THE EFFECT OF GIBBERELLIC ACID ON FREE NUCLEOTIDE METABOLISM IN ALEURONE CELLS OF WHEAT,

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December, 1970.

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SUMMARY

The production of α-amylase by isolated wheat aleurone tissue was investigated and the results found to be similar to those published elsewhere for wheat, barley, and other cereals. After a lag period of about 6 hours, enzyme activity was stimulated in the presence of gibberellic acid (GA), and by 24 hours was more than 100-fold greater than that in control tissue. Production of α-amylase was linearly related to the logarithm of the concentration of GA applied, and could be almost completely inhibited by the application of Actinomycin D.

A method was developed for extracting free nucleotides from wheat aleurone layers. The nucleotides found in measurable quantities were NAD (50 nmoles/gm. dry weight), ADP (20), ATP (218), CTP (34), GTP (34), UTP (72), UDPG (204). Changes in the levels of these compounds were examined at intervals up to 24 hours in the presence and absence of GA. Whilst only small effects of GA were detected throughout the incubation period, major changes occurred during the first 6 hours which were almost completely independent of GA treatment. These changes were found to be closely connected with the method used to separate the aleurone layers from the starchy endosperm, and most probably represented a response to tissue damage. Further investigations of the effect of GA on free nucleotides were therefore carried out on tissue pre-incubated in water for

6 hours after isolation.

The uptake of ^{32}P by the tissue was found to be a relatively slow process, but incorporation of radioactivity into the nucleotides occurred rapidly. The specific activities of ATP and UTP were approximately equal and were the highest of all the nucleotides. The specific activities of GTP and CTP were 92% and 61% respectively of the values for ATP and UTP.

When GA was added, the mean incorporation of ^{32}P into nucleotides was depressed by 6% at 15 min., and then increased by 19% at 30 min., both effects being significant. The effect then declined. ATP, UTP, GTP, and CTP were found to show the greatest response. Moreover, while the effect on ATP, UTP and GTP could no longer be detected at 90 min. the specific activity of CTP was still 27% above the control at that time. There was, therefore, a unique response by CTP.

The maximum increase in the specific activities of the nucleoside triphosphates was about 25% and it was concluded that the magnitude of this response argues against a large scale increase in the metabolism of RNA as has been reported to occur in other tissues treated with hormones.

The uniqueness of the response of CTP to GA was shown to be consistent over a range of treatments and times of incubation, and it was reasoned that the most probable explanation to account for this result was an increase in

phospholipid synthesis. Membrane formation is known to be associated with an increase in the rate of protein synthesis, and it was postulated that, because phospholipids are an integral part of the structure of membranes, one of the first manifestations of the effect of GA is an increase in membrane formation. The new membranes then act as a participant in a system, already provided with sufficient m-RNA, which is capable of large scale protein synthesis.

DECLARATION

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

ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation to Professor L.G. Paleg for suggesting this line of research, and to both Professor Paleg and Dr. C.F. Jenner for guidance and helpful suggestions throughout this study and in the preparation of the manuscript. The author is also grateful to the Australian Wool Board for their financial support, and to the Department of Plant Physiology, Waite Agricultural Research Institute for supplying the materials and facilities necessary for this study.

LIST OF ABBREVIATIONS

GA	Gibberellic acid, GA_3
NAD	Nicotinamide-adenine dinucleotide
NADP	Nicotinamide-adenine dinucleotide phosphate
AMP	Adenosine-5'-monophosphate
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
CMP	Cytidine-5'-monophosphate
CDP	Cytidine-5'-diphosphate
CTP	Cytidine-5'-triphosphate
GMP	Guanosine-5'-monophosphate
GDP	Guanosine-5'-diphosphate
GTP	Guanosine-5'-triphosphate
UMP	Uridine-5'-monophosphate
UDP	Uridine-5'-diphosphate
UTP	Uridine-5'-triphosphate
ADPG	Adenosine-5'-diphosphate glucose
CDPG	Cytidine-5'-diphosphate glucose
GDPG	Guanosine-5'-diphosphate glucose
UDPG	Uridine-5'-diphosphate glucose
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
m-RNA	Messenger-ribonucleic acid
r-RNA	Ribosomal-ribonucleic acid
t-RNA	Transfer-ribonucleic acid
P_i	Inorganic orthophosphate
TCA	Trichloroacetic acid
R_F	Mobility relative to solvent front
R_{AMP}	Mobility relative to AMP

LITERATURE REVIEW

1. Introduction

The growth of abnormally tall rice seedlings in Japanese crops was associated by Sawada, in 1912, with the presence of a fungus on the affected plants (Phinney and West, 1960). By 1929, a cell-free culture filtrate of this fungus, Gibberella fujikuroi, was shown by Kurosawa to reproduce the symptoms of abnormal elongation (Bakanae disease) noted in the field. Yabuta, in 1935, named the active principle, gibberellin, and four years later Japanese chemists crystallised "Gibberellin A" (Brian, 1966). This is now known to have been a variable mixture containing GA_1, GA_2, GA_3, GA_4, GA_7, GA_9.

Very little further work was done for the next 10 or 12 years until interest was revived outside Japan, and since then an extensive amount of literature has been published.

2. Distribution of Gibberellins

Evidence has now been obtained for the presence of gibberellins in such widely diversified plant forms as barley seed, citrus shoots, ferns, fungi, brown and green algae (Brian, 1966), Zea mays (Paleg, 1965), 28 species of Podocarpaceae and 9 gymnosperms (Aplin, Cambie and Rutledge, 1963), leaves and immature seeds of grasses (Jones, Macmillan and Radley, 1963) and bamboo shoots (Tamura, Takahashi, Murofushi and Kato, 1966). The methods employed for identification include co-chromatography against known gibberellins, measurement of extinction at 270 nm (Abdel-Wahab and Badawi, 1966), gas-liquid chromatography

(G.L.C.) (Aplin, Cambie and Rutledge, 1963), nuclear magnetic resonance (Cross, 1966), and combined G.L.C. - mass spectrum analysis. (Binks, MacMillan and Pryce, 1969)

The structure of GA has been fully elucidated (Cross et al, 1961), and in all, 29 naturally occurring gibberellins characterised (Lang, 1970). By definition, all the gibberellins have a structure based on the gibbane skeleton differing only in the nature of the ring substituents and the presence or absence of the lactone ring, (Fig.1).

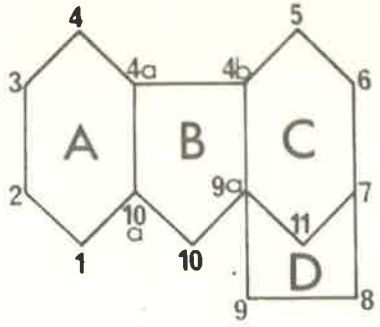
3. Physiological Effects of the Gibberellins

The ways in which gibberellins can modify the phenotypes of various plants have been reviewed by Brian (1959, 1964, 1966), Paleg (1965) and Linser (1966), the latter listing a number of effects as follows:-

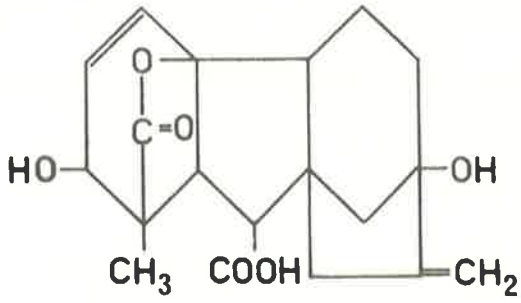
1. increase in internode length of rosette and dwarf plants.
2. promotion of hypocotyl growth.
3. promotion of germination, especially of those seeds requiring a pre-treatment with light.
4. promotion of sexual development of flowers.
5. promotion of flower formation.
6. formation of parthenocarpic fruit and promotion of fruit flesh growth.
7. breaks dormancy of buds and underground storage organs.
8. influences flower formation and the photoperiodic behaviour of flowering plants.
9. influences the shape and size of leaves.

Fig. 1

Schematic structures of gibbane skeleton and gibberellic acid.



Gibbane Skeleton



Gibberellic Acid

(GA_3)

10. promotes cell division in the cambial zone.

Although some stimulation of root growth has been reported (Paleg et al, 1964), mostly the hormone is either inhibitory or without much effect (Bastin, 1967; Brian, 1964; Brian, Hemming and Lowe, 1960).

Abscission in the cotyledonary petiole of cotton explants can be accelerated by GA, the mechanism advanced involving an increase in cell number and starch content in the abscission zone with subsequent hydrolysis and release of soluble sugars and uronic acids (Bornman, Addicott and Spurr, 1966).

An enhancement of cell size and division has been reported for leaf discs of broad bean (Humphries and Wheeler, 1960), while an increase in the amount of auxin-like substances extractable from the hypocotyls of Impatiens balsamina was shown to be under the influence of GA, although involving, to some extent, the effect of light. (Bastin, 1967).

Brian, Hemming and Lowe (1962) compared the effectiveness of a number of the gibberellins in various test systems. Their trials, and those of Paleg et al (1964), indicated that there are large differences in responses, and no one gibberellin exhibited maximal effect in all systems.

4. The Mode of Action of Gibberellic Acid

A number of investigations have been made to ascertain

the primary site of hormone action in various plant tissues treated with GA.

In endosperm milk extracted from green coconuts, GA enhanced the incorporation of ^{32}P into both nuclear and "cytoplasmic" (endosperm) RNA. (Roychoudhury, Datta and Sen, 1965; Roychoudhury and Sen, 1965). The effect of GA was evident to a slight degree in the nuclear fraction after 15 min., and to a much greater extent, in both fractions, after 4 hours. Radioactivity was not detected in the "cytoplasm" unless nuclei were present. When nuclei containing ^{32}P were incubated with nuclei-free endosperm, GA increased the amount of labelled RNA in the endosperm, this being accompanied by a progressive decrease in nuclear RNA.

In another system of isolated nuclei, from dwarf pea seedlings, Johri and Varner (1968) found that, under certain conditions, GA could stimulate the production of a different type of RNA to that extracted from controls. The unique RNA was detected only if all stages of the extraction of nuclei were carried out in the presence of GA. As the hormone was added progressively later, its effectiveness declined, the reason advanced being either the gradual loss of some factor(s) from the nucleus, or alternatively, a decreased availability of this factor(s) to the nucleus. It was found, also, that tritiated CTP was incorporated into isolated nuclei over a period of 6-9 min., and that this could be effectively

inhibited by Actinomycin D, DNase, and RNase, indicating that the incorporation utilised DNA as template. Nearest neighbour analysis of the RNA produced in the presence of GA showed a higher frequency of adenine-guanine pairs and a lower frequency of uracil-cytosine pairs. Of the total counts recovered, 75-80% were in a fraction corresponding to carrier-free ribosomal RNA, while the specific activity of the "RNA associated with DNA" and the fraction "tenaciously bound to methylated albumin keiselguhr" were 20-25% higher than controls, suggesting that a smaller type of RNA had been preferentially enhanced by GA.

Zolotov and Leshem (1968) observed the production of α-amylase in isolated barley aleurone layers treated with RNA extracted from barley embryos. The effect of the RNA extract was significantly greater than that of 25 p.p.m. GA. In view of this finding, and that of Briggs (1964) concerning α-amylase production by the embryo, it would appear that there may be a hormonal response in the embryo similar to that found in the aleurone tissue, although on a smaller scale.

Further evidence for a relationship between the application of GA and an alteration in nucleic acid metabolism has been shown by Gamburg et al (1966), Giles and Myers (1966), Nitsan and Lang (1966), and Kamisaka et al (1967), for a number of different tissues.

5. The Role of Gibberellic Acid in Cereal Germination

Perhaps the effect of GA that has received most

attention and is best, although still incompletely understood, is its participation in the germination of cereal seeds. In a monograph in 1887, von Sachs noted - "If the young embryo is removed from the seed of maize, barley or other plant, and the endosperm alone laid in moist warm earth, its starch is not dissolved and transformed into sugar". The production of diastase (amylase) during germination in seeds of barley and other grasses was known at this time, its potent action as a ferment (enzyme)had been noted, and a parallel drawn between the in vitro and in vivo effects of diastase on starchy endosperm.

Paleg (1960) noted that GA applied to embryo-less half seeds of barley stimulated the secretion of reducing sugars, especially maltose and glucose. Pretreatment of the half seeds by heating for an hour at 100°C abolished this stimulation. The intensity of the response could be altered by the type and strength of buffer used, and was sensitive to pH, having an optimum at about 5.5. The conditions thus resembled those necessary for enzyme action.

Further work, (Paleg, 1960), incorporating a differential inactivation of β -amylase indicated that α -amylase was being produced under the stimulation of GA and it was suggested that the production of this enzyme would normally be under the control of an embryonic gibberellin. Half seeds treated with GA for 3 days lost 50% of their initial dry weight, and of this loss, 80% could be traced to

the production of reducing sugars and protein nitrogen. Also, the magnitude of the response was proportional to the concentration of GA applied (Paleg, 1961).

Subsequently, other aspects of the response were investigated, for example in the malting industry, where GA had been used to increase the efficiency and evenness of barley germination on the malting floor (Briggs, 1963; Macey and Stowell, 1961).

McLeod and Millar (1962) found that fragments of barley endosperm separated from embryos could be stimulated to secrete proteinase, amylase, endo-β-glucanase, and pentosanase. Briggs (1964) reported that production of enzymes which could hydrolyse sugar phosphates, oligosaccharides, peptides and various proteins. Varner, Chandra and Chrispeels (1965) have also reported the GA -enhanced production of α-amylase, endo-β-glucanase and protease and, in addition, have described an increase in ribonuclease activity (Chrispeels and Varner, 1967). Pollard and Singh (1968) and Pollard (1968) have found evidence for the stimulation of esterase, α- and β-galactosidase, phosphomonoesterase, phosphodiesterase, α- and β- glucosidase, amylase, peroxidase, ATPase, GTPase and phytase. Evidence for a fructose-1, 6- diphosphatase has also been reported (Scala, Patrick and Macbeth, 1969).

