations of GA for 24 hr at 30°C. Lysosomes resuspended in a medium containing 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose. Incubation time: 30 min.

△ 100 µg/ml GA-aleurone lysosomes

□ 1 µg/ml GA-aleurone lysosomes

▩ 0.1 µg/ml GA-aleurone lysosomes

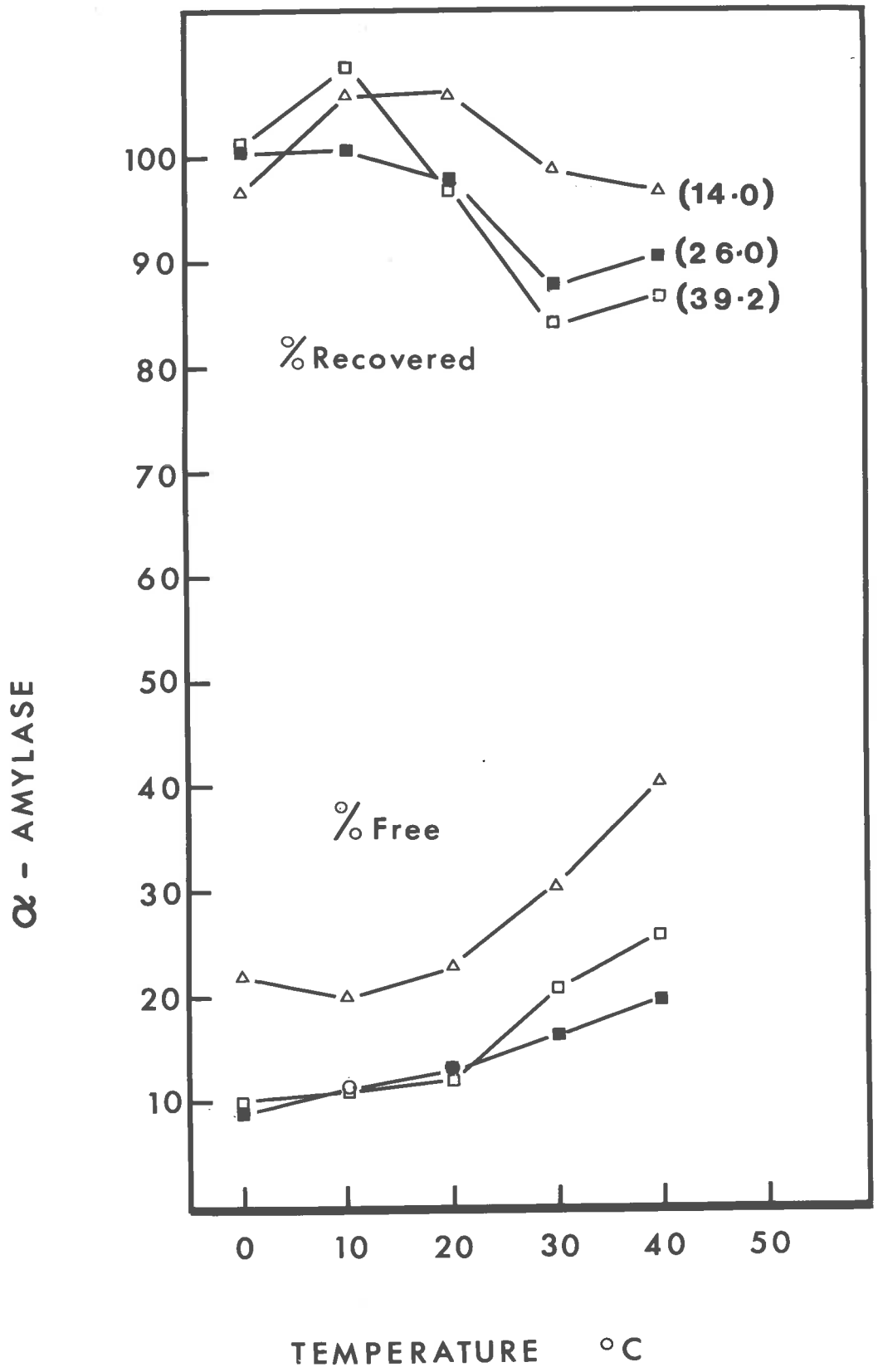

100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
(14·0)
(26·0)
(39·2)
% Recovered
% Free
α - AMYLASE
TEMPERATURE °C

Table IV.12

Effect of temperature on leakage of α-amylase from lysosomes isolated from aleurone tissue treated with various (100, 1 or 0.1 µg/ml) concentrations of GA. Lysosomes resuspended in a medium containing 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose, and incubated for 30 min at the temperature indicated.

Lysosome source	Temperature (°C)	α-Amylase % Free	α-Amylase % Recovered
100 µg/ml GA-aleurone	0	22.1	97
	10	20.0	106
	20	22.8	106
	30	31.2	99
	40	41.2	97
			100% = 14.0 units
1.0 µg/ml GA-aleurone	0	8.9	101
	10	10.9	110
	20	11.9	97
	30	20.7	84
	40	26.0	87
			100% = 39.2 units
0.1 µg/ml GA-aleurone	0	9.8	101
	10	11.4	101
	20	12.8	98
	30	16.5	88
	40	20.2	91
			100% = 26.0 units

Figure IV.9

Effect of temperature on leakage of α-amylase from lysosomes isolated from aleurone tissue treated with various concentrations of GA for 24 hr at 30°C. Lysosomes resuspended in a medium containing 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose. Incubation time: 30 min.

