GIBBERELLIC ACID AND THE GERMINATION OF BARLEY.

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Presented by

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"There were things which he stretched, but mainly he told the truth".

Mark Twain about Huckleberry Finn.

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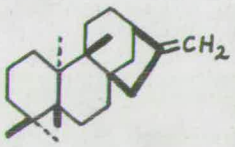
PLATES

INTRODUCTION.

The Naturally-Occurring Gibberellins.

The discovery of the gibberellins resulted from studies of the soil-borne "bakanae" disease of rice, caused by infection with the fungus, Gibberella fujikuroi (Stodola, 1957).

Kurosawa in 1926 showed that cell-free filtrates from cultures of the fungus contained a growth promoting principle and Yabuta and Hayashi in 1939 obtained an active crystalline material which they called gibberellin. This was a mixture of compounds and elucidation of the structure of these was not possible until gibberellic acid (gibberellin A_3) was isolated in good yield independently by Curtis and Cross (1954) and Stodola et al.(1955). The structure of this compound was established by Brian, Grove and MacMillan (1960) and it seems probable (Birch et al., 1958, 1959) that its biosynthesis follows substantially the same path as that of steroids and terpenes with kaurene (below) as an intermediate (Cross et al., 1964).



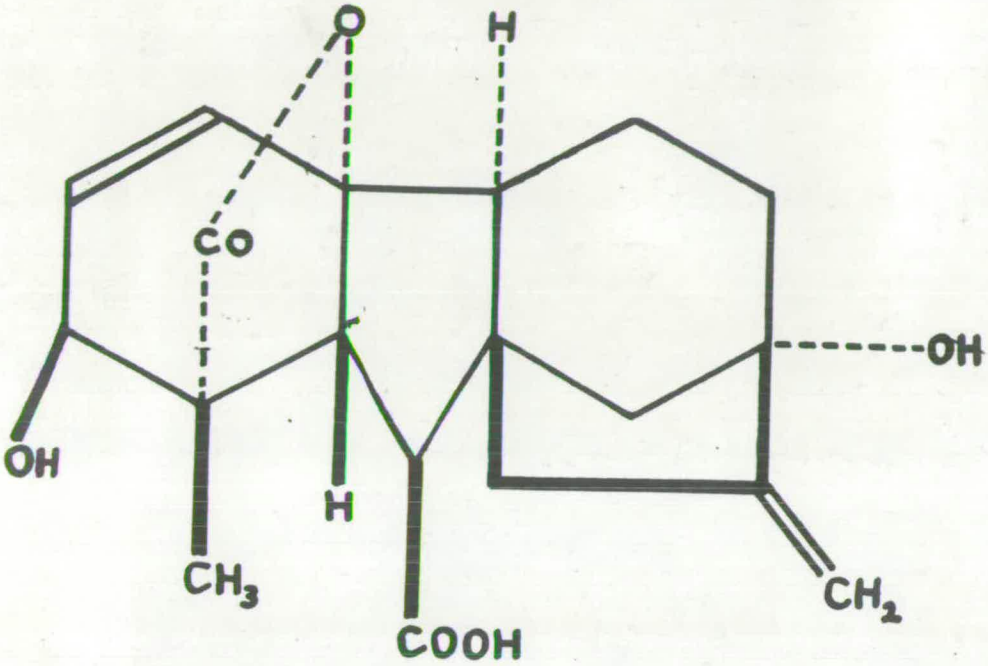
Kaurene

The structure of gibberellin A_3 along with those of the other gibberellins occurring in fermentation broths of Gibberella fujikuroi is shown in Figure I (Brian, 1961).

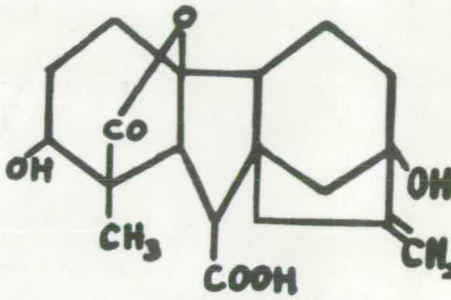
The natural occurrence of gibberellins in plants is now becoming more and more clearly established. Adler et al. using paper chromatography in conjunction

FIGURE I

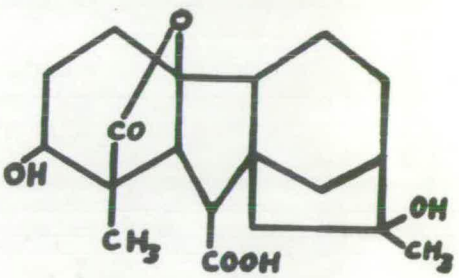
THE KNOWN GIBBERELLINS.



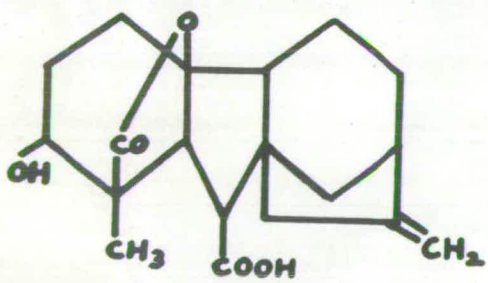
A_3



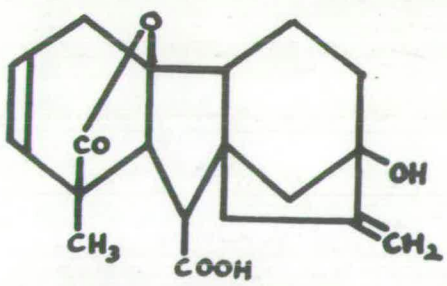
A_1



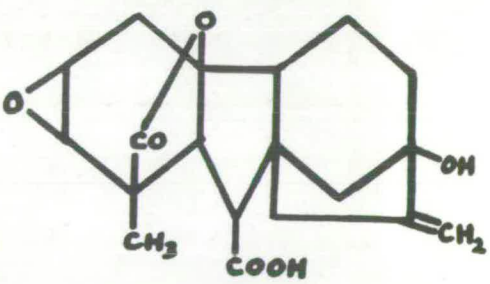
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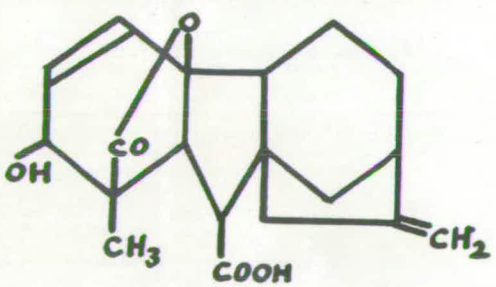
A_4



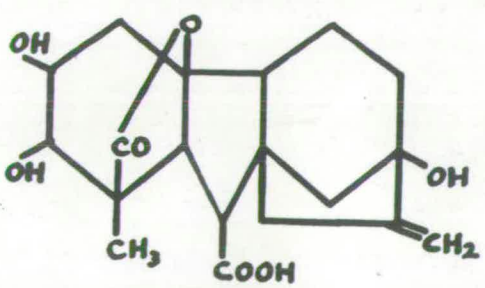
A_5



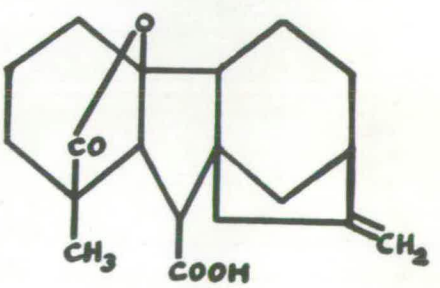
A_6



A_7



A_8



A_9

with bio-assay and a sulphuric acid spray detected the presence of a gibberellin having R_f values similar to gibberellic acid in two solvent systems, in extracts of the following plants: Kudzu vine, pinto bean, alfalfa, sweet corn, dandelion and lima bean. Radley (1959) using similar techniques, produced evidence of the presence of gibberellic acid in malt. Further evidence of this has come from the isotope dilution analysis of Lazer et al. (1961). In addition, Jones, MacMillan and Radley (1963) have presented strong evidence for the presence of gibberellic acid in immature barley. Fleming and Johnson (1964) have reported chromatographic evidence for the presence of gibberellins, including gibberellic acid, in wheat. Phaseolus multiflorus has been particularly closely investigated in respect of its gibberellin content. MacMillan et al. (1958, 1959, 1960, 1961) found gibberellins A_1, A_5, A_6 and A_8. Jones (1964) using thin layer chromatography, found A_3 in addition to these and two new gibberellins which he designated a and b. In the same paper, Jones reports the presence of A_3 and an isomer in Zea Mays. Gibberellins A_1, A_3, A_5, A_6 and A_8 together with 5 other gibberellin-like substances, "Phaseolus α — ε", have been recorded in extracts of Phaseolus coccineus (Sembder et al., 1964). In Echinocystis macrocarpa, gibberellins A_1, A_3, A_4 and A_7, together with a derivative of A_7, appear to be present (Elson et al., 1964) Finally, gibberellin A_1 has been isolated from sprouts of Citrus unshui

(Kawarada and Sumiki, 1959) and possibly from excised tomato roots (Butcher, 1963). Thus only gibberellins A_2 and A_9 have not been located so far in plant extracts.

All the gibberellins show qualitatively similar biological activity. Quantitatively, however they may be placed in the following order on the basis of their effect on dwarf pea stem and lettuce hypocotye extensions (Brian, 1961):

$$\left.\begin{matrix} A_3 \\ A_5 \\ A_7 \end{matrix}\right\} > \left.\begin{matrix} A_1 \\ A_4 \\ A_6 \end{matrix}\right\} > \begin{matrix} A_2 \\ A_8 \end{matrix}$$

In barley, on the other hand, the order of activity in stimulating germination is as follows (Griffiths et al., 1964):

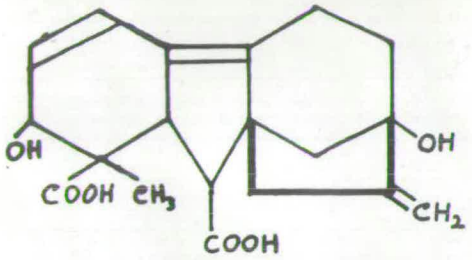
$$A_1 > A_7 > A_4 > A_3 > A_8 > \underset{\text{Acid}}{\text{Gibberellenic}} > A_5 > \underset{\text{Acid}}{\text{Allogibberic}} > A_9 > \underset{\text{(Inactive)}}{\underset{\text{gibberic Acid}}{\underset{\text{and}}{\text{Gibberic Acid}}\ \text{Epi-allo-}}}$$

Gibberellenic acid, allogibberic acid, gibberic acid and epi-allogibberic acid are all derivatives of gibberellic acid which have so far not been isolated from natural sources (Figure II).

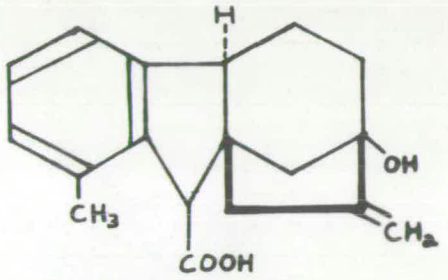
Griffiths et al, have also studied the order of effectiveness of the above compounds in stimulating reducing sugar release from de-embryonated barley. This is shown below and will be seen to differ from that with respect to germination.

FIGURE II

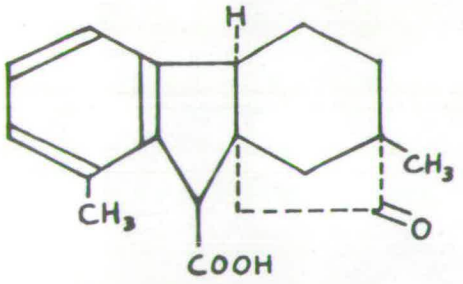
DERIVATIVES OF GIBBERELLIC ACID.



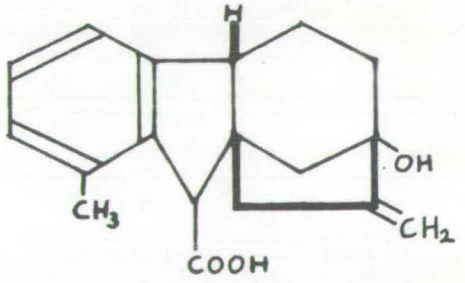
GIBBERELLENIC ACID



ALLOGIBBERIC ACID



GIBBERIC ACID



EPI-ALLOGIBBERIC ACID

$A_1 = A_3 = A_4 = A_7$ > Gibberellenic Acid > A_8 = Allogibberic Acid > A_5 > A_9 > Gibberic Acid and Epi-allogibberic Acid

100% 75% 40% 30% 20% 0%

Physiological Effects of Gibberellins.

Stem growth is enhanced in many plants, normally by an increase in internode length without an increase in internode number, and only those internodes which are expanding at the time of application respond, (Brian et al., 1958). In treated stems, apical dominance is accentuated but, if the apical bud is excised, lateral buds are stimulated (Brian et al., 1954). Root growth is, if anything, slightly inhibited.

The leaves of gibberellin treated plants appear relatively elongated but the final leaf area is usually unaffected (Humphreys, 1958).

In many plants dormancy is broken by treatment with gibberellins (Barton et al., 1957; Liverman and Johnson, 1957; Lona, 1956; Rappaport, 1956).

Rosette plants which normally require vernalisation or long days to induce flowering are in a large number of cases induced to bolt by gibberellins under non-inductive conditions, and form flowers, although the initiation of flower primordia is not detected until considerable stem extension has taken place and thus there is a general delay in flowering (Lang, 1956).

Caulescent plants, which normally require vernalisation or long days to induce flowering may respond to gibberellin treatment under non-inductive conditions

by the typical long-day response of longer internodes but they will not flower (Chouard, 1956).

Short-day plants, under long-day conditions, cannot be induced to flower by gibberellin treatment but, with short-day conditions, vegetative growth in response to gibberellin is similar to that caused by long days, with little or no effect on the initiation of flower primordia (Greulach and Haesloop, 1958).

Treatment of certain long-short day plants, e.g. Bryophyllum spp., causes flowering in short days but not in long days (Bünsow and Harder, 1956)

Parthenocarpic development of tomato fruit has been induced by gibberellins (Wittwer et al., 1957)

Seedless grapes respond to gibberellic acid treatment by swelling (Weaver and McCune, 1959).

Gibberellins have been reported to cause chlorosis but it is not certain that this is a direct effect and Brian et al. (1954) have shown that it can be somewhat alleviated by ensuring that mineral nutrients are non-limiting.

There are indications that gibberellins can cause sex reversal. Treated monoecious Cucumis sativus shows a 58% increase in the number of staminate plants while genetically female plants of di-oecious Cannabis sativa show sex reversal in the early stages of flowering (Bukovac and Wittwer, 1961; Atal, 1961).

Ivy, in the adult stage, when treated with gibberellin reverts to the juvenile morphology for any new growth.

Cuttings from this new growth produce the characteristic long internodes, lobed leaves, aerial roots and general non-flowering juvenile form (Robbins, 1960).

At present it is quite impossible to suggest any one first cause of all these, apparently unrelated phenomena, but it is reasonable to suppose that one exists though it may be difficult to detect owing to the complexity of the systems involved.

Translocation of Gibberellins.

The gibberellins are completely inactive in the Went pea test, the Avena (coleoptile bending) test and the tomato leaf epinasty test (Kato, 1953). It has therefore been suggested that, unlike indole-acetic acid, gibberellins can rapidly translocate in the stalk.

That translocation is rapid and non-directional is further indicated by the fact that apical application affects the development of branches farther down the stem and application to a lower leaf has as great an effect on the upper internodes as would an apical application (Brian, 1959).

Studies of the Cellular Effects of Gibberellic Acid in Plants Other than Barley.

It is observed with intact plants that, when increased growth occurs after gibberellin treatment it is due both to increased cell multiplication and to cell extension. However, isolated sections show little extra growth except where the tissue chosen has a high endogenous rate of growth (Brian,1959) or where indole-acetic acid is present (Brian and Hemming,1957,

1958). It is worth noting that indole-acetic acid itself, while producing large effects on cell extensions of sections, has little or no effect on whole plants.

The effects of gibberellin and of auxin treatment are additive in etiolated sections (Kato, 1958) and synergistic in green sections (Brian and Hemming, 1958). Brian and Hemming suggest that gibberellins block the action of some inhibitor and make auxin fully active. However, a more probable explanation is that gibberellins reduce the synthesis of auxinase or cause the production of an auxinase inhibitor. Evidence of reduced auxinase activity in response to gibberellins has been produced for lupins (Stuty and Watanabe, 1957), clover (Pilet and Wurgler, 1958), excised pea stems (Housley and Deverall, 1960) and cucumber hypocotyes (Halevy, 1963). Galston (1957) claims to have found an increase in an auxinase inhibitor in peas following gibberellin treatment. Sagi and Garay (1961) present evidence for a dialysable auxinase inhibitor in lupin leaves and suggest a correlation between increase in phenol content and decrease in auxinase activity.

Gibberellin and auxin show synergistic activity in intact plants as well as in sections. This may be seen in the bolting response of Silene armeria (Lang, 1959). However, they show antagonism in their effects on the bolting response of Impatiens balsamina (Weizer, 1959).

Although gibberellin has little effect on roots of intact plants, Street (1959) has reported interesting interactions with auxins in excised tomato root cultures. He concluded that if endogenous auxins in the root were supra-optimal for growth, gibberellin accelerates the normal loss of meristematic activity (ageing) and that it is similarly inhibitory if endogenous auxin is markedly sub-optimal for growth. At intermediate auxin levels, gibberellin may be stimulatory to growth.

Zinc (Dancer, 1959) and glutathione (Marrè and Arrigoni, 1958) show synergisms with gibberellic acid in stem extension. Possibly the function of the former here is its part in tryptophan synthesis and hence indole-acetic acid production. Glutathione seems to be necessary for the optimal activity of many enzymes as well, possibly, as being involved in certain respiratory processes.

The Effects of Gibberellins on Barley.

In 1940 Hayashi reported an increase in α-amylase activity in germinating cereals grown in the presence of gibberellin A.

In 1957, Munekata and Kato reported that gibberellin A increased acrospire growth in barley as well as stimulating the activity of protease, amylase and catalase. This activity was important only initially since controls tended later to catch up on treated samples. Sandegren and Beling (1959) obtained similar results and found also an increase in "cellulase"

activity. Paleg (1960) showed that this action was mediated through the endosperm and not the embryo. This was verified by MacLeod and Millar (1962) who located the site of action more precisely in the aleurone layer. Briggs (1963) independently came to the same conclusion. MacLeod and Millar also added endo-β-glucanase and endo-pentosanase to the list of enzymes showing increased activity in the presence of gibberellic acid in early stages of germination and in endosperm slices. The only non-hydrolytic enzyme claimed to have enhanced activity so far is glutamate-oxalacetate transaminase (Bergquist et al., 1959).

There are various possible modes of action for gibberellic acid leading to an increased enzymic activity. There is no evidence that the compound acts directly on any of the enzyme systems studied. It has already been mentioned that gibberellic acid may stimulate the production of an auxinase inhibitor in some tissues. By analogy, either it could reduce the concentrations of inhibitors of other enzymes or stimulate synthesis of activators. In this connection, one must consider the possibility of zymogen activation such as occurs in the mammalian intestine. However, all zymogens known are precursors of proteases and their activation generally requires proteolytic activity. Thus the finding of MacLeod and Millar (1962) that apparent inhibition of proteolysis with potassium bromate has no effect on the increase in endo-β-glucanase activity

or on sugar release from endosperm slices tends to militate against this possibility. In the same paper, MacLeod and Millar suggested that gibberellic acid might act by causing the rupture of lysosomes but this suggestion has so far received no experimental support.

The possibility that gibberellic acid acts by stimulating synthesis of the various enzymes is the one which has received most attention. This was first suggested by Briggs (1963). However, the evidence he puts forward to support this view is unsatisfactory since it depends largely on the use of metabolic inhibitors at concentrations at which they probably have multiple effects. In particular, one may single out 2,4-dinitrophenol which he uses in a millimolar solution, a concentration at which it has been shown to inhibit respiration completely in a number of tissues (Simon, 1953). Further, he states that gibberellic acid stimulates the incorporation of labelled amino-acids into the proteins released from endosperm into the incubation medium, but he presents no evidence to show that the labelled amino-acids have actually been incorporated into polypeptide chains rather than non-specifically adsorbed.

Yomo and Iinuma (1964a) and Varner and Ram Chandra (1963,1964) have also carried out studies with metabolic inhibitors. The results suggest that protein synthesis of some kind is required for gibberellic acid activity but they are very difficult to explain in their entirety. Yomo and Iinuma found that the amino-acid

analogues, ethionine and norleucine, both inhibited the increase in α-amylase activity induced by gibberellic acid. The inhibitions were reversed by methionine and leucine respectively. However, this applied only if the inhibitors were present in the medium from the start of the incubation. If endosperm pieces were incubated in gibberellic acid for 48 hours at 17°C and then transferred to solutions of the inhibitors, the inhibitors had virtually no effect, even at concentrations as high as 4×10^{-3}M for ethionine and 2×10^{-2}M for norleucine. Despite these high concentrations, it seems possible that the failure of these compounds to inhibit may have been due to the liberation of competing amino-acids as a result of gibberellic acid induced protease action.

The effects of purine and pyrimidine analogues seem to depend upon whether endosperm pieces or isolated aleurone layers are being studied. The release of reducing sugar from barley endosperm which is caused by 1.5×10^{-5}M gibberellic acid is inhibited by 28% by 2×10^{-2}M 6-mercapto-purine and by 44% by 2×10^{-2}M 5-bromo-uracil (Briggs, 1963). The concentrations of these inhibitors are such that one would have expected a very powerful inhibition of protein synthesis if the inhibitors could reach the appropriate sites. The small degree of inhibition suggests either that sugar release is not dependent on de novo enzyme synthesis

or that these inhibitors do not penetrate to the sites of protein synthesis.

In agreement with Briggs (1963), Yomo and Iinuma (1964a) report that $5x10^{-2}$M 5-bromo-uracil and $5x10^{-3}$M 8-azzguanine both produce only slight inhibitions of $6x10^{-6}$M gibberellic acid action on endosperm. In addition, Varner (1964) found that when azaguanine, thiocytosine or thio-uracil was added with 10^{-6}M gibberellic acid to endosperm halves which had been pre-incubated for three days on moist sand , no inhibition of the gibberellin-induced increase in α-amylase activity was observed. The same was true if, following pre-incubation, the endosperm halves were incubated with inhibitors for 24 hours prior to adding gibberellic acid. Only if the endosperm halves were pre-incubated from the air-dry state on sand moistened with the inhibitors were the inhibitors effective. Varner and Ram Chandra (1964) repeated this work with aleurone layers isolated from endosperm halves after three days pre-incubation on moist sand and found that 10^{-4}M 5-bromo-uracil produced 45% inhibition of 10^{-6}M gibberellic acid action. 6-Azaguanine and 8-aza-adenine at the same concentrations produced 12% and 34% inhibitions respectively. Although Varner (1964a) does not quote the concentrations of these inhibitors he used with endosperm halves, it is clear by comparison with the results obtained by other workers that these inhibitors are much more effective on the isolated aleurone layers. This supports the suggestion that

lack of action of these inhibitors on endosperm is due to their poor penetration to the sites of protein synthesis.

The effectiveness of Actinomycin D too is greater on isolated aleurone than intact endosperm. Paleg (1964) found no inhibition by Actinomycin D, at concentrations up to 500$\mu g.\ ml^{-1}$, of gibberellic acid (10^{-6}M) activity in endosperm slices cut from grain which had not been presoaked (apart from 2 hours surface sterilisation). Varner and Ram Chandra (1964), however, report that Actinomycin D, present from the start of a gibberellic acid incubation, inhibits α-amylase formation in aleurone isolated from endosperm presoaked for three days. It does not do so if added after seven hours. This is explained on the basis of an inhibition of "messenger" ribonucleic acid synthesis. While this seems quite plausible, it would be more satisfactory if some direct evidence of such an inhibition were available in view of the relatively high concentration of the compound used ($100\mu g.\ ml^{-1}$).

Varner and Ram Chandra (1964) also studied the incorporation of labelled amino-acids into protein in the presence of gibberellic acid. In particular, they studied α-amylase which they isolated in a pure state using a crystalline preparation as a carrier, following the method of Loyter and Schramm (1962). After incubation of endosperm halves in 10^{-6}M gibberellic with 1 c of L-leucine-C^{14} for 30 hours, pure

α-amylase was found to contain at least 12% of the total counts incorporated into protein. Further, most of the peptides produced from this enzyme by partial hydrolysis with trypsin and separated by the "finger printing" technique were found to be labelled. Thus it is clear that α-amylase synthesis had taken place. However, quantitative aspects of this are difficult to analyse. The total label of amino-acid incorporated into protein decreased considerably (by nearly two thirds) in endosperm halves incubated with gibberellic acid compated with endosperm halves incubated in water with the same amount of labelled amino-acid. This is attributed to the gibberellin dependent increase in proteolytic activity, causing the release within the tissue of free amino-acids which compete with the labelled acids. It means that it is impossible to compare the amount of protein synthesised in gibberellic acid treated endosperm halves with the amount synthesised in endosperm halves incubated in water for the same period. Similar results were obtained with isolated aleurone.

In conclusion, one may say that while de novo protein synthesis appears to be necessary before gibberellic acid can exert a detectable effect, there is still no definite evidence that gibberellic acid itself acts on the protein synthesising process.

Introduction to the Thesis

While the bulk of the work to be described in this thesis is devoted to elucidating the mode of action of gibberellic acid, it seemed that the possible role of this substance in normal germination had not been very closely studied. Accordingly, Section I of this thesis describes an investigation of the gibberellic acid contents of embryo and endosperm in the mature grain.

Section II of the thesis is devoted to a study of the effects of various inhibitors on the response of endosperm slices to gibberellic acid. The main inhibitors used were uncoupling agents, inhibitors of terminal oxidases and inhibitors of protein synthesis. Thus it was hoped to discover something of the energy requirements for the increase in enzyme content of the endosperm and also whether the apparent requirement for *de novo* protein synthesis found by other workers could be verified using different inhibitors.

Interpretation of the results obtained with inhibitors required a determination of the time-course of increase in enzyme activity. Further, it became clear that to investigate the various possible facets of the increase in enzyme activity (synthesis, secretion, release from a bound form or activation etc.), isolated aleurone must be studied. Both of these aspects are considered in Section III.

Since it became clear from the work reported in Section III that at least α-amylase and endo-β-glucanase were secreted from the aleurone layer in response to

gibberellic acid, an investigation of the ultrastructural changes in the aleurone in response to gibberellic acid was undertaken. These changes were correlated with the enzyme synthesis and secretion observed. This work is described in Section IV.

SECTION I.

The Location of Gibberellic Acid in Mature Barley.

Brown and Escombe (1898) showed that barley aleurone was implicated in cell-wall digestion in the endosperm and that this took place most rapidly when the embryo was present. Schander (1934) confirmed the importance of this role of the aleurone cells in normal germination of rice and Stipa. He found that removal of a ring of aleurone directly above the scutellum prevented growth and edosperm modification and concluded that some substance must move from the embryo to the aleurone to permit this modification. When this experiment was repeated with barley (MacLeod and Millar, 1962), it was found that gibberellic acid could replace this substance. Since gibberellic acid is almost certainly present in the barley grain (Lazer et al., 1961; Jones, MacMillan and Radley 1963) it is reasonable to conclude that it may in fact be identical with the unknown substance. However, while gibberellic acid has been fairly thoroughly characterised in immature barley (Jones, MacMillan and Radley, 1963) and more tentatively in malt (Lazer et al., 1961) difficulty has been experienced in trying to demonstrate its presence in the mature grain (Jones, 1964b). This suggests that the amount of gibberellic acid, like that of some enzymes (Johnston, 1964), decreases almost to zero during the later stages of maturation and that it is resynthesised during germination.