By applying different concentrations of GA to intact barley seeds, Paleg et al (1962) showed that only those

concentrations greater than 2.9×10^{-8} M would produce a response and concluded that this probably represented the endogenous level. McLeod, Duffus and Horsfall (1966) similarly arrived at a figure of 4×10^{-8} M. Paleg, Coombe and Buttrose (1962) further showed that the "endosperm mobilising hormone" was located in the embryo and demonstrated the generality of the mechanism among cereal grains by examining the production of reducing sugars in barley, oats and wheat under the influence of exogenous GA.

Using embryos removed from barley seeds germinated for various lengths of time, Radley (1967) was able to demonstrate that if the embryos were subsequently cultured on agar, a substance could be extracted from the agar which would stimulate the release of reducing sugars from barley endosperm. This she identified as GA_1 and concluded that the scutellum was the major site of production of the hormone, at least for the first 48 hours. Further work (Radley, 1969) has supported this view. Yomo and Iinuma (1966) also showed the presence of a gibberellin-like substance in the embryo of barley, which was present in appreciable amounts after 3 days of culture, and which then rose markedly over the next two days.

When the medium from a culture of the halves of barley seeds containing embryos was ultra-filtered, freeze-dried and applied to endosperm preparations for six days, Briggs (1963) found a 5-fold increase in release of reduc-

ing sugars above preparations which received no such addition. However, the application of GA at 5 mg/litre resulted in a further 10-fold increase in response. When endosperm halves, lacking embryos, were placed on agar blocks containing GA, a dissolution of the starchy endosperm commenced at the regions adjacent to the aleurone layer and gradually extended inwards, in the manner observed in vivo. The dorsal endosperm liquified more rapidly than that around the ventral furrow, also as in the intact grain. Preparations of endosperm attached to the aleurone layer, from different parts of the grain, were incubated with solutions of GA to determine whether different parts of the grain varied in their capacity to release reducing sugars. Although the dorsal region was most responsive (45.1 mg reducing sugar/ml) and the ventral the least (33.6 mg/ml), the differences were too small to account for the variation observed in normal germinating grain. Therefore it was reasoned that the progressive release of enzyme along the endosperm was most likely due to a diffusion gradient of GA. Briggs (1964) cultured embryos and endosperms together, but separated by dialysis tubing, and determined the distribution of α-amylase. The results showed that approximately 7% of the total enzyme was located in the embryo, 6.5% originated in the embryo, and the remaining 86.5% was produced in the endosperm.

McLeod, Duffus, and Horsfall (1966) pretreated endosperm slices in water, and after subsequent treatment with

10^{-5} M GA, compared the production of ∝-amylase with intact barley seeds similarly pretreated. Pretreatment in water for 4 hours resulted in a doubling of enzyme activity. However, 24 hours pretreatment of intact seeds, followed by 24 hours in GA produced a 7-fold increase in activity indicating that the presence of the embryo had accentuated the response. The conclusion drawn from these results was that some additional factor, deriving from the embryo, may be contributing to the release of enzymes. Other work (Petridis, Verbeek and Massart, 1965) seems to substantiate this, but since isolated endosperm responds more rapidly to exogenous GA than intact seed, it may be that the rate of production of enzyme in the former reaches its maximum earlier and begins to decline before production in intact seeds has become maximal.

The situation, however, may be even more complicated for according to a report by Eastwood, Taverner and Laidman (1969) there is a suggestion that cytokinin is present in the starchy endosperm, and that this substance influences the production of ∝-amylase, acting during the period of imbibition preceding treatment with GA. Thus, bran particles, isolated after only four hours of imbibition, would not respond to GA unless pretreated with cytokinin.

Studies of the respiration of intact and dissected endosperm showed that the aleurone layer was metabolically active, whilst the starchy endosperm was almost inert (Paleg,

1963; Varner, 1964; Varner and Chandra, 1964). A comparison of the effect of GA on pearled barley, in which the aleurone layer was mechanically removed, with normal and hand-peeled seed showed the aleurone layer to be the site of hormone action and enzyme production (Paleg, 1963).

Examination of isolated barley aleurone cells with the electron microscope revealed that GA initiated conspicuous modifications in the ultra-structure of the cytoplasm, whereas tissue incubated in water remained relatively unchanged. The aleurone grains enlarged, fused and vacuolated, whilst globoids within the grains disappeared. There was extensive degradation of spherosomes and a progressive erosion of cell walls. These changes, occurring in the presence of an exogenous supply of GA were identical with those occurring during the normal germination of entire barley grain (Paleg and Hyde, 1964; van der Eb and Nieuwdorp, 1967; Jones, 1969). A report by Jones (1969a) indicates that the spatial arrangement of sub-cellular particles may be significant in enzyme production, since a redistribution of these particles, by ultra-centrifugation of isolated aleurone layers, renders the layers relatively insensitive to GA for a distinct time interval, after which they respond normally.

Radioactive α-amylase was produced in the presence of labelled amino-acids (Varner, 1964; Briggs, 1964) and digestion with trypsin suggested that the entire molecule had been newly synthesised and not merely released or activated (Varner, Chandra and Chrispeels, 1965). To ascertain whether all the

α-amylase was produced by de novo synthesis, induction of the enzyme was carried out in the presence and absence of deuterium. During the hydrolysis of reserve protein, the peptide linkages take up water, and the released amino-acids, if incorporated into newly synthesised protein in the presence of deuterium, would impart to that protein a higher density than would otherwise occur. By means of this technique, and taking precautions against either the attachment of an oxygen-rich carbohydrate component or an exchange of ^{18}O with ^{16}O in preformed protein, Filner and Varner (1967) concluded that all of the GA-induced α-amylase arises by de novo synthesis.

Jacobsen and Varner (1967) have reported that the GA induced synthesis of protease, which is also synthesised de novo in the aleurone, parallels α-amylase in time of release, dose-response to GA and susceptibility to inhibitors.

Some of the events occurring after the application of GA to isolated aleurone layers have been described by Pollard and Singh (1968) -

(a) within 2 to 4 hours there was an increased secretion of soluble carbohydrates.

(b) by 4 hours oxygen uptake was increased.

(c) between 4 and 5 hours adenosine triphosphatase production increased.

(d) from 5 to 6 hours there was an increase in guanosine triphosphatase.

(e) by 6 hours phytase secretion increased.

(f) after 7 hours α-amylase could be detected.

The increase in soluble carbohydrates in the medium was accompanied by a decrease in the levels in the tissue.

Thus, the events leading to the dissolution of the reserves of the starchy endosperm, at least in mature cereal grain, consist of the imbibition of water by the seed, the production of GA in the region of the embryo and scutellum, and the diffusion of the hormone through the endosperm to the metabolically active aleurone layer. At this site or hormone action, several enzymes are induced and secreted into the endosperm where hydrolysis occurs.

6. Mode of Action of Gibberellic Acid on Cereal Aleurone Tissue.

There seems little doubt that the increase in enzyme activity that occurs in the aleurone layer as a result of treatment with GA is due to protein synthesis and that, as such, it is an energy requiring process. In general, the effects of GA on respiration are not pronounced. Respiration of barley, timothy, rape and pea seeds was increased by applications of GA during germination (Nielsen and Bergqvist, 1958), and a slight rise in respiration by endosperm pieces (including aleurone) after GA treatment, has been reported (Briggs, 1964). Working with embryo-less barley half seeds, Pollard (1968) noted that GA caused an increase in both oxygen consumption and secretion of inorganic phosphate after 2 hours of treatment. The oxygen uptake became maximal after 6 hours, and thereafter remained steady, with uptake, in the presence of GA, 10 to 30%

higher than in its absence. Dinitrophenol increased oxygen uptake and inhibited the release of P_i to the same extent whether or not hormone was present. Respiration was inhibited by puromycin, Antimycin A, and cycloheximide, the latter also preventing the release of P_i. Other work (Pollard and Singh, 1968) has substantiated these results.

However, Varner and Chandra (1964) could find no increase in respiration before or during α-amylase production. Further, they noted that no qualitative change could be determined in the incorporation of ^{32}P into acid-soluble compounds with GA treatment, and also that aleurone tissue, in the absence of GA, could incorporate significant amounts of labelled amino acids into protein. From these results, they concluded that GA accelerates the synthesis of certain specific proteins in a system already supplied with precursors and an energy source.

Attempts have been made with different types of metabolic inhibitors to learn more about the mode of action of GA. Experiments with inhibitors of nucleic acid synthesis, such as Actinomycin D and nucleotide analogues, have given variable results. Paleg (1963) could find no effect of Actinomycin D, up to 500 µg/ml, on the GA-induced release of reducing sugar, whilst Varner and Chandra (1964) observed a complete inhibition of α-amylase production at 100 µg/ml, but only if the inhibitor was added at the same time as the hormone. There was no effect when the inhibitor was added 7 hours later. Pollard and Singh (1966), on the other hand, found

no effect unless Actinomycin D was introduced 8 hours after the beginning of hormone treatment.

Nucleotide analogues imbibited enzyme production only if present during the entire 3 day period of imbibition, during which the starchy endosperm softened enough to allow the removal of aleurone layers (Chandra and Varner, 1963). Labelled RNA precursors were incorporated quantitatively into RNA in response to GA treatment under similar conditions. However, the hormonal effect lasted only 24 hours, after which there was a loss of label, whilst the control treatments continued to incorporate precursors up to 48 hours, the limit of the experiment. Application of 5-fluorouracil inhibited the incorporation of ^{32}P into RNA by 50%, and the uptake of ^{14}C-uridine, ^{14}C-adenine and ^{32}P could be prevented by both Actinomycin D and 5-bromouracil, (Varner, Chandra and Chrispeels, 1965).

Cycloheximide inhibited both amino acid incorporation and α-amylase production without significantly affecting the incorporation of ^{14}C-leucine into protein (Varner, Chandra and Chrispeels, 1965). Also, the activity of proteolytic enzymes could be inhibited by the addition of potassium bromate to the incubation medium, whilst that of α-amylase remained unaffected (Macey and Stowell, 1961; McLeod and Millar, 1962). The participation of specific m-RNA molecules was therefore suggested.

Barley half seeds were treated, from the dry state,

in the presence and absence of GA for 30 hours (Chandra and Duynstee, 1968). The production of α-amylase was found to be greatest in the presence of 10^{-7}M GA. Various fractions of RNA, isolated 16 hours after treatment of dry half seeds with and without GA in the presence of ^{14}C-uridine, showed maximal labelling with 10^{-12}M GA. At higher concentrations of GA, the amount of label incorporated decreased and it was suggested that dilution of the radioactive precursor occurred due to an increase in the activity of ribonuclease. It was also shown that when half seeds were imbibed in buffer alone for 12 hours and then treated with GA at 10^{-6}M, the amount of ^{32}P incorporated into total RNA was stimulated. The maximum effect occurred 30 min. after treatment with GA, when the specific activity of RNA was 88% greater than the control. Thereafter incorporation in the presence of GA fell, until at 4 hours it was lower than the control. When the RNA was fractionated on sucrose density gradients, it was revealed that after treatment with GA for 30 min. both soluble and ribosomal RNA were labelled, but no incorporation of ^{32}P into ribosomal RNA was detected in the control. By 90 min. all fractions of RNA were labelled in the presence and absence of GA, and after 4 hours, the controls were more highly radioactive.

The RNA from barley aleurone was further characterised by chromatography on methylated albumin keiselguhr columns. Half seeds which had been imbibed for 12 hours and

then treated with GA for 4 hours showed the presence of an attitional species of r-RNA. This new RNA was not detected in the controls and could be almost completely inhibited by Actinomycin D, (Chandra and Duynstee, 1968). Analysis of the base ratios of the various fractions of RNA which were examined revealed that GA had very little effect. Only in the case of the soluble RNA component was any change observed, and this had a higher AG/CU ratio after GA treatment.

These results have not been supported by Pollard and Singh (1968). These workers could show no incorporation of either labelled adenine or uracil into the RNA of barley aleurone layers in response to GA, and only a small incorporation of labelled leucine into protein during the first 8 hours of hormonal treatment. Their conclusion was that GA had no stimulatory effect on RNA synthesis and only slightly affected protein synthesis during this time.

These contradictory results raise the question of the permeability of the tissues to the labelled precursors at the time of treatment.

7. <u>The Metabolic Roles of the Free Nucleotides.</u>

The previous discussion on the effects of GA on nucleic acid metabolism presupposes the involvement of free nucleotides as precursors of the nucleic acids. If, as has been suggested earlier, GA causes an increase in, or change in spectrum of, RNA, the assumption is always made that the free nucleotides are in plentiful supply and in no way limit

or regulate the expression of the hormonal response. In fact, the rapid interconversions which are known to occur between nucleotide species tends to indicate that the nucleotide balance in the living cell is well buffered against drastic changes. However, as suggested by Commoner (1964 a, b), the availability of free nucleotides may well be an important factor in determining the rate of synthesis of nucleic acids. Little evidence is available so far to support this hypothesis.

In addition to acting as precursors for nucleic acid metabolism, free nucleotides also seem to play indispensable roles in the regulation of other cellular metabolic processes. For example, many biochemical reactions require the participation of these compounds as coenzymes. As a general classification, the adenosine, uridine, and cytidine nucleotides are involved as group transfer agents in reactions with substrates at the acyl, aldehyde, and alcohol levels of oxidation, respectively (Mahler and Cordes, 1967).

Many cellular processes are endergonic and require a supply of energy to proceed. In most cases this energy is provided by the high energy bonds of ATP, which are derived primarily by electron transfer to molecular oxygen, or other terminal electron acceptor, by way of the NAD-linked cytochrome system (Bonner, 1965). Each of the 2 terminal phosphate ester bonds of ATP have a free energy of hydrolysis of about -7 kcal/mole. Thus ATP may be involved in the transfer of orthophosphate, pyrophosphate or the complete adenosyl

moiety. In most tissues, the level of ATP is very much greater than that of AMP and therefore a small amount of degradation of ATP to AMP results in a relatively large change in the concentration of AMP. It has been suggested that the relative proportion of AMP is important in determining the rate at which many biochemical reactions proceed (Krebs, 1964; Atkinson, 1966). In addition, ATP participates in the metabolism of sulphur by the formation of adenosine phosphatosulphate (Bandurski, 1965), and in protein synthesis in which amino acids are first "activated" prior to forming a complex with transfer RNA, (Holley, 1965).