△ 100 µg/ml GA-aleurone lysosomes

□ 1 µg/ml GA-aleurone lysosomes

▩ 0.1 µg/ml GA-aleurone lysosomes

▲ 0.01 µg/ml GA-aleurone lysosomes

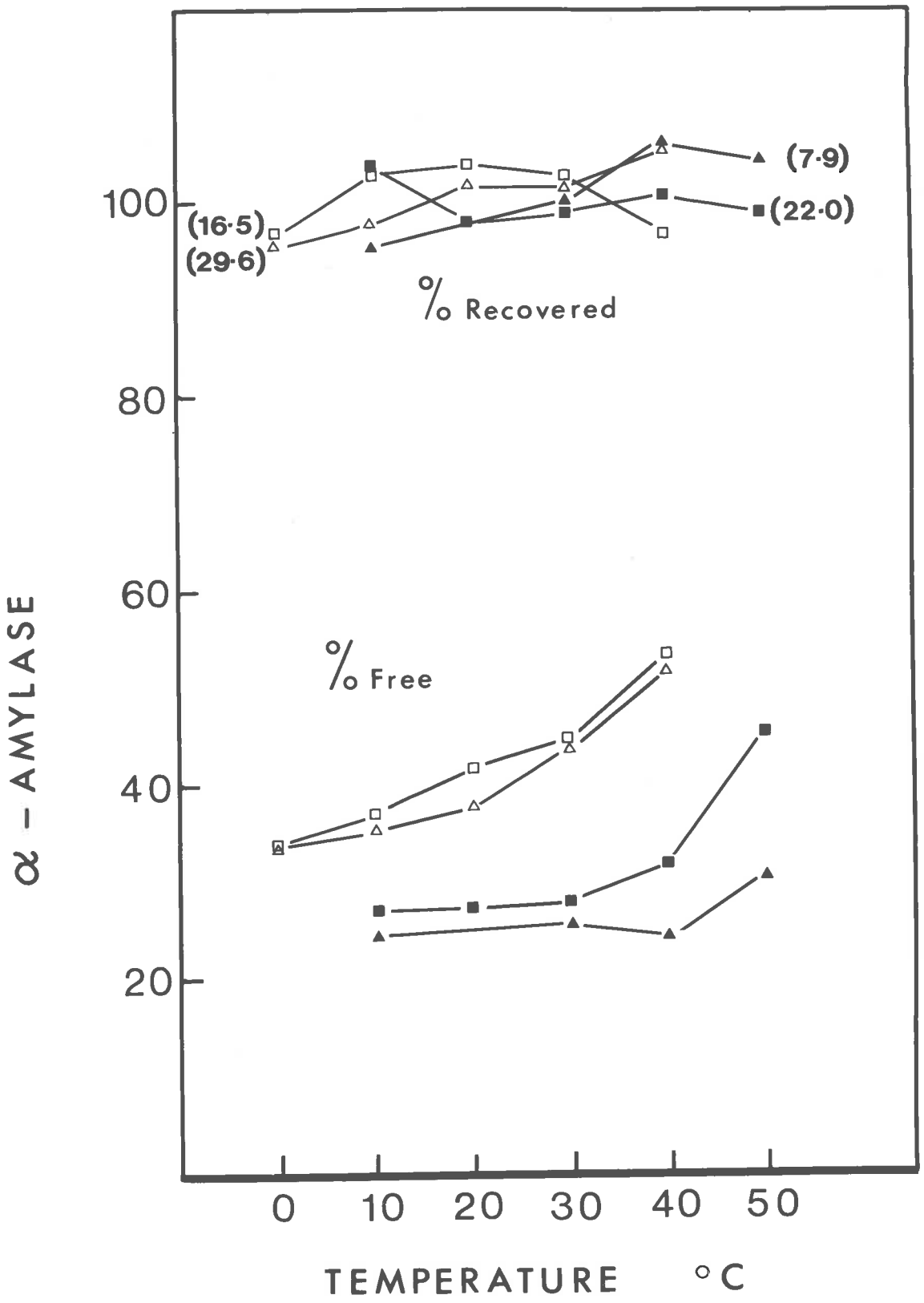

100
80
60
40
20
α - AMYLASE
(16·5)
(29·6)
(7·9)
(22·0)
% Recovered
% Free
0
10
20
30
40
50
TEMPERATURE °C

Table IV.13

Effect of temperature on leakage of α-amylase from lysosomes isolated from aleurone tissue treated with various (100, 1.0, 0.1 or 0.01 μg/ml) concentrations of GA. Lysosomes resuspended in a medium containing 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose, and incubated for 30 min at the temperature indicated.

Lysosome source	Temperature (°C)	α-Amylase % Free	α-Amylase % Recovered
100 μg/ml GA-aleurone	0	33.5	96
	10	35.2	98
	20	37.9	102
	30	43.8	102
	40	51.7	106
			100% = 16.5 units
1 μg/ml GA-aleurone	0	34.1	97
	10	37.2	103
	20	42.0	104
	30	45.0	103
	40	53.7	97
			100% = 29.6 units
0.1 μg/ml GA-aleurone	10	27.0	104
	20	27.4	98
	30	28.1	99
	40	31.9	101
	50	45.7	99
			100% = 22.0 units
0.01 μg/ml GA-aleurone	10	24.3	95
	30	25.3	100
	40	24.1	106
	50	30.5	104
			100% = 7.9 units

Figure IV.10

Effect of temperature on leakage of α-amylase from lysosomes isolated from aleurone tissue treated with various concentrations of GA for 24 hr at 30°C. Lysosomes resuspended in a medium containing 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose. Incubation time: 30 min.

△ 100 µg/ml GA-aleurone lysosomes

□ 1 µg/ml GA-aleurone lysosomes

▣ 0.1 µg/ml GA-aleurone lysosomes

▲ 0.01 µg/ml GA-aleurone lysosomes

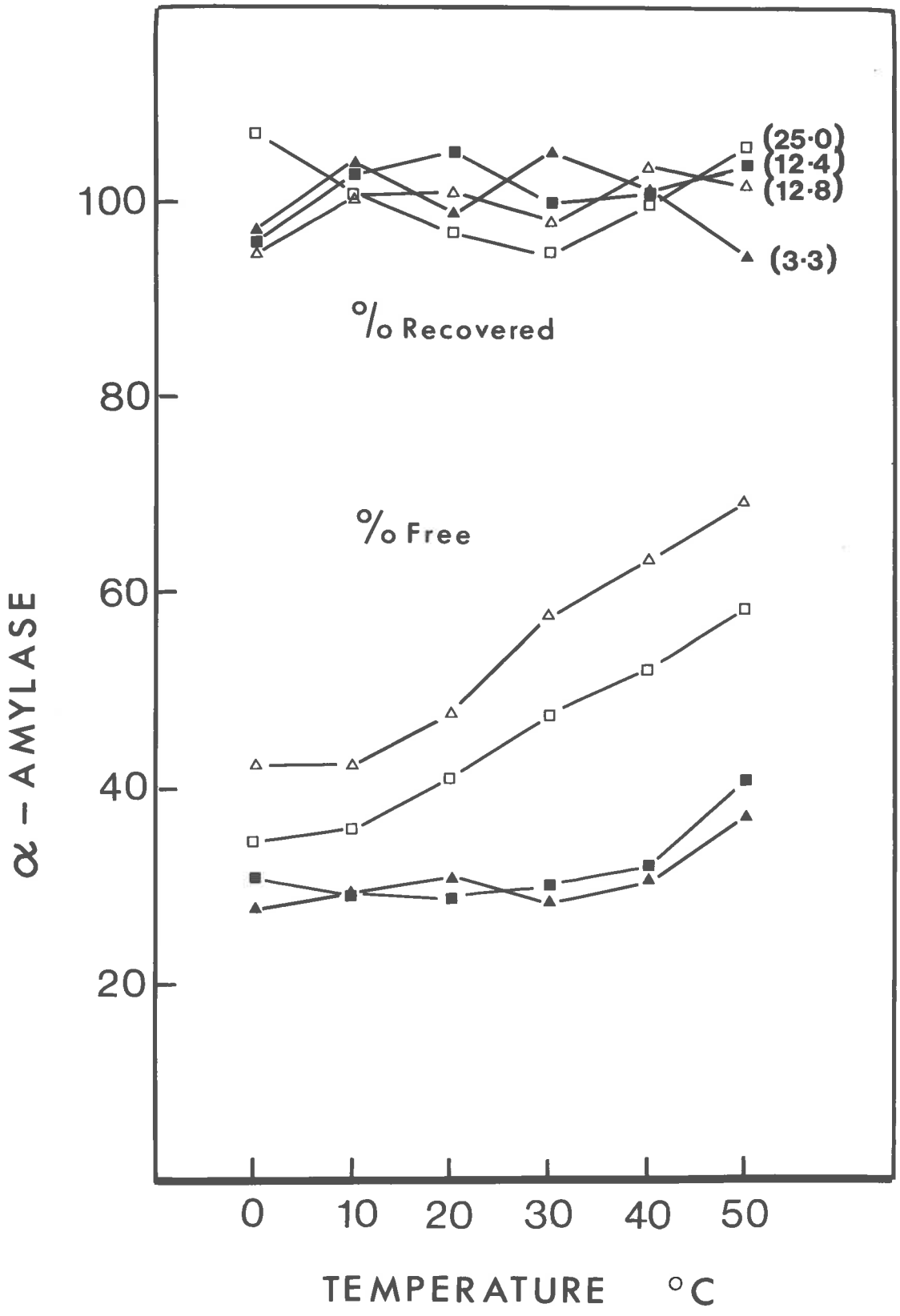

(25·0)
(12·4)
(12·8)
(3·3)
% Recovered
% Free
α – AMYLASE
100
80
60
40
20
0
10
20
30
40
50
TEMPERATURE °C

Table IV.14

Effect of temperature on leakage of α-amylase from lysosomes isolated from aleurone tissue treated with various (100, 1.0, 0.1 or 0.01 µg/ml) concentrations of GA. Lysosomes resuspended in a medium containing 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose, and incubated for 30 min at the temperature indicated.