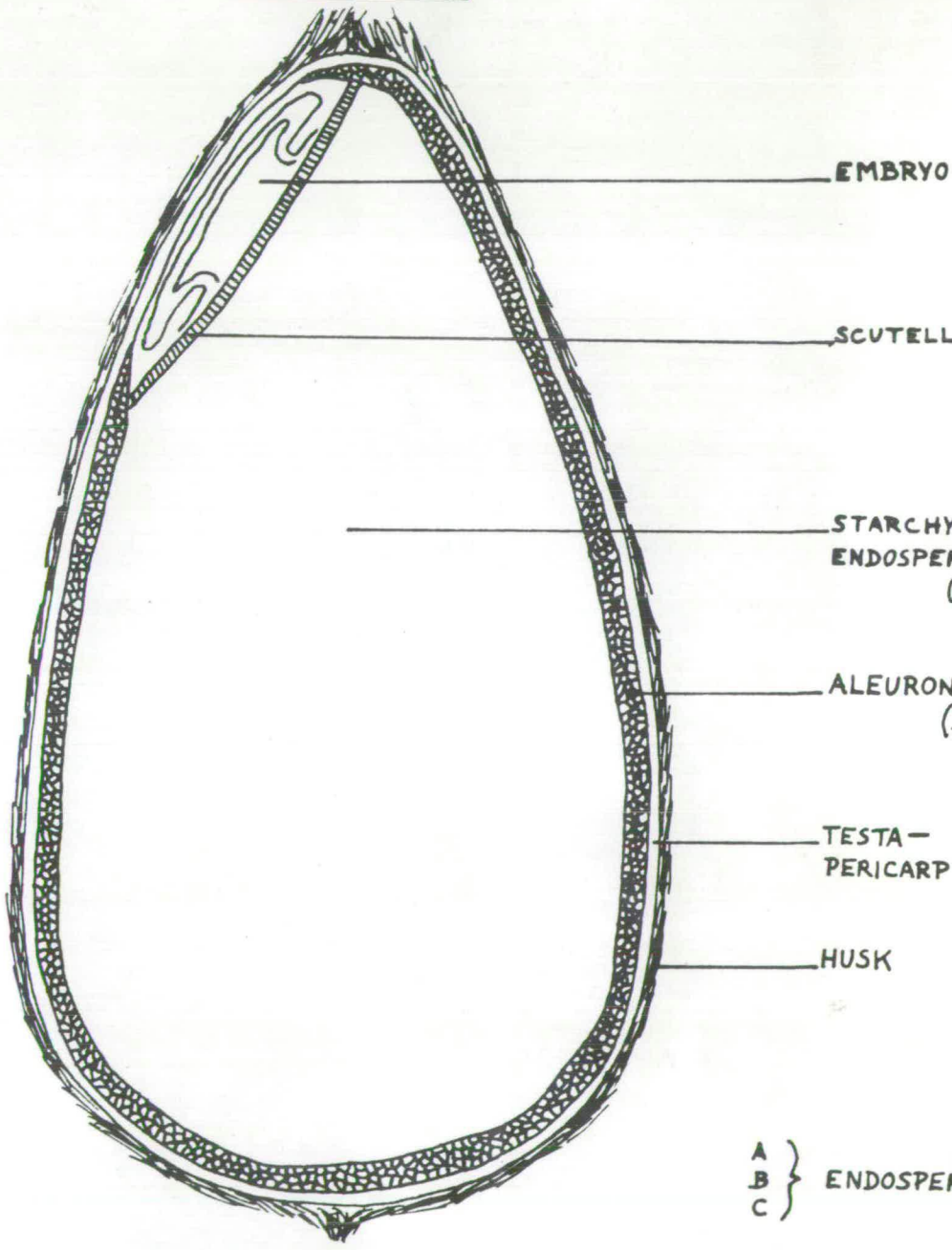
On the assumptions that there is a very small amount of gibberellic acid in the mature grain and that gibberellic acid fulfils the hormonal function suggested, it seems probable that the gibberellic acid may be localised in the embryo. To test this, embryos were dissected out from the grain and they and the remaining tissues were extracted separately. The extracts were partially purified and examined for gibberellins by thin layer chromatography. Compounds tentatively identified by this method were eluted from the chromatograms and their activity in causing an increase in α-amylase content of endosperm slices was determined. This provided a rough estimate of their concentrations in terms of gibberellic acid.

Experimental.

General

The barley used in locating gibberellic acid in the mature grain was Proctor from the 1962 harvest. Before use, the barley was dehusked by treating with 50% sulphuric acid for three to four hours at room temperature. Thereafter it was washed thoroughly and treated with a suspension of calcium carbonate to neutralise any remaining traces of acid. The grain was then washed free of calcium carbonate, dried on filter paper, and stored at $4^{\circ}C$ in a sterile container. No results quoted are for samples stored for more than three months after dehusking though it was found that the barley could be stored for periods of 5-6 months

FIGURE III



BARLEY GRAIN (L.S.)

SECTION THROUGH THE MEDIAN GROOVE TO SHOW THE RELATIO

before detectable deterioration started.

Embryos were dissected out by hand from the dry grain using a dissecting needle with a cutting edge. This was inserted under the embryo at the tip of the grain and moved round with gentle leverage, cutting through the starchy endosperm just below the scutellum (Figure III). Batches of 2,000 grains each were treated in this way and the embryos and endosperm were extracted separately as described below. Latterly, since it appeared that biologically active material was localised in the embryo, the grain was dissected simply by means of a vertical incision behind the embryo. This technique provides a pure endosperm preparation but an embryo preparation which contains a quantity of endosperm as well.

The embryoswere ground in ethanol in a glass homogeniser, about 500 being extracted in 5 ml. of the solvent. The extraction was repeated twice and then another 500 embryos were extracted in the same way and so on until all 2,000 had been treated. The bulked extract was evaporated to dryness at the water-pump. The residue was suspended in 10 ml. water to which a large spatula tip of activated charcoal had been added. This mixture, after addition of 1 ml. 0.1M phosphate-citrate buffer, pH2.4, was extracted thrice with 10 ml. ethyl acetate and the bulked extract evaporated to dryness at the water-pump as before. The oily residue was further purified chromatographically (see next page)

Crude embryo preparations of 2,000 embryos, containing endosperm, were ground in a coffee mill prior to extraction. Extraction was carried out in an MSE top-drive blender with 50 ml. ethanol. This was repeated twice and the bulked extract was treated as for the pure embryo preparation.

Endosperm too was ground in the coffee mill prior to extraction. One thousand endosperms were extracted at a time in about 70ml. ethanol using the MSE top-drive blender. Again the extraction was repeated twice and the bulked extract treated as for the pure embryo preparation.

Thin Layer Chromatography.

The methods followed were those described by Jones, MacMillan and Radley (1963). Thin layers (300 microns thick) of Kieselguhr G and Silica Gel G (E. Merck, A.G. Darmstadt) were prepared using a Shandon Unoplan Leveller.

The solvent systems used were as follows:

1. Benzene: acetic acid : water (8:3:5)
2. Di-isopropyl ether: acetic acid (95:5)
3. For two dimensional chromatography on Silica Gel G:
   a) Ethyl Acetate
   b) Water

Solvent system I was used on Kieselguhr G plates which were equilibrated overnight with the lower phase and then developed with the upper phase. Solvent system 2 was used on Silica Gel G plates.

TABLE I.

R_f Values of the Known Gibberellins on Thin Layers of Kieselguhr and Silica Gel.

Gibberellin	Kieselguhr Layer	Silica Gel Layer	
	$C_6H_6/HOAc/H_2O$	$(C_3H_7)_2O/HOAc$	$C_2H_5OAc : H_2O$ *
A_1	0.26	0.11	1.00
A_2	0.30	0.04	0.89
A_3	0.18	0.11	1.00
A_4	1.00	0.37	0.89
A_5	0.88	0.31	0.83
A_6	0.76	0.25	0.93
A_7	1.00	0.37	0.89
A_8	0.06	0.04	1.07
A_9	1.00	0.75	0.78

*Mobilities relative to gibberellic acid (A_3) which has an R_f = about 0.90.

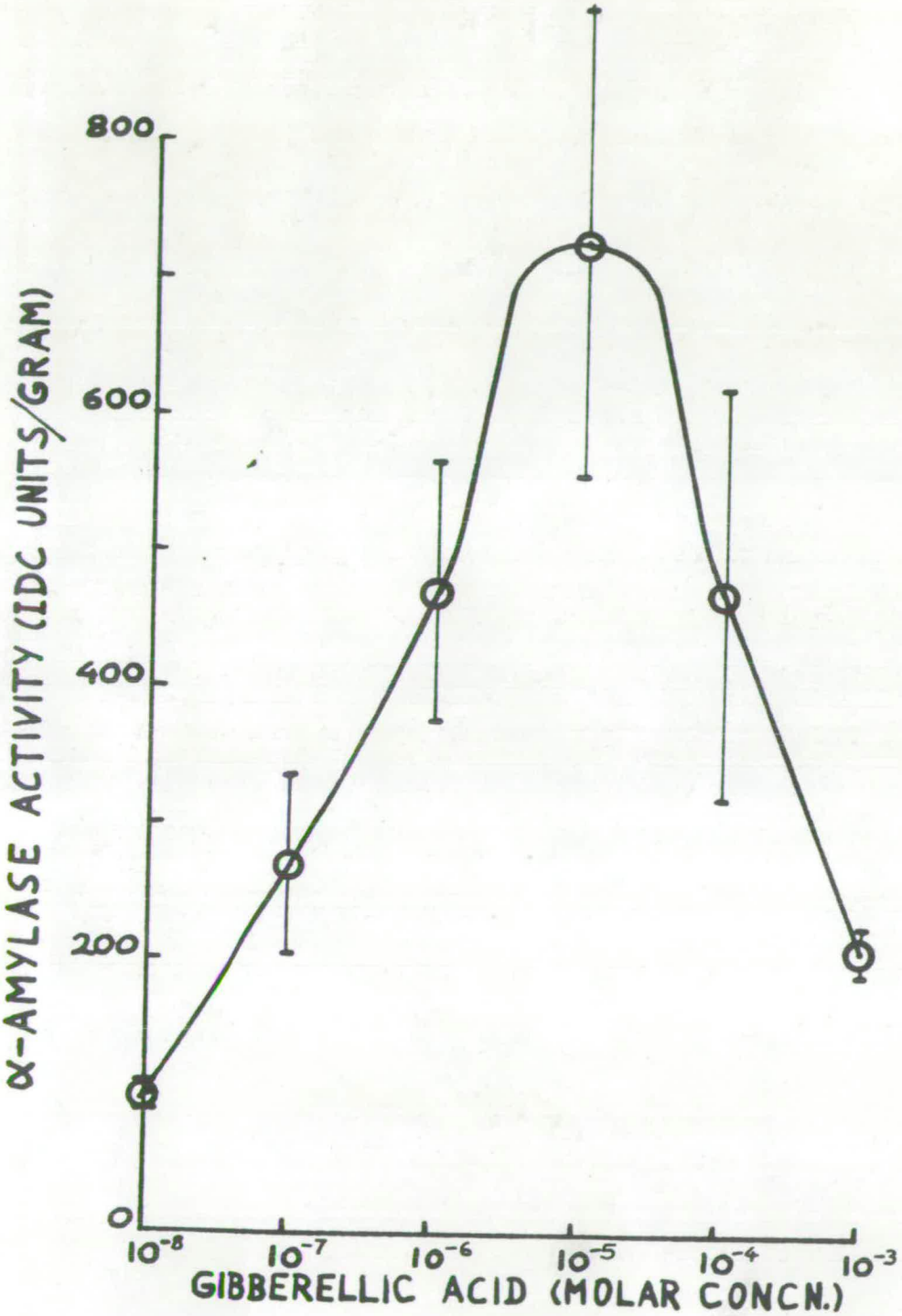
Solvent system 3 has the advantage that the ethyl acetate elutes lipid contaminants without affecting gibberellins which are subsequently separated by development in water.

The plates were dried at room temperature and sprayed with 70% sulphuric acid. With this spray, before heating, only gibberellins A_3 and A_7 are visible in ultraviolet light, if present in sufficient quantity. After heating at 120°C for ten minutes, all the gibberellins are visible in ultraviolet light. Gibberellins A_1, A_3, A_6, and A_8 give green-blue fluorescent spots; gibberellins A_2, A_4 and A_9 give blue-purple fluorescent spots; gibberellins A_5 and A_7 give light yellow and brown fluorescent spots respectively. The sensitivity of the spray is reported to range from 0.00025µg. gibberellin A_3 to 0.01µg. gibberellin A_6 (MacMillan and Suter, 1963).

The normal R_f values of the gibberellins in the solvent systems used are shown in Table I. Since they are somewhat variable, a gibberellic acid control was run where-ever possible.

Solvent system 1 was used for further purification of the oily residues obtained from embryo and endosperm fractions following the treatment already described. The residues were streaked across one or more Kieselguhr plates and these were developed with this solvent system (Plate 1). After drying, bands of Kieselguhr were scraped off at appropriate R_f values and each was extracted thrice with 5ml acetone. The total extract

FIGURE IV



TEMP.=25°C. PROCTOR BARLEY (1963).
INCUBATION PERIOD=42 HRS.

CHANGE IN α-AMYLASE ACTIVITY WITH INCREASING GIBBERELLIC ACID CONCENTRATION.

from each band was evaporated to dryness under vacuum. The residues were tested for biological activity and were rechromatographed using solvent systems 2 and 3.

Biological activity was assayed by suspending each residue in 1 ml. of water and adding 10 endosperm slices. The flasks were sealed with Parafilm and incubated at 25°C. for 42 hours. After this period the slices were extracted by grinding in 2.5 ml. 0.1M sodium chloride in a glass homogeniser. The homogenate was allowed to stand for two hours to ensure complete extraction. Then solid material was removed by centrifugation and the α-amylase activity of the supernatant was determined using the Iodine-Dextrin Colour method described on pp.38 - 41. Comparison of this activity with that obtained using known concentrations of gibberellic acid (Figure IV) enable an approximate estimation of the amount of active material in terms of this compound to be made. Provided the concentration of active material in the medium corresponded to less than 10^{-6}M gibberellic acid, the amount of enzyme extracted into the medium was negligible and could be ignored.

TABLE II

Summary of the Results of Thin Layer Chromatographic Studies of Ethanol Extracts of Barley Embryos.

Kieselguhr Layer $C_6H_6/HOAc/H_2O$ R_f of Band	Enzyme Activity Assay μg. G.A.*/kg. fresh wt. of embryo.	Silica Gel Layer $(C_3H_7)_2O/HOAc$ Gibberellins?	Silica Gel Layer $C_2H_5OAc : H_2O$ Gibberellins?
0.00 0.05	0 (2)	- (3)	- (2)
0.05 0.15	0.7 ± 0.1 (2)	A_3 (3)	- (2)
0.15 0.25	0.7 ± 0.1 (2)	A_3A_1 (3)	- (2)
0.25 0.40	0 (2)	- (3)	- (2)
0.40 0.55	0 (2)	- (2)	- (2)
0.55 0.70	0 (2)	- (2)	A_4, A_9 (2)
0.70 0.85	0 (2)	- (2)	A_4, A_9 (2)
0.85 1.00	0 (2)	- (2)	A_4, A_9 (2)

* Gibberellic Acid Equivalent.

Numbers of experiments are given in parentheses

Gibberellic acid has an R_f value of 0.18 on the Kieselguhr layer.

Results

Embryo Extracts (Table II)

Of the fractions obtained from the crude extracts by thin layer chromatography on Kieselguhr, only those eluted from the two bands between the R_f values 0.05 and 0.25 caused an increase in the α-amylase activity of endosperm. These fractions would be expected to contain gibberellic acid if it were present. Chromatographic analysis of these fractions on a Silica Gel thin layer developed with di-isopropyl ether/acetic acid solvent showed the presence of a component which corresponded to gibberellic acid in its R_f value and fluorescence in response to a 70% sulphuric acid spray (Plate 2). However, further analysis on a Silica Gel thin layer developed with the two dimensional system described earlier yielded no gibberellin-like spots from these fractions (Plate 4B,C).

The three fractions eluted from bands between the R_f values 0.55 and 1.00 after chromatography on Kieselguhr had no effect on endosperm slices nor could any gibberellin-like spots be detected following chromatography on Silica Gel with the di-isopropyl ether/acetic acid solvent probably because of the complexity of the fractions (Plate 2). However, chromatography of these fractions on Silica Gel thin layers using the two dimensional system revealed two spots which, from their R_f values and response to the sulphuric acid spray, appeared to correspond to

TABLE III

Summary of the Results of Thin Layer Chromatographic Studies of Ethanol Extracts of Barley Endosperm.

Kieselguhr Layer $C_6H_6/HOAc/H_2O$ R_f of Band	Enzyme Activity Assay $\mu g.$ G.A.*/kg. Fresh wt. of endosperm.	Silica Gel Layer $(C_3H_7)_2O/HOAc$ Gibberellins?	Silica Gel Layer $C_2H_5OAc : H_2O$ Gibberellins?
0.00 0.05	0 (2)	- (2)	- (2)
0.05 0.15	0 (2)	A_3 (2)	- (2)
0.15 0.25	0 (2)	A_3A_1 (2)	- (2)
0.25 0.40	0 (2)	- (2)	- (2)
0.40 0.55	0 (2)	- (2)	- (2)
0.55 0.70	0 (2)	- (2)	A_9? (2)
0.70, 0.85	0 (2)	- (2)	A_9? (2)
0.85 1.00	0 (2)	- (2)	A_9? (2)

*Gibberellic Acid Equivalent

Numbers of experiments are given in parentheses.

Gibberellic acid has an R_f of 0.18 on the Kieselguhr Layer.

gibberellins A_4 and A_9 (Plate 4 F,G,H). These spots were eluted with three 5 ml. portions of ethyl acetate from an aqueous suspension (5 ml.) of the appropriate part of the Silica Gel layer. The ethyl acetate was removed under vacuum and the residue tested for its effect on α-amylase activity in endosperm slices. No response was observed.

Endosperm Extracts (Table III).

None of the fractions obtained after thin layer chromatography on Kieselguhr caused an increase in endosperm α-amylase activity. On the other hand, the two obtained from bands between the R_f values 0.05 and 0.25 did contain a substance which chromatographed like gibberellic acid on Silica Gel layers developed with di-isopropyl ether/acetic acid solvent and which responded similarly to the sulphuric acid spray (Plate 3). These fractions, like the corresponding ones from embryo extracts, showed no gibberellin-like components following chromatographic analysis on silica gel layers with the two dimensional system (Plate 5 B,C).

The fractions eluted from bands between the R_f values 0.55 and 1.00 after chromatography on Kieselguhr, again like those from embryo extracts, had no effect on endosperm slices. Chromatography on Silica Gel layers revealed nothing when they were developed with the di-isopropyl ether/acetic acid solvent. However, development with the two dimensional system revealed a component which gave a blue fluorescence in response to the sulphuric acid spray. Its R value lay between 0.59

and 0.64. This is slower than any of the known gibberellins although one might have been severly retarded by the other components of the fraction (Plate 5 F, G, H).

Discussion.

The only fraction active in promoting an increase in ∝-amylase activity in endosperm slices was obtained from the embryo extracts. From its R_f values on the Kieselguhr thin layers and its chromatographic analysis on Silica Gel thin layers with di-isopropyl ether/acetic acid solvent this fraction appeared to contain gibberellic acid and possibly gibberellin A_1. However, corresponding gibberellin-like components could not be detected following chromatography on Silica Gel layers using the two-dimensional system. Since this system depends upon separating lipid compounds from gibberellins with ethyl acetate, followed by separation of the gibberellins with water, it is tempting to suggest that the fraction may contain the gibberellins mentioned, bound to a lipid moiety.

The presence in both extracts of compounds which might be identified chromatographically as gibberellins but which exert no effect on endosperm slices emphasises the tentative nature of such identification. It is possible that some of these compounds are inactive derivatives of gibberellins.

As far as they go, the results are in keeping with the hypothesis that some gibberellic acid like compound, liberated from the embryo to the endosperm, has a hormonal function in the germinating grain. The quantity of this compound apparently present is of some interest. If the total amount in the embryo were dispersed

throughout the grain, this would correspond to about 0.05 micrograms of gibberellic acid per kilogram fresh weight of barley. This figure compares favorably with the 0.02 micrograms per kilogram fresh weight of mature Montcalm barley found by Lazer *et al.* (1961) using isotope dilution techniques. It contrasts with the 3 micrograms of gibberellic acid per kilogram fresh weight of immature Proctor barley (1962) found by Jones, MacMillan and Radley (1963). Possibly a correlation may be found between this apparent fall in gibberellic acid concentration during maturation and a fall in activity of enzymes such as endo-β-glucanase (Johnston, 1964) which increase in activity in gibberellic acid treated endosperm from the mature grain. Such a fall in enzyme activity during maturation has been demonstrated only in *Bromus* spp. so far but in many respects seeds of Bromus and barley are very similar (Johnston, 1964).

SECTION II.

Studies with Inhibitors.

The interpretation of results obtained in the presence of inhibitors is not easy. The main difficulty arises from the fact that no known inhibitor is completely specific. Even in cases where only one action of an inhibitor has been reported, one may reasonably feel that this is due simply to the impossibility of testing all the feasible systems open to inhibition. However, there are a number of ways in which one can increase the likelihood that the assumption of a fairly specific inhibition is correct.

First, if one is dealing with a hormone-like substance like gibberellic acid, it is essential to test the effect of the inhibitor both on the substance itself and on the assay system used to detect the activity of the substance.

Secondly, one must use the inhibitor at a number of different concentrations and find the smallest required to give a reasonable degree of inhibition. At this concentration, secondary effects should be minimal and the primary effect, if one has been demonstrated, is more likely to be the sole cause of the inhibition.

Thirdly, one should ascertain, if possible, that the nature of the inhibition in the tissues being studied is the same as in other reported studies.

Fourthly, it is often of interest to determine whether an inhibition is reversible. The most obvious method of inhibition reversal in whole tissues is to try to wash the inhibitor out of the tissue. However, other methods, where applicable, may yield more information; e.g. if a synthetic pathway has been inhibited, normal metabolism may be restored by adding the product of that pathway.

Having ensured as far as possible that one is dealing with a fairly specific inhibition, one should in the first instance adopt the simplest interpretation of the results and attempt to verify it by further experimentation.

The following is a general outline of the functions of the inhibitors which have been used to modify the action of gibberellic acid.

2.4-Dinitrophenol (DNP)

This inhibitor is well known as an uncoupling agent when it is present at fairly low concentrations (ca.10^{-5}M) but at high concentrations (10^{-4}M+) it inhibits respiration (e.g. Ellis and Scholefield, 1962). So far, only the most tentative suggestion of its mode of action in uncoupling has been put forward (Lehninger, 1959). Low pH values enhance the inhibitory effects of DNP in whole tissues, probably by suppressing ionisation of the phenolic group, and thereby increasing penetration (Simon, 1953).

Sodium Salicylate

This is an uncoupling agent at concentrations similar to the uncoupling concentrations of DNP and apparently with a similar mode of action (Lehninger, 1959). At higher concentrations it inhibits respiration (Dawson et al, 1959).

It is also an antimetabolite of pantoic acid, which is required for the synthesis of coenzyme A (Maas, 1952).

Sodium Azide.

This is reported to be an uncoupling agent in certain tissues at concentrations near 10^{-4}M (Pullman and Penefsky, 1963). At higher concentrations (10^{-3}M+) it inhibits many iron-containing enzymes, including catalase and cytochrome oxidase (Warburg, 1949).

Sodium Arsenate

Like sodium azide, this compound is reported to be an uncoupling agent but at concentrations near 10^{-2}M (Cooper and Lehninger, 1956). This effect is probably related to its competing with phosphate for incorporation in labile compounds e.g. acetyl phosphate (Stadtman and Barker, 1950; Stadtman et al., 1951).

It also inhibits formate metabolism (Kalnitsky and Werkman, 1943; Strecker, 1951).

Sodium Malonate.

This compound is well known as a competitive inhibitor of succinic dehydrogenase (Quastel,1953).

At 10^{-2}M, it also inhibits fumarase, lactic dehydrogenase and malic dehydrogenase (Dawson et al., 1959).

On the other hand, it appears to be metabolised in perfused heart preparations (Fawaz, Tutunji and Fawaz, 1958) though it is said to inhibit pyruvate kinase (Fawaz and Fawaz, 1962).

Chloramphenicol.

Protein synthesis appears to be inhibited by chloramphenicol at a point beyond the assimilation of free amino-acids in bacterial but not in mammalian systems (Gale and Folkes, 1953; Gale, 1958; von Ehrenstein and Lipmann, 1961). Ellis (1964) has presented evidence suggesting that chloramphenicol does not inhibit protein synthesis in higher plants although it does inhibit permeation as had already been shown by Péaud-Lenoël and de Gournay-Margerie (1962) and Jacoby and Sutcliffe (1962). However, Molotkovskii and Smirnov (1963) have reported that isolated roots of Medicago sativa, grown in the presence of chloramphenicol, showed a diminished growth and reduced protein synthesis accompanied by an accumulation of free amino-acids. Respiration and cytochrome oxidase activity actually became greater. Moreover, Hodges, Stoner and Hanson (1964) report that chloramphenicol causes uncoupling in maize mitochondria at a site distinct from that involved with DNP and obgomycin.

5-Methyl Tryptophan.

5-Methyl tryptophan inhibits protein synthesis, possibly as a result of inhibiting the enzyme which produces tryptophanyl adenylate (Sharon and Lipmann, 1957) since it is not incorporated into the proteins of Escherichia coli, though it inhibits the growth of the organism (Munier and Cohen, 1957; Pardee et al., 1957; Pardee and Prestidge, 1958).

It also, apparently, inhibits the biosynthesis of tryptophan between anthranilic acid and indole since its inhibitory effect on Escherichia coli is reversed by tryptophan or indole but not by anthranilic acid (Anderson, 1945; Beerstecher, 1948, 1954). In Lactobacillus plantarum, anthranilic acid reverses the toxicity of 5-methyl tryptophan in a competitive manner. In Aerobacter aerogenes, 5-methyl tryptophan inhibits the enzymic conversion of 5-phosphoshikimic acid and glutamine to anthranilic acid (Doy and Pittard, 1960; Moyed, 1960).

Puromycin.

Puromycin is generally taken to be an inhibitor of protein synthesis, possibly by preventing the transfer of leucine from soluble ribonucleic acid into protein (Yarmolinsky and de la Haba, 1959). It may also effect nucleic acid metabolism (Bortle and Oleson, 1954; Hutchings, 1957).

However, it must be noted that at 10^{-4}M it proved to be a growth stimulant for Chlorella pyrenoidosa

though it acted as an inhibitor at 10^{-2}M (Tomisek et al., 1957). Also, it has been shown to stimulate incorporation of radio-activity from L-leucine-^{14}C and from acetate -2-^{14}C into protein fractions of uredo-spores of the corn rust fungus, Puccinia sorghi, more than twice as fast as in spores treated with water (Staples et al., 1961). This may be connected with the observation that in reticulocytes puromycin promotes the rapid non-enzymatic release of soluble protein from the ribosomes (Morris and Schweet, 1961; Morris et al., 1962; Allen and Zamecnik, 1962). It appears that the puromycin is covalently bound to the soluble protein released, in the ratio of one residue to each polypeptide chain. It is suggested that puromycin substitutes for amino-acyl soluble ribonucleic acid in binding to peptidyl ribonucleic acid, interrupting further extension of the peptide chain and causing it to separate from the ribosome (Zamecnik, 1962).

Potassium Cyanide.