A nucleotide which has been receiving increasing attention is cyclic AMP, or adenosine - 3' , 5' - monophosphate. It was first noted that the hormones epinephrine and glucagon act in liver to stimulate the formation of cyclic AMP. In turn, the increased level of cyclic AMP activated a phosphorylase to degrade glycogen. Many other cellular processes are now known to be influenced by either an increase in the concentration of cyclic AMP, brought about by the enzyme adenyl cyclase, or a decrease due to its hydrolysis to 5'- AMP by a specific phosphodiesterase (Robison, Butcher and Sutherland, 1968). It has been reported that α-amylase production by barley aleurone is stimulated albeit erratically, by the presence of cyclic AMP (Galsky and Lippincott, 1969). The addition of GA, up to $2X10^{-9}$ M, increased the stimulation, but at higher concentrations

production was inhibited.

The uridine nucleotides are of major importance as nucleotide-sugar complexes. This was first observed by Paladini and Leloir (1952), and a wide range of UDP-sugars have now been isolated from living tissues (Hassid, Neufeld and Feingold, 1959; Mandel, 1964). These coenzymes are involved in a variety of glycosyl transfer reactions and epimerisations in carbohydrate metabolism such as the synthesis of glycogen in animals, and cell wall polysaccharides in plants. However, other nucleotides are also known to function in polysaccharide synthesis e.g. ADP-glucose in starch synthesis (Jenner, 1968), and GDP-mannose in cell wall synthesis (Villemez, 1970).

The major role of the guanine nucleotides occurs in protein synthesis. One of the first steps in this process is the activation of amino acids to the amino-acyl level requiring the high energy bonds of ATP. The amino-acid so activated is then transferred to the ribosome by its specific t-RNA, where it is incorporated into the nascent peptide chain. This process requires the energy from 2 molecules of GTP and is catalysed by a number of protein factors. The initiation of the peptide chain by formyl-methionyl t-RNA also requires the cleavage of GTP before binding to the ribosome (Ono, Skoultchi, Waterson and Lengyel, 1969; Prichard et al, 1970; Brown and Smith, 1970.

The function of cytidine nucleotides and derivatives

was recognised by Kennedy and Weiss (1956) as being primarily concerned with lipid synthesis. Intermediates such as CDP-choline and CDP-ethanolamine participate in the production of lecithin and other phospholipids (Mandel, 1964). Since phospholipids are an integral component of cellular membranes, it would seem highly probably that CTP is necessary for the synthesis of these structures.

8. The Role of Free Nucleotides in Germination.

Little work has been done on relating changes in the free nucleotide pool to the process of germination, and most of the results available are derived from long-term studies extending over several days after the commencement of imbibition.

Ingle, Beevers and Hageman (1964) and Ingle and Hageman (1965) followed changes in the amounts of nucleic acids and soluble nucleotides in germinating corn. After 24 hours, DNA, RNA and soluble nucleotides were increased by de novo synthesis since they found only small amounts of these metabolites stored in the dry grain. After 48 hours of germination, the level of acid-soluble nucleotides in axis, scutellum and endosperm was approximately the same, whereas after 120 hours the axis accounted for 71% of the total.

Bergkvist (1956) measured the amounts of acid-soluble nucleotides in wheat plants of unstated age and concluded that 90% of the total were derivatives of adenosine and uridine, and that these were present in practically equimolar amounts. No attempt was made to follow changes, with time, in individual

components.

The metabolism of mucleotides in corn germinated under conditions which would induce water stress has been studied by West (1962). Their findings were similar to those of Ingle and Hageman (1965), showing an exponential rise with time up to 6 days. Accumulation was slower in seedlings subjected to water stress.

Keys (1963) extracted the acid-soluble nucleotides from wheat seedlings over 5 days after imbibition of the seeds. The content of nucleotides in the embryo was 20-30 times that of the endosperm, on a dry weight basis, after 2 days imbibition, whilst over the first 2 to 3 days of development there was an increase of 30-fold in the embryo and only 2- to 3-fold in the endosperm. UDPG was a major component in the embryo.

Elnaghy and Nordin (1966) also found UDPG to be a major component of wheat seedlings, and in addition identified AMP, ADP, GMP, and UMP.

The changes occurring in nucleotides derived from nicotinic acid in wheat seeds during germination have been investigated by Mukherji, Dey and Sircar (1968). They identified the oxidised and reduced forms of both NAD and NADP and followed their levels over a 5 days period. NAD reached a maximum after 24 hours, then declined progressively whilst NADH accumulated. NADP rose rapidly for 48 hours and

then more slowly up to 72 hours. After 72 hours, the level fell, and it was suggested that the decline in NADP was related to the increase in NADH, since NADP is dependent for its production and maintenance on a relatively high NAD to NADH ratio.

Changes in nucleic acids during germination provide indirect evidence for the involvement of free nucleotides. For example, Vold and Sypherd (1968, 1968a) extracted total amino-acyl transfer RNA from Wheat seedlings and determined that quantitative changes occurred, within the different species of t-RNA, during germination. Further evidence involving nucleic acid metabolism in germination has been presented by Cherry (1963), Chroboczek and Cherry (1965), and Olsson and Boulter (1968).

Thus it appears that part, at least, of the full RNA complement is produced during germination, probably by de novo synthesis from stored precursors.

Evidence for the existence, in cotton seeds, of an inactive form of m-RNA has been presented by Waters and Dure (1966), and by Chen, Sarid and Katchalski (1968) for germinating wheat embryos. The latter workers found no new m-RNA synthesised during the first 24 hours of germination, although there was marked protein synthesis, and concluded that the pre-formed m-RNA is activated upon imbibition and then supports early protein synthesis.

One of the earliest effects of imbibition is that

reported by Spedding and Wilson (1968). Labelled γ-amino-butyric acid and aspartic acid could be detected 5 min. after soaking Sinapis alba seeds in tritiated water.

9. Effects of Hormones on Nucleotide Metabolism.

The increase in respiratory activity in etiolated pea seedlings, resulting from treatment with indoleacetic acid, led Marre and Forti (1958) to investigate changes in ATP levels. The amount of ATP in their material, was estimated by extracting the adenine nucleotides and measuring the labile phosphorus liberated on hydrolysis. Treatment with indoleacetic acid significantly increased the fraction termed "high energy phosphate" during the first 30 min., but thereafter the fraction declined to almost control levels.

The effect of the growth substance 2,4-dichlorophenoxy-acetic acid (2,4-D), and of GA, on phosphorus metabolism of Trifolium spp. has been studied by Ormrod and Williams (1960). An increase in acid-soluble organic phosphorus and a decrease in inorganic phosphorus was detected as early as 1 min. after application of 50 µg of either substance was made to petioles and stems. After 1 or 2 days, levels returned to normal.

Using soybean seeds and hypocotyls, Key and Hanson (1961) showed that within 3 hours of treatment with 2,4-D, ATP increased by about 45% without any corresponding decrease in ADP or AMP, and again the effect was ephermeral. Other nucleotides isolated showed small increases after treatment.

A decrease in ATP concentration, expressed as a depression in the ATP to ADP ratio, has been shown after treatment of oat coleoptile sections with indoleacetic acid, naphthylacetic acid and 2,4-D. This again was a transitory effect, the depression reaching its maximum after 10 min. and returning to control levels after 4-6 hours (Trewavas, Johnston and Crook, 1967). UDPG showed increased labelling with ^{32}P in the presence of indoleacetic acid, whereas this was depressed with naphthylacetic acid.

Endo and Yosizawa (1968) examined the effect of the animal hormones oestrogen and progesterone, on the levels of sugar nucleotides in rabbit uteri. Ovariectomised rabbits were pretreated with hormone, their uteri removed, and incubated with radioactive glucose. The levels of sugar nucleotides were found to rise 1.4 to 1.9 times that of controls, whilst turnover rate was enhanced 5- to 7-fold. The authors suggest that the enzymes involved are strongly activated by hormone treatment.

Bone cells, isolated from the 20-day old rat foetus, incorporated more labelled uridine in the presence of insulin than in its absence. (Peck and Messinger, 1970). The effect was maximal within 60 min. and thereafter declined to control values by 4 hours. Radioactivity was detected in both the acid-soluble extract and RNA, and the time-course for the response was identical for each. Fresh insulin, administered again after 4 hours, induced another stimulation

and it was concluded that the disappearance of the effect of the first dose was due to an inactivation of the hormone.

No published reports have been found which describe an effect of GA on free nucleotides.

10. Summary.

Considerable evidence is available that hormones influence both protein and nucleic acid metabolism, although the mechanism by which they do so is obscure. It is clear that an investigation of the hormonal response in any tissue is greatly aided if the tissue is a homogeneous one. Thus, the response of the cereal aleurone layer to the presence of GA makes it an ideal system for a study of this kind. Phillips (1968) has developed a method for the isolation of viable wheat aleurone tissue in amounts up to 20 gm. at one time and has described in detail the GA induced production of α-amylase by this material.

Much of the work carried out on the metabolism of nucleic acids in cereal aleurone shows some contradiction. Reports suggesting the presence of m-RNA in an inactive form in allied tissues prior to hormone action, raise the question of whether changes in RNA metabolism which are observed to occur after the application of GA to aleurone tissue, are a reflection of changes in nucleotide precursor level. Furthermore, the fact that in one instance Actinomycin D was shown to be completely without effect on the GA-induced production of α-amylase raises serious doubts about the participation

of RNA metabolism in the GA response.

It was desired to investigate more fully the response of cereal aleurone to GA to determine if a reproducible, as well as biologically important, effect of the hormone could be detected before a stimulation in the activity of α-amylase occurred. The latter condition was considered necessary because it was clear that α-amylase was synthesised de novo in the presence of GA and as such represented the culmination of a number of biochemical events. It was hoped that the question of the participation of RNA metabolism could be elucidated during the investigation, and that other facets of the response might be revealed.

As free nucleotides participate as co-factors in many biochemical reactions, and in addition, act as precursors for the synthesis of nucleic acids, it appeared probable that a study of these compounds would clarify some aspects of the mode of action of GA. Accordingly, an investigation was carried out to examine the effects of GA on the metabolism of the free nucleotides of wheat aleurone tissue.

MATERIALS AND METHODS

1. Plant Material

Wheat of the variety Olympic, kindly supplied by the Victorian Department of Agriculture, was used throughout. The grain was stored over a saturated solution of Ca Cl_2.2 H_2O which was reported by Merry and Goddard (1941) to maintain a relative humidity of 32% in the gas phase. Grain stored in this manner had a moisture content of 10.3%. Seeds were bisected transversely and the embryo halves discarded.

2. Chemicals

The following solvents were re-distilled before use: triethylamine, iso-butyric acid, ethanol, diethyl ether (over $FeSO_4$ and CaO) and n-propanol (over KOH). Water was distilled twice and de-ionised.

Nucleotides were purchased from P-L Biochemicals Inc., Milwaukee, Wisc., U.S.A.; Actinomycin D from Mann Research Laboratories, New York, N.Y.; Gibberellic acid from Merck and Co. Inc., New Jersey, U.S.A. (97.4% GA_3); ^{32}P from the Australian Atomic Energy Commission, Sydney, Australia.

All other chemicals and solvents used were of reagent grade.

Gibberellic acid was sterilised before use by filtering through equipment supplied by the Millipore Filter Corporation, Bedford, Massachusetts, U.S.A. (filter porosity 0.22 μ).

In experiments with ^{32}P, glassware was treated with Siliclad, a silicone preparation manufactured by Clay-Adams Inc., New York, N.Y.

3. Preparation of Tissue

All equipment and water was sterilised before use by autoclaving at 250°C and 15 lb pressure for 20 min. A sterilising solution for the half-seeds was prepared by suspending 5 gm calcium hypochlorite in 100 ml water, the suspension shaken for 10 min. and then filtered (Wilson, 1915). The half-seeds were soaked in the filtrate for two hours, rinsed ten times in water and then transferred to a 14 cm petri dish containing 4 sheets of Whatman No. 1 filter paper beneath a disc of fibre-glass gauze. Sufficient water was added to the petri dish to cover the filter paper, so that aeration of the half-seeds was not impeded; the petri dish and contents were then left for 24 hours in an incubator at 30°C to allow the endosperm to take up water and soften.

The technique to separate the aleurone layer (including testa and pericarp) from starchy endosperm was developed by Phillips (1968). Prior to this, aleurone layers were hand-peeled, making the preparation of relatively large amounts of tissue a very tedious process. With the method to be described, up to 16 gm fresh weight of aleurone tissue can be isolated from the starchy endosperm in about 60 min.

After imbibition for 24 hours, the half-seeds were

aseptically transferred to a glass jar (12 cm diam. and 24 cm long) in a small cabinet, previously sterilised by vaporising propylene glycol. A polyethylene bottle (sterilised with 70% ethanol) 17 cm long and 7 cm diam., filled with sand to a total weight of 1200 gm, was then placed in the jar, which, after capping, was laid on two rollers driven by a small electric motor. 30 ml of water was injected through a hole in the cap and the jar rotated at 50 r.p.m. for 30 min. with an additional 10 ml aliquot of water injected every 5 min.

After this time, the contents of the jar were transferred to a sieve supported in a Buchner funnel and rinsed clean of adhering starch. The tissue was returned to the jar, rolled for a further 7-8 min., then the washing repeated. The tissue was next sandwiched between two layers of 12 sheets of Whatman No. 1 paper and one of fibre-glass gauze in a 9 cm petri dish and, after excess moisture had been taken up, weighed out for use.