Lysosome source	Temperature (°C)	α-Amylase % Free	α-Amylase % Recovered
100 µg/ml GA-aleurone	0	42.3	95
	10	42.3	101
	20	48.0	101
	30	58.0	98
	40	63.5	104
	50	69.5	102
			100% = 12.8 units
1 µg/ml GA-aleurone	0	34.5	107
	10	36.0	101
	20	41.0	97
	30	47.5	95
	40	52.0	100
	50	58.2	106
			100% = 25.0 units
0.1 µg/ml GA-aleurone	0	31.0	96
	10	29.0	103
	20	28.5	105
	30	30.0	100
	40	32.0	101
	50	41.0	104
			100% = 12.4 units
0.01 µg/ml GA-aleurone	0	27.5	97
	10	29.5	103
	20	31.0	97
	30	28.0	105
	40	30.5	100
	50	37.0	95
			100% = 3.3 units

100 µg/ml GA-treated tissues again showed similar α-amylase release curves and lysosomes from both these sources leaked more enzyme into the medium than lysosomes from tissue treated with a sub-optimal GA concentration. Again, temperature had little effect on the leakage of α-amylase from 0.1 µg/ml GA-lysosomes up to 30°C. The recovery of α-amylase was pleasingly constant for all treatments (Table IV.13). The percentage of free enzyme detected at 0°C was higher for all treatments than found in the previous experiment. The experiment was repeated a third time and comparable results were obtained (Figure IV.10 and Table IV.14).

The data from the three above experiments can be illustrated in another way, by plotting the log of the percent leakage against the reciprocal of the absolute temperature (Figure IV.11). Although this is not a true Arrhenius plot, it has been used to demonstrate thermal transitions in liposomal systems (Wood and Paleg, 1974). Although the data is equivocal, thermal transitions appear to occur (as indicated by plot discontinuities) and these thermal transitions appear to be affected by the concentration of GA in which the tissue was incubated. If these transitions are real, it would tend to indicate that the lysosomal membranes from tissue treated with optimal and supra-optimal GA concentrations go through a phase change at lower temperatures (≃ 10°C) than similar membranes isolated from tissue treated with sub-optimal levels of GA (30-40°C).

Similar curves to those shown in Figure IV.10 have been

Figure IV.11

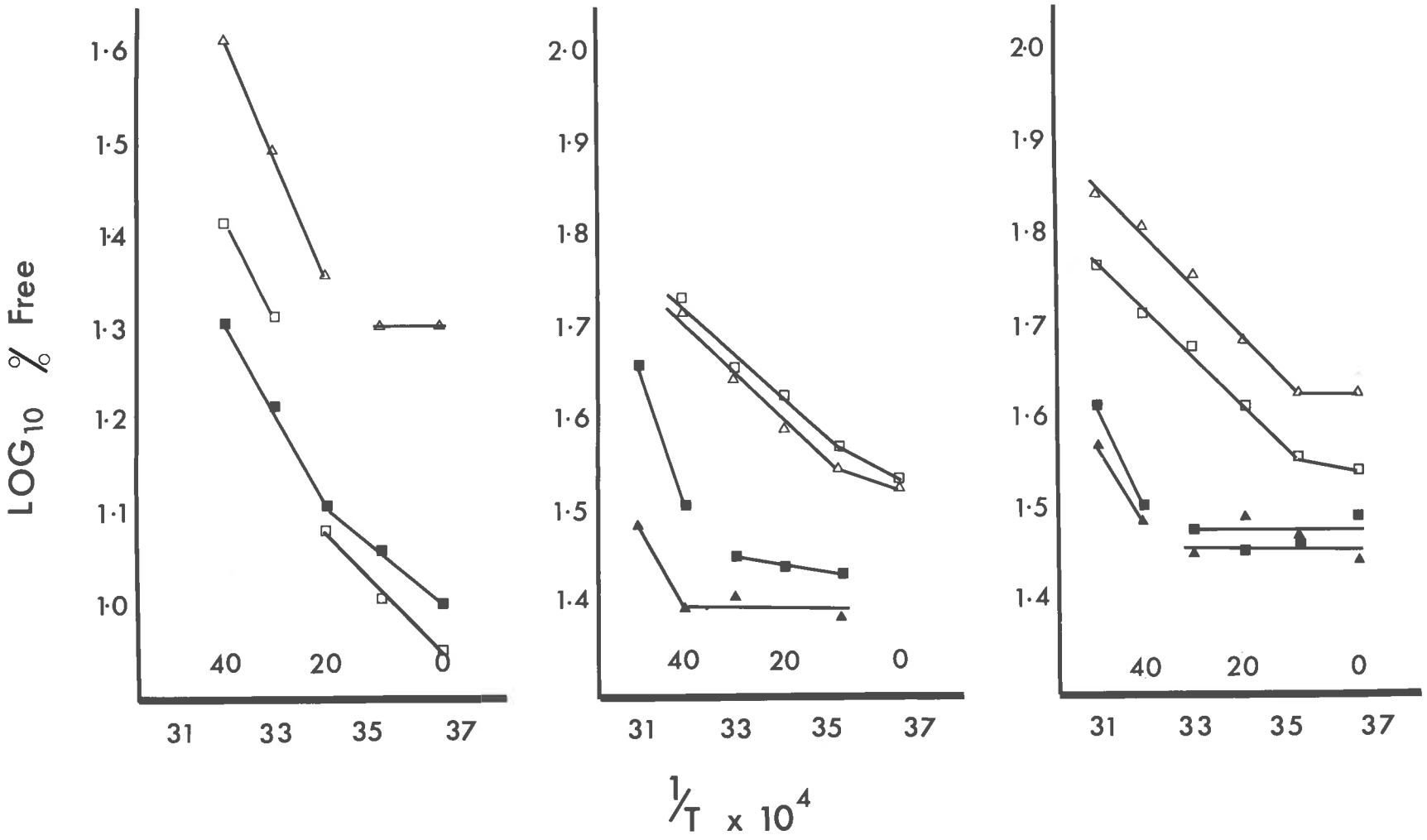
Plot of log of % free α-amylase against reciprocal of the absolute temperature of data contained in Figure IV.8 (left), Figure IV.9 (centre) and Figure IV.10 (right).