Best known as an inhibitor of cytochrome oxidase (Warburg, 1949), this substance also inhibits many metal-containing enzymes and enzymes dependent upon carbonyl groups for their activity. However, it activates a number of proteolytic enzymes e.g.papain (Bersin and Logemann, 1933) presumably because of its ability to reduce disulphide bonds.

Sodium Sulphide.

Sodium sulphide is another inhibitor of cytochrome oxidase (Warburg, 1949). In solution it generates

hydrogen sulphide which can inhibit a number of enzymes by reducing their disulphide groups.

Carbon Monoxide.

This compound is reputed to inhibit polyphenol oxidase irreversibly (Kubowitz, 1937; Keilin and Mann, 1939). This contrasts with its effect on cytochrome oxidase which it inhibits photoreversibly (Warburg, 1949).

General Considerations.

Clearly the uncertainty which surrounds the mode of action of even the most frequently used inhibitors makes it dangerous to offer a simple explanation of phenomena observed in the presence of these inhibitors without further confirmatory evidence as to the inhibitory process. As far as possible such evidence has been sought and where it is lacking, this is due either to lack of suitable techniques or the appearance of a complex inhibition, investigation of which might require time which could be better spent attacking the main topic of this thesis, i.e. the mode of action of gibberellic acid in promoting hydrolysis of endosperm materials, in a different fashion.

Experimental

General.

In these studies three samples of barley were used, Wisa (1960 harvest) and Proctor (1962 and 1963 harvests). The first sample was used because it had previously been shown to give a vigorous response to gibberellic acid. The other samples were used to verify the results obtained and to extend them.

The criteria used to assess gibberellic acid activity were the increases in endo-β-glucanase and α-amylase activities in treated endosperm slices. These were felt to be at least one step nearer to the primary action of gibberellic acid than the increased production of hydrolysis products of the endosperm carbohydrates, measurement of which was used earlier by Paleg (1960, 1961, 1964), MacLeod and Millar (1962) and Briggs (1963). The β-glucan substrate used in the assay of the glucanase was prepared by the method of Preece and MacKenzie (1952) and the enzyme activity was measured in terms of the fall in viscosity of the substrate (Preece and Hoggan, 1956). The α-amylase activity was determined by the iodine-dextrin colour method devised by Briggs (1961). The effect of each inhibitor at the highest concentrations used was determined on both enzyme systems.

Before use, the barley was dehusked by treating with 50% sulphuric acid as described in Section I.

For each estimation of inhibitor - gibberellic

acid interaction, ten endosperm slices (ca.2mm. thick) were incubated in a 5cm. diameter Petri dish in a total volume of 4ml. incubation medium. Incubation was normally for 18 hours at 25°C with the Wisa (1960) and Proctor (1962) samples but the Proctor (1963) sample required a 36 hour incubation at this temperature to give a similar response.

Each inhibitor solution was adjusted to pH7 with sodium hydroxide or hydrochloric acid and, in the majority of cases, no buffer was used since it was found, as had been shown by Paleg (1960) that the commonly used buffers cannot be regarded as being inert in the systems being studied. The gibberellic acid was not neutralised since it appeared that it would be difficult to avoid inactivation in the process owing to the instability of the compound in aqueous solution. Moreover, although the pH of the medium is altered at different concentrations of gibberellic acid, the pH inside the tissue can be expected to remain fairly constant owing to the buffering power of the vast quantity of storage tissue present.

After incubation in the appropriate medium, the slices were dried on filter paper and then ground in 5ml. 0.1M sodium chloride solution in a glass homogeniser. The homogenate was left for two hours at room temperature to complete extraction and then the particulate residue was removed by centrifugation and the enzymic activity of the supernatant was determined. This technique was used for all the work with Wisa

(1960) and Proctor (1962) samples involving 18 hour incubation periods since no enzyme was liberated into the incubation media under these circumstances. Where longer incubation periods were involved it was found that varying quantities of enzyme appeared in the medium and so the slices were extracted in the medium to which 1ml. 0.5M sodium chloride was added.

Oxygen uptake in the presence of uncoupling agents was determined by Warburg manometry.

Aleurone was isolated as described in Section III.

Preparation of β-Glucan.

Finely ground Ymer barley was extracted thrice with 1½ times its own volume of boiling 80% ethanol under reflux, thirty minutes being allowed for each extraction. Finally, after drying under suction in a Buchner funnel, the grain was left in air for 48 hours to evaporate last traces of ethanol. This treatment inactivates cytolytic enzymes and extracts free sugars and oligosaccharides.

The dry residue was extracted in twice its own volume of water at 40°C. The aqueous suspension was filtered through muslin and the residue subjected to two further extractions. The bulked extracts were centrifuged, filtered through Celite 541 and treated overnight with barley malt α-amylase (kindly provided by Mr C.S. Johnston), free of cytolytic activity, to remove contaminating starch. The extracts were now concentrated to about 200ml. by evaporation and

ammonium sulphate was added to a concentration of 20% ($^{w}/_{v}$). The precipitate was filtered off on a sintered glass funnel and redissolved in a small quantity of water. The resulting solution was dialysed in the presence of thymol till free of sulphate (ca. 3 days). An equal volume of acetone was added to the dialysed solution and the precipitate was filtered off as before and dried with acetone.

The acceptable product had a specific viscosity between 2 and 3 (0.5% aqueous solution) and on hydrolysis yielded only glucose.

Endo-β-Glucanase Assay.

This involved following the drop in viscosity of a standard reaction mixture containing 1.6ml. 0.687% ($^{w}/_{v}$) B-glucan, 0.2ml., 0.1M acetate buffer, pH5, and 0.4ml. enzyme extract. All solutions were pre-adjusted to 25°C and the reaction was carried out at this temperature in a miniature Ostwald viscometer. Readings were taken at 10-15 minute intervals over a period of up to 90 minutes. The slope of the line obtained by plotting the reciprocal of the specific viscosity against time gives a measure of the enzyme activity in arbitrary units.

α-Amylase Assay.

β-Amylase solution was prepared by extracting 10g soy bean flour with 100ml. of a solution 0.008M with respect to sodium chloride and 0.0128M with respect to pH5 acetate buffer.

Soluble starch solution was prepared as follows. A suspension of 1g. soluble starch in 10ml. distilled water was stirred into 50ml. boiling distilled water and after boiling for a further minute, was cooled rapdily. To the cold solution was added 8 ml. 0.1M sodium chloride and 12.8ml. 0.1M pH5 acetate buffer and the total volume was mde up to 100ml.

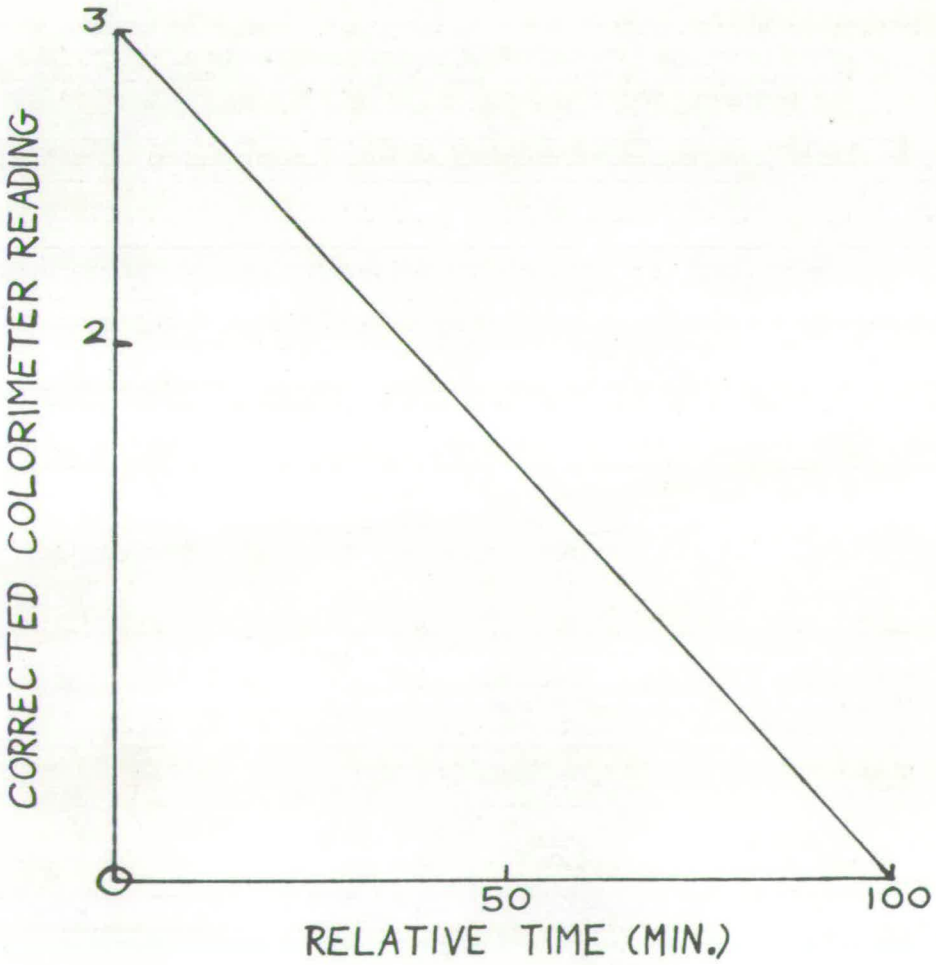
The iodine reagent used consisted of 0.254g. iodine dissolved in a solution of 4.0g. potassium iodide and diluted to 1 litre.

The assay was carried out in test-tubes in a water bath at 25°C. Substrate was prepared by adding 4ml. β-amylase solution to 20ml. starch solution and incubating for at least an hour at this temperature. Thus the substrate actually used in the assay was β-limit dextrin, which is not affected by any β-amylase or, over the incubation period used, by R enzyme admixed with the α-amylase being estimated. To 1.5ml. of the substrate solution was added 1ml. of the enzyme extract which had previously been brought to the same temperature. A 0.2ml. sample was taken out at zero time and mixed with 10ml. iodine reagent. Three further samples were taken at suitable intervals over a period of up to ten minutes and treated identically. The colour produced in each sample was measured in an E.E.L colorimeter, using an Ilford 608 filter.

The calculation of α-amylase activity from these results depends upon the β-limit dextrin concentration

FIGURE V.

α-AMYLASE STANDARD GRAPH (AFTER BRIGGS)



being substantially less than the Km for α-amylase. This being so, if x is the concentration of substrate and t is the time, the following applies:

$$-\frac{dx}{dt} = kx$$

$$\frac{dx}{x} = -kdt$$

$$\therefore \ln x = -kt + C.$$

if $x = x_o$ when $t = 0$

$$\ln x_o = C$$

$$\therefore \ln x = -kt + \ln x_o$$

$$\text{i.e. } \ln \frac{x}{x_o} = -kt.$$

In this equation, x_o and x may be determined in colorimeter units while t is known and thus k, the rate constant, which is proportional to enzyme concentration, may be found. By comparison with the rate constant for a standard enzyme solution under the same conditions, the enzymic activity of an unknown solution may be defined in arbitrary units.

In practice, the α-amylase activity was determined as follows. The experimental times (t_e) and the corresponding colorimeter readings (minus enzyme blank if necessary) were tabulated. The colorimeter readings, which correspond to the log of the substrate concentration, were adjusted relative to the zero time control being set = 3.00. Using the adjusted readings and the standard graph (Fig V) the times (t_r) taken by the standard enzyme solution to produce corresponding

substrate concentrations were found. Then the activity of each sample was calculated as $^{tr}/_{t}$ Iodine Dextrin Colour (I.D.C.) units. Each assay represents the average of three such samples.

Warburg Manometry.

Oxygen uptake was determined using the standard method (Dixon, 1951) at 25°C. Each flask contained 4ml. incubation medium with 20 2mm. endosperm slices (volume equal to about 0.4ml.) and 0.2ml. 10% potassium hydroxide in the centre well. Wherever possible, any pre-incubations involved were carried out in the Warburg flasks themselves to avoid transference of material with accompanying possibilities of contamination or loss.

Test of Inhibitor Effect on Enzyme Systems.

The effect on endo-β-glucanase was tested with an acetone precipitated preparation from Proctor barley supplied by Mr J.M. Bowden (Bowden, 1963). The enzyme was incubated in 0.1M sodium chloride solution in the presence of inhibitor for the period over which changes in its activity had been studied in endosperm slices. The final activity found was compared with that of the enzyme incubated in 0.1M sodium chloride solution alone.

For α-amylase, a preparation from Proctor malt by Mr C.S. Johnston was treated in a similar fashion.

TABLE IV

Effects of 2,4-Dinitrophenol and Sodium Salicylate on Glucanase Activity in 1960 Wisa Barley Endosperm.

(18 Hr. Incubation at 25°C)

Incubation Medium	Activity†	Relative Activity.
Water	59±44 (20)	100
DNP* (10^{-5}M)	16±7 (3)	27
Salicylate (5×10^{-4}M)	12±7 (4)	20
G.A.** (1.5×10^{-5}M)	342±61 (14)	100
G.A.(1.5×10^{-5}M)+DNP(10^{-5}M)	163±33 (3)	48
G.A.(1.5×10^{-5}M)+Salicylate (5×10^{-4}M)	171±30 (3)	50
G.A.(7.5×10^{-4}M)	362±87 (3)	100
G.A.(7.5×10^{-4}M)+Salicylate (5×10^{-4}M)	46±11 (3)	13
G.A.(1.4×10^{-3}M)	332±86 (3)	100
G.A.(1.4×10^{-3}M)+DNP(10^{-5}M)	58±9 (3)	17

* 2,4-Dinitrophenol

** Gibberellic Acid

† Δ^{1}/η_{sp}/100g. fresh wt. endosperm x hr.

Each result is the mean ± standard deviation.

Numbers of experiments are given in parentheses.

TABLE V

Effect of 2,4-Dinitrophenol on Glucanase and α-Amylase Activities in Endosperm from Proctor Barley (1962 Harvest) Sample (a).

(18 Hr. Incubation at 25°C).

Incubation Medium	Endo-β-Glucanase		α-Amylase	
	Activity†	Rel. Activity	Activity††	Rel. Activity
Water	55±17 (3)	100	39±6 (5)	100
P.Buffer*	86±7 (3)	156	31±2 (3)	79
DNP** (10^{-5}M)	27±10 (3)	49	39±3 (3)	100
P.Buffer + DNP (10^{-5}M)	23±13 (3)	42	25±6 (3)	64
G.A.*** (10^{-6}M)	384±80 (5)		413±134(5)	
G.A.(1.5×10^{-5}M)	378±27 (3)	100	351±89 (4)	100
G.A.(1.5×10^{-5}M)+ P.Buffer	241±37 (3)	64	178±34 (5)	51
G.A.(1.5×10^{-5}M)+ DNP(10^{-5}M)	118±22 (3)	31	76±36 (7)	22
G.A.(1.5×10^{-5}M)+ P.Buffer + DNP(10^{-5}M)	104±14 (5)	28	139±29 (8)	40
G.A.(7.5×10^{-4}M)	397±62 (3)	100	309±105(3)	100
G.A.(7.5×10^{-4}M)+ P.Buffer	289±25 (3)	73	172±17 (3)	56
G.A.(7.5×10^{-4}M)+DNP(10^{-5}M)	20±8 (3)	5	61±26 (7)	20

cont..

TABLE V (Cont'd).

Incubation Medium.	Endo-β-Glucanase		α-Amylase	
	Activity†	Rel.Activity.	Activity††	Rel.Activity.
G.A.(7.5×10^{-4}M) +P.Buffer+DNP (10^{-5}M).	66±26 (5)	17	74±21 (8)	24

* 10^{-2}M Phosphate Buffer, pH7.

** 2,4-Dinitrophenol.

*** Gibberellic Acid.

† Δ^1/hsp/100g. fresh wt. endosperm x hr.

†† IDC units/g. fresh wt. endosperm.

Each result is the mean ± standard deviation.

Numbers of experiments are given in parentheses.

TABLE VI

Effect of 2,4-Dinitrophenol on α-Amylase Activity In 1963 Proctor Barley Endosperm.

(42 Hr. Incubation at 25°C)

Incubation Medium	Activity†		Relative Activity
Water	0	(17)	
DNP* (10^{-5}M)	0	(3)	
G.A.** (10^{-8}M)	100±9	(3)	
G.A.(10^{-7}M)	269±65	(3)	100
G.A.(10^{-7}M)+DNP (10^{-5}M)	95±32	(4)	35
G.A.(10^{-6}M)	471±94	(3)	100
G.A.(10^{-6}M)+DNP (10^{-5}M)	211±15	(3)	45
G.A. (10^{-5}M)	726±172	(3)	100
G.A.(10^{-5}M)+DNP (10^{-5}M)	235±32	(5)	32
G.A.(10^{-4}M)	469±149	(3)	100
G.A.(10^{-4}M)+DNP (10^{-5}M)	133±27	(4)	28
G.A.(10^{-3}M)	209±18	(3)	100
G.A.(10^{-3}M)+DNP (10^{-5}M)	42±16	(3)	15

cont'd

TABLE VI (Cont'd).

* 2,4-Dinitrophenol

** Gibberellic Acid

† IDC units/g. fresh wt. endosperm.

Each result is the mean ± standard deviation.

Numbers of experiments are given in parentheses.

TABLE VII

Time Course of Increse in α-Amylase Activity† in Proctor Barley (1963 Harvest) Endosperm in the Presence of Gibberellic Acid and 2,4-Dinitrophenol.

Medium \ Time (Hr)	0	18	21	24	27	30	33	36	39	42
Water	0	0	0	0	0	0	0	0	0	0
DNP* (10^{-5}M)	0	0	0	0	0	0	0	0	0	0
G.A.** (10^{-5}M)	0	0	49±3	108±32	194±66	252±15	362±26	417±75	497±91	726±198
G.A. (7.5×10^{-4}M)	0	0	0	0	68±32	81±32	107±37	139±61	282±94	326±94
G.A. (10^{-5}M)+DNP(10^{-5}M)	0	0	0	0	0	51±13	50±5	147±37	185±8	222±39
G.A. (7.5×10^{-4}M)+DNP(10^{-5}M)	0	0	0	0	0	0	0	30±8	42±9	60±35

Temp. = 25°C:

† Expressed as IDC units /g. fresh wt. endosperm.

* 2,4-Dinitrophenol.

** Gibberellic Acid

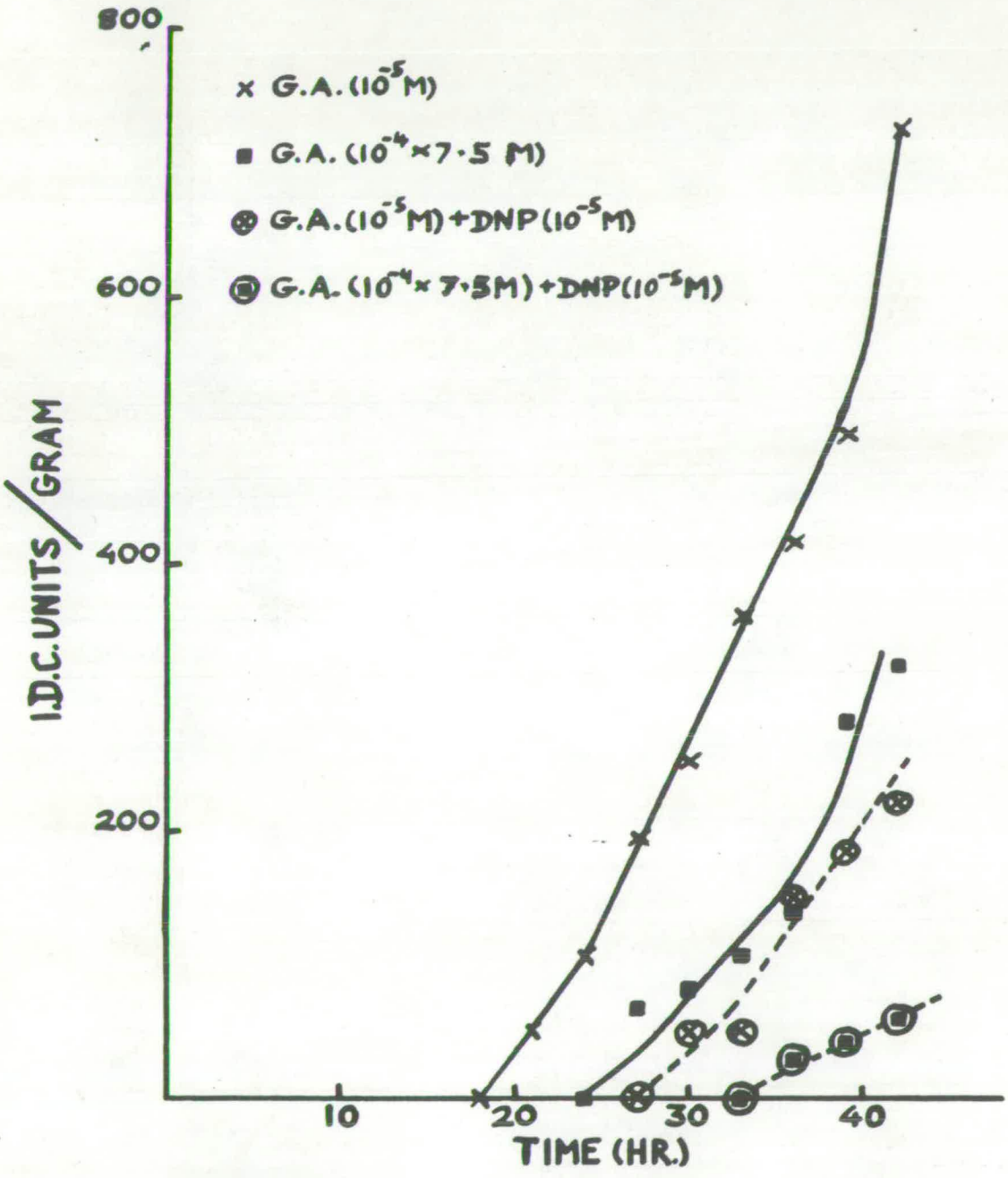
Each result is the mean of three estimations and is quoted with the standard deviation.

FIGURE VI

TIME-COURSE OF INCREASE IN α-AMYLASE ACTIVI

IN MEDIA OF GIBBERELLIC ACID AND

2,4-DINITROPHENOL (DN



TEMP.=25°C

PROCTOR BARLEY (1963)

TABLE VIII

Oxygen Uptake† of Endosperm Slices of Proctor Barley (1962 Harvest), Sample (a), in the Presence of Gibberellic Acid and 2,4-Dinitrophenol

(After 18 Hr. at 25°C. in the Given Media)

Medium \ Time (Min)	0	20	40	60	80	100	120
Water	0(5)	27±9	54±6	80±16	113±12	149±22	179±38
G.A.* (1.5×10^{-5}M)	0(4)	33±9	70±14	117±26	180±18	233±22	272±32
G.A. (7.5×10^{-4}M)	0(4)	43±7	77±17	115±32	158±44	204±59	248±75
DNP** (10^{-5}M)	0(3)	75±13	166±26	256±37	344±51	422±65	521±73
DNP (10^{-4}M)	0(3)	-	-	-	-	-	52±12
G.A.(1.5×10^{-5}M)+DNP(10^{-5}M)	0(3)	97±18	191±34	299±43	409±65	518±93	627±116
G.A.(7.5×10^{-4}M)+DNP(10^{-5}M)	0(4)	19±9	32±11	48±14	61±17	73±16	85±18

* Gibberellic Acid

** 2,4-Dinitrophenol

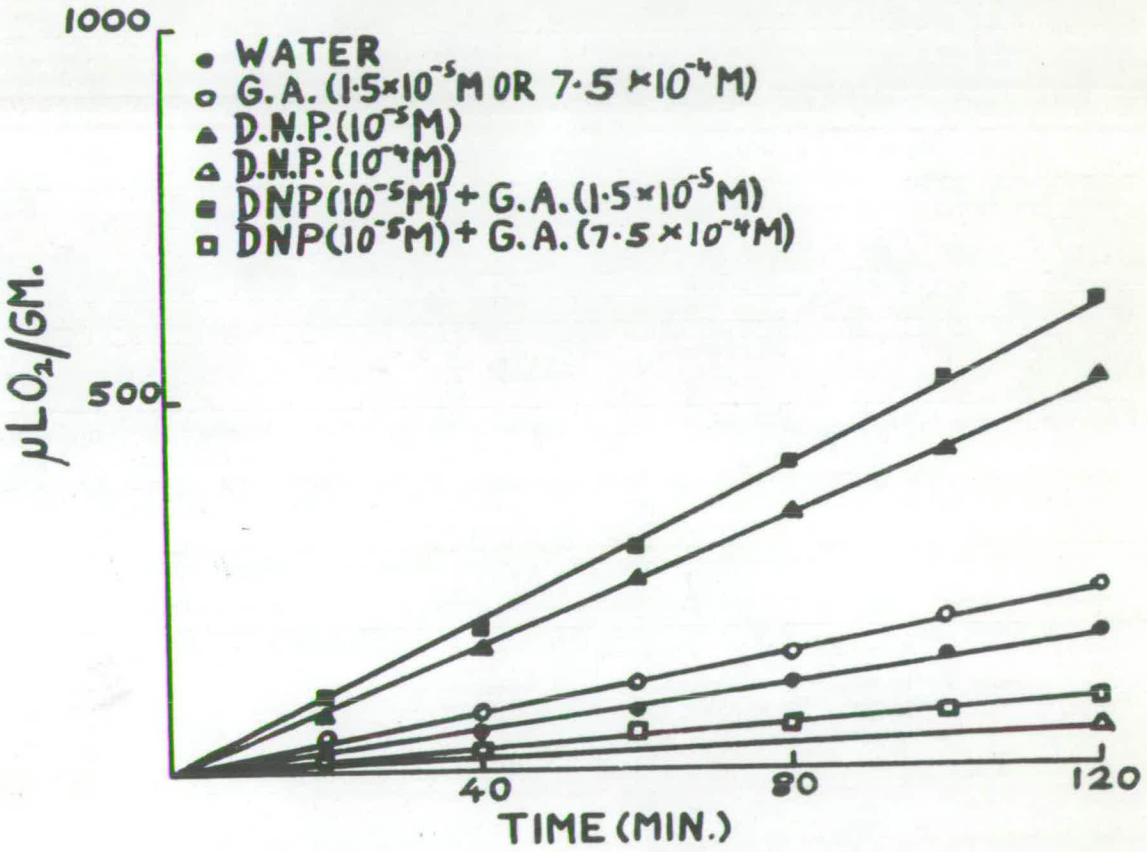
† µl.Oxygen/g. fresh wt. endosperm.

Numbers of experiments are given in parentheses in the zero-time column.

The values given are means ± standard deviation.

FIGURE VII

OXYGEN UPTAKE OF ENDOSPERM SLICES IN MEDIA OF GIBBERELLIC ACID AND 2,4-DINITROPHENOL*



TEMP.=25°C. PROCTOR BARLEY, 1962.

*AFTER 18 HOURS INCUBATION

FIGURE VIII

ULTRA-VIOLET ABSORPTION SPECTRA OF SUBSTANCES RELEASED FROM ISOLATED ALEURONE.