4. Extraction of Nucleotides

The technique eventually adopted was a modification of the methods used by Cole and Ross (1966), Isherwood and Barrett (1967), and Jenner (1968). This involves acid extraction in the cold, which appears to be essential to inactivate phosphatases, followed by ion-exchange and paper chromatography. Ethanol, although easier to remove than acid, does not inactivate phosphatases over the temperature range of 4°C to boiling (Bieleski, 1964; Wilson and Harris,

1966). Thin layer chromatography, as described by Randerath and Randerath (1964) for the separation of nucleotides on polyethylene-imine cellulose, could not be effectively used with extracts of aleurone tissue. Reference to the use of charcoal as an adsorbent for nucleotides can be found (Brown, 1962; Weinstein et al, 1969), but experimentation with this technique showed yields to be variable, the recovery depending not only on the extracting medium used, but also on the base moiety of the nucleotide.

The method used was as follows:

After treatment, the tissue was taken to a cold room and all operations, unless otherwise stated, were carried out at 4°C. In early experiments, disintegration was achieved by grinding in a Kontes all-glass power-driven homogeniser with 5% (w/v) trichloroacetic acid containing 0.15% (w/v) 8-hydroxyquinolene. Subsequently, the use of an Ultra-Turrax high-speed blender increased efficiency and shortened the time taken for disintegration from 3-4 min. to 30 sec. with no discernable alteration in the amounts of nucleotides extracted. Where the power-driven all-glass homogeniser was used, the ground tissue was transferred, with rinsings, to an 80 ml centrifuge tube whilst with the Ultra-Turrax, the tissue was disintegrated directly in the centrifuge tube.

Each extract was left to stand for 15 min. and an equal volume of a solution of chloroform and iso-amyl alcohol (24:10 v/v) then added. After shaking vigorously, the

mixture was centrifuged at 4000 r.p.m. for 5 min. (Sevag, Lackman and Smolens, 1938). The mixture became biphasic, with the cell debris at the interface. The upper aqueous layer was transferred to twice its volume of diethyl ether, shaken, allowed to settle and the aqueous phase again extracted with twice its volume of ether. The cell debris was washed three times with small portions of water, the washings extracted with ether and finally added to the bulk of the extract.

A method (Warner and Finamore, 1967) for the removal of trichloroacetic acid with trioctylamine in chloroform was examined, but poor recoveries of nucleotides were obtained.

4(a) Preparative Column Chromatography

To remove much of the non-nucleotide material, the acid extract was passed through columns of cation- and anion-exchange media. Initially, the cation exchanger used was Dowex AG 50 W-X8, 200-400 mesh in the hydrogen form, but as about 50% of the total NAD was irreversibly adsorbed on the resin, a powdered cellulose (Whatman P11) was later used instead. The ~~cation~~ anion-exchange medium was DEAE cellulose (Whatman DE11).

The cellulose exchange materials were prepared for use as recommended in Whatman Technical Bulletin C5. Coarse and fine particles were first removed, then the exchanger washed in acid and alkali and finally water until the effluent was neutral. Whatman P11 was stored and used in the hydrogen form, while Whatman DE11 was stored in the hydroxyl form and

converted to bicarbonate just before use by washing with 15% $KHCO_3$ and then water until neutral.

The aqueous layer, after ether extraction, was passed down a column of cation-exchange material (0.6 meq.) and washed through with 12-15 bed volumes of water. The eluate was adjusted to pH 4.6 (indicator paper) with 2M ammonium hydroxide, extracted with an equal volume of diethyl ether and passed down a column of Whatman DE11 (2.5 meq). The ether was washed with a small volume of water, which was also passed through the column, then the column was washed with about 10 bed volumes of water.

Finally, the nucleotides were eluted off with 6 bed volumes of 0.5M triethylammonium bicarbonate at pH 7.4 (Porath, 1955).

4(b) Preparative Paper Chromatography

The effluent from the DEAE cellulose columns was evaporated to dryness in vacuo at a temperature not exceeding 30°C, transferred in a small volume of 20% ethanol to a 25 ml flask, again dried, and applied as a thin band 20 cm long onto acid-washed Whatman 3MM paper. At first, the procedure described by Bieleski and Young (1963) was used for washing the paper. However, 0.1M oxalic acid appeared to permit better and more consistent resolution than 0.01M EDTA, pH 8, in the subsequent chromatography.

Many different solvents were tested for their suitability at this stage of the procedure, and the one

which allowed further chromatography to give successful separations was adapted from that used by Isherwood and Barrett (1967). This was n-propanol : ammonia : water : 0.2M EDTA :: 72.5 : 0.1 : 27.3 : 0.1 (v/v/v/v). However, in this form the solvent reduced the yield of UDPG by 27%. When adjusted to pH 7.0 with acetic acid, it was just as effective as in the original form but without deleterious effect on UDPG.

The extract was developed in this solvent for 16 hours, the U-V absorbing bands cut from the paper and soaked in absolute ethanol for 2-3 min. to remove excess ammonium acetate. The paper strips were then eluted with water, and the uluate taken to dryness.

4(c) Two-Dimensional Paper Chromatography

The combination of paper chromatography and electrophoresis employed by Jenner (1968) did not successfully separate the nucleotides, largely because residual non-nucleotide material interfered with the electrophoresis.

The combination found most effective was iso-butyric acid : ammonia : water (57 : 4 : 39 v/v/v) followed by 95% ethanol : 1M ammonium acetate pH 7.5, 0.01M with respect to EDTA (70:30 v/v). The first solvent was developed by Pabst Laboratories (P-L Biochemicals Circular OR-10); the second is a modification by Pabst Laboratories of a solvent developed by Paladini and Leloir (1952)

The eluate from the preparative chromatogram was spotted onto Whatman No. 1 paper as a band 6 cm long and

developed for 24 hours in the iso-butyric acid solvent. Ammonium isobutyrate was removed either by handing the papers over a steam-bath for about 2 hours (this had no effect on nucleotide recovery), or by exposing them to a strong flow of air for 24 hours or longer.

The resolved bands of nucleotides were compressed by running water along the paper by capillary action from both ends in a direction at right angles to the flow of the previous solvent (Wade and Morgan, 1955). After drying, the paper was developed in the ethanol-ammonium acetate system for about 20 hours.

Nucleotides were located by ultra-violet light (Mineralight Ultra-Violet Products, Inc., California. 254 mμ), cut from the paper and eluted in 0.01 N HCl for a minimum of 4 hours. Three blank areas of about the same size as the nucleotides were removed from each chromatogram and similarly eluted. Optical densities were measured, in 1 cm quartz cells, at 260 and 296 mμ for the adenine and uracil series, and at 260 and 320 mμ for the guanine and cytosine series.

Correction for the absorbance of the blanks was made as follows:

$$E_{260}\ (\text{corr.}) = E_{260}\ (\text{sample}) - \left[E_{296}\ \text{sample} \times \frac{E_{260}(\text{blank})}{E_{296}(\text{blank})} \right]$$

where,

E_{260} (corr.) = corrected absorbance of sample at 260 mμ.

E_{260} (sample)= recorded absorbance of sample at 260 mμ.

E_{296} (sample)= recorded absorbance of sample at 296 mμ.

E_{260} (blank) = recorded absorbance of blank at 260 mμ.

E_{296} (blank) = recorded absorbance of blank at 296 mμ.

(For the guanine and cytosine series substitute 320 for 296).

The amount of each nucleotide was estimated, by means of its extinction coefficient, from the corrected absorbance at 260 mμ. The extinction coefficients used were ($x10^{-3}$) -

ATP	14.3	UTP	9.9
ADP	14.5	UDP	9.9
CTP	6.0	UDPG	9.9
GTP	11.8	NAD	17.8

(Bock, Nan-Sing, Morell and Lipton, 1956).

5. Determination of α-amylase

The method used to assay α-amylase production is a slightly modified version of the technique reported by Filner and Varner (1967). It is based on the enzymic degradation of starch, the course of the reaction being followed by measuring the decreasing intensity of the colour of the blue starch-iodine complex.

Starch was prepared by boiling, for 1 min., a 0.15% suspension of native potato starch in 100 ml of 0.04M

KH_2PO_4 in 1 mM calcium acetate. After cooling, the denatured amylopectin was centrifuged off, and the supernatent of crude amylose used for the assay.

IKI indicator solution was made by dissolving 1.016 gm KI and 0.1016 gm I_2 in 100 ml water. This was stored in the dark and, prior to use, diluted 10-fold with water.

After treatment, both the surrounding medium and the tissue were assayed for the presence of α-amylase. An aliquot of the medium was mixed with an equal volume of 0.01M calcium acetate and the volume then made up to a standard amount with 0.005 M calcium acetate. Tissue was rinsed in cold water, ground with either an all-glass homogeniser or an Ultra-Turrax high-speed blender in 0.005M calcium acetate, centrifuged at 5000 r.p.m. for 10 min. and the supernatant decanted.

The crude enzyme preparations from both tissue and ambient solution were next heated to 70°C for 20 min. to inactivate β-amylase, cooled, and centrifuged at 5000 r.p.m. for 10 min. (Kneen, Sandstedt and Hollenbeck, 1943).

To assay α-amylase, an aliquot of the enzyme preparation was added to an equal volume of starch substrate, then at intervals, 1 ml of this mixture was added to 1 ml of dilute IKI solution. After diluting to 5 ml with water, the optical density was measured in a Unicam spectrophotometer in 1 cm glass cells at 620 mμ. When necessary, the

enzyme preparations were diluted with 0.005M calcium acetate to slow down the reaction rate to workable levels. All solutions, including the reaction mixture, were held at 30°C, the optimum for α-amylase activity (Paleg, 1961).

Zero time samples were prepared by adding 0.5 ml enzyme to 1 ml of IKI, then adding 0.5 ml starch and diluting to 5 ml. The spectrophotometer blank was obtained by adding 0.5 ml enzyme to 1 ml IKI and making to 5 ml.

The data were expressed as percentages of the zero time sample, and enzymic activity was then calculated by taking the reciprocal of the time to reach 50% of the original colour intensity, multiplying by any dilution made, and expressing this as Starch-Iodine Colour (SIC) units per gm wet weight of tissue (Sandstedt, Kneen and Blish, 1939; Briggs, 1962; Briggs, 1967).

6. Starch Determinations on Aleurone Tissue

After extraction with trichloroacetic acid, the residue was washed in ethanol, air-dried and finely ground with a mortar and pestle. In some cases, the determination was made on oven-dried material which was subsequently ground in a power-driven all-glass homogeniser.

50-250 mgm of material was heated at 100°C in 4 ml water for 15 min., cooled, and 3 ml 72% perchloric acid added slowly with agitation (Pucher, Leavenworth and Vickery, 1948). The mixture was incubated at 25°C, stirring occasionally, for 20 min. 20 ml water were then added,

mixed and centrifuged. The supernatent was decanted and the residue re-extracted as above, the two extractions being bulked together and the final volume made to 50 ml. This was again centrifuged and the supernatent stored in the cold for up to 48 hours.

Aliquots were taken, and made to 10 ml before adding 5 ml of 20% aqueous NaCl and 2 ml of IKI solution (7.5 gm iodine and 7.5 gm KI, ground with 150 ml water, made to 250 ml and filtered).

After mixing, the solution was left for at least 30 min. to precipitate starch, then centrifuged and decanted carefully. The precipitate was suspended in 5 ml ethanolic NaCl (350 ml ethanol, 80 ml water, 50 ml 20% aqueous NaCl, diluted to 500 ml with water), centrifuged and the precipitate washed with 5 ml ethanolic NaOH (350 ml ethanol, 100 ml water, 25 ml of 5/N NaOH, diluted to 500 ml with water and filtered). The colour was discharged, with gentle shaking, the precipitate centrifuged, suspended in 5 ml ethanolic NaCl and after centrifuging, dissolved in 2 ml 0.5 N NaOH with a little warming (McCready and Hassid, 1943). The solution was made slightly acid with 6 N HCl and the glucose content determined by the anthrone method. The amount of starch was calculated from the optical density by using the mass extinction coefficient obtained for glucose and multiplying by a factor of 0.90 (McCready, Guggolz, Silviera and Owens, 1950).

7. Estimation of Phosphate

Two different procedures were followed in assaying for phosphorus:-

(a) Inorganic phosphorus was measured after formation of the phosphomolybdate complex, and development of the blue colour by reduction with stannous chloride. Total phosphate was estimated after digestion with sulphuric and perchloric acids (Martin and Doty, 1949; Weil-Malherbe and Green, 1951; Martin and Morton, 1956).

(b) Inorganic phosphorus was precipitated from solution as the calcium salt and estimated as the vanadomolybdophosphate. Total phosphate was measured following digestion with sulphuric acid and hydrogen peroxide (Lipmann and Tuttle, 1944; Bartlett, 1959; Parvin and Smith, 1969).

The details of the procedures are as follows:-

(a) Tissue to be assayed for inorganic phosphorus was first deproteinised with silicotungstic acid at 4°C (3.88 gm sodium metasilicate. 4 H_2O and 79.4 gm sodium tungstate, 2 H_2O dissolved in 500 ml water; after adding 15 ml conc. H_2SO_4, the mixture was boiled for 5 hours, cooled and diluted to 1 litre). 2 gm of tissue were homogenised with an Ultra-Turrax, in 7.5 ml water, 3 ml silico-tungstic acid and 2 ml 5% acid ammonium molybdate (5% ammonium molybdate, 4 H_2O in 4 N H_2SO_4). 20 ml of iso-butanol were added to the homogenate, blended in with

the Ultra-Turrax and the resultant mixture centrifuged at 5000 r.p.m. for 10 min. 5 ml of the upper layer of iso-butanol was removed, 5 ml of freshly prepared acidified ethanol added (10 ml conc. H_2SO_4 and 490 ml abs. ethanol), and then, within 10 min., 0.2 ml of dilute stannous chloride reagent added (10 gm stannous chloride in 25 ml conc. HCl, diluted 40-fold with N HCl just before use). The optical density at 730 mμ was measured in 1 cm cells in a Unicam spectrophotometer within 15 min.

Samples for total phosphate were digested on a hot plate at 150-160°C with 0.5 ml of 10 N H_2SO_4 and two drops of 70% perchloric acid, until quite clear. Following digestion, the samples were neutralised with 2 N NaOH, using p -nitrophenol as indicator, then back-titrated until just acid. After adjusting the volumes to the same level with water, 6 ml iso-butanol and 1 ml acid molybdate reagent were added, the mixture shaken for 15 sec. and the layers allowed to separate. 5 ml of the upper iso-butanol layer was removed and treated as described above.