100 µg/ml GA-aleurone lysosomes


1 µg/ml GA-aleurone lysosomes


0.1 µg/ml GA-aleurone lysosomes


0.01 µg/ml GA-aleurone lysosomes


LOG10 % Free
1·6
1·5
1·4
1·3
1·2
1·1
1·0
2·0
1·9
1·8
1·7
1·6
1·5
1·4
40
20
0
31
33
35
37
1/T x 10^4

obtained for hydrolytic enzyme release from lysosomes of animal origin (Lee and Fritz, 1972) and the increased percentage of free enzyme from lysosomes from treated animals compared with that from control animals has been explained in terms of a change in the permeability of the lysosomal membrane. Although it is tempting to suggest that this is also occurring in aleurone lysosomes, the basis for doing so would be considerably strengthened if one could also demonstrate a change in a physical property of lysosomal membranes that could be correlated with the enzyme leakage data.

An attempt to do this was made by determining the effect of temperature on the rotational motion of a "spin-label" incorporated into the hydrophobic regions of membranes. Spin-labels are paramagnetic molecules and as such can be detected in an electron spin resonance (esr) spectrometer. The signal obtained varies with the physical state of the membrane.

As the majority of the membrane-enclosed α-amylase appeared to be associated with the ER (Section II), a microsomal pellet was prepared from aleurone tissue treated with 0.1, 1.0 or 100 µg/ml GA. The microsomal pellet was obtained by removing contaminating organelles with a preliminary centrifugation at 12,000 *g* and subjecting the subsequent supernatant to a final centrifugal force of 60,000 *g*. This lysosome preparation was similar in properties (Figure IV.12 and Table IV.15) to those isolated by the normal method

Figure IV.12

Leakage of α-amylase from 12,000 - 60,000 *g* lysosomes used for esr spectrometry illustrated in Figures IV.13 and IV.14. Conditions of experiment described in the text. Resuspension medium: 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose. Lysosomes isolated from aleurone treated with GA at concentration indicated.

□ 100 µg/ml GA-aleurone lysosomes

△ 1 µg/ml GA-aleurone lysosomes

○ 0.1 µg/ml GA-aleurone lysosomes

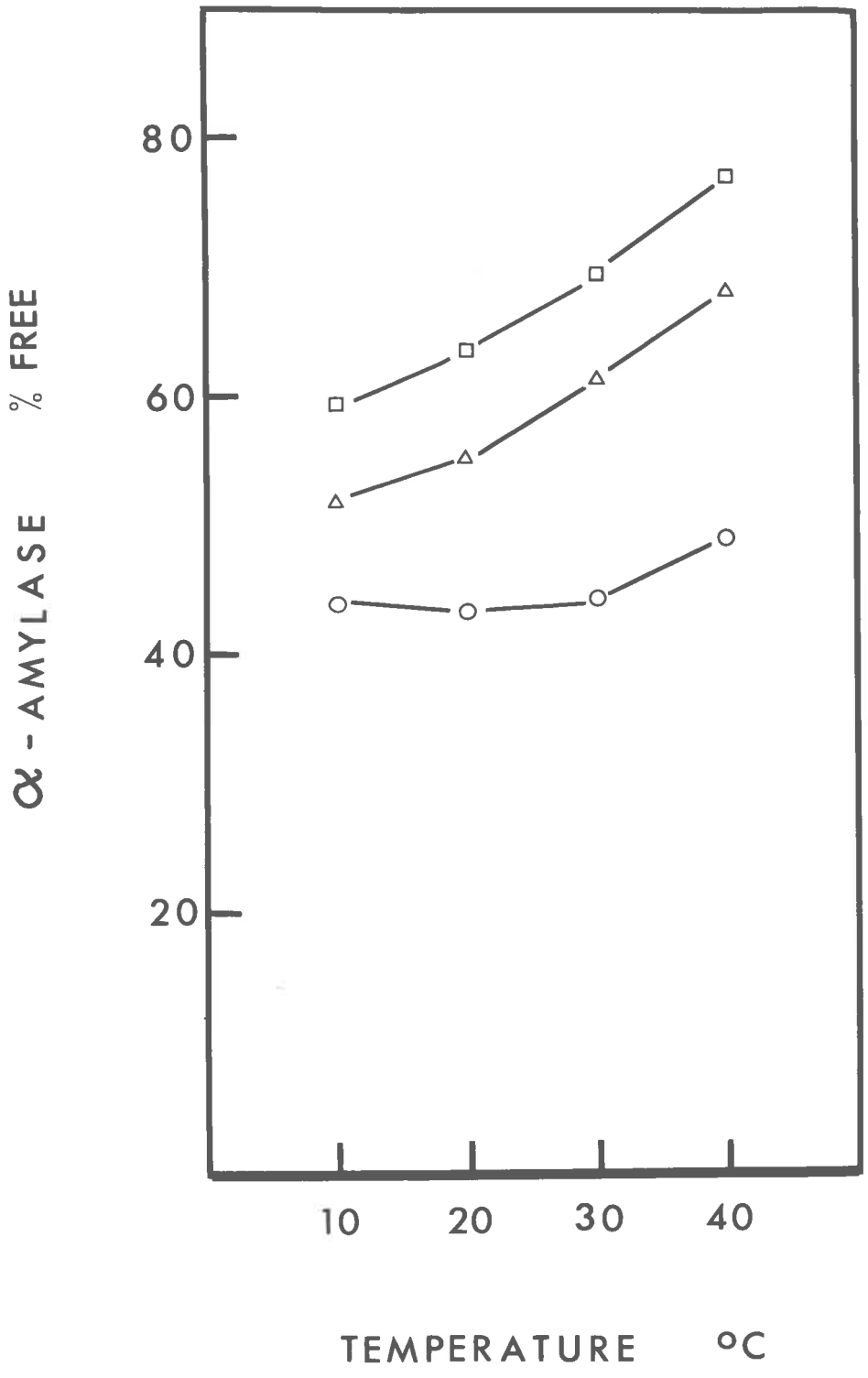

80
60
40
20
α - AMYLASE % FREE
10
20
30
40
TEMPERATURE °C

Table IV.15

Effect of temperature on leakage of α-amylase from 12,000 *g* - 60,000 *g* lysosomes from aleurone tissue treated with various (100, 1.0 or 0.1 μg/ml) concentrations of GA. Lysosomes resuspended in a medium containing 0.01 M Tris-HCl (pH 7.0) and 0.4 M sucrose, and incubated for 30 min at the temperature indicated.

Lysosome source	Temperature (°C)	α-Amylase % Free	α-Amylase % Recovered
100 μg/ml GA-aleurone	10	51.8	99
	20	55.0	99
	30	61.5	98
	40	68.0	101
			100% = 27.2 units
1 μg/ml GA-aleurone	10	59.0	97
	20	63.2	101
	30	69.3	100
	40	76.5	102
			100% = 41.3 units
0.1 μg/ml GA-aleurone	10	44.0	102
	20	43.4	103
	30	44.3	98
	40	49.0	100
			100% = 29.8 units

(60,000 *g* only, Figure IV.9).

The data from the esr spectra are complex (Figures IV.13 and IV.14). The individual points show about the same scatter from a straight line plot regardless of the source of the lysosomal membrane (Figure IV.13). When the data are plotted so that points in a common line are connected, certain discontinuities are seen (Figure IV.14). Such discontinuities have been called thermal transitions and are thought to be due to a temperature-induced phase change in the membrane lipids (Raison *et al.*, 1971). Although there may be a thermal transition in the membranes of 100 and 1 µg/ml GA-lysosomes in the 0-10°C range which is not present in lysosomal membranes from 0.1 µg/ml GA aleurone, there is too little data for definite conclusions to be drawn.