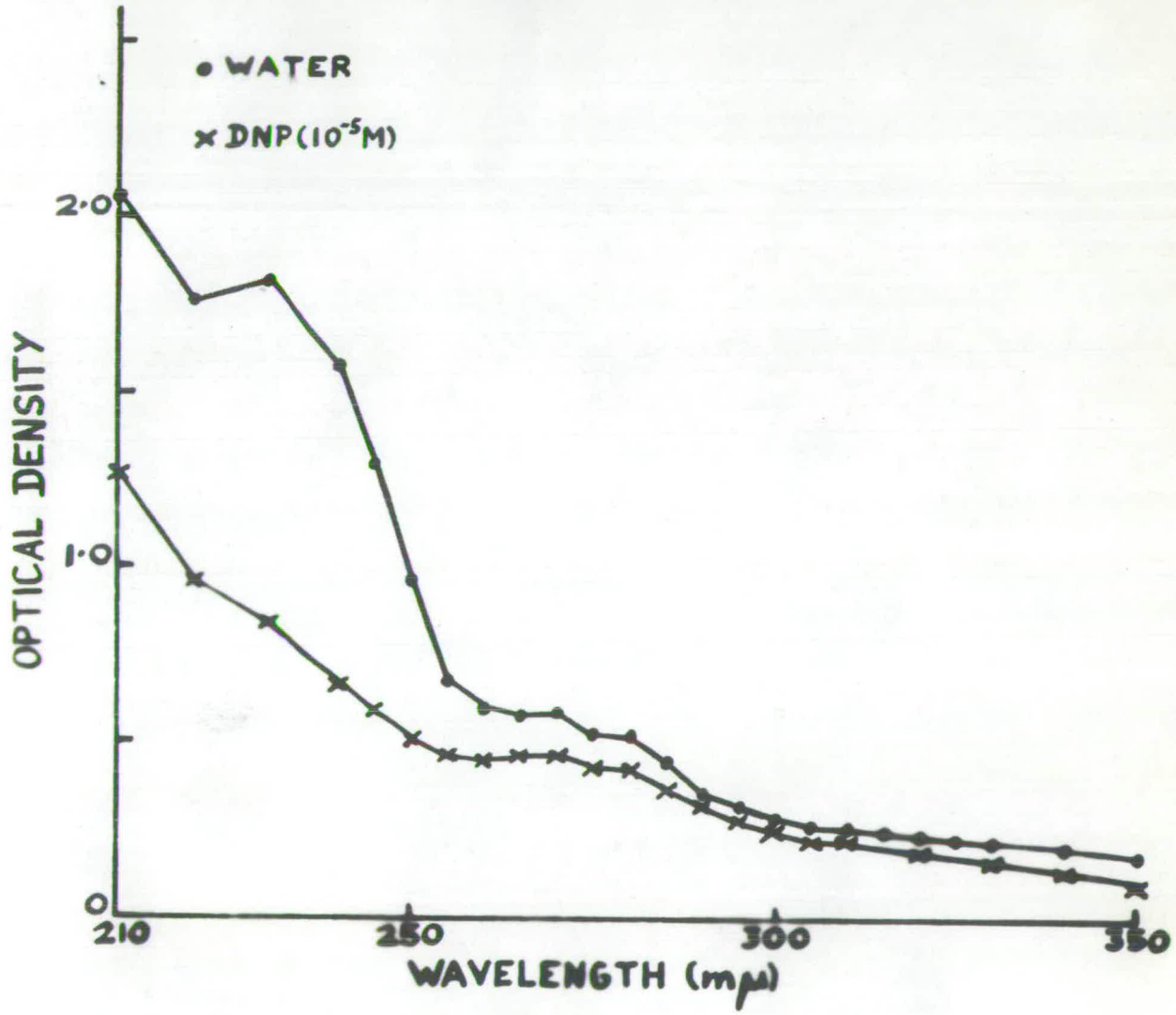


FIGURE IX

ULTRA-VIOLET ABSORPTION SPECTRA OF SUBSTANCES RELEASED FROM ISOLATED ALEURONE.

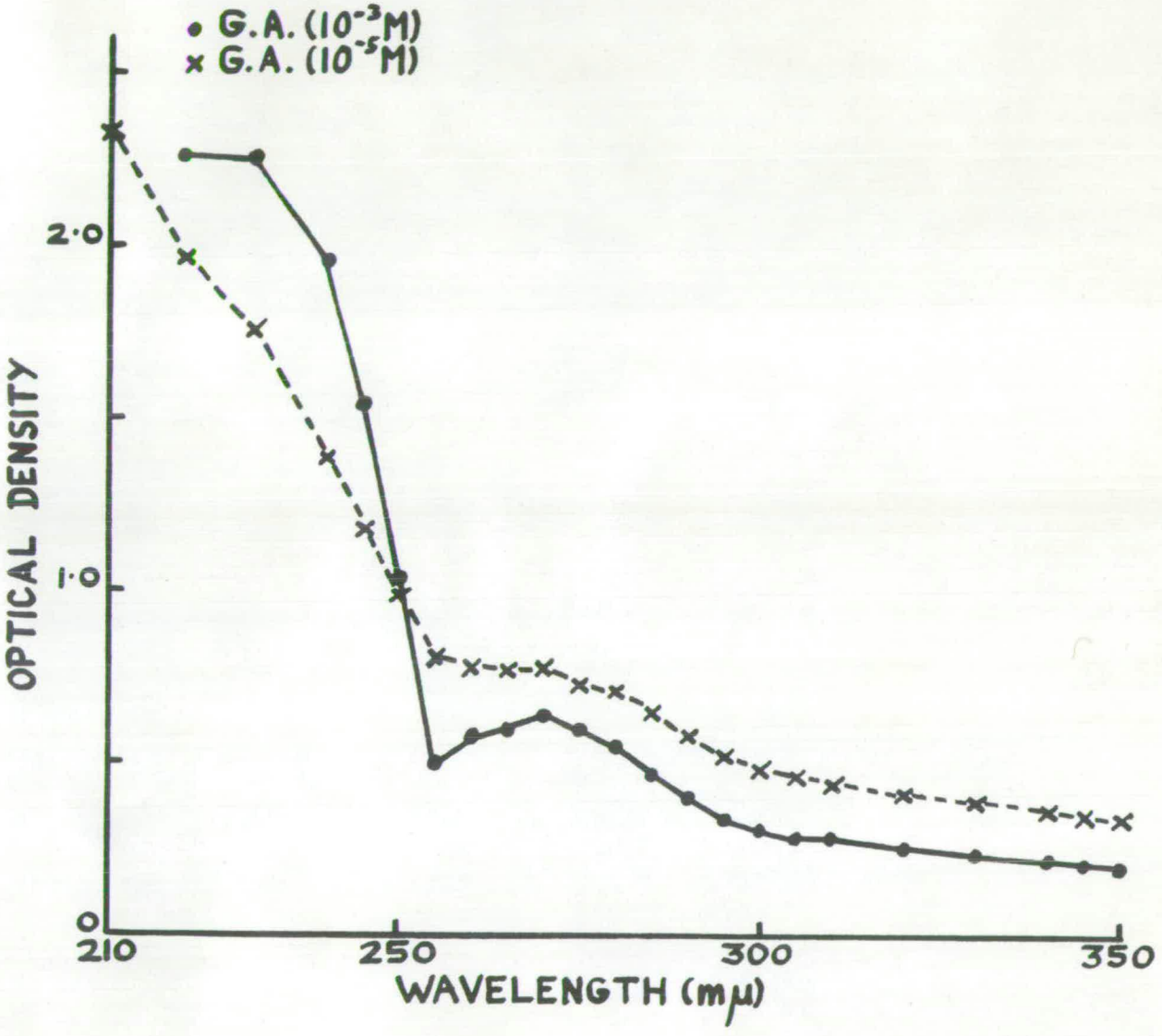


FIGURE X

ULTRA-VIOLET ABSORPTION SPECTRA OF SUBSTANCES RELEASED FROM ISOLATED ALEURONE.

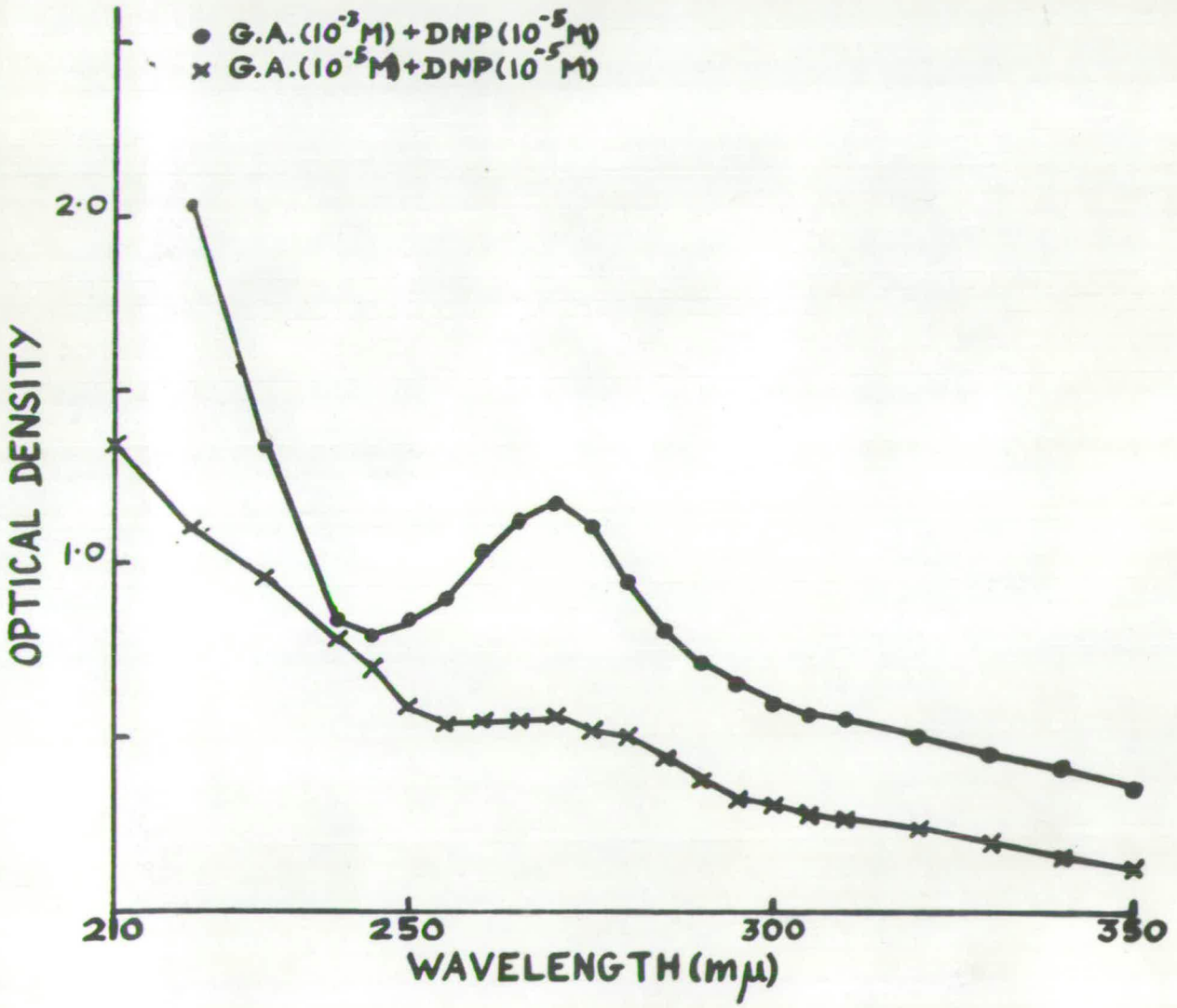


TABLE IX

Effect of Sodium Salicylate on α-Amylase Activity In Endosperm of 1962 Proctor Barley, Sample (b)

(42 Hr. Incubation at 25°C).

Incubation Medium	Activity†		Relative Activity
Water	24±4	(3)	100
Salicylate (5×10^{-4}M)	0	(3)	0
G.A.*(1.5×10^{-5}M)	540±28	(3)	100
G.A.(1.5×10^{-5}M)+Salicylate (5×10^{-4}M)	130±61	(3)	24
G.A.(7.5×10^{-4}M)	744±40	(3)	100
G.A.(7.5×10^{-4}M)+Salicylate (5×10^{-4}M)	183±75	(3)	25

* Gibberellic Acid

† IDC units/g. fresh wt. endosperm.

Each result is the mean ± standard deviation.

Numbers of experiments are given in parentheses.

TABLE X

Oxygen Uptake† of Endosperm Slices of Proctor Barley (1962 Harvest), Sample (b), in the Presence of Gibberellic Acid and Sodium Salicylate.

(After 18 Hr. at 25°C. in the Given Media).

Medium \ Time (min).	0	20	40	60	80	100	120
Water	0	30±5	61±10	84±9	116±12	145±9	174±16
G.A.* (1.5×10^{-5}M)	0	28±10	61±19	88±22	118±27	148±29	182±40
G.A. (7.5×10^{-4}M)	0	32±9	57±10	82±6	115±14	141±8	171±15
Salicylate (5×10^{-4}M)	0	38±11	74±14	118±18	160±17	215±25	270±32
Salicylate (10^{-3}M)	0	59±13	113±18	176±24	250±33	345±44	415±61
Salicylate (5×10^{-3}M)	0	7±1	7±3	10±2	17±6	18±6	20±7
G.A.(1.5×10^{-5}M)+Salicylate (5×10^{-4}M)	0	46±9	93±12	143±15	187±22	246±30	291±37
G.A.(7.5×10^{-4}M)+Salicylate (5×10^{-4}M)	0	22±2	41±6	58±6	77±11	100±18	116±16

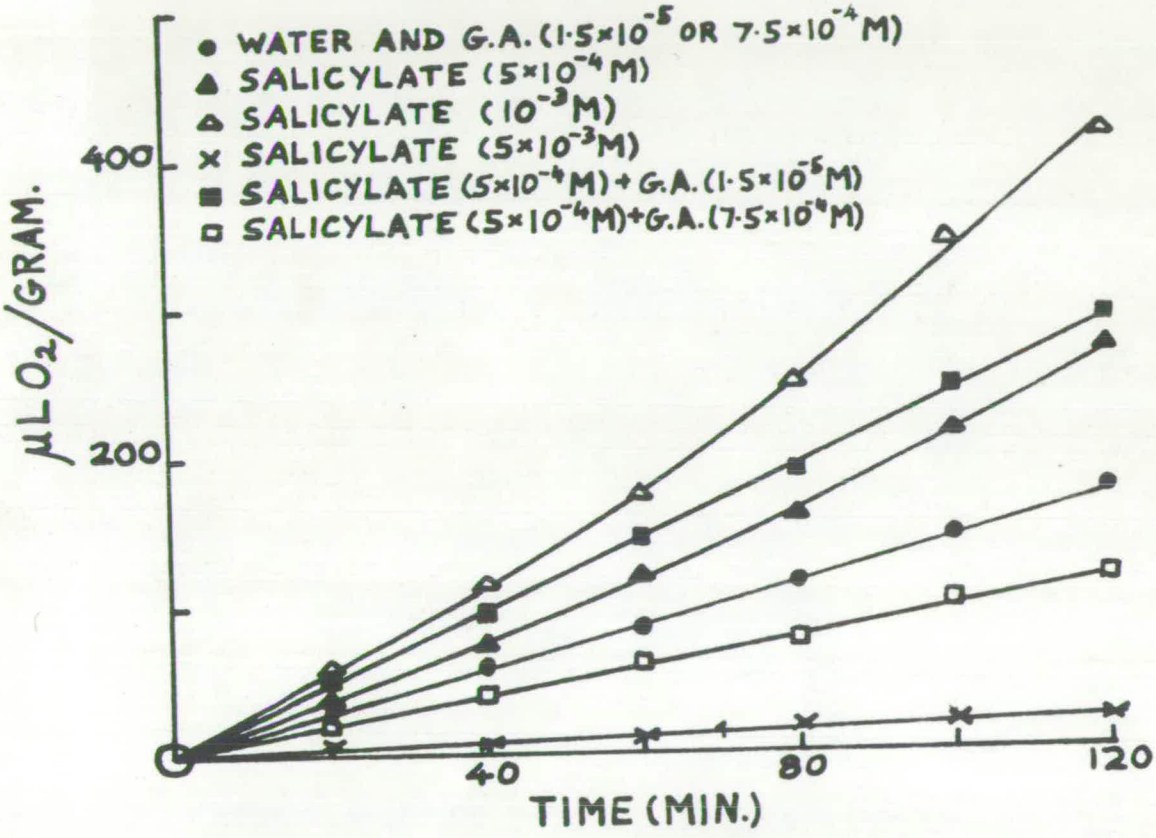
* Gibberellic Acid

† µl. Oxygen /g. fresh wt. endosperm.

Each result is the mean of three experiments ± standard deviation.

FIGURE XI

OXYGEN UPTAKE OF ENDOSPERM SLICES IN MEDIA OF GIBBERELLIC ACID AND SODIUM SALICYLATE.



TEMP.=25°C. PROCTOR BARLEY (1962) b.

*AFTER 18 HOURS INCUBATION.

TABLE XI

Oxygen Uptake† of Endosperm Slices of Proctor Barley (1962 Harvest), Sample (b), in the Presence of Gibberellic Acid and Sodium Azide.

(After 18 Hr. at 25°C. in the Given Media)

Medium \ Time (min)	0	20	40	60	80	100	120
Water	0	30±5	61±10	84±9	116±12	145±9	174±16
G.A.* (1.5×10^{-5}M)	0	28±10	61±19	88±22	18±27	148±29	182±40
G.A. (7.5×10^{-4}M)	0	32±9	57±10	82±6	115±14	141±8	171±15
Azide (10^{-4}M)	0	8±1	15±4	22±6	30±5	37±8	43±14
G.A.(1.5×10^{-5}M)+Azide(10^{-4}M)	0	6±2	9±1	11±2	18±8	23±10	25±7
G.A.(7.5×10^{-4}M)+Azide(10^{-4}M)	0	6±2	12±4	16±5	19±9	34±10	34±12

* Gibberellic Acid

† µl. Oxygen /g. fresh wt. endosperm.

Each result is the mean of three estimations ± standard deviation.

TABLE XII

Effect of Sodium Azide on Glucanase Activity in 1960 Wisa Barley Endosperm.

(18 Hr. Incubation at 25°C).

Incubation Medium	Activity †		Relative Activity
Water	59±44	(20)	100
Azide (10^{-4}M)	13±10	(5)	22
G.A.* (1.5×10^{-5}M)	342±61	(14)	100
G.A. (1.5×10^{-5}M) + Azide (10^{-4}M)	43±33	(4)	13
G.A. (7.5×10^{-4}M)	362±87	(3)	100
G.A. (7.5×10^{-4}M) + Azide (10^{-4}M)	247±95	(4)	68

* Gibberellic Acid

† Δ^1/η_{sp}/100g. fresh wt. endosperm x hr.

Each result is the mean ± standard deviation.

Numbers of experiments are given in parentheses.

TABLE XIII

Effect of Sodium Azide on α-Amylase Activity in Endosperm of 1962 Proctor Barley, Sample (b).

(42 Hr. Incubation at 25°C)

Incubation Medium	Activity†		Relative Activity
Water	24±4	(3)	100
Azide (10^{-4}M)	0	(3)	0
G.A. (1.5×10^{-5}M)	540±28	(3)	100
G.A.(1.5×10^{-5}M)+Azide (10^{-4}M)	93±50	(3)	17
G.A.(7.5×10^{-4}M)	744±40	(3)	100
G.A.(7.5×10^{-4}M)+Azide (10^{-4}M)	452±44	(3)	61

* Gibberellic Acid

† IDC units/g. fresh wt. endosperm.

Each result is the mean ± standard deviation.

Numbers of experiments are given in parentheses.

TABLE XIV

Effect of Sodium Arsenate in the Presence of Phosphate Buffer on the Oxygen Uptake† of Endosperm Slices of Proctor Barley (1962 Harvest), Sample (b).

(After 18 Hr. at 25°C. in the Given Media)

Medium \ Time (min)	0	20	40	60	80	100	120
Water	0	30±5	61±10	84±9	16±12	145±9	174±16
G.A.* (1.5×10^{-5}M)	0	28±10	61±19	88±22	118±27	148±29	182±40
G.A. (7.5×10^{-4}M)	0	32±9	57±10	82±6	115±14	141±8	171±15
P. Buffer**	0	81±11	165±15	265±20	353±30	-	553±36
G.A. (1.5×10^{-5}M)+P. Buffer	0	81±12	163±17	259±23	352±26	-	564±33
G.A. (7.5×10^{-4}M)+P. Buffer	0	65±5	133±12	222±13	308±10	-	512±10
Arsenate (8×10^{-5}M)	0	20±8	40±11	67±13	101±17	-	165±20
Arsenate (8×10^{-5}M). P. Buffer	0	77±8	153±5	244±4	332±4	-	529±16
G.A.(1.5×10^{-5}M)+Arsenate (8×10^{-5}M)	0	18±5	35±8	63±11	95±15	-	151±17
G.A.(7.5×10^{-4}M)+Arsenate (8×10^{-5}M)	0	22±7	44±12	66±13	94±16	-	147±21

continued next page

TABLE XIV (Cont'd)

Medium \ Time (min)	0	20	40	60	80	100	120
G.A. (1.5×10^{-5}M)+Arsenate (8×10^{-5}M)+P.Buffer	0	87±16	173±29	265±44	371±41	-	606±41
G.A. (7.5×10^{-4}M)+Arsenate (8×10^{-5}M)+ P. Buffer	0	95±14	189±25	292±36	394±45	-	626±60

* Gibberellic Acid

** 10^{-2}M Phosphate Buffer, pH7.

† μl. Oxygen/g. fresh wt. endosperm.

Each result is the mean of three estimations ± standard deviation.

TABLE XV

Effect of Sodium Arsenate on Glucanase and α-Amylase Activities in Endosperm from Proctor Barley, 1962 Harvest. Sample (a).

(18 Hr. Incubation at 25°C)

Incubation Medium	Endo-β-Glucanase		α-Amylase	
	Activity.†	Rel. Activity.	Activity††	Activity
Water	55±17 (3)	100	39±6 (5)	100
P. Buffer*			31±2 (3)	79
Arsenate ($8x10^{-5}M$)	54±28 (3)	100	49±9 (3)	126
Arsenate ($8x10^{-5}M$)+P. Buffer			37±11 (3)	95
G.A.** ($1.5x10^{-5}M$)	378±27 (3)	100	351±89 (4)	100
G.A.($1.5x10^{-5}M$)+P. Buffer			178±34(5)	51
G.A.($1.5x10^{-5}M$)+Arsenate($8x10^{-5}M$)	168±4 (3)	44	139±48 (3)	40
G.A.($1.5x10^{-5}M$)+P.Buffer+Arsenate ($8x10^{-5}M$)			154±15 (4)	44
G.A.($7.7x10^{-4}M$)	397±62 (3)	100	309±105 (3)	100
G.A.($7.5x10^{-4}M$)+P.Buffer			172±17 (3)	56
G.A.($7.5x10^{-4}M$)+Arsenate($8x10^{-5}M$)	177±31 (3)	45	93±31 (3)	31
G.A.($7.5x10^{-4}M$)+P.Buffer+Arsenate ($8x10^{-5}M$)			74±11 (4)	24

cont'd ...

TABLE XV (Cont'd).

* 10^{-2}M Phosphate Buffer, pH7.

** Gibberellic Acid

† $\Delta^1/\eta sp/100g.$ fresh wt. endosperm x hr.

†† IDC units/g. fresh wt. endosperm.

Each result is the mean ± standard deviation.

Numbers of experiments are given in parentheses.

TABLE XVI

Effect of Sodium Malonate on Glucanase Activity In 1960 Wisa Barley Endosperm.

(18 Hr. Incubation at 25°C.)

Incubation Medium.	Activity †		Rel. Activity.
Water	59±44	(20)	100
Malonate (10^{-1}M)	10±5	(3)	17
G.A.* (1.5×10^{-5}M)	342±61	(14)	100
G.A.(1.5×10^{-5}M)+Malonate(10^{-1}M)	30±6	(3)	9
G.A.(1.5×10^{-5}M)+Malonate(10^{-1}M) +Succinate** (0.7M)	8±9	(3)	2
G.A.(1.5×10^{-5}M)+Malonate(10^{-1}M) +Succinate (1.4M)	3	(1)	1
G.A.(7.5×10^{-4}M)	362±87	(3)	100
G.A.(7.5×10^{-4}M)+Malonate(10^{-1}M)	35±8	(3)	10

* Gibberellic Acid

** Sodium Salt

† $\Delta 1/\eta sp$/100g. fresh wt. endosperm x hr.

Each result is the mean ± standard deviation.

Numbers of experiments are given in parentheses.

TABLE XVII

Effect of Chloramphenicol on Glucanase Activity In 1960 Wisa Barley Endosperm.

(18 Hour Incubation at 25°C)

Incubation Medium	Activity†	Relative Activity.
Water	59±44 (20)	100
CAM* ($3x10^{-3}$M)	10±8 (3)	17
G.A.** ($1.5x10^{-5}$M)	342±61 (14)	100
G.A. ($1.5x10^{-5}$M) + CAM ($3x10^{-3}$M)	88±37 (3)	26
G.A. ($7.5x10^{-4}$M)	362±87 (3)	100
G.A. ($7.5x10^{-4}$M) + CAM ($3x10^{-3}$M)	113±35 (3)	31

* Chloramphenicol

** Gibberellic Acid

† $\Delta^{1}/_{\eta}$sp/100G. fresh wt. endosperm x hr.

Each result is the mean ± standard deviation.

Numbers of experiments are given in parentheses.

TABLE XVIII

Effect of 5-Methyl Tryptophan on Glucanase Activity In 1960 Wisa Barley Endosperm.

(18 Hr. Incubation at 25°C).

Incubation Medium	Activity†	Rel. Activity.
Water	59±44 (20)	100
5-MT*(10^{-3}M)	272±86 (3)	461
5-MT (5×10^{-3}M)	30±19 (3)	51
G.A.**(1.5×10^{-5}M)	342±61 (14)	100
G.A.(1.5×10^{-5}M)+5MT(10^{-3}M)	417±140 (4)	122
G.A.(1.5×10^{-5}M)+5MT(5×10^{-3}M)	47±27 (3)	14
G.A.(7.5×10^{-4}M)	362±87 (3)	100
G.A.(7.5×10^{-4}M)+5MT(10^{-3}M)	387±141 (4)	107
G.A.(7.5×10^{-4}M)+5MT(5×10^{-3}M)	18±10 (3)	5

* 5-Methyl Tryptophan

** Gibberellic Acid

† $\Delta^1/_1$sp/100g. fresh wt. endosperm x hr.

Each result is the mean ± standard deviation.

Numbers of experiments are given in parenthesis.

TABLE XIX

Effect of 5-Methyl Tryptophan on α-Amylase Activity in Endosperm of 1962 Proctor Barley, Sample (a).

(18 Hr. Incubation at 25°C)

Incubation Medium	Activity †	Rel. Activity.
Water	39±6 (5)	100
G.A.* (10^{-6}M)	413±134 (5)	1060
5-MT** ($5x10^{-5}$M)	62±4 (3)	159
5-MT (10^{-4}M)	106±22 (3)	272
5-MT ($5x10^{-4}$M)	189±47 (3)	485
5-MT (10^{-3}M)	130±31 (3)	333
5-MT ($5x10^{-3}$M)	28±5 (3)	72

* Gibberellic Acid

** 5-Methyl Tryptophan

† IDC units/g. fresh wt. endosperm.

Each result is the mean ± standard deviation.

Numbers of experiments are given in parentheses.

TABLE XX

Effect of Puromycin on α-Amylase Activity in the Endosperm of Proctor Barley (1962 Harvest), Sample (a)

(18 Hr. Incubation at 25°C)

Incubation Medium	Activity †	Rel. Activity.
Water	39±6 (5)	100
Puromycin (10^{-4}M)	0 (3)	0
Puromycin (10^{-3}M)	0 (3)	0
Puromycin (10^{-2}M)	0 (3)	0
G.A.* (1.5×10^{-5}M)	351±89 (4)	100
G.A.(1.5×10^{-5}M)+Puromycin (10^{-4}M)	158±15 (3)	45
G.A.(1.5×10^{-5}M)+Puromycin (10^{-3}M)	243±41 (3)	69
G.A.(7.5×10^{-4}M)	309±105 (3)	100
G.A.(7.5×10^{-4}M)+Puromycin (10^{-4}M)	99±31 (3)	32
G.A.(7.5×10^{-4}M)+Puromycin (10^{-3}M)	154±6 (3)	50

* Gibberellic Acid

† IDC units/g. fresh wt. endosperm.