(b) 2 gm of tissue was homogenised at 4°C in 5 ml of 5% trichloroacetic acid, centrifuged, and 0.5 ml aliquots of the supernatant taken for estimation of Pi. To these were added 1 drop of thymol blue indicator, and the extract rapidly brought to pH 8 with neutralising solution (100 ml conc. ammonia, 40 ml glacial acetic acid made to 1 litre with water, then 100 ml of 0.4M sodium bicarbonate added).

2.5 ml of ethanolic calcium chloride (3.3% anhydrous $CaCl_2$ in 33% ethanol) were immediately added and mixed. The precipitate was centrifuged for 5 min. at 4000 r.p.m., washed with 2 ml of ethanolic $CaCl_2$ and re-centrifuged. Finally, the precipitate was dissolved in 0.5 ml of 0.5 N HCl, made to 10 ml with water, and aliquots taken to estimate P_i.

Since the precipitate also contained the Ca salts of phosphate esters, it was stored in the cold and dissolved in acid just before the estimation. The yield of prepared solutions of P_i was quantitative, and P_i added to tissue prior to extraction gave 92% recovery.

Aliquots containing 1-10 µg P_i were made to 2 ml with water. 4 ml of n-butanol were added, then 2 ml of the vanadomolybdate reagent (14 ml 10 N HCl, 10 ml 0.234% ammonium metavanadate and 20 ml 3.53% ammonium molybdate diluted to 1 litre with water). The mixture was stirred on a Vortex mixer for 10 secs., the two phases allowed to separate, and the opical density of the upper n-butanol layer measured at 310 mµ in 1 cm quartz cells.

Measurement of total acid-soluble phosphorus was made by hydrolysing 0.5 ml portions of the TCA extract with 0.5 ml of 10 N H_2SO_4 at 150-160°C for at least 3 hours. After this, 2 drops of 30% H_2O_2 were added and the samples returned to the oven for a further 1½ hours. After cooling, and diluting to 40 ml. aliquots were taken for determination of P_i by vanadomolybdate.

8. Detection of Phosphate Esters on Paper

Developed chromatograms were dipped in a reagent made by mixing 5 ml 60% perchloric acid, 5 ml 20% (w/v) ammonium molybdate, 10 ml N HCl and 80 ml acetone. After drying, papers were exposed to ultra-violet light for a few minutes to develop the blue complex. To fix the colour and reduce background, the papers were dipped in a solution of 2.5% (w/v) benzoin α-oxime in methanol (Harrap, 1960).

9. Sugar Determinations

For solutions having a range of 10-100 µg glucose/ml the anthrone method of McCready, Guggolz, Silviera and Owens (1950) was used. The anthrone reagent was prepared by dissolving 0.2 gm anthrone in 100 ml H_2SO_4 (2 litres conc. H_2SO_4 and 400 ml water). 5 ml of anthrone reagent was placed in a test tube held in an ice bath and 1 ml of the sample slowly layered on top. When all the samples had been prepared, they were mixed rapidly one at a time and placed in a boiling water bath for 10 min. After cooling, the optical density was measured in 1 cm glass cuvettes, at 625 mµ.

For samples over the range 1-10 µg glucose per ml the more sensitive test described by Guinn (1967) was used. 1 ml of sample was mixed with 1 ml ferricyanide (0.5 gm potassium ferricyanide/litre water) and 1 ml carbonate-cyanide (5.3 gm sodium carbonate and 0.65 gm potassium cyanide/litre water), and heated on a boiling water bath

for 15 min. After cooling, 5 ml of ferric reagent (1.5 gm ferric ammonium sulphate and 1.0 gm sodium lauryl sulphate/ litre of 0.05 N H_2SO_4) was added and the optical density measured in 1 cm glass cuvettes at 680 mμ after 15 min.

10. Detection of Sugars on Paper

Papers were dipped in a solution of 2 gm silver nitrate, 20 ml water and acetone to 1 litre. After drying, they were dipped in a solution of 5 gm NaOH dissolved in 25 ml water and made to 250 ml with ethanol. To fix the developed papers, they were immersed, after drying, in a solution of 100 gm sodium thiosulphate, 15 gm sodium metabisulphite and water to 1 litre, then washed in water for 2 hours. This technique is modified from Ahet and Reynolds (1954).

11. Identification of Nucleotides

The absorption spectra in 0.01 N HCl (pH2) were determined in a recording spectrophotometer, and compared with the spectra published in P-L Biochemicals Circular OR-10 (1956).

R_F valves were compared against authentic nucleotides in the following solvent systems:

(i) Iso-butyric acid : ammonia : water (66:1:33 v/v/v)

(ii) Iso-butyric acid : ammonia : water (57:4:39 v/v/v)

(iii) 95% ethanol : 1M ammonium acetate pH 7.5 0.01M in EDTA (70:30 v/v).

(iv) 95% ethanol : 1M ammonium acetate pH 3.8 0.01M in EDTA (70:30 v/v).

(v) n-butanol : acetic acid : water (50:20:30 v/v/v)

(vi) Electrophoresis for 60 min. in 0.05M citrate buffer (tris, pH4.8) at 30 v/cm.

(Solvents (i)-(iv) are described in P-L Biochemicals Circular OR-10; (v) Smith (1960); (vi) Jenner (1968)).

Co-chromatography was done by adding mixtures of authentic nucleotides to aleurone tissue before extraction, and the extracts separated in the two dimensional combinations of solvents (ii) and (vi), and (ii) and (iii) listed above.

Hydrolysis of nucleotides was carried out either by the method of Wyatt (1951) with 98% formic acid in sealed tubes held at 175°C for 30 min., or by heating at 100°C in N HCl for 60 min. (Amos and Korn, 1958). The former method hydrolyses all nucleotides to their corresponding bases, while the latter yields guanine, adenine, cytidylic acid and uridylic acid. The hydrolysis products were run in 95% iso-propanol : conc. HCl (65:17.4 made to 100 ml with water) against authentic markers for identification(Wyatt, 1951).

Ultra-violet absorbing compounds indistinguishable from uridine diphosphate glucose by the method described above were hydrolysed at pH2 for 15 min. at 100°C (Caputto et al, 1950). The hydrolysate was passed through Amberlite IR-120 (H^+) and IR 4-B (OH^-) resins, the effluent evaporated to dryness, and developed in ethyl acetate : pyridine : water

(10:4:3 v/v/v) for 18 hours on Whatman No. 1 paper. With the aid of markers of authentic sugars, the sugar-containing areas were cut from the paper, eluted with water and estimated by the anthrone method (Jenner, 1968).

12. Photography and Autoradiography

U-V absorbing regions on paper chromatograms were photographed by passing U-V light through the papers onto Ilford Reflex Contact Document paper. The document paper was then developed and fixed with commercial reagents (Markham and Smith, 1949).

Autoradiographs were prepared by fastening chromatograms to Kodak Envelope-Packed X-Ray film, leaving for several hours, then developing and fixing the film with commercial reagents.

13. Detection of Radioactivity

Radioactive samples were either plated onto copper planchets and counted in a Nuclear-Chicago Planchet counter, or mixed with scintillation fluid (Kennedy, 1969) and counted on a Packard Tri-Carb scintillation counter. On one occasion, samples of tissue were loaded onto copper planchets and counted in an Ecko scaler connected to a Geiger-Mueller tube.

RESULTS

1. Isolation of Wheat Aleurone Tissue

The aleurone layer from barley has been extensively used in studies of the stimulation of enzyme production by gibberellic acid. To remove this layer from the starchy endosperm, it is necessary to first soften the half-seeds, a process requiring 3 days imbibition of water. The tissue is then peeled from the endosperm by hand. The preparation of large samples of homogeneous material from barley is thus lengthy and laborious. Phillips (1968) overcame these difficulties by using wheat, which requires only 26 hours imbibition, and by separating the aleurone from the starchy endosperm by mechanical means. In this way she was able to isolate 16-20 gm tissue from sterilised, imbibed half-seeds, in about 45 min. and accordingly, her methods have been used here.

2. Residual Starch Adhering to Isolated Tissue

Some starchy endosperm remains attached to the isolated aleurone tissue. This residual contamination was determined, as described under "Materials and Methods", as being 56% of the total dry weight after 30 min. of the isolation procedure and 15% after a further 7-8 min. (average of duplicate results). The latter value is somewhat higher than the figure of 9% obtained by Phillips (1968).

3. Conditions of Sterility

The procedure used to minimise microbial contamination is described under "Materials and Methods", and is the

same as used by Phillips (1968). She found that bacterial and fungal colonies were almost completely absent even 36 hours after the isolation of the tissue. Since most incubations in this present study were limited to 6-7 hours, no further precautions were taken.

4. Reproducibility of Isolation Procedure

The ratio of fresh weight to dry weight of the isolated tissue affords some measure of the reproducibility of the isolation procedure. This ratio, over three separate occasions, varied from 3.9 to 4.1 which is in good agreement with the results that Phillips (1968) obtained.

5. The Production of α-amylase by Isolated Aleurone in Response to Different Concentrations of GA.

Samples of aleurone tissue (2 gm in each) were weighed into 9 cm petri dishes, 7 ml of solution added, and incubated at 30°C. After 24 hours, the tissue and ambient solution were assayed separately for α-amylase and the results are shown in Table 1.

The amounts of α-amylase synthesised are linearly related to the logarithm of the concentration of GA up to a level of about 10 µg/ml. Above 100 µg/ml the production of enzyme apparently declines, (Fig 2).

Fig. 2.

The production of ∝-amylase by wheat aleurone in response to different concentrations of GA. Tissue was treated immediately after isolation and enzyme activity measured 24 hours later.

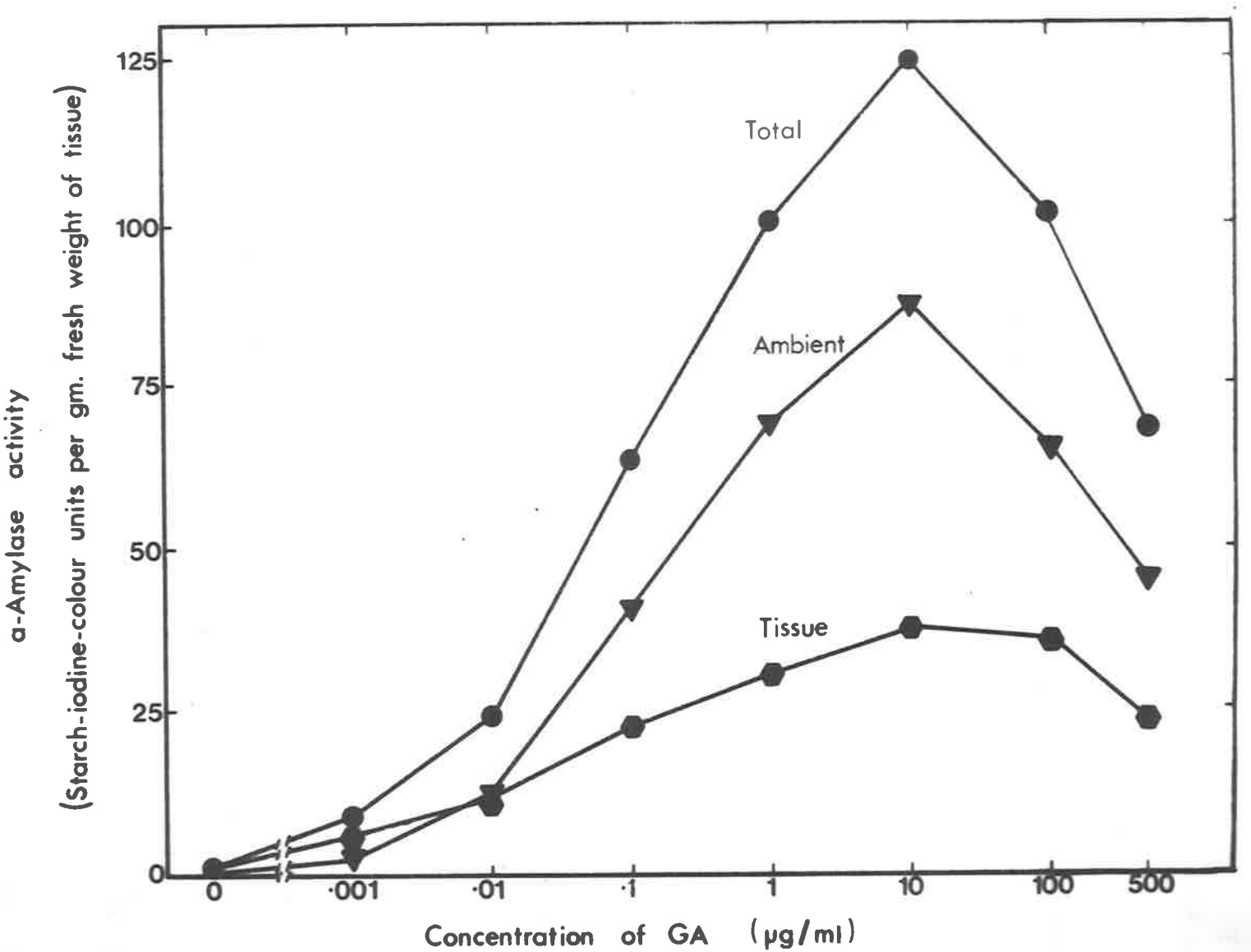

α-Amylase activity
(Starch-iodine-colour units per gm. fresh weight of tissue)
125
100
75
50
25
0
Total
Ambient
Tissue
0
·001
·01
·1
1
10
100
500
Concentration of GA (µg/ml)

Table 1

Production of α-amylase by wheat aleurone tissue in response to different concentrations of GA. Time of incubation: 24 hours. Enzyme activity: starch-iodine-colour units per gm of tissue (fresh weight). (Average of two determinations).