Lyons and Raison (1970) demonstrated that by measuring the activity of membrane-bound enzymes over a range of temperatures it was possible to illustrate a phase change in the lipid of the membrane by a sudden discontinuity in enzyme activity when examined as an Arrhenius plot. The method is ideal in that changes in the membrane can elicit changes in the conformation of the enzyme and thus alter its activity. Thus, membrane-bound enzymes can amplify small changes in the physical properties of the membrane.

An experiment was performed using $NADH_2$ cytochrome c reductase, which has been shown to be associated with the ER (Moore *et al.*, 1973)

Figure IV.13

Arrhenius plot of T_o values obtained by esr spectrometry as described in the text. Lysosome preparations obtained from GA-treated tissue at the concentration indicated. Data are plotted for a best-fit straight line.

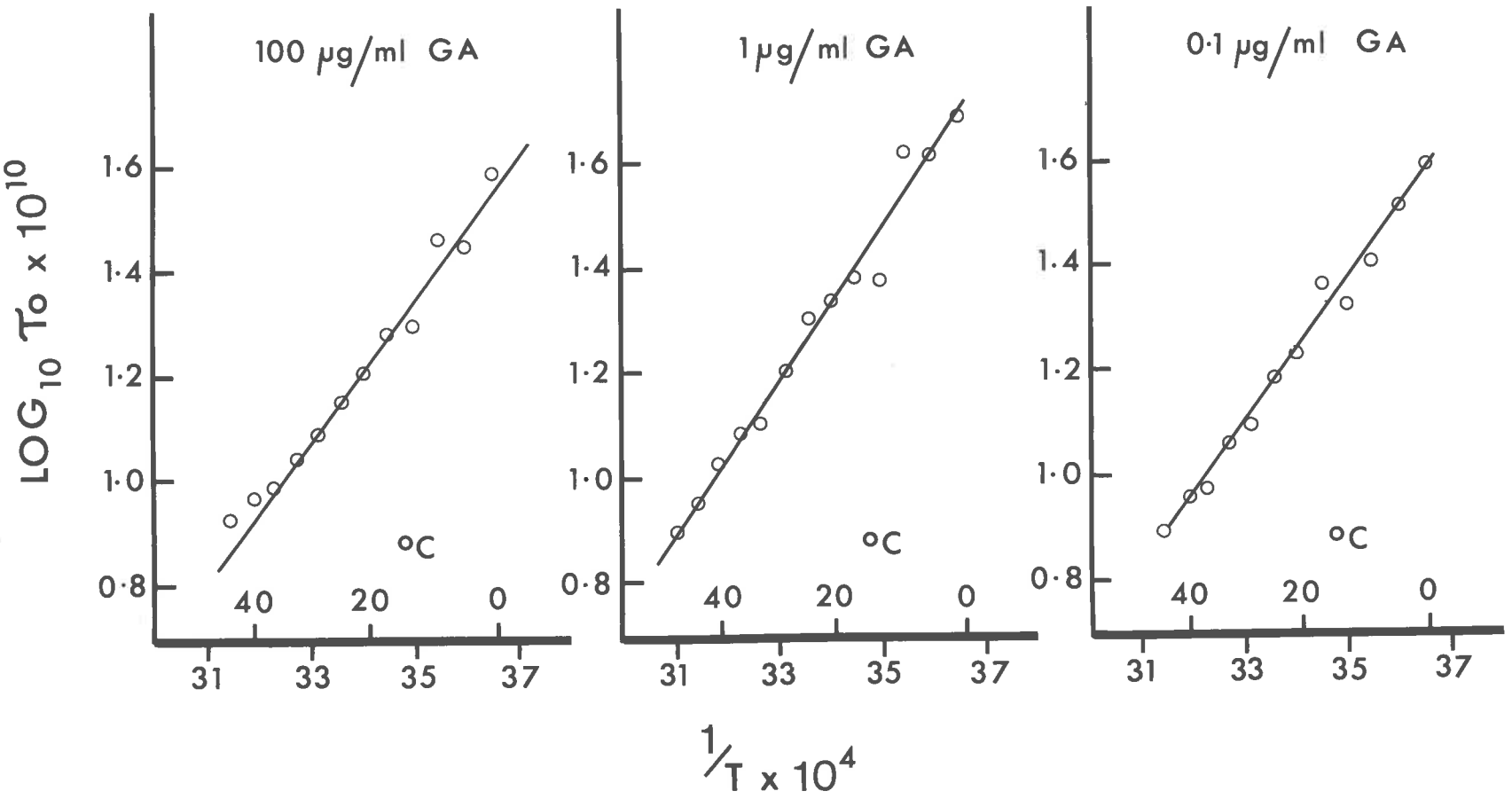

100 µg/ml GA
1 µg/ml GA
0·1 µg/ml GA
LOG10 To x 10^10
1·6
1·4
1·2
1·0
0·8
°C
40
20
0
31
33
35
37
1/T x 10^4

Figure IV.14

Arrhenius plot of T_o values obtained by esr spectrometry as described in the text. Lysosome preparations obtained from GA-treated tissue at the concentration indicated. Data are plotted so that points in a common line are connected.