Each result is the mean ± standard deviation

Numbers of experiments are given in parentheses.

Results.

2,4-Dinitrophenol (DNP)

No direct interaction between gibberellic acid and DNP could be observed spectrophotometrically nor was there any evidence of endo-β-glucanase or α-amylase inhibition directly by DNP at the highest concentrations used.

With 10^{-5}M DNP, it was found that increasing the concentration of gibberellic acid above 10^{-6}M caused a progressive decrease in endosperm response to the gibberellic acid as measured by the change in endo-β-glucanase and α-amylase activities (Tables IV,V,VI) after a fixed incubation period. The use of such a fixed incubation period depends upon the assumption that the increase in these activities follows a simple, nearly linear pattern over this period. This was verified for α-amylase activity in the presence of gibberellic acid and DNP (Table VII. Fig VI). These results show that DNP not only reduces the rate of increase in α-amylase activity but also delays the appearance of activity. It is worth noting that taking the values of enzyme activity at an arbitrary time may give a misleading picture if that time is not carefully chosen. For example, here values measured at 27 hours would suggest a complete inhibition of gibberellic acid activity by 10^{-5}M DNP. The apparently different rate of increase in α-amylase activity in 7.5×10^{-4}M gibberellic acid was shown to be due to

inactivation of the enzyme at the pH of the medium since 10^{-5}M gibberellic acid adjusted to the same pH gave similar results. The increase in endo-β-Glucanase activity in response to gibberellic acid also follows a near linear pattern (Table XXII, Fig. XIII).

Decrease in pH facilitates DNP inhibition but that this is not a major factor here is shown by the persistence of the peculiar pattern of results in the presence of phosphate buffer at a concentration sufficient to overcome the increase in acidity produced by the higher concentration of gibberellic acid (Table V).

The oxygen uptake of endosperm slices was determined over a period of two hours, immediately after the slices had been subjected to the usual incubation procedure (Table VIII, Fig.VII). It was verified that DNP stimulates oxygen uptake at an external concentration of 10^{-5}M, as would be expected if it were uncoupling, but inhibits oxygen uptake almost completely at 10^{-4}M. Gibberellic acid itself stimulates oxygen uptake slightly. At a concentration of 10^{-5}M in a 10^{-5}M DNP medium, gibberellic acid causes only a slight further increase in oxygen uptake above that of the 10^{-5}M DNP control. However, at a concentration of 7.5×10^{-4}M in the same medium, it apparently causes a massive inhibition. In this case the oxygen uptake is only slightly greater than that of endosperm slices in 10^{-4}M DNP.

In an attempt to see whether DNP penetration of

gibberellic acid concentration, isolated aleurone was incubated with 10^{-5}M DNP in the presence of 10^{-5}M and 10^{-3}M gibberellic acid and the media were examined spectrophotometrically before and after incubation (Figs VIII,IX,X). Unfortunately the absorption spectra of the substances released during the incubation period masked any changes in DNP concentration. As will be seen in Section III, these substances do not apparently include the hydrolytic enzymes studied in any quantity. It was therefore decided to plot the absorption spectra of the media after incubation, making corrections for known components and assuming no change in gibberellic acid or DNP content.

The same general pattern of results was obtained on three separate occasions but only one set of results is shown here. The spectra are too complex to give an obvious indication of the nature of the substances appearing in the media. However, the peak obtained at 270mμ from media which had contained aleurone, gibberellic acid and DNP appears to mark an extension of a similar, much smaller peak from media with aleurone and gibberellic acid alone. It may possibly be relevant that various purines and pyrimidines have absorption maxima in this region.

Sodium Salicylate.

Broadly speaking, this substance behaves in the same way as DNP. It will be seen (Table IV) that the inhibition of gibberellic acid stimulated increase in glucanase activity in Wisa endosperm by 5×10^{-4}M

sodium salicylate increases with increasing gibberellic acid concentration. Although the same cannot be said for Proctor barley (1962), sample b, with respect to α-amylase release (Table IX), measurement of oxygen uptake of endosperm slices of this barley, after 18 hours incubation in media of gibberellic acid and salicylate, gives results in keeping with those obtained with media of gibberellic acid and DNP (Table X, Fig XI). Thus it is found that salicylate stimulates oxygen uptake at $5x10^{-4}$M, presumably by uncoupling; it exerts a greater stimulatory effect at 10^{-3}M and inhibits strongly at $5x10^{-3}$M. Gibberellic acid ($1.5x10^{-5}$ M) does not alter the stimulatory effect of $5x10^{-4}$M salicylate on oxygen uptake but $7.5x10^{-4}$M gibberellic acid converts this effect to an inhibition.

Again no direct interaction of the inhibitor with gibberellic acid could be observed spectrophotometrically nor was there any evidence of endo-β-glucanase or α-amylase inhibition at the highest inhibitor concentrations used.

Sodium Azide.

Despite the low concentration of this inhibitor used, it appears from the oxygen uptake figures (Table XI) that it is inhibiting terminal oxidases strongly. This being the case, it is surprising that $7.5x10^{-4}$M gibberellic acid should produce a response of the magnitude observed (Tables XII,XIII). The other surprising feature of these results is the increasing response of the tissue with increasing gibberellic

acid concentration in the presence of this inhibitor. This is the exact opposite of the interaction obtained with DNP and salicylate.

The inhibitor had no direct effect on endo-β-glucanase, α-amylase or gibberellic acid.

Sodium Arsenate.

At the concentration used, this compound has little effect on respiration (Table XIV). It inhibits response of endosperm slices to 1.5×10^{-5}M gibberellic acid and this inhibition is overcome by phosphate (Table XV). However, inhibition of response to 7.5×10^{-4}M gibberellic acid is not overcome by phosphate.

The compound has no effect on either gibberellic acid or the enzyme directly.

It is worth noting that phosphate buffer, while inhibiting response of the tissue to gibberellic acid, stimulates oxygen uptake markedly.

Sodium Malonate.

This compound at 10^{-1}M inhibits response of endosperm to gibberellic acid completely (Table XVI). This inhibition is not reversed by sodium succinate. The compound does not interact with either gibberellic acid or the enzyme.

Chloramphenicol (Table XVII).

Chloramphenicol inhibits the gibberellic acid stimulated increase in endo-β-glucanase activity considerably. It has no effect on gibberellic acid or the enzyme.

DL-5-Methyl Tryptophan

At high concentrations ($5x10^{-3}$M), 5-methyl tryptophan inhibits the increase in endo-B-glucanase activity in response to gibberellic acid (Table XVIII). At lower concentrations, it mimics the effect of gibberellic acid (Tables XVIII,XIX). However, it is active over a much narrower range of concentrations.

The related compounds, DL-tryptophan and indole acetic acid had no stimulatory effect on α-amylase activity in endosperm slices from 1962 Proctor barley incubated under the same conditions. The concentrations of DL-tryptophan used were $5x10^{-6}$M, 10^{-5}M, $5x10^{-5}$M, 10^{-4}M, $5x10^{-4}$M and 10^{-3}M. The indole acetic acid was used over the range of concentrations 10^{-9}M, 10^{-8}M, 10^{-7}M etc. to 10^{-3}M.

5-Methyl tryptophan has no effect on either of the enzyme systems studied or on gibberellic acid.

Puromycin.

This compound inhibits response of endosperm slices to gibberellic acid (Table XX). The results appear to show that a greater inhibition of the response to a fixed concentration of gibberellic acid is produced by using a lower concentration of the inhibitor. However, the significance of this is doubtful since attempts to verify it using Proctor barley from the 1963 harvest produced completely haphazard results though the general effect was inhibitory. Since 42 hour incubation periods were used with the latter

sample, it was thought that this might reflect instability of the inhibitor in aqueous solution and it was indeed found that its inhibitory properties decreased quite rapidly on storage. The sample of inhibitor used was supplied by Nutritional Biochemicals Incorporated.

The inhibitor had no effect on ∝-amylase or gibberellic acid directly.

Potassium Cyanide and Sodium Sulphide.

It was hoped to verify by the use of these compounds that the terminal exidases of the aleurone were required in a functional state for response to gibberellic acid of the tissue. However, it was found that both compounds at 5×10^{-2}M inhibited endo-β-glucanase and so the investigation was not pursued further.

Carbon Monoxide

Exposure of endosperm slices to carbon monoxide in coal gas in the light for periods of up to five hours produced no inhibition of their response to gibberellic acid.

Discussion.

2,4-Dinitrophenol and Sodium Salicylate.

Both of these compounds are uncoupling agents, apparently with a similar mode of action (Lehninger, 1959). This is borne out by the similarity of the results obtained in this investigation.

In general terms, if a suitable concentration of these inhibitors is chosen, increasing the concentration of gibberellic acid above a certain value increases their apparent inhibitory power. A possible explanation may be formulated if one makes the assumption that gibberellic acid at high concentrations alters the steady state situation between extracellular and intracellular concentrations of these compounds so that the effective intracellular concentration for any given extracellular concentration is increased. This possibility is supported by the oxygen uptake measurements. For example, 10^{-5}M DNP in the presence of 7.5×10^{-4}M gibberellic acid has an inhibitory effect comparable to that of 10^{-4}M DNP on its own. This contrasts with the marked stimulation by 10^{-5}M DNP on its own or in the presence of 1.5×10^{-5}M gibberellic acid. The pattern of results with sodium salicylate and gibberellic acid is similar.

Sodium Azide and Sodium Arsenate.

These compounds may be considered together since both have been reputed to act as uncoupling agents (Pullman and Penefsky, 1963). This being the case, it

was hoped that some comparison might be made with the organic uncoupling agents used. Unfortunately neither gave evidence of uncoupling activity in barley endosperm at the concentrations used.

Sodium azide appeared to inhibit terminal oxidases strongly. However, in the presence of this inhibitor, increasing gibberellic acid concentration produced increasing tissue response. Again an explanation could be suggested in terms of penetration, this time of gibberellic acid, but there is no supporting evidence that azide inhibits this.

The inhibition by sodium arsenate appears to be due to a simple competition with phosphate for phosphate requiring systems. There is no obvious explanation for the one result which appears to be anomalous in this respect.

Sodium Malonate

This inhibitor was used in the hope of verifying the role of the tricarboxylic acid cycle in supplying energy required for the response of the tissue to gibberellic acid. Unfortunately the inhibition it exerted proved to be more complex than that expected as a result of competitive inhibition of succinic dehydrogenase. It is worth noting that a similar type of malonate inhibition, not reversed by succinate, has been found by James (1963) to occur in barley roots.

Chloramphenicol, DL-5-Methyl Tryptophan and Puromycin

All these compounds have been reported to inhibit protein synthesis, probably at the ribosomal level. All, at suitable concentrations, inhibit the response of endosperm slices to gibberellic acid indicating that this requires an active protein synthesising system.

The gibberellin-like effect of 5-methyl tryptophan, at concentrations just below those producing inhibition, is rather puzzling. It is apparently not due to its being converted into a derivative of tryptophan or indole-acetic acid. A possible explanation is that at these concentrations, 5-methyl tryptophan inhibits some energy requiring process other than protein synthesis, thereby making more energy available for protein synthesis would presumably accelerate the increase in hydrolytic enzyme activity which occurs slowly even in endosperm slices incubated in water (MacLeod, Duffus and Millar, 1963). It must be recorded that Proctor barley from the 1963 harvest does not respond to 5-Methyl tryptophan in this fashion (Johnstone, 1965). Possibly this is because this barley is less mature than the other samples studied. This is in agreement with the observation that the two samples used in the work described here responded markedly to gibberellic acid within 18 hours whereas Proctor (1963) endosperm slices required 36 hours to give the same response.

Carbon Monoxide.

The lack of effect of this inhibitor under the

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conditions used indicates that polyphenol oxidase activity in the tissue is not essential for its response to gibberellic acid.

Summary

Compounds which inhibit oxidative phosphorylation and protein synthesis have been shown to inhibit endosperm response to gibberellic acid. The results appear to suggest that the penetration of two of these compounds, DNP and salicylate, into the actively respiring cells is enhanced by high concentrations of gibberellic acid.

SECTION III

Studies of the Time-Course of Activity Change of Endo-β-Glucanase, α-Amylase, Acid Phosphatase and Protease in Endosperm Slices and Isolated Aleurone.

This investigation followed directly from that described in the previous section. Before interpreting the results obtained there it was essential to establish that the time-course of increase in enzyme activity followed a simple, near-linear pattern.

In addition, it seemed desirable to study isolated aleurone to determine which enzymes, if any, were being secreted from this tissue in response to gibberellic acid. This necessitated comparison with endosperm slice response under similar conditions and the comparison was extended to endosperm slices cut from normally germinating grain.

The enzyme systems chosen for comparison were those which appear to be of most importance in hydrolysing the reserve material of the starchy endosperm.

Experimental.

General

The barley used throughout the following experiments was Proctor from the 1963 harvest. This was dehusked and stored as described in Section I.

The grain was allowed to germinate in 10cm. diameter Petri dishes on filter paper saturated with distilled water at 25°C. After suitable periods, samples were taken and 2mm. endosperm slices cut from these. This was done in order that the enzyme activities might be referred back to the fresh weight of comparable slices cut from the dry grain. For ten such slices, the mean fresh weight was found to be 0.1830g. with a standard deviation of 0.0129g (72 samples were taken). The slices were ground in a glass homogeniser in 5ml. 0.1M sodium chloride solution and the homogenate left for two hours at room temperature to complete extraction. The particulate residue was removed by centrifugation and the activity of the supernatant determined.

Endosperm slices cut from the dry grain were incubated in 10^{-5}M gibberellic acid in 5cm. Petri dishes at 25°C. After appropriate times, they were extracted in the same way as those from the germinating grain. However, in this case, extraction was carried out in the ambient medium to which sodium chloride had been added to give a final concentration of 0.1M. This was done because it was found that, after long incubation periods, an appreciable amount of enzyme was liberated into the medium.

Aleurone was isolated from endosperm slices by halving them after incubation and then grinding them in distilled water in a glass homogeniser until all the starchy endosperm was suspended. The mixture was then passed through a sieve and the aleurone thus separated

was washed thoroughly with more distilled water. This aleurone preparation was microscopically free of starchy endosperm, reduced tetrazolium dyes and had an oxygen uptake, with respect to endogenous substrate, comparable with that of the tissue in intact endosperm slices. It will be seen that this aleurone preparation contained pericarp-testa (Fig III).

The aleurone was incubated in 10^{-5}M gibberellic acid in 5cm. Petri dishes at 25°C. After appropriate times, the enzyme activities were assayed in both the tissue and the medium. The tissue was ground in a glass homogeniser in the presence of ground glass until a cell-free homogenate was obtained. Solids were centrifuged off and the activity of the supernatant was determined without further delay.

Before interpreting the results obtained with this aleurone preparation, it was thought necessary to discover whether the period of presoaking affected the response of the tissue to gibberellic acid. Therefore 2mm. endosperm slices were incubated for 18 hours and 24 hours in water at room temperature (18-20°C). and then transferred to 10^{-5}M gibberellic acid at 25°C and the time-course of increase in α-amylase activity determined. Simultaneously, the enzyme content of the aleurone was measured. The results obtained were compared with those for slices which had not been presoaked prior to incubation in gibberellic acid.

Endo-β-Glucanase Assay

See Section II.

α-Amylase Assay

See Section II.

Acid Phosphatase Assay.

This enzyme was assayed colorimetrically by measurement of the release of p-nitrophenol from p-nitrophenyl phosphate at pH5 using "Calsuls" supplied by the Californian Biochemical Company Incorporated.

Each "Calsul" contains the substrate and buffer substances. One "Calsul" is dissolved in 10ml. of distilled water to make the substrate reagent. To 1ml. of substrate reagent at 25°C was added 0.2ml. of the enzyme extract at the same temperature. A blank containing only 1ml. of the substrate reagent was also prepared. Both samples were incubated for a suitable period at 25°C and then 4ml. of 0.05N sodium hydroxide solution was added. Now, 0.2ml. of enzyme extract was added to the blank and the optical density at 405mμ of the assay solution read against the blank set at zero. The time of incubation was chosen so as to give an optical density of the assay solution of less than one. Where t is the time of incubation in minutes, multiplying the optical density by 0.0933t gives the enzyme activity as millimoles of p-nitrophenol released per litre of extract per hour at 25°C.

Protease Assay

This was essentially a modification of the method suggested by Massart (1946). The standard substrate solution contained 6.875% gelatin and for each assay the following reaction mixture was prepared:

1.6ml. substrate solution
0.2ml. 0.1M acetate buffer, pH5
0.4ml. enzyme extract.

All at 40°C.

From this mixture, 2ml. was placed in a miniature V-tube viscometer in a waterbath at the given temperature. The viscosity of the mixture was then determined at suitable intervals (10-15 min.) over 1-1½ hours. The drop in specific viscosity is linear over at least the first 0.4 units and the rate of drop is proportional to the enzyme concentration. Hence the activity of the enzyme may be expressed as the change in specific viscosity per hour per gram fresh weight of endosperm.

This method gives a measure of endo-protease activity only.

TABLE XXI

Time Course of Increase in α-Amylase Activity in Aleurone and Starchy Endosperm of Prehydrated Endosperm Slices in Response to Gibberellic Acid.

(Proctor Barley, 1963 Harvest: Incubations at 25°C.)

18 Hour Prehydration at Room Temperature

Time (Hr).	Aleurone Contents	Secreted Enzyme
0	0	0
3	-	-
6	0	0
9	21±2	0
12	33±14	92±12
16	75±11	243±72

24 Hour Prehydration at Room Temperature

Time (Hr).	Aleurone Contents	Secreted Enzyme
0	0	0
3	8±7	0
6	11±2	0
9	38±4	55±22
12	74±10	101±12
16	170±25	480±70

N.B. (1) α-Amylase activity is expressed as IDC units per g. fresh wt. endosperm.

(2) Each result is the mean of three estimations ± standard deviation.

(3) All incubations in 10^{-5}M Gibberellic Acid.

TABLE XXII

The Increase in Activity of Enzymes in Barley Endosperm in Response to 10^{-5}M Gibberellic Acid and in the Course of Normal Germination

	Aleurone	Starchy Endosperm							
	(G.A.* (10^{-5}M)	G.A.* (10^{-5}M)				Germination			
Time (Hr)	A	G	A	Ph	Pr	G	A	Ph	Pr
0	0	0	0	6.3±0.3	75±15	0	0	6.3±0.3	75±15
6	-	0	-	5.1±0.4	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
12	0	14±5	-	5.4±0.2	-	-	-	-	-
15	-	48±7	-	5.7±0.2	-	-	-	-	-
18	47±2	114±25	0	7.5±1.3	50±32	-	-	-	-
21	-	-	49±3	8.2±0.9	-	0	0	5.4±0.3	-
24	-	230±50	108±32	8.2±0.8	75±22	-	-	-	25±20
27	-	300±20	194±66	7.1±1.2	-	42±13	0	4.6±0.5	-
30	90±4	-	252±15	9.8±0.6	200±18	52±7	13±4	5.2±0.6	50±16
33	-	867±163	362±26	10.6±0.8	-	-	-	-	-
36	-	874±237	417±75	10.6±1.2	750±73	264±20	87±10	6.2±0.4	125±50

cont'd.

TABLE XXII (Cont'd)

	Aleurone		Starchy Endosperm						
	G.A.* (10^{-5}M)		G.A.* (10^{-5}M)			Germination			
Time (Hr)	A	G	A	Ph	Pr	G	A	Ph	Pr
39	-	1458±509	497±91	9.7±0.8	-	-	-	-	-
42	218±86	1239±300	726±198	11.7±1.1	-	528±31	452±68	6.8±0.7	600±86
48	-	1350±162	-	-	1500±198	695±72	802±208	7.7±0.3	700±100
54	-	-	2080±132	12.7±0.5	1675±241	861±120	1304±193	7.7±0.6	-
60	-	-	-	-	3000±387	-	-	-	1875±268

*Acting on 2mm. Endosperm Slices

Proctor Barley (1963)

Temp = 25°C.

G = Endo-β-Glucanase. Activity as $\Delta^1/_1$sp/100g. fresh wt. endosperm x hr.

A = α-Amylase. Activity as IDC units/g. fresh wt. endosperm.

Ph= Acid phosphatase. Activity as millimoles p-nitrophenol/100g. x hr.

Pr=Protease. Activity as $\Delta 1/_1$sp/kg. fresh wt. endosperm x hr.

N.B. Each result is the mean of three estimations ± standard deviation.

TABLE XXIII

The Increase in Activity of Enzymes in Isolated Aleurone in Response to Gibberellic Acid (10^{-5}M).

	Aleurone Contents				Content of Medium			
Time (Hr)	G	A	Ph	Pr	G	A	Ph	Pr
0	0	0	0.3±0.2	50±25	-	-	-	-
6	0	0	0.5±0.1	-	-	-	-	-
10	-	0	-	-	-	-	-	-
12	37±3	10±16	0.8±0.1	-	-	-	-	-
15	78±3	33±11	0.5±0.1	-	-	-	-	-
18	-	54±6	-	-	-	-	-	-
21	104±16	-	0.9±0.1	-	-	-	-	-
24	-	63±5	-	50±6	-	-	-	-
27	-	-	-	-	-	-	-	-
30	159±24	96±6	1.5±0.2	175±25	0	-	-	-
33	-	-	-	-	-	-	-	-
36	180±15	178±18	2.0±0.2	225±30	18±8	-	-	-

continued next page

TABLE XXIII (Cont'd)

Time (Hr)	Aleurone Contents				Content of Medium			
	G	A	Ph	Pr	G	A	Ph	Pr
39	-	-	-	-	-	-	-	-
42	260±13	312±40	4.2±0.4	-	-	-	-	-
48	260±18	463±88	5.2±0.3	260±36	40±17	0	0	0

Proctor Barley (1963) Temp. = 25°C

G = Endo-β-Glucanase. Activity as $\Delta^{1}/_{1}$sp/100g. fresh wt. endosperm x hr.

A = α-Amylase. Activity as IDC units/g. fresh wt. endosperm.

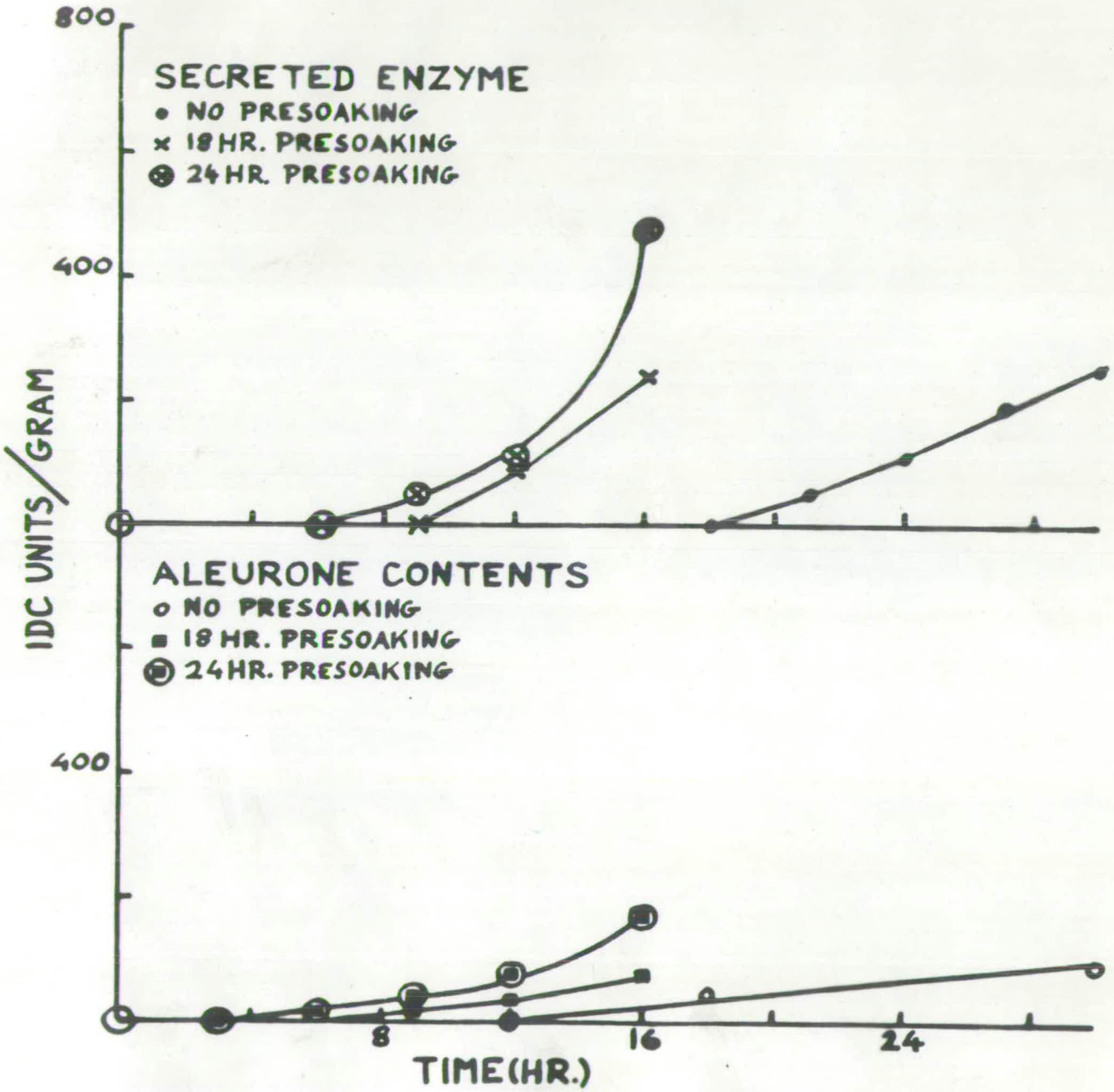
Ph= Acid phosphatase. Activity as millimoles p-nitrophenol/100g. x hr.

Pr= Protease. Activity as $\Delta^{1}/_{1}$sp/kg. fresh wt. endosperm x hr.

N.B. Each result is the mean of three estimations ± standard deviation.

FIGURE XII

EFFECT OF HYDRATION ON ENDOSPERM SLICE RESPONSE TO GIBBERELLIC ACID (10^{-5}M). (AS SHOWN BY α-AMYLASE ACTIVITY).