Concentration of GA	α-amylase activity			Ratio of α-amylase activity in
µg/ml	Ambient	Tissue	Total	ambient to tissue
0	0.2	0.8	1.0	0.25
0.001	2.6	6.3	8.9	0.41
0.01	12.6	12.0	24.6	1.05
0.1	40.7	22.8	63.5	1.78
1	69.0	30.8	99.8	2.24
10	87.0	37.6	124.6	2.31
100	65.2	35.6	100.8	1.83
500	45.1	23.6	68.7	1.91

The ratio of the amount of enzyme in the ambient solution to that in the tissue also increases with concentration of GA up to about 1 µg/ml. Above this, there is little further change.

Accordingly, a concentration of 100 µg/ml was used in all further experiments as at that level it could be assumed that the hormone was not limiting.

6. The Identity of U-V Absorbing Compounds isolated from Wheat Aleurone

The U-V absorbing compounds separated by paper chromatography in two dimensions with iso-butyric acid: ammonia: water (57:4:39 v/v/v) and 95% ethanol: IM ammonium acetate pH7.5 (0.01M in EDTA) (7:3 v/v) respectively, can be seen from Fig.3. Nine major U-V absorbing areas are

Fig. 3.

U-V photograph of two-dimensional chromatogram

Solvent 1 : iso-butyric acid : ammonia : water
(57 : 4 : 39 v/v/v).

Solvent 11: 95% ethanol : IM ammonium acetate
pH 7.5 (0.01 M in EDTA) (7:3 v/v).

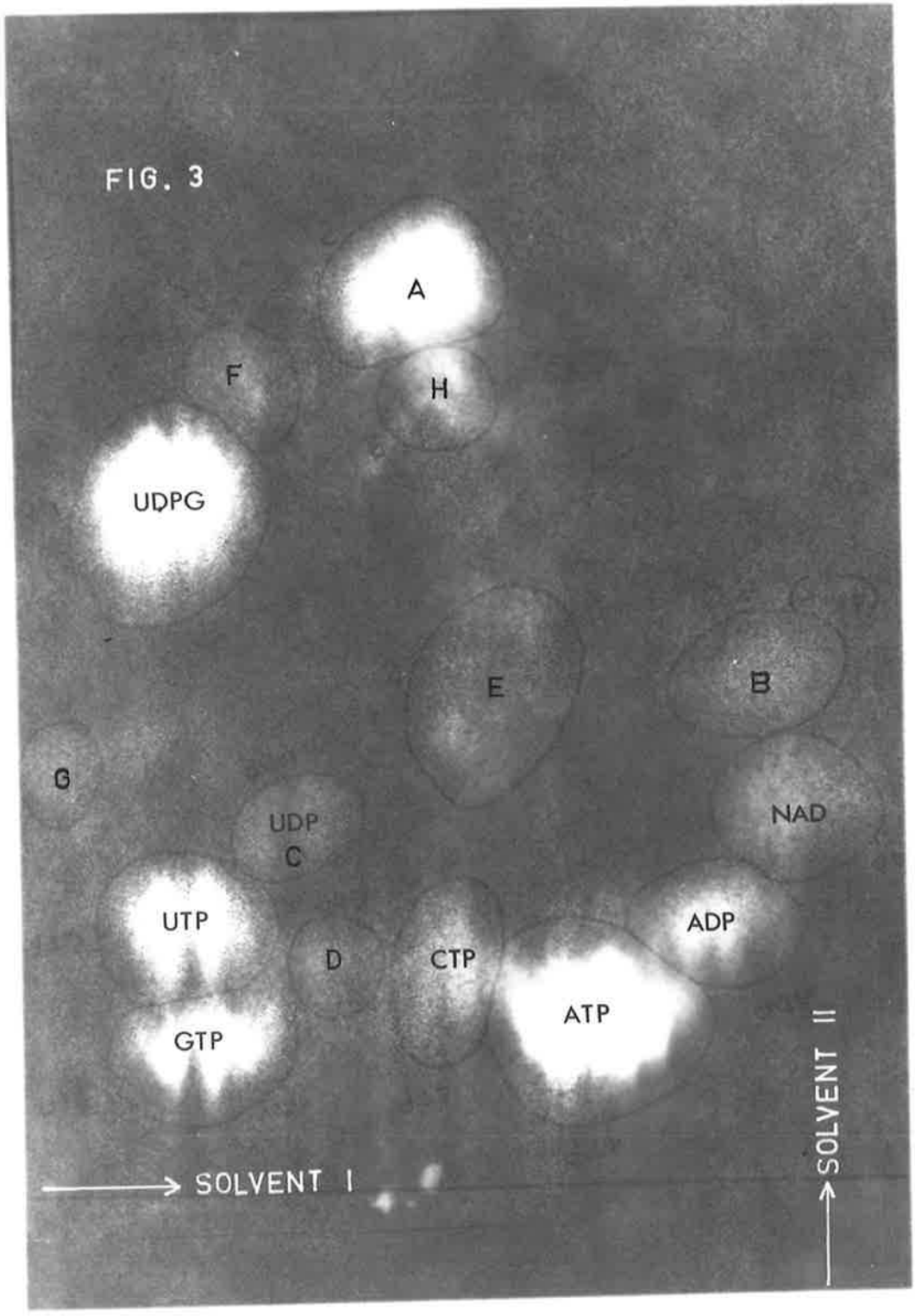


FIG. 3
A
F
H
UDPG
E
B
G
UDP
C
NAD
UTP
ADP
D
CTP
ATP
GTP
SOLVENT I
SOLVENT II

clearly discernable. These were eluted in 0.01 N HCl (pH2) and their absorbance measured at wavelengths of 260,296, and 320 nm in 1 cm quartz cuvettes.

Two of the major components had a ratio of absorbance at 260 to 296 nm of 0.88-0.96, a value too high to be characteristic of nucleotides. These migrated to positions with R_F 0.37 and 0.60 in the iso-butyric acid solvent, and R_{AMP} 2.26 and 1.27 respectively in the ethanol-ammonium acetate system, (Compounds A and B in Fig.3). The absorption spectra of the other seven compounds were determined with a Unicam SP 800 recording spectrophotometer and the wavelengths of maximum absorption, together with relative chromatographic mobilities, are shown in Table 2.

Table 2

Wavelengths of maximum absorbance, and relative chromatographic mobilities of several of the U-V absorbing compounds extracted from wheat aleurone tissue.

Compound	R_F* Iso-butyric acid: ammonia:water	R_{AMP}+ Ethanol:Ammon. acetate pH7.5	Wavelength of max. absorbance nm
ADP	0.56	0.65	258
ATP	0.48	0.44	257 - 258
CTP	0.39	0.56	280
GTP	0.22	0.39	255 - 256
UTP	0.24	0.70	262
UDPG	0.28	1.75	262 - 263
NAD	0.62	0.95	256 - 258

*Mobility relative to solvent front.

+Mobility relative to AMP.

Six or seven additional U-V absorbing areas can be seen in Fig. 3, but these were too weakly absorbing to be measured accurately. One of these, with R_F and R_{AMP} of 0.31 and 0.94 respectively, corresponded to UDP, (Compound C in Fig 3). Another, with mobilities of 0.33 and 0.57 in the two solvent systems, corresponded to GDP, (Compound D in Fig. 3). These minor components were always present in small amounts and were not observed to change to any detectable degree by the treatments subsequently applied to the tissue. The following nucleotides were not detected: AMP, UMP, CDP, CMP, GMP, CDPG, ADPG, GDPG, NADP.

One substance, which migrated with R_F 0.47 in iso-butyric acid:ammonia and R_{AMP} 1.27 in ethanol:ammonium acetate, gave a light-blue fluorescence under U-V light (Compound E in Fig.3). Other fluorescent material was confined mainly to the origins of the chromatograms.

Six of the seven most abundant compounds shown in Table 2 were further characterised by acid hydrolysis and identification of the bases liberated. The methods and results are described in Table 3.

Table 3

Base analysis of U-V absorbing compounds. Hydrolysis was carried out with 98% formic acid in sealed tubes heated at 175°C for 30 min., or with N HCl at 100°C for 60 min. The hydrolysates were developed in iso-propanol:HCl using authentic bases and nucleotides as markers. The percentage recovery after hydrolysis in N HCl is given.

Compound	Method of hydrolysis		Per cent recovery
	98% formic acid	N HCl	
ADP	adenine	adenine	-
ATP	adenine	adenine	88
	(trace of cytosine)		
CTP	cytosine	CMP	93
	(trace of adenine)		
GTP	guanine	guanine	84
UTP	uracil	UMP	86
UDPG	uracil	UMP	85

Traces of adenine were found in hydrolysates from CTP, and of cytosine from ATP indicating that these compounds were not completely separated. The absorbance of ATP at 296 nm was about 5% of that at 260 nm. Since, at these two wavelengths, the extinction value of CTP is very nearly equal, it was assumed that the contamination of ATP by CTP amounted to approximately 5%. No correction was made for this in the levels of either nucleotide.

The compound resembling UDPG was subjected to mild acid hydrolysis, followed by paper chromatography of the neutral fraction. Four reducing substances were separated, with mobilities identical to glucose, fructose, galactose and xylose. The molar ratio of these substances was 1.00: 0.44:0.44:0.38 respectively, and the molar ratio of UDP

to total reducing substance was 1.00:0.94. The recovery of reducing compounds was 90%.

A minor U-V absorbing component, (Compound F in Fig.3), which appears at R_F 0.32 in iso-butyric acid:ammonia and R_{AMP} 2.08 in ethanol:ammonium acetate was also subjected to mild acid hydrolysis. Chromatography of the neutral fraction revealed reducing substances with mobilities identical to glucose and fructose. These were present in the molar ratio 1.0 to 3.2 respectively. A compound corresponding to UMP was identified after hydrolysis of this component in N HCl. The molar proportion of UDP to total reducing substance was 1.0 to 2.5 respectively.

Authentic nucleotides, corresponding to the compounds shown in Table 2, were added to aleurone tissue which was then extracted for free nucleotides. The final chromatogram was compared to one derived from tissue without such addition, and the pattern of U-V absorbing areas was found to be identical in each case.

The relative mobilities of the seven most abundant compounds were examined in a total of 7 different chromatographic systems (see "Materials and Methods"). In each case the R_f value of the compound, and an authentic sample of the compound it was presumed to be, corresponded closely.

7. The Recovery of Authentic Nucleotides

An aliquot of authentic nucleotides was added to one

of two otherwise identical batches of aleurone tissue. Both lots of tissue were then extracted for free nucleotides. Another aliquot of the authentic mixture, equal to that added to the tissue, was developed in the 2-dimensional system of paper chromatography used for the tissue extracts. The U-V absorbing compounds corresponding to the added nucleotides were eluted from the three papers and measured. The recoveries, calculated by difference, are shown in Table 4, where it is clear that

Table 4

Per cent recovery of authentic nucleotides added to wheat aleurone tissue before extraction of the tissue for free nucleotides.

ATP	96*	UTP	96*	CTP	92*	GTP	86*
ADP	98*	UDP	84	CDP	96	GDP	82
AMP	95	UMP	93	CMP	87+	GMP	96
		UDPG	85*	NAD			

*Average of duplicate determinations
+CMP and NAD were not separated from each other in the solvent system used, viz. iso-butyric acid : ammonia : water (57:4:39 v/v/v) followed by 95% ethanol: IM ammonium acetate pH 7.5 (0.01M in EDTA) (7:3 v/v).

for most nucleotides more than 90% of the amount added was subsequently recovered.

No corrections, on the basis of the recovery data, have been made on subsequent measurements.

8. The Level of Free Nucleotides in Residual Starch.

Since about 15% of the dry matter of isolated aleurone layers consisted of starch, it was necessary to determine how much nucleotide the starchy material contained.

Samples of tissue were removed after half-seeds had been rolled for 30 min., and again after a further 4 min. These were analysed for dry weight, starch content and nucleotide level.

In Table 5 it is clear that the sample with the greater amount of starch has the smaller amount of nucleotide. To calculate the weight of aleurone in each sample, the assumption was made that starch accounts for 80.9% of the total weight of the endosperm (Shetlar, Rankin, Lyman and France, 1947). The estimated weight of endosperm was subtracted from the total weight of the sample, and the amounts of each nucleotide are expressed in Table 6 on the basis of the weight of aleurone alone. From these data it is concluded that the vast majority, if not all of the nucleotides are derived from the aleurone and essentially none from the adhering endosperm.

Table 5

Dry weight and nucleotide level of aleurone tissue prepared with different levels of residual starch.

		Sample A	Sample B
Dry weight (gm)		1.545	1.140
Starch content (gm)		0.828	0.320
Level of nucleotide (nmoles/sample)	ATP	141	194
	ADP	14	16
	CTP	20	24
	GTP	17	27
	UTP	48	82
	UDPG	148	197

Table 6

Nucleotide levels from Table 5 adjusted on the basis of equal weight of aleurone in each sample. (nmoles/gm) dry weight of aleurone).

Nucleotide	Sample	
	A	B
ATP	271	262
ADP	27	22
CTP	38	32
GTP	33	36
UTP	92	111
UDPG	284	266
Total	745	729

9. Changes in the levels of Free Nucleotides in response to Gibberellic Acid

Aleurone tissue was isolated and weighed into three portions, each of 4 gm. One of these was extracted immediately for free nucleotides, and the other two were incubated with either 10 ml of water or GA in 9 cm petri-dishes at 30°C, and then also extracted.

As only sufficient material for three samples could be prepared at one time, separate experiments were done for each time of incubation. The results are shown in Table 7.

Since 4 gm fresh weight of tissue were used for the determinations, and the dry weight is 25% of the fresh weight, the values correspond approximately to nmoles per gm dry weight of tissue. Each experiment was repeated three times, thus the mean values for the zero time control, representing the nucleotide status of the tissue immediately after

isolation, are the average of 18 separate measurements. Values for NAD have been omitted because of poor recoveries from the cation exchange resin column (see "Materials and Methods").

Reference to Table 7 shows that 88% of the nucleotides measured contain the bases adenine and uracil. ATP and UDPG are the most abundant individual components and together account for 72% of the total. The molar proportions of the four nucleoside triphosphates, ATP, UTP, GTP and CTP are 1.00:0.33:0.16:0.16 respectively and the molar ratio of ATP to ADP is approximately 11 to 1. No AMP was detected at any stage in the experiment.