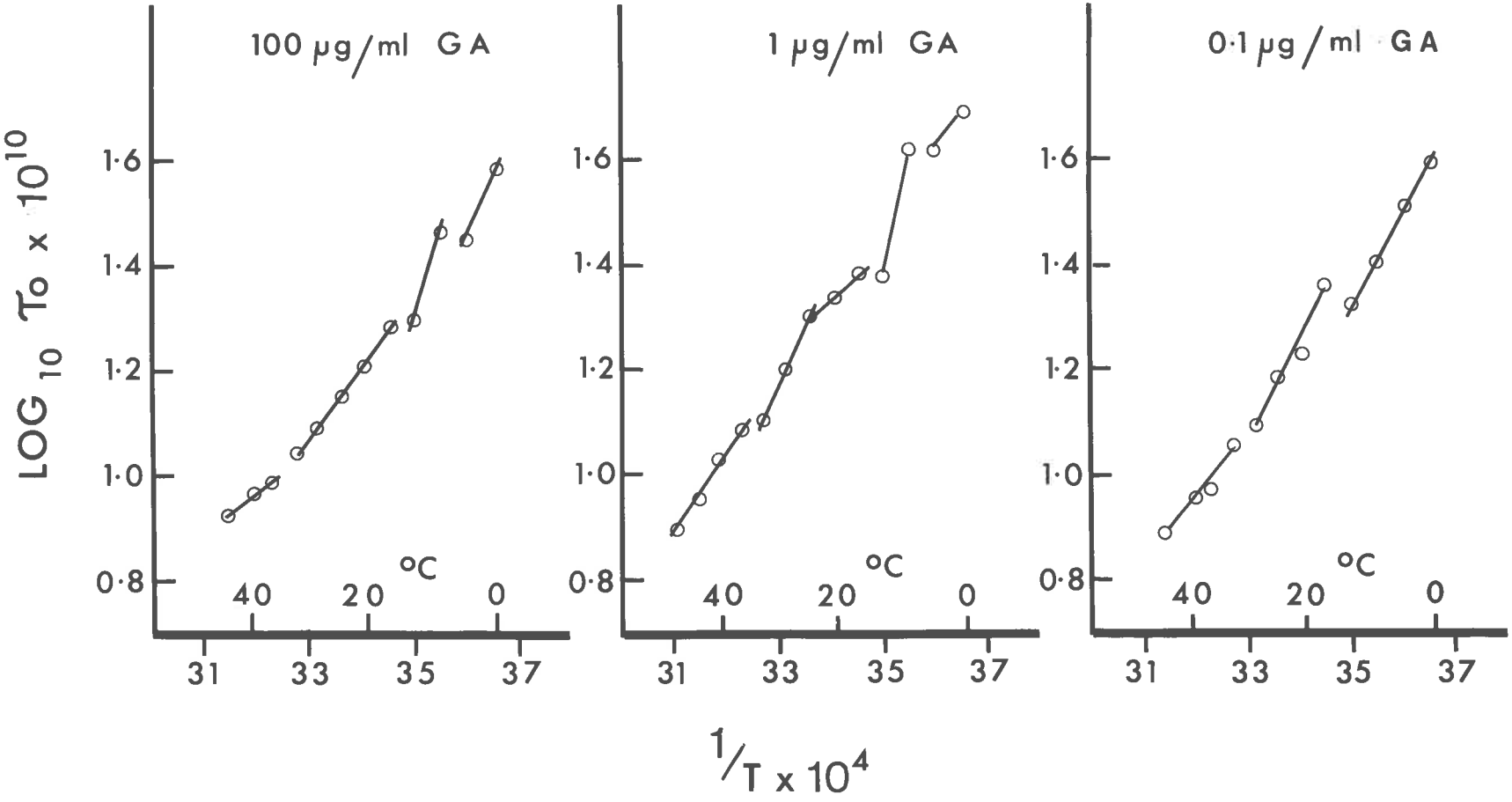

100 µg/ml GA
1 µg/ml GA
0·1 µg/ml GA
LOG 10 To x 10^10
1·6
1·4
1·2
1·0
0·8
°C
40
20
0
31
33
35
37
1/T x 10^4

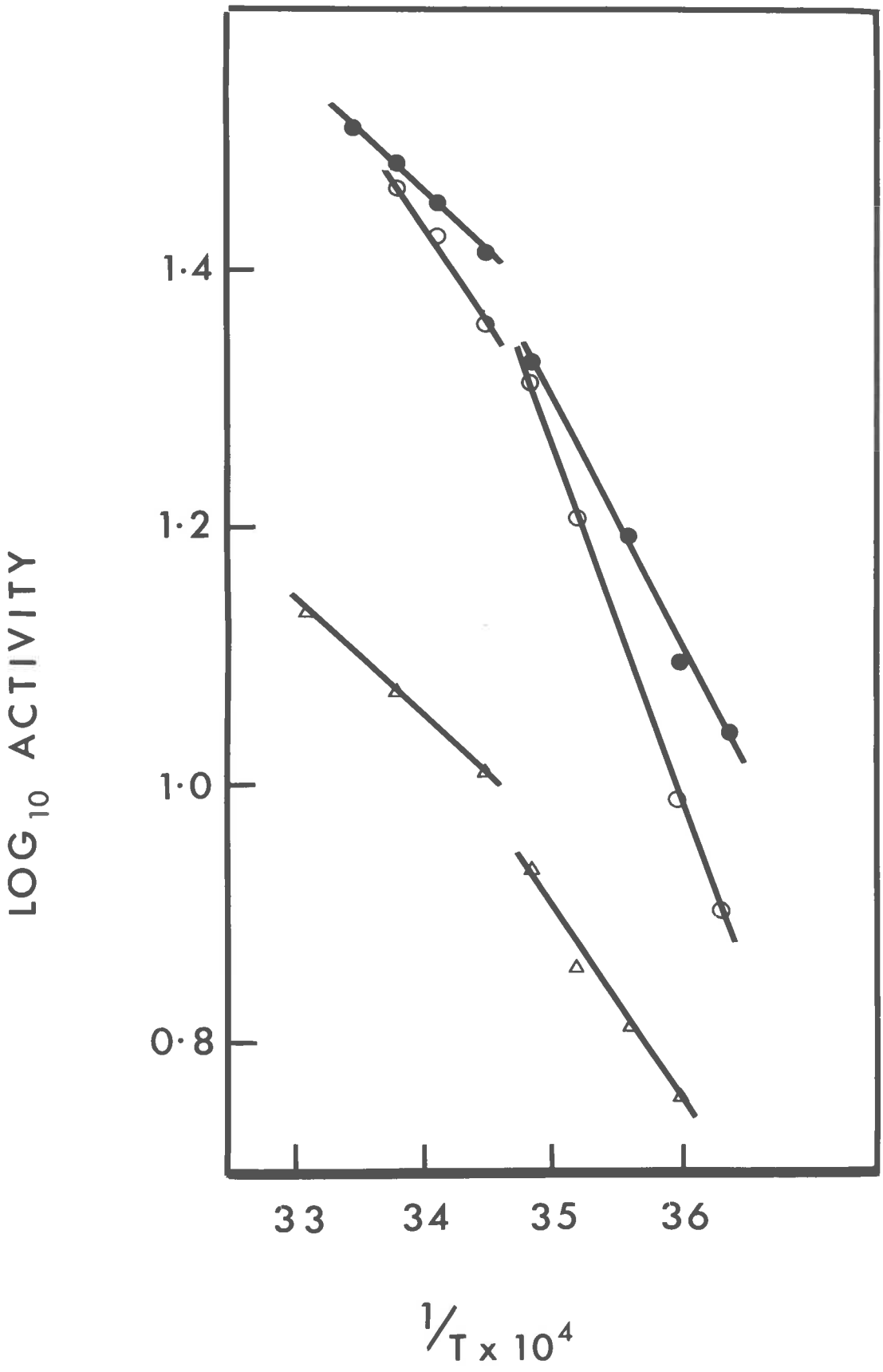
Figure IV.15

Arrhenius plot of $NADH_2$-cytochrome c reductase activity in lysosome preparations from both control and 1 µg/ml GA-treated tissue. Enzyme activity was also determined in the post-60,000 *g* supernatant (soluble enzyme) from GA-treated tissue.


+GA lysosomes


-GA lysosomes


Soluble enzyme


LOG10 ACTIVITY
1·4
1·2
1·0
0·8
33
34
35
36
1/T x 10^4

from both control and GA-treated aleurone. There was a clear discontinuity in the Arrhenius plot at about 16°C corresponding to a change in the activation energy (E_a) of cytochrome c reduction (Figure IV.15). There was no difference in the temperature at which this discontinuity occurred whether the enzyme was isolated from control or GA-treated tissue. A similar break in an Arrhenius plot for cytochrome c reductase has been reported by Vernon *et al.* (1952).

6. Discussion

It seems incongruous to be talking of leakage of hydrolytic enzymes from lysosomes after exhaustive experiments to establish that they are prevented from attacking the cell because they are lysosomal (i.e. membrane enclosed). After all, why should the cell go to all the trouble of packaging hydrolytic enzymes only to have them leak out again. No such suggestion is made or inferred by this work. The behaviour of lysosomes in a medium vastly different from the controlled conditions of the cytoplasm, can only be equated with their behaviour in the cell with difficulty. Rather, the leakage of α-amylase from lysosomes has been determined with a view to examining whether or not the lysosomal membrane is affected by GA.