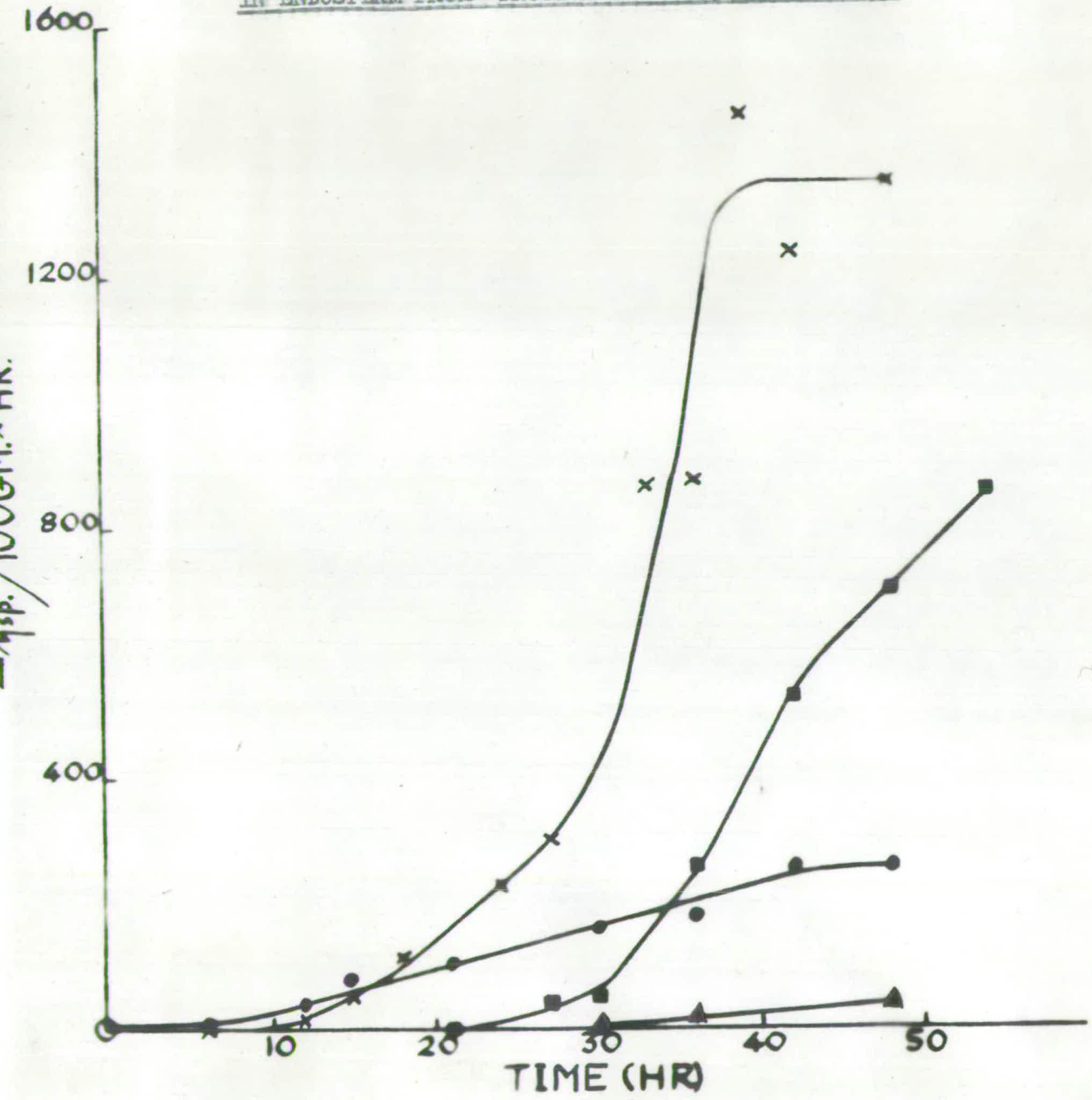


TEMP.=25°C

PROCTOR BARLEY (1963)

FIGURE XIII

THE TIME-COURSE OF INCREASE IN ENDO-β-GLUCANASE ACTIVITY IN ENDOSPERM FROM PROCTOR BARLEY (1963 HARVEST).



X – Extract of G.A.-treated endosperm slices.

● – Extract of G.A.-treated isolated aleurone.

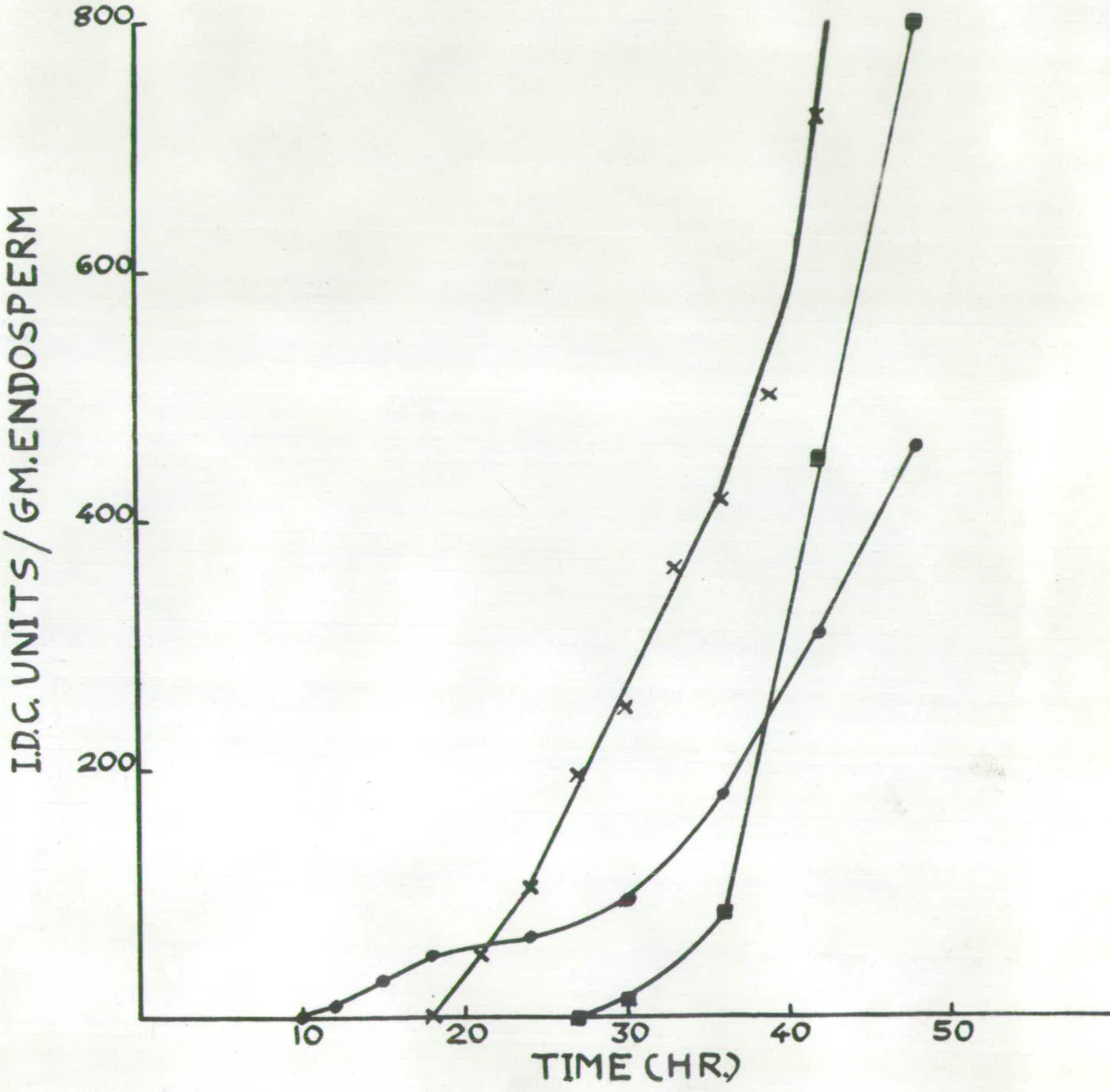
▲ – Enzyme secreted from G.A.-treated isolated aleurone.

■ – Extract of endosperm from germinating grain.

G.A. = 10^{-5} M Gibberellic Acid. Temp. = 25°C.

FIGURE XIV

THE TIME-COURSE OF INCREASE IN α-AMYLASE ACTIVITY IN ENDOSPERM FROM PROCTOR BARLEY (1963 HARVEST).



× - Extract of G.A.-treated endosperm slices.

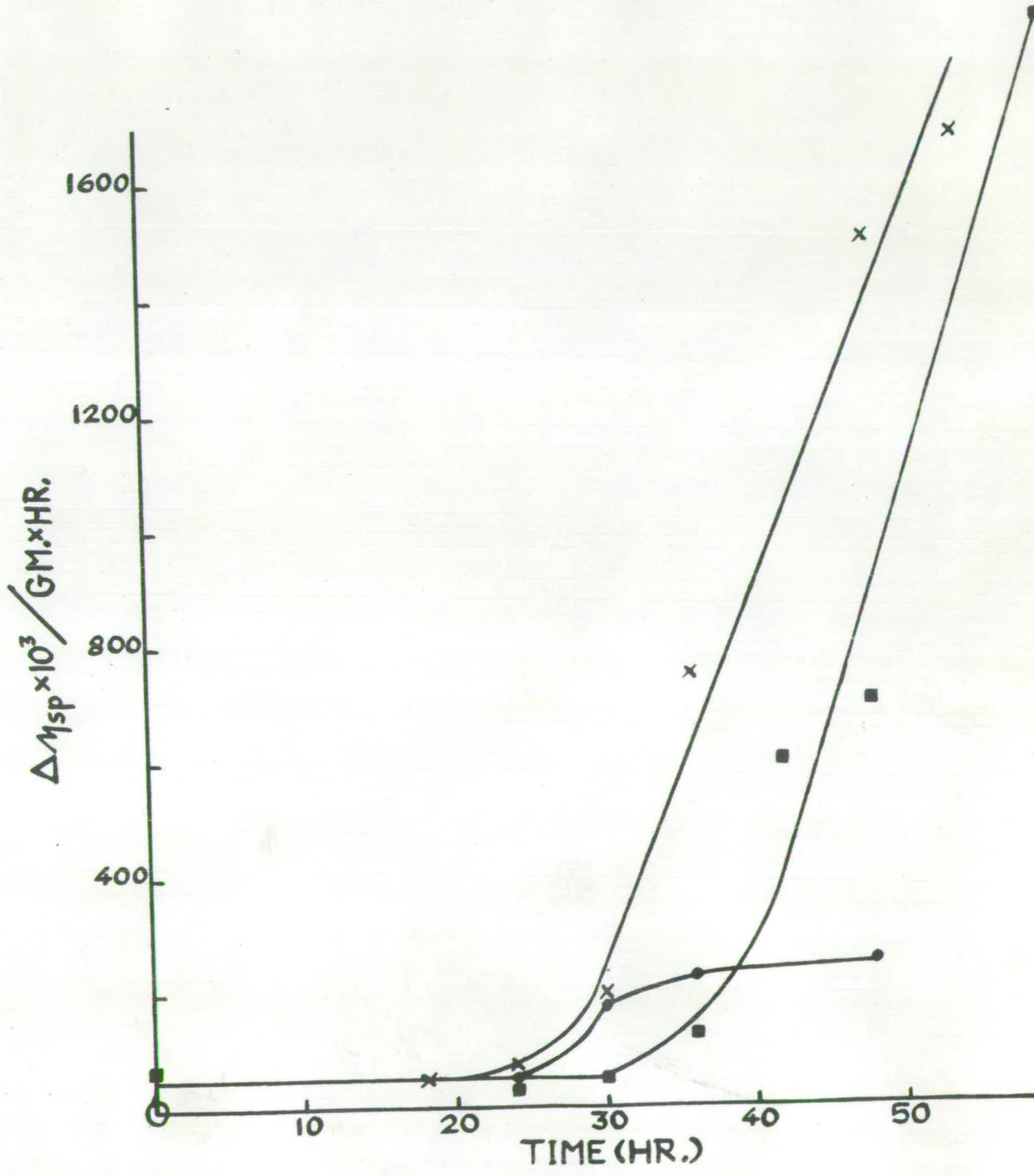
● - Extract of G.A.-treated isolated aleurone.

■ - Extract of endosperm from germinating grain.

G.A.= 10^{-5} M. Gibberellic Acid. Temp.=25°C.

FIGURE XV

THE TIME - COURSE OF INCREASE IN PROTEASE ACTIVITY IN ENDOSPERM FROM PROCTOR BARLEY (1963 HARVEST).



×- Extract of G.A. -treated endosperm slices.

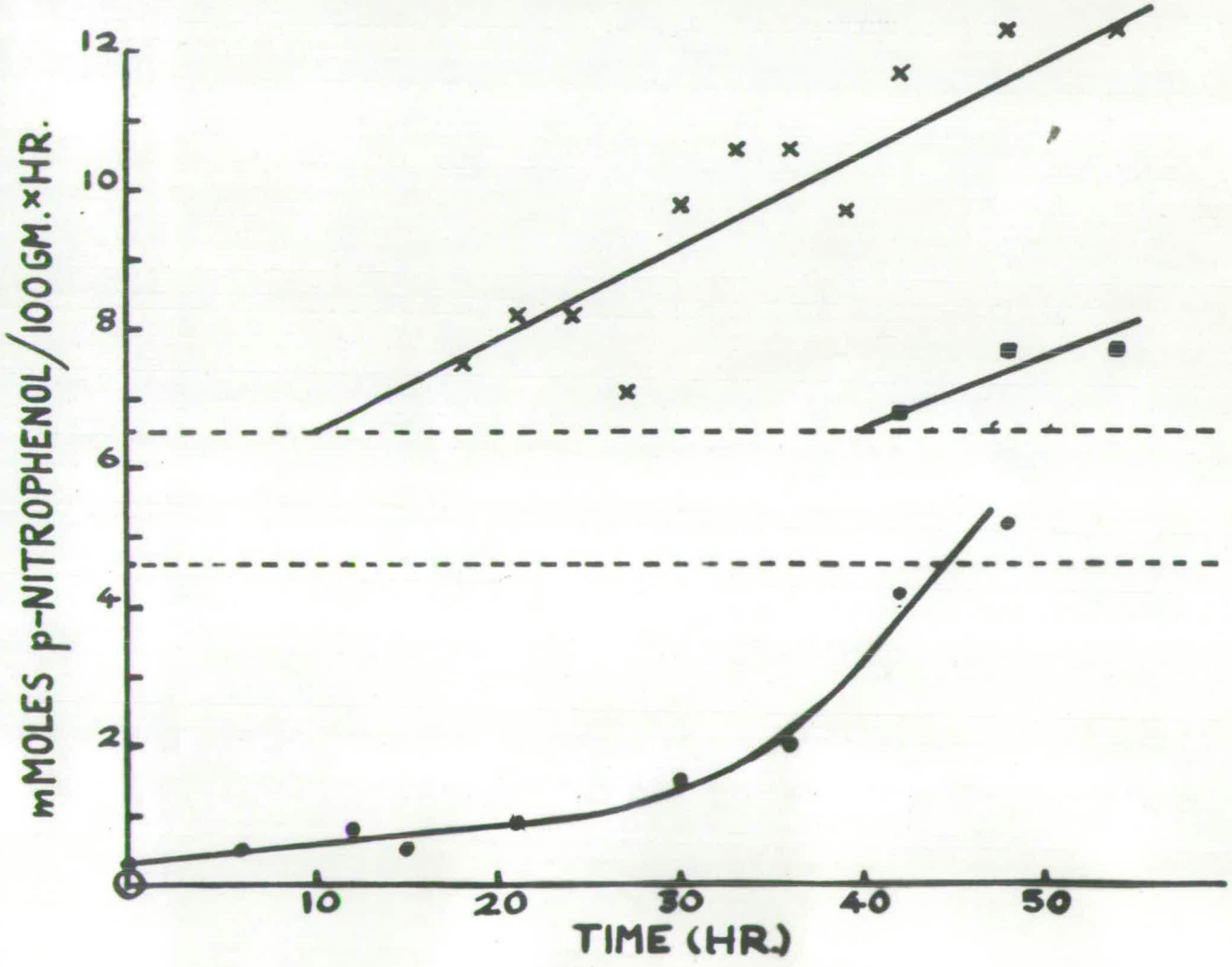
●- Extract of G.A. -treated isolated aleurone.

■- Extract of endosperm from germinating grain.

- 10^{-5} M. Gibberellic Acid. Temp. = 25°C.

FIGURE XVI

THE TIME-COURSE OF INCREASE IN ACID PHOSPHATASE ACTIVITY IN ENDOSPERM FROM PROCTOR BARLEY (1963 HARVEST).



X- Extract of G.A.-treated endosperm slices.

●- Extract of G.A.-treated isolated aleurone.

■- Extract of endosperm from germinating grain.

G.A. $=10^{-5}$M. Gibberellic Acid. Temp. $=25^{\circ}$C.

TABLE XXIV

Times of Appearance of Increased Enzyme Activities in Hours†.

(Proctor Barley, 1963 Harvest; Incubation at 25°C).

	Endo-β-Glucanase	α-Amylase	Protease
Germinating Barley	21	27	30
Endosperm Slices*	10	18	21
Isolated Aleurone*	6	10	24

* In 10^{-5}M Gibberellic Acid.

† From Figs. XIII, XIV and XV.

TABLE XXV

Time Course of Increase in Enzyme Activities in Barley Endosperm Incubated in Water.

(Proctor Barley, 1963 Harvest: Incubation at 25°C)

	Isolated Aleurone				Endosperm Slices			
Time (Hr)	G	A	Ph	Pr	G	A	Ph	Pr
0	0	0	0.3±0.2	50±25	0	0	6.3±0.3	75±15
48	11±3	0	0.9±0.1	15±10	88±17	0	5.1±0.5	50±11

G = Endo-β-Glucanase. Activity as Δ^{1}/η_{sp}/100g. fresh wt. endosperm x hr.

A = α-Amylase. Activity as IDC units/g. fresh wt. endosperm.

continued next page.

TABLE XXV (Cont'd)

Ph = Acid Phosphatase. Activity as m Moles p-nitrophenol/ 100g. x hr.

Pr= Protease. Activity as Δ sp/kg. fresh wt. endosperm x hr.

N.B. Each result is the mean of three estimations ± Standard deviation.

Results

The Effect of Prehydration

Presoaking reduces the lag period between commencing incubation in gibberellic acid and the appearance of α-amylase activity (Table XXI, XXII, Fig. XII). Further, presoaking reduces the time required for a given rate of increase in α-amylase activity to be attained as compared with slices cut from the dry grain.

It is of interest to note that in all the samples studied α-amylase activity can be detected in the aleurone before it appears in the endosperm.

General Time-Course Studies (Tables XXII, XXIII, Figs. XIII, XIV, XV, XVI).

The results obtained show that, in each of the three systems, the order of appearance of increased activity of the enzymes studied is, from first to last, endo-β-glucanase, α-amylase and then protease (Table XXIV). Where acid phosphatase fits into this sequence is difficult to determine owing to the variability of the results which necessitated the use of the method of least squares in drawing the linear graph shown for endosperm slice activities (Fig XVI).

Comparison of the rates of increase in enzyme activity in the three systems is difficult owing to the varying patterns of increase. However, the rates in germinating grain endosperm and endosperm slices in gibberellic acid are fairly similar except possibly in

the case of endo-β-glucanase. In isolated aleurone, on the other hand, the rates appear to be decreased, most markedly in the case of protease. Acid phosphatase may be an exception here but the variability of the results leaves this uncertain.

Studies with Isolated Aleurone.

The most remarkable feature of the results obtained with isolated aleurone is that, despite pronounced formation of α-amylase and acid phosphatase, only endo-β-glucanase is secreted and it is in very small quantities (Table XXIII). This is not because the isolated tissue is incapable of secretion. Aleurone, isolated from 1963 Proctor barley endosperm after 42 hours incubation in gibberellic acid, secretes 70 IDC units of α-amylase per gram fresh weight endosperm equivalent in a further 18 hours in 0.1M sodium chloride solution.

Aleurone from 1962 Proctor barley endosperm behaves similarly. However, its secretory ability following isolation from gibberellic acid treated material is much more marked (Appendix 1).

Discussion

The Effect of Prehydration.

The reduced lag period in response to gibberellic acid and the more rapid attainment of a high rate of increase in enzyme activity produced by prehydration may be partly explained by increased metabolic acitivity of the presoaked slices. The rate of oxygen uptake of endosperm slices is observed to increase markedly over the first 18 hours of soaking, being negligible in the dry state (Paleg, 1964).

The fact that α-amylase activity can be detected in the aleurone before it appears in the endosperm is in keeping with its being secreted from the former tissue.

General Time-Course Studies.

The sequential appearance of increased enzyme activity within the aleurone suggests that gibberellic acid exerts some specific effect on the synthesis of the enzymes involved. If gibberellic acid simply stimulated general protein synthesis or caused the release of pre-formed enzymes from lysosomes of the type postulated by de Duve (1963), one would expect a simultaneous increase in enzyme activities.

The decreased rates of increase in enzyme activities within the isolated aleurone relative to the rates in endosperm slices may be due to a mass action effect. The accumulation of enzymes within the aleurone cells may tend to prevent dissociation of newly synthesised

molecules from the synthesising sites, presumably polysomes.

Despite the virtual non-secretion of the enzymes studied from isolated aleurone, the observation that the sequential appearance of increased enzyme activity is the same in isolated aleurone and endosperm slices supports the view that they are secreted from the former tissue into the starchy endosperm. That the observed sequence is followed in endosperm slices from germinating barley as well as those incubated in gibberellic acid indicates that the latter phenomenon approximates closely to the natural situation.

Studies with Isolated Aleurone.

It is reasonable to suppose that failure in enzyme secretion by aleurone separated from slices which were not treated with gibberellic acid is due to the lack of a third factor which is essential for the reaction between gibberellic acid and aleurone, and which is normally transferred from the starchy endosperm to the aleurone in the presence of gibberellic acid. However attempts to demonstrate such a factor directly were unsuccessful, even with aleurone from 1962 Proctor barley, the endosperm of which responds rapidly to gibberellic acid (See Appendix 1).

Similar results have been obtained by other workers. Briggs (1964) found that aleurone isolated from Proctor barley (1961) by treatment of endosperm slices with snail juice, secretes α-amylase only after 4 days

incubation. Intact slices yield comparable amounts in two days. Yomo and Iinuma (1964b) prepared aleurone by treating endosperm slices with a hydrolase preparation from Trichoderma viride at 17°C. for three days. They found that after three days further incubation with gibberellic acid only endo-β-glucanase was secreted in appreciable quantity though large amounts of both α-amylase and protease were produced within the tissue. The protease they assayed was probably different from that assayed here using gelatin. They measured the release of dinitrophenyl-amino-acids from dinitrophenol-haemoglobin. This would determine mainly exoprotease activity whereas the gelatin method is specific for endoproteases.

Different results have been obtained by Varner and Ram Chandra (1964) and Paleg (1964). Both claim that aleurone stripped off barley endosperm after between two and three days presoaking responds to gibberellic acid by secreting α-amylase in amounts comparable with those extractable from similarly treated endosperm. There are various possible explanations. This may simply be a varietal difference. Bearing in mind the secretion of α-amylase from aleurone isolated from gibberellic acid treated endosperm slices, it may reflect the stage of maturation attained by the grain. It may also reflect the method of isolation of the aleurone. Unfortunately Varner and Ram Chandra do not give a detailed description of this but Paleg does.

Paleg soaked his barley in 5% calcium hypochlorite for two hours to surface sterilise it. During this period, some interaction between the various tissues may well have occurred. Further, Paleg remarks that he presoaked endosperm slices from this grain in distilled water at 30°C., an unusually high temperature for plant tissues. After soaking the tissue he stripped off the aleurone and he comments that this was contaminated with a small amount of adhering starchy endosperm. If this starchy endosperm constituted a fairly continuous layer, however thin, it could well have a significant effect, since slices of aleurone with a thin adhering starchy endosperm layer do indeed respond to gibberellic acid in a comparable fashion to intact endosperm (MacLeod and Millar, 1962).

In conclusion, one may say that the results reported in this section seem fairly well substantiated by those obtained by other workers, but that there are involved a number of variables which remain to be clearly defined.

SECTION IV

Ultrastructural Changes in the Aleurone Layer Following Gibberellic Acid Treatment.

Since gibberellic acid produces considerable metabolic changes in the aleurone layer, akin to differentiation, it seemed probable that these changes might be reflected at the ultrastructural level. Accordingly, endosperm slices were incubated for varying periods in gibberellic acid and the aleurone isolated and examined, after suitable preparation, in the electron microscope. This work was carried out in close co-operation with Mr C.S. Johnston to whom I am indebted for advice on techniques and for the electron micrographs shown.

Experimental

General

The barley used was Proctor from the 1963 harvest, the sample being identical with that used for the work described in Section III.

Following each incubation, aleurone was prepared as described in Section III.

Electron Microscopy.

In most cases, the isolated aleurone was fixed in 0.6% potassium permanganate over a range of times from 15 minutes to 6 hours. Similar results were obtained whether the permanganate was buffered with pH7.4 veronal

buffer or not. Also, fixation between 0°C and 4°C., though slower, did not produce appreciably different results from that at room temperature.

The fixed material was dehydrated in increasing concentrations of ethanol according to the following schedule:

30% Ethanol - 15-30 minutes
50% Ethanol - 15-30 minutes
70% Ethanol - 15-30 minutes
90% Ethanol - 1 hour
100% Ethanol - 2 x 1 hour.

It was found that more gradual dehydration did not affect the results, nor did leaving overnight in 70% ethanol at 0-4°C. if necessary.

After dehydration, the material was embedded in a methacrylate mixture (88% butyl methacrylate + 12% methyl methacrylate) under reduced pressure as below:

Methacrylate mixture/Ethanol 1:1 - 2hrs
Methacrylate mixture - 2hrs
Methacrylate mixture (fresh) - 18hrs
Methacrylate mixture + catalyst* - 48hrs to 1 week.

* Dichlorobenzoyl peroxide (1% w/v in the mixture).

The mixture plus catalyst is stored out of direct sunlight since ultraviolet rays promote polymerisation.

Finally, the sections were transferred to gelatin capsules (size 00) and these were filled with methacrylate mixture plus catalyst. Polymerisation took place overnight at 60°C.

The polymerised blocks were trimmed and sections were cut on to 15% ethanol by a "Cambridge" ultramicrotome. The sections were usually of 500A° thickness though on occasions sections of 250A° were examined. Any ripples present in the sections were evened out by treatment with xyol vapour. The sections were mounted on uncoated grids and subsequently coated with a thin layer of carbon on one side only.

The specimens were examined and photographed in an Akashi TRS 50 EI electron microscope fitted with a specially modified D.C. filament at 50 kv. E.H.T.

Latterly, fixation in glutaraldehyde followed by staining with osmic acid and embedding in Araldite has been employed.

Sections were fixed in 6% ($^{v}/_{v}$) glutaraldehyde in 0.1M phosphate buffer, pH7. They were then transferred to the buffer alone and soaked for three hours with three changes to elute excess glutaraldehyde. Finally the sections were stained in 2% osmic acid for 30 minutes or 1 hour.

After treatment with osmic acid, the sections were washed with water and dehydrated with increasing concentrations of acetone according to the following schedule:

25% acetone - 15 minutes
50% acetone - 15 minutes
75% acetone - 30 minutes (or overnight)
100% acetone - 1 - 2 hours.

After dehydration, the material was transferred to xylene for 30 minutes and then embedded as follows:

Araldite/Xylene 1:1 - 30 minutes

Araldite mixture - 2 x 30 minutes (50°C)

Lastly the sections were transferred to gelatin capsules as before. The capsules were filled with Araldite mixture and the araldite polymerised for 1 - 2 days at 55°C.

Results

The Aleurone Layer of the Mature Grain.

Studies with the light microscope have shown that the aleurone cells, besides having apparently normal nuclei and very thick walls, contain a number of unusual inclusions (Haberlandt, 1914). Use of the electron microscope has enabled these inclusions to be characterised more accurately. It has also shown that some at least of the nuclei in both untreated and gibberellin treated aleurone are abnormal in showing after permanganate fixation a mottled appearance due to deposits of electron dense material (Plates 6,9-11).

One marked difference between aleurone cells from the mature grain and other more typical plant cells is that the former appear to have no mitochondria and no clearly defined endoplasmic reticulum (Plates 6-8).

In every cell examined, deposits of material of medium electron density (Pr) are found (Plates 6-11). These deposits are generally irregular and each is enclosed in a unit membrane. Within these deposits are often found regular, electron transparent, ovoid structures (V), each again enclosed in a unit membrane. These resemble typical plant vacuoles (Poux, 1962; Graham *et al.*, 1962). There are also dense structures (I) and structures of intermediate density, e.g. I_1 and I_2 (Plate 7) similar in shape to the transparent structures (V). This variation in density cannot be due to an artifact of fixation since the relative

positions of the inclusions vary from cell to cell.