The experimental data, given in Appendix 1, were analysed by non-parametric statistics using the Mann-Whitney U test. The significant effects, (5% level) are described below.

During the first two hours, the levels of UDPG in both water- and GA-treated tissue are depressed below those of the control. Initially, both treatments fall at the same rate. However, after the first hour, the level with GA falls more rapidly and by 2 hours has reached a lower value than the water-treated tissue. Both treatments rise to control levels by 3 hours and remain relatively unchanged until 15 hours. At this time, the level with GA rises above the corresponding value for water. The effect is transient and disappears by 24 hours.

The level of UTP in tissue treated with water rises

Table 7.

Time-course of changes in the levels of free nucleotides of wheat aleurone tissue in response to GA. Concentration of GA: 100 µg/ml. Nucleotide levels, expressed as nanomoles per 4 gm. fresh weight, are means of triplicate values (c.f. Appendix table 1). Values in parentheses are the levels relative to the control as 100.

Nucleotide	Treatment	Time of incubation (Hrs.) 0*	1	2	3	6	15	24
ATP	Control	218	209	246	209	227	205	214
	-GA		214(102)	225(91)	192(92)	229(101)	211(103)	208(97)
	+GA		195(93)	213(86)	188(90)	230(101)	225(110)	249(116)
ADP	Control	20	20	18	27	17	19	21
	-GA		21(105)	27(150)	32(118)	20(118)	18(95)	18(86)
	+GA		20(100)	25(139)	31(115)	23(135)	21(110)	17(81)
UDPG	Control	204	219	254	181	173	203	196
	-GA		183(84)	178(70)	157(87)	200(116)	206(101)	218(111)
	+GA		172(78)	163(64)	147(81)	214(124)	253(125)	236(120)
UTP	Control	72	80	93	64	59	60	75
	-GA		100(125)	121(130)	82(128)	83(141)	86(143)	66(88)
	+GA		96(120)	129(139)	85(133)	97(164)	88(147)	119(159)
GTP	Control	34	30	30	34	42	33	33
	-GA		35(117)	30(100)	30(88)	42(100)	31(94)	37(112)
	+GA		32(107)	29(97)	32(94)	46(110)	38(115)	40(121)
CTP	Control	34	28	35	29	41	33	37
	-GA		37(132)	37(106)	30(103)	45(110)	48(145)	54(146)
	+GA		35(125)	40(114)	34(117)	46(112)	45(136)	59(159)

* These values are the means of 18 samples.

during the first hour and continues above the control value up to the 24th hour. A similar rise in tissue treated with GA, apparent by 2 hours, persists throughout the experiment and the level at 24 hours is higher than that following treatment with water.

In the presence of GA, the level of ATP falls relative to that in both control and water-treated tissue. This effect, detectable at 2 hours, disappears during the next hour. However, by 15 hours the level with GA treatment rises above the control value, and at 24 hours is higher than the corresponding treatment with water.

The amount of ADP in tissue incubated with water for 2 hours is higher than the control. Although the level following treatment with GA rises also, the difference is only significant at the 10% level. By 3 hours, this effect has been abolished and no further change occurs until 15 hours when the level in water-treated tissue falls and that in GA-treated tissue rises relative to the control. These effects are not significant at 24 hours.

No significant treatment effects occur in the levels of CTP until 15 hours of incubation. At this time the level in tissue treated with water has risen compared to the control. However, by 24 hours, both treatments are higher than the control value.

The amount of GTP does not change significantly with either time or treatment.

Two apparently distinct effects of GA can be discerned. The first is evident after 2 hours of incubation when both UDPG and ATP levels are depressed. The effect is transient and disappears after a further hour. The second is discernable at 15 hours when, in the presence of GA, the level of UDPG rises more rapidly than in its absence; at 24 hours there are similar trends in both UTP and ATP. Although not significant, small increases also occur in the levels of CTP and GTP after treatment of tissue with GA for 24 hours.

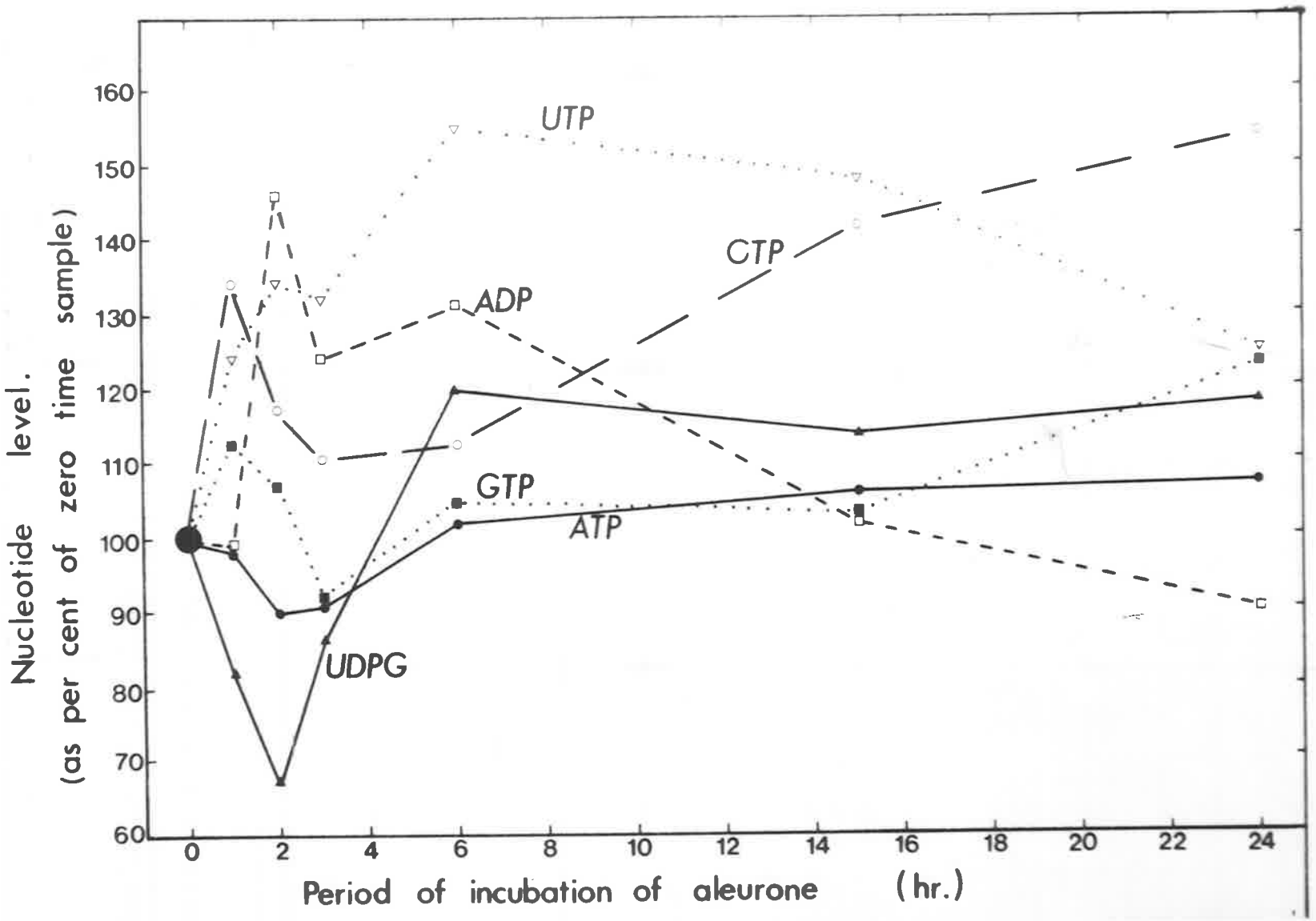
Inspection of Table 7 shows that the nucleotide levels of both water- and GA-treated material exhibit similar changes with time of incubation. This is especially evident during the first 6 hours. Analysis between times by non-parametric statistics is difficult to interpret because of fluctuations in the level of the control treatment. To stabilise this variance, the values of Table 7 were transformed by making the control equal to 100 and adjusting the other treatments accordingly. The adjusted values were then analysed as a 6 x 2 x 3 factorial design. The mean values for each time are shown in Table 8, and Fig.4

Table 8

Nucleotide	Time of incubation (hrs.) 0	1	2	3	6	15	24	Least significant difference 5% level	1% level
ATP	100	98	90	91	102	106	107	7	10
ADP	100	99	146	124	131	102	90	32	43
UDPG	100	82	67	86	120	114	118	15	20
UTP	100	124	134	132	155	148	125	21	N.S.
GTP	100	113	107	92	105	103	123	N.S.	N.S.
CTP	100	134	117	111	113	142	154	N.S.	N.S.

Fig. 4.

Nucleotide levels at intervals during incubation of aleurone for 24 hours. Levels in the presence and absence of GA have been averaged and are expressed relative to the zero time sample as 100.


Nucleotide level.
(as per cent of zero time sample)
160
150
140
130
120
110
100
90
80
70
60
UTP
CTP
ADP
GTP
ATP
UDPG
0
2
4
6
8
10
12
14
16
18
20
22
24
Period of incubation of aleurone (hr.)

The variance ratios for time of incubation were significant for ATP, ADP, UDPG and UTP. Comparison of the mean values for each time with the least significant differences (Table 8) shows that, over the first two hours, the levels of ATP and UDPG decrease, whilst those of ADP and UTP increase. Although UDPG falls by 33% during this period, there is a rapid recovery and by 6 hours UDPG has risen to a value 20% above the control. This is equivalent to an increase of 121 nmoles. The decrease in the amount of ATP of 10% or 27 nmoles does not match the rise in ADP since the increase of 46% in the latter is equivalent to only 8 nmoles. UTP rises to a level 34% above the control by 2 hours, but, in contrast to UDPG, ATP and ADP, does not return to control values between 3 and 6 hours. The difference of approximately 30 nmoles is maintained throughout.

It is apparent that modifications in the nucleotide pool are initiated during the first two hours of incubation of aleurone tissue in water alone. These fluctuations could represent wounding responses of the tissue, and consequently, the changes occurring shortly after the isolation of the tissue were investigated in greater detail.

10. Response of Free Nucleotides to Isolation of Tissue.

Four lots of aleurone tissue (4 gm in each) were prepared from each of three batches of half-seeds previously imbibed for periods of 24, 26 and 30 hours. One portion of tissue from each preparation was immediately extracted

for free nucleotides. The other three were incubated in 10 ml water at 30°C for times of 2, 4, and 6 hours and then also extracted. Changes in the levels of ATP, UTP and UDPG were followed and are shown in Table 9. A graphical representation is given in Fig. 5.

In general, the changes observed over the 6 hours of incubation were similar for each time of imbibition of half-seeds, and also corresponded to those shown in Table 8. It was clear that the technique used to prepare the aleurone tissue was,in itself, causing changes in the amounts of nucleotides that represent a wound response.

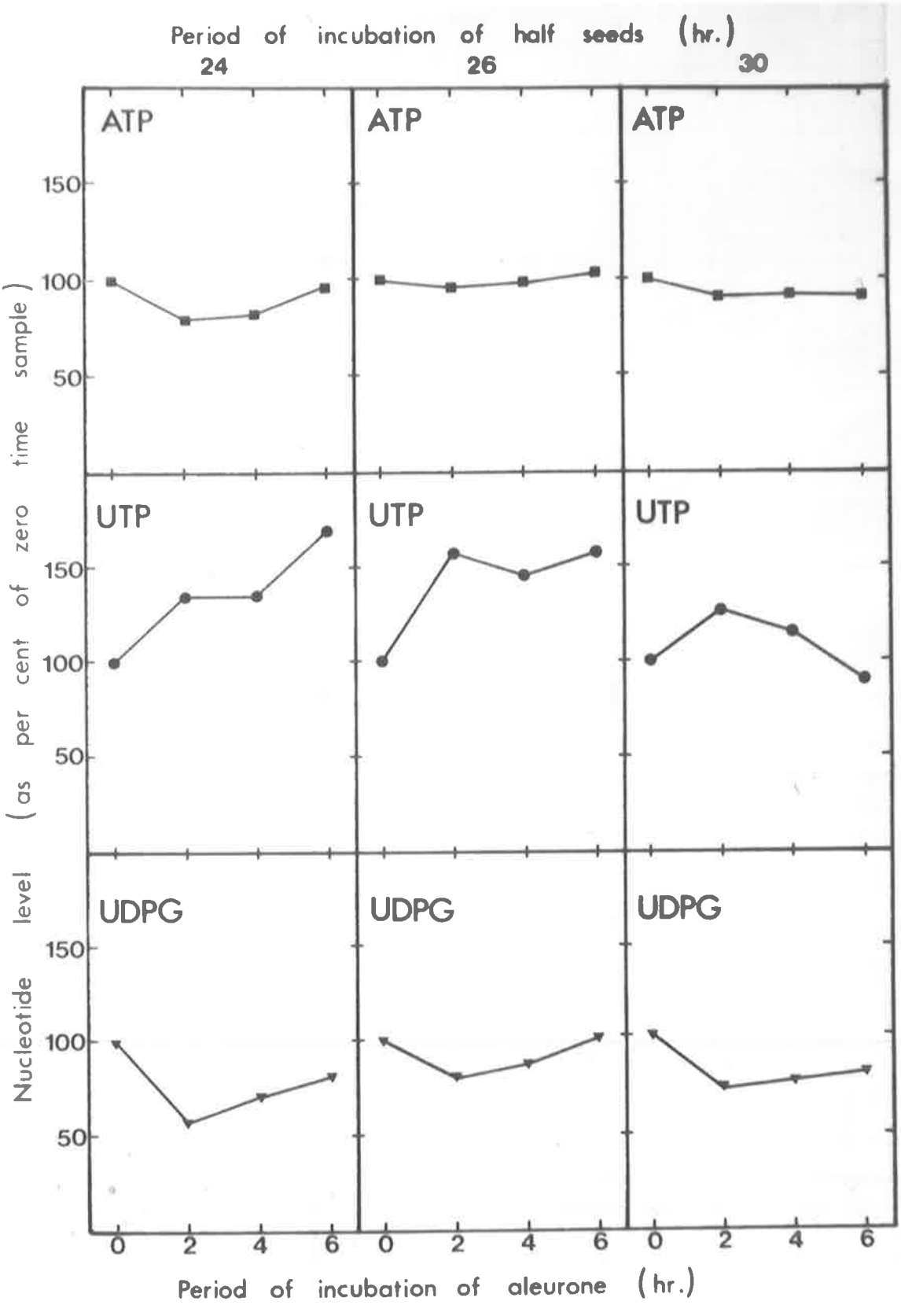
Table 9

Aleurone tissue (4 gm) was prepared from half-seeds after different periods of imbibition. The tissue was then incubated in water and examined for free nucleotides at intervals up to 6 hours. Figures in parentheses are the results relative to the zero time control. Nucleotide levels in nmoles/sample. The results for 26 hours imbibition are the average of duplicates; other results are a single observation.