To examine hormonal effects on membranes one requires a property which is indicative of known membrane behaviour, is specific

for a particular membrane and is easily measurable. The crude lysosomal preparations used in this study (60,000 *g* pellet) contain most of the organelles present in aleurone cells. By assaying the easily measurable enzyme, α-amylase, the effect of various treatments are monitored in only those membranes that enclose α-amylase (i.e. the GA-induced lysosomal membrane) regardless of the effects these same treatments may have on other membranes. Most importantly, permeability is a well defined property of membranes.

Attempts to establish whether or not direct addition of GA affected the leakage of α-amylase from isolated lysosomes (Table IV.1 and Figure IV.1) suggested that the resuspension medium used to incubate the isolated lysosomes must be critically examined. Those compounds in the medium which appeared to be without effect on α-amylase leakage such as BSA, potassium bromate, 2-mercaptoethanol and lecithin (Table IV.2) were not included in subsequent experiments. In addition, calcium nitrate (5 mM) which was found to promote leakage of α-amylase from isolated lysosomes (Tables IV.2 and IV.3) was removed. As 0.4 M sucrose appeared to be an entirely satisfactory osmoticum (Table IV.4), this sugar was retained in the medium. Thus a standard resuspension-incubation medium was developed, containing only 0.4 M sucrose and 0.05 M Tris-HCl buffered at pH 7.0 (Figure IV.2).

When phosphate was substituted for tris buffer, much lower amounts of α-amylase leaked from the lysosomes (Table IV.6). This

effect could possibly be interpreted as being due to a tightening of the lysosomal membrane. Certainly the effect seems to be specific for inorganic phosphate for neither arsenate nor phosphorylated nucleotides such as ATP or ADP had any significant effect (Table IV.7). However, phosphate proved to be unsuitable for use in leakage experiments as above 30°C losses of α-amylase were high (Table IV.10). As these losses in enzyme activity were probably due to removal of calcium from the α-amylase molecule an attempt was made to counter the losses with additions of calcium and magnesium (Table IV.11). This was largely unsuccessful and the experiments with phosphate were not pursued.

The effect of phosphate on decreasing the amount of free α-amylase is nevertheless interesting for it represents the only treatment found in this study that reduced the leakage of α-amylase from lysosomes. Many such compounds have been reported for animal lysosomes including the steroids, cholesterol and cortisone (Koenig, 1969). Such compounds have been referred to as stabilizers, in contrast to those compounds that increase leakage, which are called labilizers, such as the steroid oestrogen (Szego and Seeler, 1973) and the metallic ions calcium and mercury (Sawant *et al.*, 1964). Both groups of compounds have been interpreted to act by changing the permeability of the lysosomal membrane. Since phosphate clearly

reduces the leakage of α-amylase and calcium appears to increase this process (Tables IV.3 and IV.6) the role of these ions on membranes in general deserves further attention. This is particularly true in the case of cereal aleurone cells where both these ions appear to leak from the tissue in response to GA (Eastwood and Laidman, 1971).

Attempts to demonstrate an *in vitro* effect of GA on lysosomal membranes were unsuccessful. It may well be that to attempt to determine an effect of a hormone on lysosomal membranes which require the hormone for their synthesis is somewhat self-defeating. In other words, since GA must be applied to aleurone cells to induce lysosomal α-amylase any hormone-membrane interaction may have already occurred.

Several experiments were performed which were designed to circumvent this problem. Additions of GA to lysosomes from tissue incubated in low concentrations of GA (1.0 and 0.1 µg/ml) had no effect on the leakage of α-amylase (Table IV.8, Figures IV.3 and IV.4). Because it could have been reasonably suggested that any GA attachment sites on the membrane were already occupied the experiment was repeated using lysosomes from 48 hr control tissue (Figure IV.6). Again no effect of added GA was observed. A similar experiment performed with a phosphate resuspension-incubation medium failed to demonstrate a significant effect of added GA on control or GA-treated lysosomes (Figure IV.7).

It was therefore concluded that either there was no direct effect

of GA on lysosomal membranes, or the techniques used were unsuitable. It is interesting to note in this regard, that the increased leakage of lysosomal hydrolases by steroid hormones reported by de Duve *et al.* (1962) was only achieved at hormone concentrations well above physiological levels (Szego, 1972a, b).

Although no *in vitro* effect of GA on α-amylase leakage could be detected, there was a significant difference in the leakage of enzyme from lysosomes obtained from tissue incubated in different GA concentrations (Figure IV.8). This difference was only observable at 30°C but pointed to the fact that a range of temperatures must be used to illustrate differences in leakage of α-amylase from lysosomes from different tissue treatments. Subsequent experiments (Figures IV.8, IV.9 and IV.10) confirmed that temperature-dependent leakage of lysosomal enzymes was a function of the GA concentration used to treat the tissue.

Although there is much variation in absolute percentages of enzyme found free in the supernatant for a given temperature in the various experiments, certain features are constant. More α-amylase leaks from lysosomes from tissue treated with optimal (1.0 µg/ml) or high (100 µg/ml) levels of GA than from equivalent lysosomes isolated from tissue incubated with lower (0.01 and 0.1 µg/ml) levels of GA (Figures IV.8, IV.9 and IV.10). This difference is greater at higher temperatures (30-50°C) than at lower temperatures (0-20°C).

The question must therefore be posed, if GA has no effect on membranes why are the leakage curves so different for lysosomes from tissue treated with different concentrations of the hormone? One possible explanation is that the leakage of α-amylase is merely a function of the level of enzyme in the lysosomes. This proposal must be rejected in view of the data presented in Figure IV.10 and Table IV.14. In this particular experiment the amount of enzyme in 100 µg/ml GA-lysosomes was identical to that found in 0.1 µg/ml GA-lysosomes and yet their leakage curves are distinctly different. A GA-induced change in the permeability of lysosomal membranes seems an inescapable conclusion.

Furthermore, the data also suggest that this change in lysosomal membrane permeability may be related to a phase change in the membrane at a certain temperature which is again dependent on the GA concentration used to treat the aleurone tissue (Figure IV.11). Such a phase change, or change in the phospholipid portion of the membrane from one mesomorphic state to another, is referred to as a thermal transition and is well documented in a variety of systems. For example, thermal transitions have been observed in model membrane systems (liposomes) (Papahadjopolous and Bangham, 1966) and tissue systems (Overath *et al.*, 1970). In addition, changes in the thermal transition of liposomal membranes in response to steroids have been

correlated with steroid-induced changes in lysosome permeability (Bangham *et al.*, 1965). Even more pertinent is the recent report of Wood and Paleg (1974) who have shown a GA-induced change in the thermal transition of liposomes composed of plant phospholipids and sterols. The data plotted in Figure IV.11 indicate that GA *in vivo*, causes a shift in the thermal transition temperature of lysosomal membranes from 30-40°C (0.1 and 0.01 µg/ml GA) to around 10°C (100 and 1 µg/ml GA).

If, as the data suggest (Figure IV.11), this GA-induced change in the thermal transition of aleurone lysosomal membranes is real, what does such an effect mean in terms of the secretion of hydrolytic enzymes? Since at temperatures above the transition temperature membranes are more fluid than at temperatures below this point (Chapman and Wallach, 1968), it follows that any treatment that lowers the observed transition temperature of a membrane also increases the flexibility of that membrane at a given temperature.