Small regular vesicles (UB), 0.3 - 0.4μ in diameter, are found either surrounding the medium density material (Pr) or aggregated along the cell wall, just inside the plasmalemma (Plates 6-11). These vesicles are electron transparent and have a fine limiting membrane. When the tissue is fixed in glutaraldehyde followed by osmic acid staining and embedding in Araldite, these vesicles acquire considerable electron density (Plate 12).

In addition to the above, there are smaller electron dense inclusions (DB) (Plates 6-11).

The Aleurone Layer from Gibberellic Acid Treated Endosperm.

Essentially the same organelles as in aleurone from the mature grain are present in aleurone isolated from endosperm slices which have been incubated in 10^{-5}M gibberellic acid for 30 hours at 25°C (Plates 9-11). However, examination of a number of micrographs suggests that the intense staining of localised portions of the nucleus, including the nucleolus, is more common in cells from the gibberellic acid treated tissue. Further, the medium density deposits (Pr), though less in total, appear to be individually more extensive. They still contain electron-dense inclusions (I) which appear in some cases to have a lamellar structure. Fixation causes many of the inclusions to harden to such an extent that they fracture when cut.

The small vesicular structures (UB) appear to have increased in number. They are still electron transparent following permanganate fixation.

Structures of a mitochondrial nature (M) appear in fairly large numbers but their origin is as yet uncertain (Plate II).

The small dense inclusions (DB) of the mature grain now seem to be distinctly vesicular when examined carefully (Plates 10).

Plasmadesmata.

The very thick cell walls of the aleurone clearly pose problems as far as the transport of any hormonal material through the tissue is concerned. More so, they pose problems as to mechanisms of enzyme secretion since they are apparently not destroyed in the process (Plate 14). The solution to both these problems would appear to lie in the plasmadesmata, strands of cytoplasm penetrating the walls and linking the aleurone cells (Plates 13,14).

Discussion.

The Aleurone Layer of the Mature Grain.

The inability to detect any typical mitochondria or endoplasmic reticulum may be due to the masking effect of the dense cytoplasm (Paleg and Hyde, 1964).

Buttrose (1963) has suggested that the medium density material (Pr) may be vacuolar in origin and that it and the inclusions present in it may constitute the aleurone grain of the light microscopists.

It is not clear whether there is any relationship between the regular, electron-transparent, ovoid structures (V) and the dense structures (I) of the same shape. The existence of intermediates, however, suggests that there may be a developmental connection. Paleg and Hyde (1964), on the other hand, in their ultrastructural examination of barley aleurone did not observe the intermediate forms shown here. They consider that the two structures are quite distinct on the basis of their reactions with potassium permanganate and osmic acid.

The small electron dense inclusions (DB) have also been observed by Paleg and Hyde (1964) in barley aleurone following osmic acid fixation, and embedding in Epon 812. They have been reported to occur in cereal embryos too (Setterfield *et al.*, 1959).

The Aleurone Layer from Gibberellic Acid Treated Endosperm and the Relation of the Ultrastructural

The Aleurone Layer from Gibberellic Acid Treated Endosperm and the Relation of the Ultrastructural Changes to Biochemical Changes.

The continued electron transparency of the small vesicular structures (UB) following gibberellic acid treatment may be somewhat anomalous. Paleg and Hyde (1964) report that in isolated barley aleurone they become electron dense following similar treatment. Further, this change in electron density has been observed in aleurone from wheat following one day's germination (Buttrose, 1963) and in aleurone from Bromus allowed to germinate for 18 hours (MacLeod et al., 1964). Since Paleg and Hyde surface-sterilised their material by soaking intact grains in calcium hypochlorite for 2 hours prior to separating the endosperm, it is just possible that the movement of some material from the embryo produced the effect observed in all three cases.

It seems probable that the medium density deposits (Pr) are proteinaceous in nature. The decrease in total amount following gibberellic acid treatment suggests that they may provide the raw material for the synthesis of secreted enzymes.

The increase in number of the small vesicular bodies (UB) in the gibberellic acid treated aleurone and their tendency to aggregate round the medium density deposits and the cell wall may indicate that they are involved in the secretory process.

GENERAL DISCUSSION

Although it still cannot be stated definitely that gibberellic acid is a natural hormone released from the embryo of the germinating grain and causing secretion of hydrolases from the aleurone, results obtained in the course of the work reported here are in keeping with this hypothesis. For example, a compound with some of the properties of gibberellic acid is present in the embryo of the mature grain and not in the endosperm. Further, the patterns of increase in activity of certain enzymes in the endosperm of the germinating grain and in endosperm slices incubated in gibberellic acid are virtually the same. That this increase in enzyme activity is at least partly the result of secretion of enzymes from the aleurone is borne out by the observed secretion of α-amylase from aleurone isolated from gibberellic acid treated endosperm. It is also borne out by the experiments with endosperm slices in gibberellic acid where it was shown that α-amylase activity appears in the aleurone before it can be detected in the starchy endosperm. In addition, this hypothesis is supported by the fact that the sequence of increase in activities of the enzymes studied is the same in isolated aleurone as in endosperm slices incubated in gibberellic acid.

Accepting that gibberellic acid does cause hydrolase secretion from aleurone, the studies with the isolated tissue indicate that the mechanism of this can

be subdivided into at least two components, formation of the hydrolases and their release from the cell. Inhibitors therefore may act on either of these two stages and this complicates interpretation of the results given in Section II. Another complicating factor is the varying response obtained from different barley samples, even samples from the same variety and the same harvest.

Despite the above complications, two general conclusions may be drawn from the studies with inhibitors. It appears that oxidative phosphorylation and protein synthesis are required for response of endosperm to gibberellic acid. Since only the aleurone layer appears to be capable of these processes (Paleg, 1964; Varner, 1964), this is in keeping with the other evidence which points to the aleurone layer as the basic site of action of gibberellic acid (MacLeod and Millar, 1962; this thesis, Section III). Further, it is possible that gibberellic acid may increase the penetration of some organic substances, like 2,4-dinitrophenol and salicylic acid, into the aleurone layer.

The sequential formation of hydrolases within the isolated aleurone in response to gibberellic acid is difficult to explain without invoking some action at the level of the gene. It is tempting to suggest that it reflects the sequential production of "messenger" ribonucleic acid (RNA) by the appropriate enzymes working along a segment of deoxyribonucleic acid, newly exposed as a result of gibberellic acid action.

If this is so, it would be interesting to apply Actinomycin D to endosperm slices at various times after commencing incubation in gibberellic acid. This compound inhibits "messenger" RNA synthesis in some systems (e.g.Goldberg and Rabinowitz,1962). Hence it may be possible to prevent increase in protease activity without affecting increase in endo-β-glucanase and α-amylase activities.

The ultrastructural studies described in Section 4 cannot be said to have cast any light on the basic function of gibberellic acid but they have indicated a role for certain organelles in the secretory process and it would clearly be of interest to isolate these using the ultracentrifuge and study their properties. The ultrastructural studies themselves are only a beginning and should be continued using more specific fixation reagents. Particular attention should be paid to those reagents which may give some indication as to how the hydrolases are distributed within the cell prior to and during secretion.

Finally, it may be remarked that so far no-one has succeeded in preparing a cell-free homogenate of aleurone or barley endosperm which will respond to gibberellic acid (Radley, 1964; Varner, 1964). This therefore is the main challenge for the future as far as determining the mode of action of gibberellic acid is concerned.

Further verification of the role of gibberellic acid or a gibberellic acid derivative in normal

germination will require a more detailed characterisation of the apparently related compound partially purified in the course of this work. It will also require a determination of the change in concentration of this compound during germination in both the embryo and the aleurone.

APPENDIX I.

Studies with Aleurone Isolated from 1962 Proctor Barley (Sample a) Endosperm.

The aleurone was isolated as described in Section III. Since endosperm slices from this barley sample showed a marked response to gibberellic acid after only 18 hours incubation at 25°C. (Table V), all incubations of isolated aleurone were for the same period. However, no secretion of α-amylase or endo-β-glucanase from the aleurone into the gibberellic acid medium could be detected.

When the aleurone was isolated from endosperm slices which had been incubated in gibberellic acid for 18 hours, it secreted up to 758 IDC units of α-amylase per gram fresh weight endosperm equivalent in a further 18 hours in 0.1M sodium chloride. Hence it is clear that the isolated aleurone is capable of secretion.

It was thought that the failure in enzyme secretion of aleurone separated from slices not pretreated with gibberellic acid might be due to lack of factor essential for secretion to take place, particularly after it had been shown (Section III) that enzyme formation within the aleurone was occurring in response to gibberellic acid. This factor was presumably transferred from the starchy endosperm to the aleurone in the presence of gibberellic acid. Accordingly, various compounds which might be expected to be supplied by starchy endosperm

were added to the aleurone preparations in the incubation medium with gibberellic acid. The substances added, together with the reasons for using each, are listed below.

Components of the Medium in Addition to Gibberellic Acid (1.5×10^{-5}M).

Glucose (5×10^{-2}M)	Normally present in endosperm.
Soluble Starch (1%)	Main endosperm constituent
Knop's Solution (Full Strength or Half Strength).	Balanced solution of ionic macronutrients.
White's Medium (Minerals)	Tissue culture medium, macro - and micronutrients
Oxoid Nutrient Broth, No.2, pH 7.5	Balanced solution of amino-acids.
Vitamin B Factors: Pyridoxine (10^{-7}M) Nicotinic Acid (10^{-6}M) Thiamine (2.3×10^{-7}M) Biotin (2×10^{-6}M)	Vitamins supplied in concentrations used in tissue culture work
Growth Factors: Kinetin (10^{-4}M, 10^{-5}M, 10^{-7}M). Indole-acetic Acid (10^{-5}M) Kinetin (10^{-5}M)+Indole-acetic acid (10^{-5}M) Adenine (2×10^{-6}M)	
Dried ethanolic extract of endosperm	Aleurone pressed on and maintained in a water - saturated atmosphere.
Freeze dried aqueous extract of endosperm.	All endosperm solubles.
Dialysate of freeze dried extract	Diffusible material.

None of the materials listed was effective in inducing, in conjunction with gibberellic acid, any secretion of α-amylase into the medium. Addition of separated barley endosperm to each of the media mentioned with gibberellic acid was ineffective too as was addition of sodium and potassium chlorides to a concentration of 0.1M.

APPENDIX 2.

The Effect of Inhibitors on Endo-β-Glucanase.

In the course of this work it was found that the following compounds inhibited endo-β-glucanase directly:

Potassium cyanide ($5x10^{-2}M$)

Sodium Sulphide ($5x10^{-2}M$)

Thioglycollic acid ($5x10^{-2}M$).

Since sodium azide at $5x10^{-2}M$ and ethylene diamine tetra-acetic acid at $10^{-2}M$ had no effect, it is clear that no metal ions are required for the activity of the enzyme. Further, hydroxylamine at $2.5x10^{-4}M$ produced no inhibition, showing that the cyanide inhibition is not due to its action as a carbonyl reagent.

It is probable therefore that the action of all the active inhibitors is to reduce disulphide bonds, thereby altering the configuration of the active centre. Such an action has been postulated for cyanide to explain its activation of several proteolytic enzymes (Bersin and Logemann, 1933). It would be of interest to find whether this inhibition is reversible by appropriate oxidising agents.

TABLE XXVI

The Response to Gibberellic Acid of Endosperm Slices Cut from Germinating Proctor Barley (1963), As Indicated By the Increase in α-Amylase Activity.

(24 Hr. Incubation at 25°C).

Pretreatment† Time(Hr.)	S or G.††	Incubation Medium	Activity**	Relative Activity
0		Water	0 (3)	0
0		G.A.*(10^{-5}M)	108±32 (3)	100
4	S	Water	0 (2)	0
4	S	G.A.(10^{-5}M)	224±60 (2)	207
4	G	Water	0 (2)	0
4	G	G.A. (10^{-5}M)	225±10 (2)	208
24	S	Water	0 (2)	0
24	S	G.A. (10^{-5}M)	206±0 (2)	191
24	G	Water	0 (2)	0
24	G	G.A.(10^{-5}M)	780±110 (2)	722

† At 25°C.

†† S = Endosperm slices (2mm.) incubated in water.

G = Grain germinated on water-saturated filter paper prior to cutting 2mm. slices for further incubation.

* Gibberellic Acid

** IDC units/g. fresh wt. endosperm.

Each result is the mean ± standard deviation

Numbers of experiments are given in parentheses.

APPENDIX 3

The Response to Gibberellic Acid of Endosperm Slices Cut from Germinating Barley.

It was thought that some of the variations in response of endosperm from different barley samples to gibberellic acid might be due to a degree of premature germination, occurring either in the field or in storage. Accordingly, it was decided to investigate the change in this response over short periods of germination. Proctor barley from the 1963 harvest was germinated for periods of 4 hours and 24 hours on water-saturated filter paper at 25°C. as described in Section III. Then 2mm. slices were cut and further incubated at 25°C. in water and gibberellic acid for 24 hours. Similar slices, cut from the dry grain, were incubated in water for times equal to those of germination and at the same temperature; then they were incubated in the same way as the slices cut from the germinated grain. These slices from the dry grain permitted allowance to be made for hydration effects. Finally, α-amylase activity in the slices was determined as described in Section II. This was referred back to the fresh weight of the slices which had previously been determined for this barley (Section III).

It will be seen from Table XXV that, under the conditions used, maximum enhancement of response to gibberellic acid resulting from hydration is attained within four hours. Further enhancement occurs

however in the germinating grain. After 24 hours germination, the response is over three times as much as that of slices from the dry grain prehydrated for the same period. Although only a small number of results is quoted here, similar results have been obtained for other barley samples (Horsfall, 1965).

The most obvious difference between endosperm in intact germinating grain and endosperm slices in water is that the former is in contact with the embryo. Hence one may suggest that there is secreted by the embryo some factor which sensitises the aleurone to gibberellic acid. There is also the possibility that the aleurone in the mature grain contains some gibberellin inhibitor which is translocated to the embryo during germination. If this were so, however, one might reasonably expect the inhibitor to pass slowly into the aqueous medium from isolated slices so that their response to gibberellic acid would increase with prehydration for periods longer than four hours though not so fast as in the intact grain.

It is interesting to note that the enhancement of response of endosperm slices to gibberellic acid caused by prehydration is less marked in this set of experiments than in those carried out with the same barley sample seven months earlier (Section III). This may reflect the different temperature of prehydration or it may simply be a symptom of aging.

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PUBLICATIONS.

"Gibberellic Acid in the Germination of Barley". by Anna M. MacLeod, J. Duffus, and A.S. Millar, Proceedings of the European Brewing Convention Brussels, 1963, 85.

Reference is made to the work described in Section I and a considerable portion of that described in Section II is reported, with particular reference to the studies with uncoupling agents.

"Ultrastructure of Caryopses of the Gramineae - I. Aleurone and Central Endosperm of Bromus and Barley". by Anna M. MacLeod, C.S. Johnston, and J.H. Duffus, J. Inst. Brew., 70 (1964), 303

Much of the work described in Section IV is included.

"Development of Hydrolytic Enzymes in Germinating Grain". by Anna M. MacLeod, J.H. Duffus, and C.S. Johnston, J. Inst. Brew., 70 (1964), 521.

Almost all the work described in Section III is reported in this paper. This material also formed the basis of a lecture given by the author at the 1964 Research Symposium of the Botanical Society of Edinburgh.

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Plates 1 - 5

Thin Layer Chromatograms

These plates show typical chromatograms following treatment with the 70% sulphuric acid spray and heating at 120°C. for 10 minutes. Fluorescent areas have been outlined and their colour indicated by the following symbols:

B = Blue

BG = Blue-Green

G = Green

Y = Yellow

Fractions obtained as described in Section I have been designated A, B, C etc., according to the following convention:

Fraction	Kieselguhr Layer R_f.
A	0.00 to 0.05
B	0.05 to 0.15
C	0.15 to 0.25
D	0.25 to 0.40
E	0.40 to 0.55
F	0.55 to 0.70
G	0.70 to 0.85
H	0.85 to 1.00

G.A. = Gibberellic Acid Control.

Plate 1

Kieselguhr thin layer used for the purification of an ethanolic extract of embryos. It will be seen that following development with benzene/acetic acid/water solvent, most of the lipid contaminants have moved near the solvent front. A similar picture is obtained with ethanolic endosperm extracts if allowance is made for the considerable increase in quantity.

Plate 2

Silica Gel thin layer chromatograms of fractions obtained from an ethanolic extract of "crude" embryos. These chromatograms have been developed with di-isopropyl ether/acetic acid solvent. The streaks obtained from fractions D,E,F,G, and H have a yellow fluorescence.

Plate 3

Silica Gel thin layer chromatograms of fractions obtained from an ethanolic endosperm extract.
The chromatograms have been developed with di-isopropyl ether/acetic acid solvent. The streaks obtained from fractions D,E,F,G,H have a yellow fluorescence.

ABBREVIATIONS

FRACTION	KIESELGUHR R_F
A	0·00 to 0·05
B	0·05 to 0·15
C	0·15 to 0·25
D	0·25 to 0·40
E	0·40 to 0·55
F	0·55 to 0·70
G	0·70 to 0·85
H	0·85 to 1·00

FLUORESCENCE

B = Blue
BG = Blue-Green
G = Green
Y = Yellow

G.A. = Gibberellic Acid

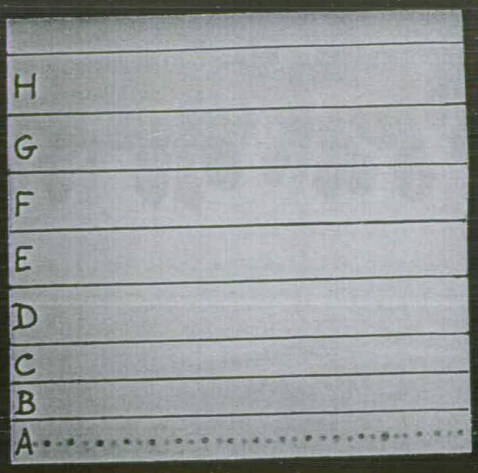


PLATE 1

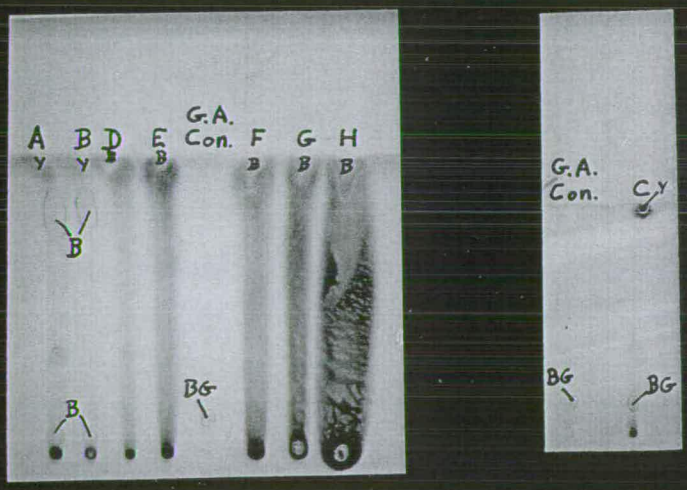


PLATE 2

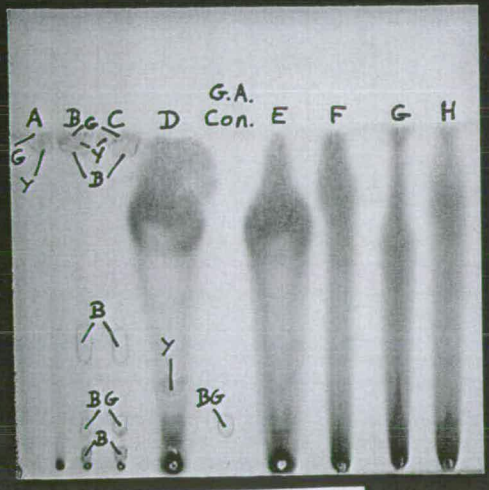


PLATE 3

Plate 4

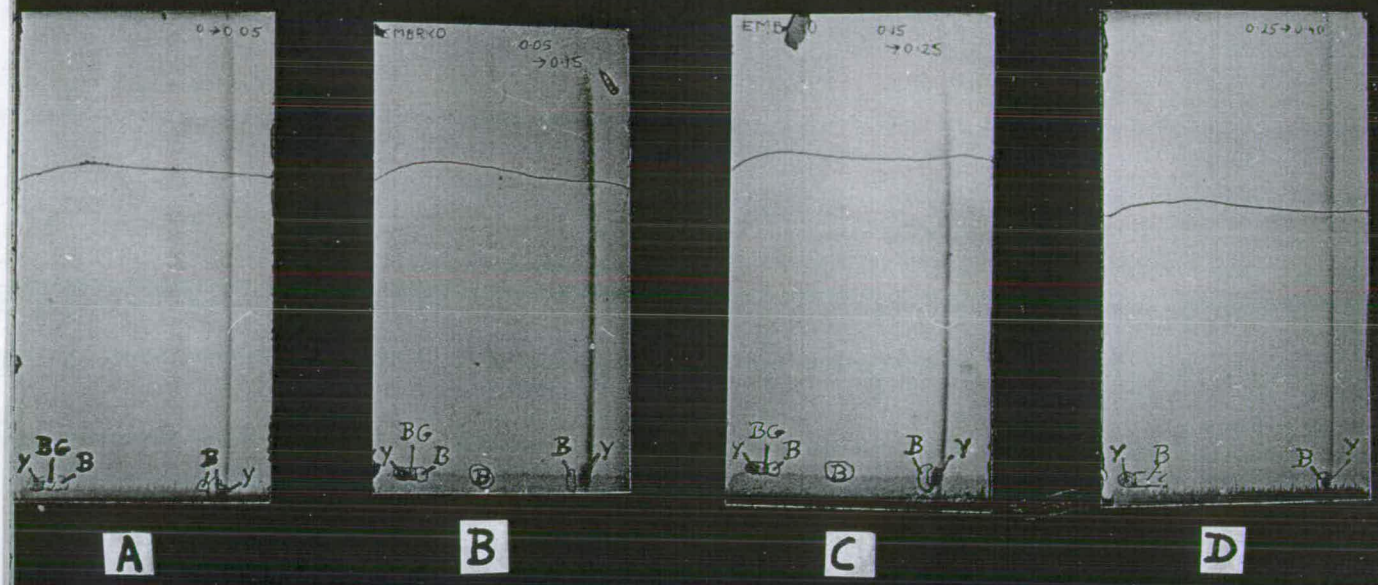
Silica Gel thin layer chromatograms of ethanolic "crude" embryo extracts. These chromatograms have been developed with the two dimensional system involving development in ethyl acetate followed by development in water.

ABBREVIATIONS

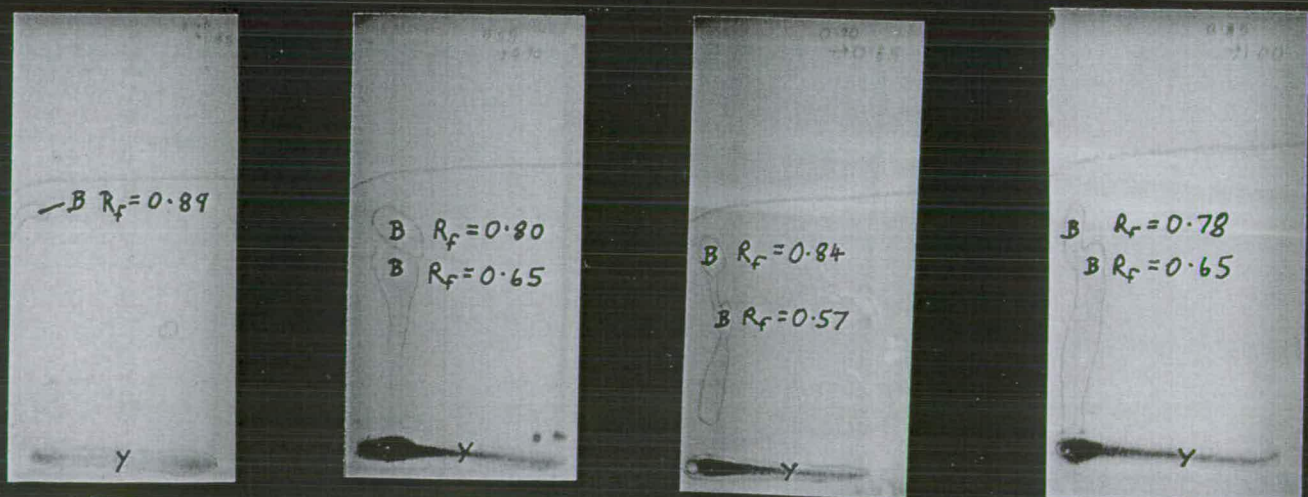
FRACTION	KIESELGUHR R_f
A	0·00 to 0·05
B	0·05 to 0·15
C	0·15 to 0·25
D	0·25 to 0·40
E	0·40 to 0·55
F	0·55 to 0·70
G	0·70 to 0·85
H	0·85 to 1·00

FLUORESCENCE

B = Blue
BG = Blue-Green
G = Green
Y = Yellow



A B C D



E F G H

PLATE 4

Plate 5

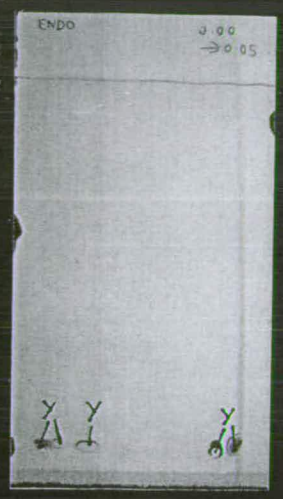
Silica Gel thin layer chromatograms of ethanolic endosperm extracts. These chromatograms have been developed with the two dimensional system involving development in ethyl acetate followed by development in water.

ABBREVIATIONS

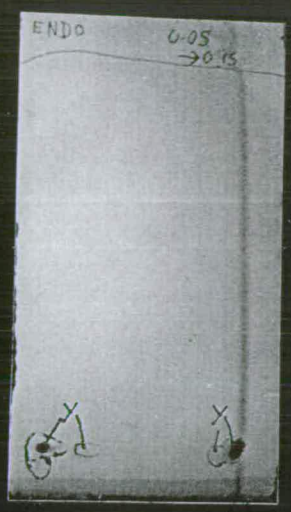
FRACTION	KIESELGUHR R_F
A	0·00 to 0·05
B	0·05 to 0·15
C	0·15 to 0·25
D	0·25 to 0·40
E	0·40 to 0·55
F	0·55 to 0·70
G	0·70 to 0·85
H	0·85 to 1·00

FLUORESCENCE

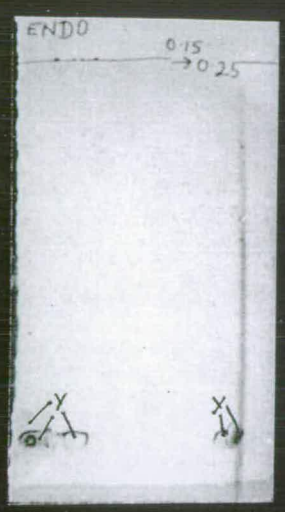
B = Blue
BG = Blue-Green
G = Green
Y = Yellow



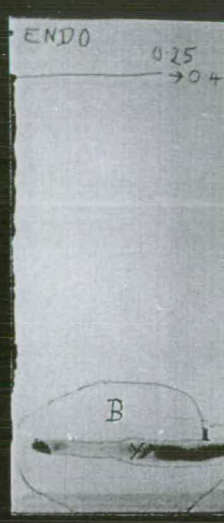
A



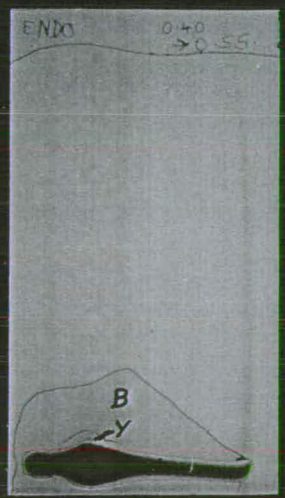
B



C



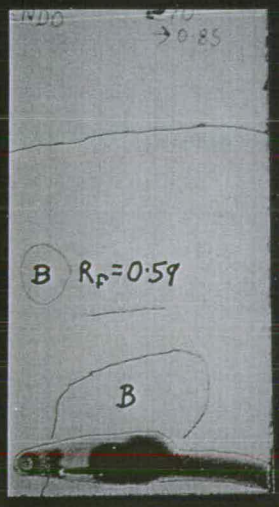
D



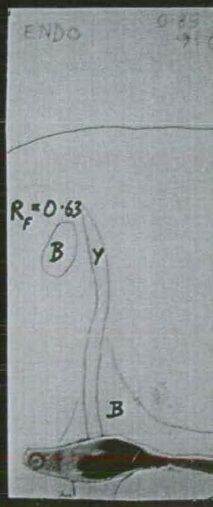
E



F



G



H

PLATE 5

Symbols Used in Plates 6-14

Electron Micrographs

DB	Dense Body.
I	Inclusion.
M	Mitochondrion.
ML	Middle Lamella.
N	Nucleus.
NE	Nuclear Envelope.
NL	Nucleolus.
Pd	Plasmadesmata.
Pl	Plasmalemma.
Pr	"Protein."
UB	Unidentified Body.
V	Vacuole (Or Transparent Vacuolar Inclusion).
VM	Vacuolar Membrane.
W	Cell Wall.

Plate 6

Aleurone cell (detail). Section from aleurone isolated from endosperm slices soaked in water for 18hours. Fixed in veronal buffered potassium permanganate, pH7.4, for two hours.
Magnification:15,000x.

Plate 7

Aleurone cell (detail). Section from aleurone isolated from endosperm slices soaked in water for 18hours. Fixed in veronal bufferer potassium permanganate, pH7.4, for two hours.
Magnification:11000x.

ABBREVIATIONS

DB = Dense Body
I = Inclusion
N = Nucleus
NE = Nuclear Envelope
Pr = "Protein"
UB = Unidentified Body
V = Vacuole
VM = Vacuolar Membrane

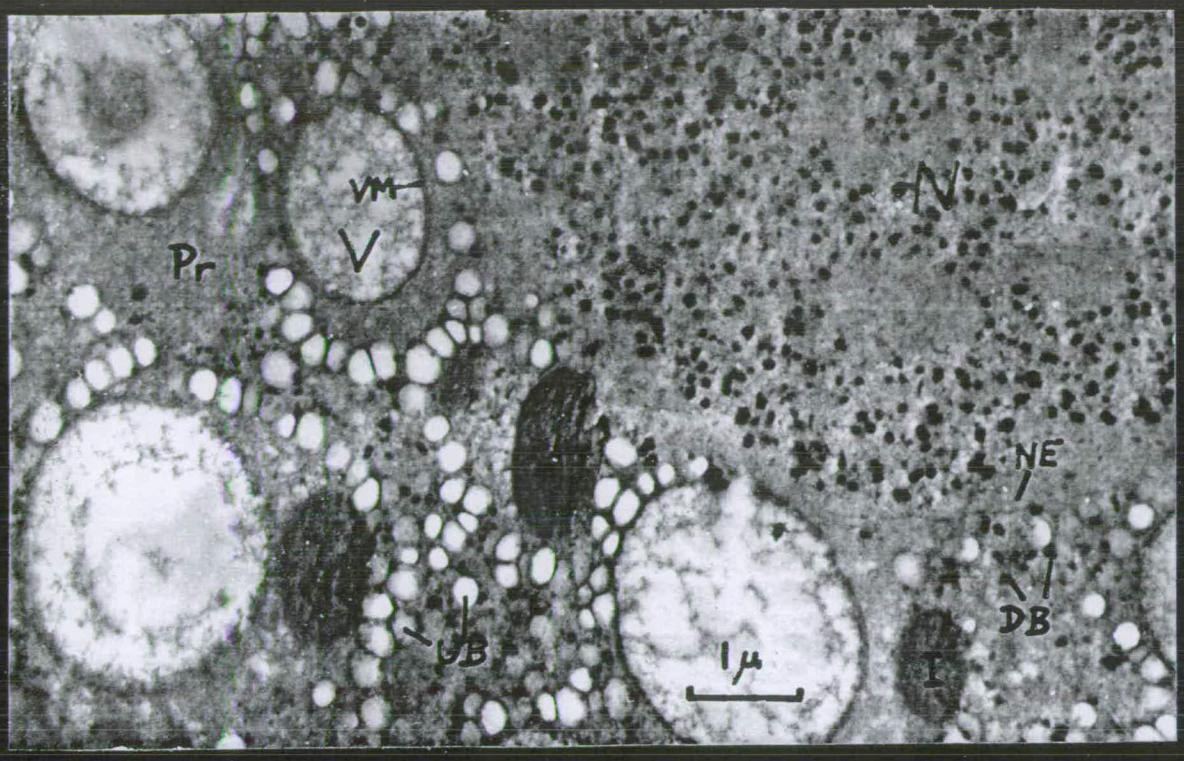


PLATE 6

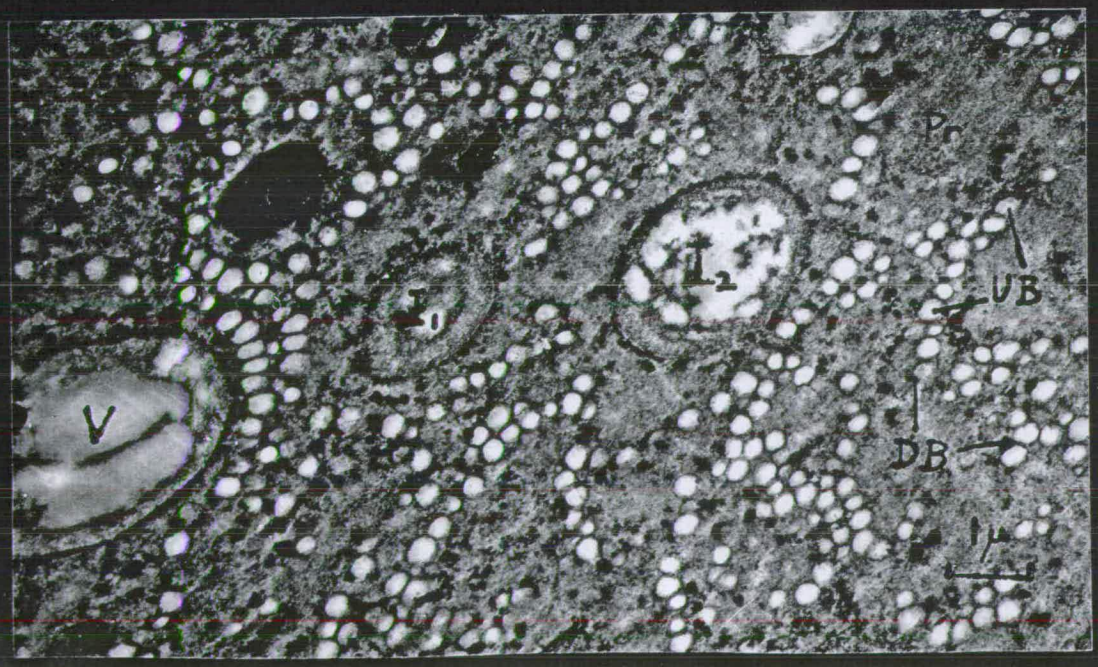


PLATE 7

Plate 8

Aleurone cell (detail). Section from aleurone isolated from endosperm slices soaked in water for 18hours. Fixed in veronal buffered potassium permanganate, pH7.4, for two hours. Magnification:13,000x.

Plate 9

Aleurone cell. Section from aleurone isolated from endosperm slices incubated in 10^{-5} M gibberellic acid for 30hours at 25 °C. Fixed in unbuffered potassium permanganate for thirty minutes. Magnification:4,800x.

ABBREVIATIONS

DB	=	Dense Body
I	=	Inclusion
N	=	Nucleus
Pl	=	Plasmalemma
Pr	=	"Protein"
UB	=	Unidentified Body
V	=	Vacuole
W	=	Wall of Cell

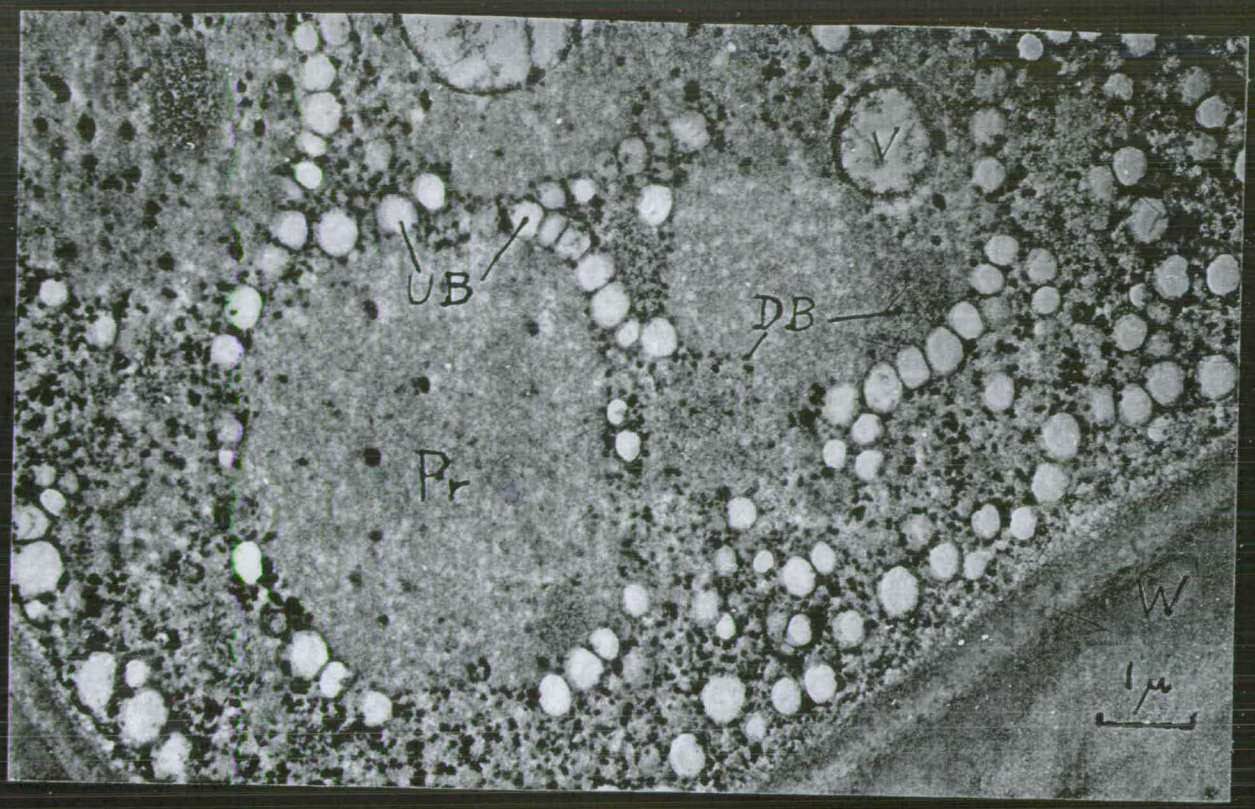


PLATE 8

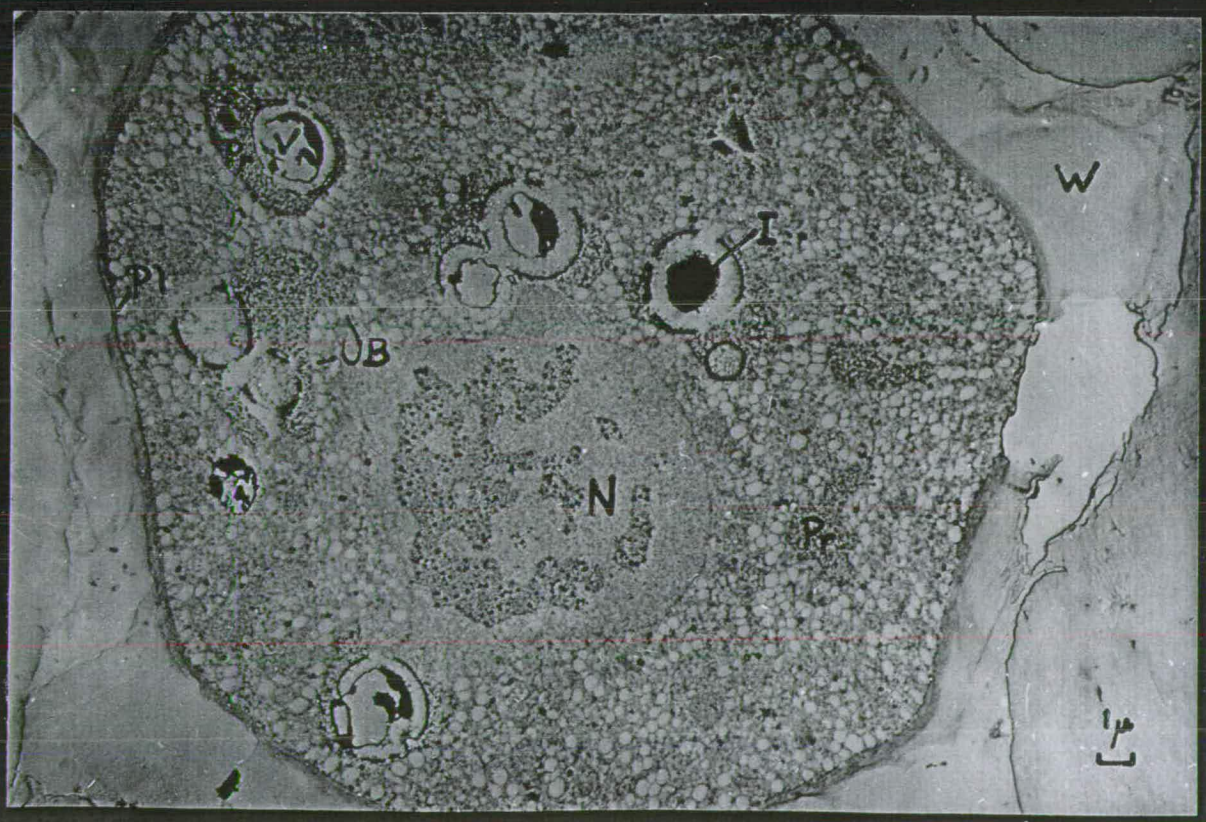


PLATE 9

Plate 10

Aleurone cell (detail). Section from aleurone isolated from endosperm slices incubated in 10^{-5}M gibberellic acid for 30 hours at 25°C. Fixed in unbuffered potassium permanganate for thirty minutes.
Magnification: 9,600x.

Plate 11

Aleurone cell (detail). Section from aleurone isolated from endosperm slices incubated in 10^{-5}M gibberellic acid for 30 hours at 25°C. Fixed in unbuffered potassium permanganate for thirty minutes.
Magnification: 7,500x.

ABBREVIATIONS

DB = Dense Body
M = Mitochondrion
N = Nucleus
NL = Nucleolus
Pr = "Protein"
UB = Unidentified Body
W = Cell Wall

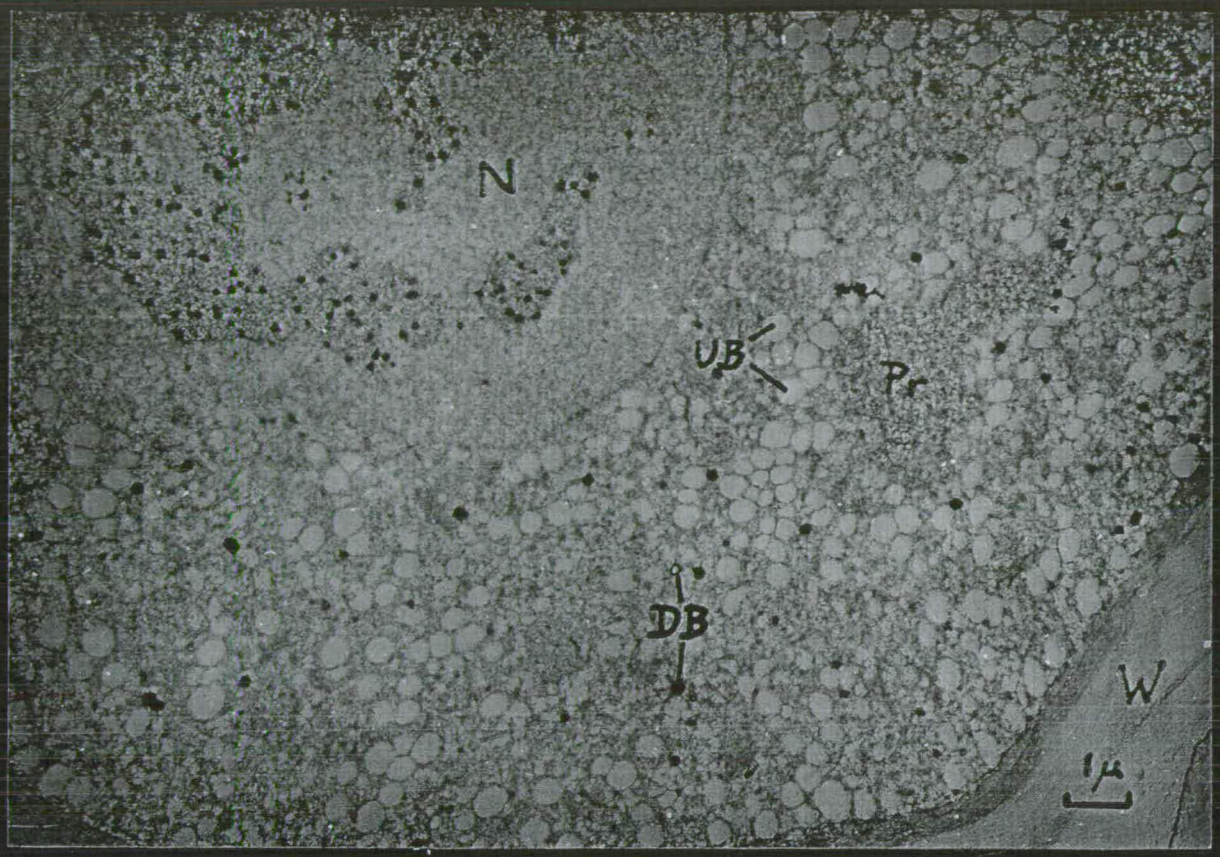


PLATE 10

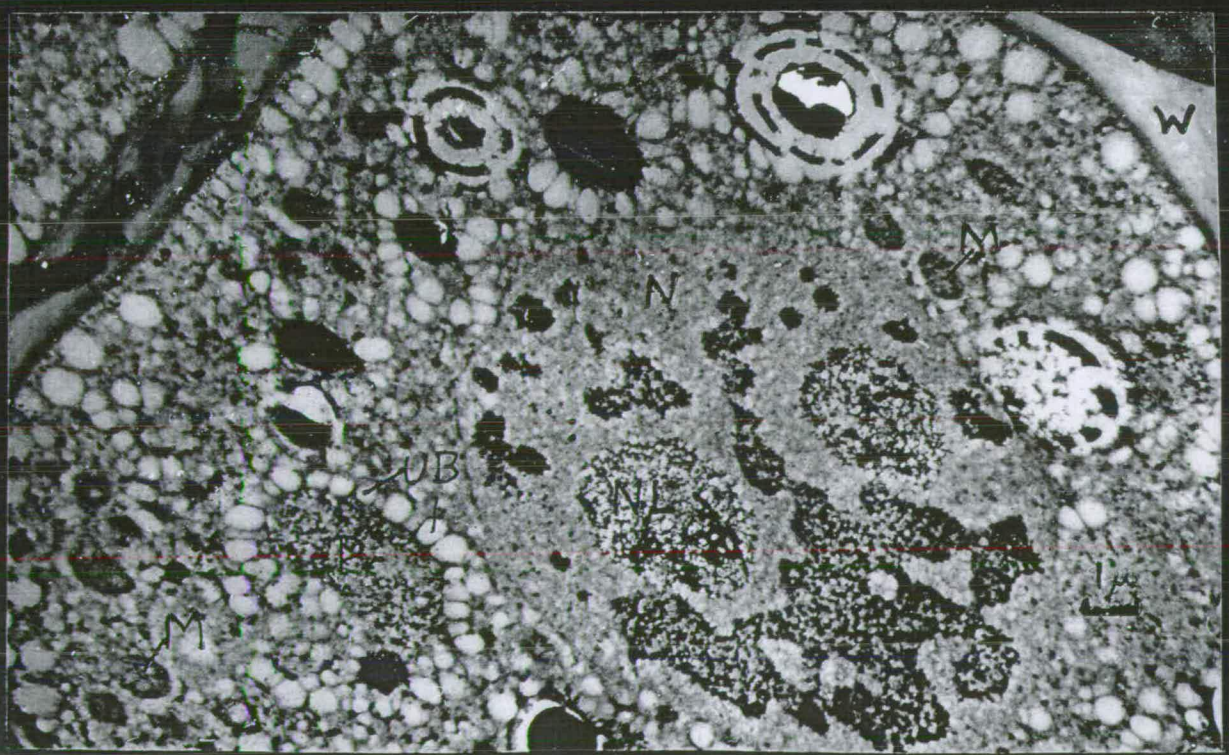


PLATE 11

Plate 12

Aleurone cell (detail). Section from aleurone isolated from endosperm slices incubated in 10^{-5}M gibberellic acid for 24 hours. Fixed in glutaraldehyde followed by staining with osmic acid.

Magnification: 10,000x.

ABBREVIATIONS

UB = Unidentified Body

W = Cell Wall



PLATE 12

Plate 13

Aleurone cell wall. Section from aleurone isolated from endosperm slices cut from barley germinated for three days. The section passes across the surface of one wall showing plasmadesmata in cross-section. Fixed in veronal buffered potassium permanganate pH7.4 for 1 hour.
Magnification : 8000x.

Plate 14

Aleurone cell wall. Section from aleurone isolated from endosperm slices cut from barley germinated for three days. The cell contents have been destroyed during fixation but the plasmadesmata are shown clearly in longitudinal section. Fixed in veronal buffered potassium permanganate, pH7.4, for 1 hour.
Magnification:4,900x.

ABBREVIATIONS

ML = Middle Lamella

Pd = Plasmadesmata

W = Cell Wall

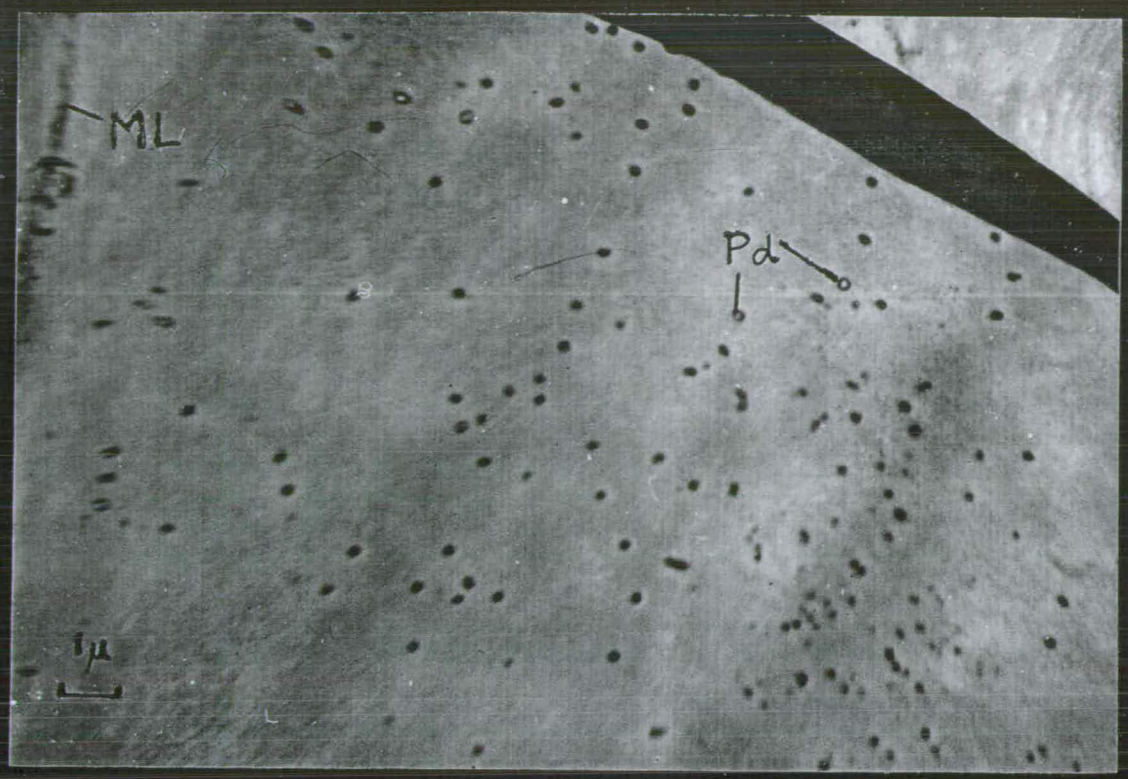


PLATE 13



PLATE 14

ABSTRACT OF THESIS

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Address C/O ANDERSON, 15 GREENHILL PL., EDINBURGH 10.

Degree DOCTOR OF PHILOSOPHY *Date* 20TH. MAY, 1965.

Title of Thesis GIBBERELLIC ACID AND THE GERMINATION OF BARLEY.

ABSTRACT OF THESIS

Other workers have shown that gibberellic acid causes an increase in the activity of hydrolytic enzymes in barley endosperm. They have also shown that gibberellic acid can be extracted from the intact barley grain. Evidence is presented here that in the mature grain gibberellic acid like activity is to be found only in embryo extracts. Further this activity appears to be associated with a substance which is indistinguishable from gibberellic acid on thin layer chromatography in two solvent systems but not in a third. It is suggested therefore that the substance may be a compound of gibberellic acid with some other moiety, possibly lipid.

Studies of the effects of various metabolic inhibitors on the response of barley endosperm to gibberellic acid have been made. The response was determined by measuring the increase in activity of α-amylase and endo-β-glucanase. Compounds reputed to uncouple oxidative phosphorylation and inhibit protein synthesis inhibit endosperm response. There is some suggestion that the penetration of two of these compounds, 2.4 - dinitrophenol and sodium salicylate, into the actively respiring cells is enhanced by high concentrations of gibberellic acid.

Interpretation of the results obtained with inhibitors required a determination of the time course of increase in activity of α-amylase and endo-β-glucanase in endosperm slices incubated in gibberellic acid. This determination was extended to include protease and acid phosphatase, enzymes of importance in the normal breakdown of starchy endosperm. The results obtained were compared

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with the results of similar studies on endosperm slices from germinating grain and on isolated aleurone incubated in gibberellic acid. These results demonstrated further the similarity between the endosperm modification occurring during germination and that resulting from gibberellic acid treatment. They were also in keeping with previously obtained evidence that gibberellic acid exerts its action on the aleurone layer, causing it to secrete hydrolases. The observation that the enzymes studied were produced sequentially seems to indicate that gibberellic acid exerts a specific effect on their synthesis. Secretion from the aleurone layer appears to require an endosperm factor in addition to gibberellic acid.

The ultrastructure of barley aleurone from endo-sperm from the mature grain and from gibberellic acid treated endosperm has been studied with the electron microscope. Several unusual organelles have been observed. A marked increase in the number of one of these following gibberellic acid treatment together with a consideration of its intracellular distribution has led to the postulate that it may be involved in the secretory process.

Evidence has also been obtained that during germination there occurs a transference from the embryo of a substance which sensitises the aleurone to gibberellic acid.

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