If we accept the data in Figure IV.11 that suggest GA lowers the transition temperature in a concentration-dependent fashion, then the transition temperature of control lysosomal membranes (by extrapolation) must be high (> 50°C). Since this investigation has adequately demonstrated that GA-induced hydrolases are membrane enclosed, active secretion from the cell must involve fusion of lysosomes with the plasmalemma. As the ability of a membrane to fuse with

another must be dependent on the physical state (fluidity) of the membranes involved, it may be that hydrolytic enzyme synthesis and secretion in aleurone cells is controlled by the physical state of the secretory system (lysosomes) rather than protein synthesis *per se*. Thus, control tissue with lysosomal membranes in a relatively static state (high thermal transition) would be less able to package and secrete (via fusion with the plasmalemma) enzymes, than tissue with lysosomal membranes (ER and derived lysosomes) in a relatively fluid state (GA-treated tissue).

It must be pointed out, however, that attempts to correlate the leakage data with a measurable physical change in the state of the lysosomal membrane by esr spectrometry (Figures IV.13 and IV.14) were inconclusive, perhaps due to the lack of purity of the preparation. Furthermore, an experiment designed to measure a change in the thermal transition of ER membranes (Figure IV.15) was also unsuccessful. Therefore the data concerning an *in vivo* effect of GA on lysosomal membrane permeability must be considered with caution.

Alternatively, the two methods chosen to corroborate the leakage data may have been inadequate. The ER-associated enzyme, $NADH_2$-cytochrome c reductase, may not alter its conformation in response to temperature-induced changes in the membrane. Indeed, the discontinuity in the Arrhenius plot (Figure IV.15) appears even in the

data for the soluble enzyme. Similar results have been reported for cytochrome c reductase (Vernon, 1952) and other soluble enzymes (Raison, 1973). Furthermore, as both the purity of the membrane fraction and the choice of spin label are critical in esr spectrometry (Raison, 1973), it is perhaps not surprising that the data obtained (Figures IV.13 and IV.14) were equivocal.

In conclusion, it seems reasonable to suggest that the data obtained in this section of the thesis can be interpreted in at least three ways.

1) There is a direct GA-membrane interaction which only occurs *in vivo* as has been suggested for steroid hormones in animals (Szego, 1972a, b).

2) GA induces a change in lysosomal membranes which is mediated by metabolic events (phospholipid synthesis, etc.).

3) GA has no effect on lysosomal membranes in aleurone cells.

In other words, can the effect of temperature on the leakage of α-amylase from lysosomes isolated from aleurone treated with different concentrations of GA be explained in any other terms than changes in membrane permeability? In the matrix binding theory advanced by Koenig (1969), the lysosome is regarded as a membrane-limited lipoprotein granule. The hydrolytic enzymes are contained

within the lysosome in an inert state by electrostatic binding to the lipoprotein matrix. Cited in favour of this proposal is the activation of lysosomal enzymes by heavy metals (Hg^{2+}) and extremes of pH (Koenig, 1969). However, Sawant *et al.* (1964) and Verity and Reith (1967) have reported similar pH curves for hydrolytic enzyme leakage to those reported by Koenig (1969) and in the present study (Figure IV.2) and concluded that such pH dependent labilization was a membrane effect.

Certainly the effects of calcium (Table IV.3) and phosphate (Table IV.7) on α-amylase leakage as well as the pH data (Figure IV.2) may equally well be explained in terms of changes in electrostatic binding as in terms of membrane effects. However, the consistent differences observed in the temperature dependent release of α-amylase (Figure IV.11) are perhaps best explained in terms of changes in membrane permeability due to an effect of GA *in vivo*.

No data were obtained in the present study that could distinguish between a direct *in vivo* interaction and a hormone induced change in the phospholipid composition of the lysosomal membrane. This crucial question must be addressed, particularly in view of the wealth of data available on GA-induced phospholipid synthesis in barley aleurone cells.

Within 2 hr of hormone addition to aleurone cells there is a significant increase in the activity of certain phospholipid synthesizing enzymes (Johnson and Kende, 1971) which are known to be associated with the ER (Moore *et al.*, 1973). Four hours after GA addition increased incorporation of ^{32}P into phospholipids is measurable (Koehler and Varner, 1973). This process reaches a maximum 10 hr after GA application.

Although Evins and Varner (1971) claimed to have measured increased phospholipid synthesis in barley aleurone ER, the more recent data of Koehler and Varner (1973) indicate that these workers were actually measuring increased turnover of phospholipids. Koehler and Varner (1973) demonstrated that the total phospholipid content of aleurone cells remained constant during GA treatment despite increased ^{32}P incorporation into phospholipids. This GA-induced increase in phospholipid turnover prompted Koehler and Varner to examine the ^{32}P incorporation into various phospholipids. The authors reported that GA caused an increase in ^{32}P incorporation into all phospholipids; no preferential synthesis of particular phospholipids was occurring.

Although such results have prompted Johnson and Kende (1971) to propose that GA-induced enzymes are only synthesized on new ER, there are no data available on this point. It may well be that the

phospholipid composition of different membranes in aleurone cells may be preferentially being changed but that the effect is not discernible when total phospholipids are examined. The recent work of Goldstone *et al.* (1973) indicates that only a small portion of rat kidney cell RER is involved in lysosomal enzyme synthesis. Since the results of the current study (Section III) suggest that GA does not cause a measurable increase in total ER, it could well be that only specific areas of aleurone cell ER undergo a change in membrane composition in response to the hormone.

The present data suggest that there is a measurable difference in the physical properties of at least one aleurone cell membrane: the lysosomal membrane. Although there is no direct evidence available to suggest a GA-induced change in aleurone cell membrane composition, the known involvement of phospholipid synthesis in this system would seem to favour such an explanation for the present results. If this is so, why is there such a difference in the permeability of lysosomal membranes isolated from 0.1 and 1.0 µg/ml GA-treated tissue? Surely after 24 hr of hormone treatment any phospholipid change would have occurred even though it could be suggested that this process is slower in 0.1 µg/ml GA-treated tissue.

It should be possible to determine whether the *in vivo* increase in lysosomal membrane permeability is due to a direct GA-

membrane interaction or is mediated by GA-induced phospholipid synthesis, experimentally. However, certain difficulties arise if α-amylase leakage is to be used as a measure of membrane permeability. Since α-amylase is essentially absent in control tissue, GA must be applied to induce the formation of α-amylase containing lysosomes. It may be that a marker, other than α-amylase, must be found to examine in detail the short term effects of GA, especially during the lag period.

One possible approach may be to incubate aleurone in low levels of GA (0.01 μg/ml) and after a suitable period (24 hr) add high concentrations of the hormone (100 μg/ml) for shorter periods. If GA directly increases the permeability of lysosomal membranes *in vivo*, then a change in the leakage rates of α-amylase from isolated lysosomes should be detectable within 30 min (time for GA to enter the cell; Goodwin and Carr, 1972). Should this process take longer, it would be reasonable to infer that some synthetic event is involved.

Although no definite conclusions concerning the mechanism of GA action can be drawn from the α-amylase leakage data presented here, it is felt that the results are sufficiently suggestive to warrant further investigation.

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