Nucleotide	Time of imbibition of half-seeds (hrs.)	Time of incubation of tissue (hrs.) 0	2	4	6
ATP	24	232(100)	186(80)	191(82)	222(96)
	26	214(100)	206(96)	211(98)	222(103)
	30	256(100)	234(91)	236(92)	234(91)
UTP	24	70(100)	95(135)	95(135)	112(160)
	26	62(100)	98(158)	91(146)	98(158)
	30	101(100)	128(126)	118(117)	91(90)
UDPG	24	264(100)	151(57)	185(70)	214(81)
	26	200(100)	160(80)	176(88)	202(101)
	30	268(100)	195(73)	210(78)	219(82)

Fig. 5

Aleurone layers were prepared from half seeds imbibed for various periods of time, incubated in water, and examined for levels of free nucleotides, at intervals of 2 hours, up to 6 hours. Results are expressed relative to the zero time sample as 100.


Period of incubation of half seeds (hr.)
24
26
30
ATP
ATP
ATP
UTP
UTP
UTP
UDPG
UDPG
UDPG
150
100
50
Nucleotide level (as per cent of zero time sample)
0
2
4
6
Period of incubation of aleurone (hr.)

The largest differences were observed at 2 hours after isolation, and during the next four hours the values for ATP and UDPG tended to return to the initial values, while the level of UTP remained relatively constant.

11. Investigation of the Ambient Solution for the Presence of Nucleotides or Bases

The possibility that the fall in the levels of some nucleotides, shortly after tissue isolation, was due to leakage into the ambient solution, was examined.

The ambient solutions from two of the experiments reported in Table 9 were removed from around the tissue and an equal volume of ice-cold 0.4 N perchloric acid was added. After 15 min. at 4°C, the extracts were neutralised with 0.33 N KOH and left at 4°C for several hours. $KClO_4$ and acid-insoluble material was then removed by centrifugation. Further $KClO_4$ was removed by evaporating the samples *in vacuo*, dissolving the residue in a small volume of water and centrifuging at 18,000 r.p.m. for 15 min. at 0°C. The supernatents were then examined by chromatography.

The samples derived from half-seeds imbibed for 24 hours were divided into two portions, one of which was developed in iso-propanol:HCl:water (170:41:39 v/v/v), the other in iso-butyric acid:ammonia:water (57:4:39 v/v/v). Samples derived from half-seeds imbibed for 26 hours were developed in 95% ethanol:1M ammonium acetate pH 7.5 (0.01M with respect to EDTA) (7:3 v/v).

Areas corresponding to authentic bases and nucleotides were eluted from the papers and measured for U-V absorbance. Those areas corresponding to guanine were eluted in 1.6 N HCl, other bases in 0.1 N HCl and nucleotides in 0.01 N HCl (Smith, 1960).

It is apparent in Table 10 that only small amounts of compounds resembling nucleotides could be detected in the ambient solution. The largest quantities were found after two hours of incubation, and no nucleotides could be detected after 4 or 6 hours. Although some U-V absorbing material was detected after chromatography with the iso-butyric acid solvent, none was found following development of the 26 hours sample with ethanol:ammonium acetate. It can be seen from Table 11 that the amounts of ATP and UDPG lost from the tissue are not of the same order as those found in the ambient solution.

On another occasion, the ambient solution from tissue incubated in water for 6 hours was removed, and examined for nucleotides by the method described for tissue. After initial chromatography with n-propanol:ammonium acetate, no U-V absorbing areas could be detected on the paper by visual means. However, treatment of the paper with phosphate reagent revealed inorganic phosphate and phosphate-containing compounds.

Table 10

Wheat aleurone tissue (4 gm) was prepared from half-seeds previously imbibed for 24 hours. After subsequent incubation in water, the resulting ambient solution was examined for the presence of bases and nucleotides. Levels in nmoles. (-) denotes level too low to be detected.

Solvent System	Compound	Period of incubation (hrs) 2	4	6
Iso-propanol : HCl	Uracil	1.5	-	-
	Cytosine	4.0	2.0	2.0
	Adenine	0.4	-	-
	Guanine	-	-	-
Iso-butyric acid : ammonia	UTP	9.6	-	-
	UDP + UDPG	4.8	-	-
	ATP	1.3	-	-
	ADPG + UMP	-	-	-
	ADP	-	-	-
	AMP	-	-	-

Table 11

Wheat aleurone tissue (4 gm) was prepared from half-seeds imbibed for 24 and 26 hours. Changes in the levels of ATP, UDPG and UTP were followed over the ensuing 6 hours in water. (Actual nucleotide levels shown in Table 9). Values in nmoles/sample.

Nucleotide	Imbibition Period(hrs.)	Period of incubation (hrs.) 0-2	0-4	0-6
ATP	24	-46	-41	-10
	26	-8	-3	8
UTP	24	25	25	42
	26	36	29	36
UDPG	24	-113	-79	-50
	26	-40	-24	2
UDPG + UTP	24	-88	-54	-8
	26	-4	5	38

Thus, although some phosphorylated compounds leak from the tissue into the ambient solution, the decline in the levels of nucleotides cannot be ascribed to the leakage of the nucleotides themselves. This work however does not rule out the possibility that the nucleotides are degraded to their corresponding bases in the ambient solution, and that these in turn are metabolised to non -U.V. absorbing compounds. Such a process seems unlikely.

12. The Effect of Tissue Equilibration on α-amylase Production in Response to GA

To examine more fully the changes in free nucleotides after short-term applications of GA, it was necessary to eliminate, as far as possible, the early fluctuations induced during the preparation of the tissue. The method adopted was to allow the tissue to equilibrate for 6 hours in water at 30°C, before applying the treatments.

Several reports had indicated that an extension of the imbibition period for barley aleurone either reduced the induction period, or increased α-amylase production in response to GA (McLeod, Duffus and Horsfall, 1966; Yung and Mann, 1967).

The induction period for α-amylase in wheat aleurone was examined by preparing four portions of 4 gm aleurone tissue two of which were incubated in 10 ml of water or GA immediately after isolation. The other two were left for 6 hours in 10 ml water, then 5 ml of the ambient solution replaced with an equal volume of water or GA (200 µg/ml).

α-amylase production was examined at intervals up to 12 hours after the addition of the hormone and the results are shown in Table 12, and Fig.6.

Table 12

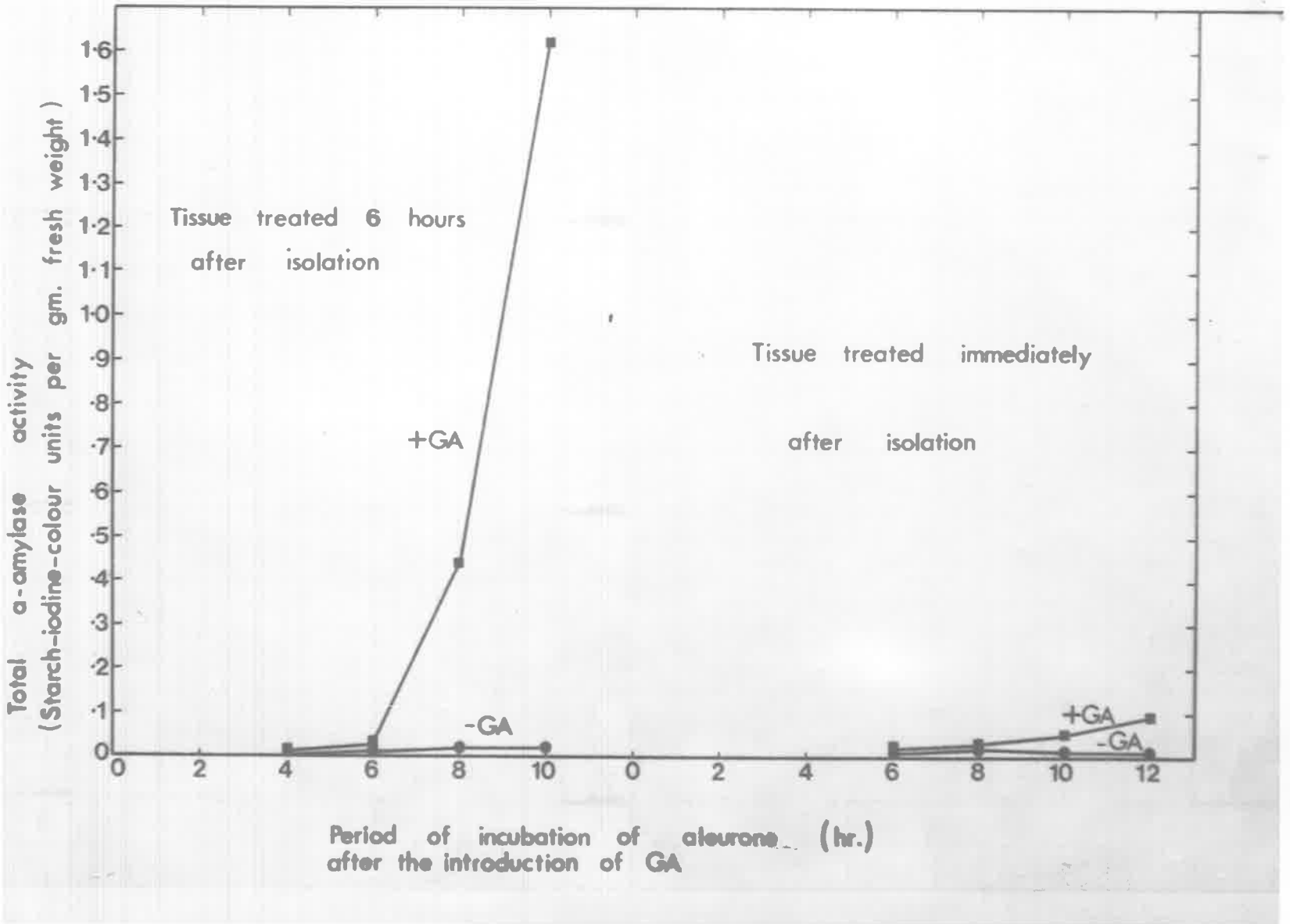
Determination of the induction period for α-amylase in response to GA. Samples (4 gm) of aleurone tissue were treated with GA either immediately after preparation or following an equilibration period of 6 hours. Values are Starch-Iodine Colour units per gm fresh weight of tissue. (-) denotes enzyme not detected.

Time after GA applied (hrs.)	Treatment -GA			+GA		
	Ambient	Tissue	Total	Ambient	Tissue	Total
(a) Tissue treated immediately after isolation						
6	0.01	-	0.01	0.01	0.01	0.02
8	0.01	0.01	0.02	0.01	0.02	0.03
10	-	0.01	0.01	0.03	0.02	0.05
12	-	0.01	0.01	0.03	0.06	0.09
(b) Tissue treated 6 hours after isolation						
4*	-	-	-	-	0.01	0.01
6*	-	0.01	0.01	-	0.03	0.03
8	-	0.02	0.02	0.06	0.38	0.44
10	-	0.02	0.02	0.62	1.00	1.62

*Average of two separate experiments.

Fig. 6.

Determination of the induction period for α-amylase in response to GA. GA was added either to freshly isolated tissue, or to tissue which had received a pre-incubation period of 6 hours in water. Enzyme activity was measured at intervals up to 12 hours after the addition of GA.


Total α-amylase activity
(Starch-iodine-colour units per gm. fresh weight)
1·6
1·5
1·4
1·3
1·2
1·1
1·0
·9
·8
·7
·6
·5
·4
·3
·2
·1
0
Tissue treated 6 hours after isolation
+GA
-GA
Tissue treated immediately after isolation
+GA
-GA
0 2 4 6 8 10 0 2 4 6 8 10 12
Period of incubation of aleurone (hr.) after the introduction of GA

A comparison of the total amounts of α-amylase produced in the presence and absence of GA shows that the induction period for tissue treated immediately after isolation is between 8 and 10 hours. For tissue treated 6 hours later, this period is between 6 and 8 hours. Thus the extra 6 hours of imbibition has resulted in a decrease of approximately 2 hours in the induction period and this is in agreement with other results for barley.

13. The Effect of Actinomycin D on the GA-Induced Production of α-amylase

It was reported by Click and Hackett (1963) that the inhibition by Actinomycin D of certain metabolic effects induced in potato tuber tissue by slicing, was progressively lower as the time of addition of the inhibitor was delayed. Similarly, Varner and Chandra (1964) observed that Actinomycin D inhibited the GA-stimulated production of α-amylase by barley aleurone if applied with the hormone, but was ineffective if added 7 hours later. Since it was proposed to allow freshly prepared wheat aleurone to equilibrate for 6 hours in water before use, the effect of this treatment on the sensitivity of the tissue to Actinomycin D was examined.

Samples of aleurone (0.5 gm) were incubated in 4 cm petri dishes with 1.6 ml of test solution. One set of treatments was applied to freshly prepared tissue, the other after the tissue had been incubated in 1.6 ml of water for

6 hours. ∝-amylase production was measured 24 hours after addition of the test solutions. The results are shown in Table 13.

Although there is a slight stimulation at 1 µg/ml, as the concentration of Actinomycin D is increased the production of ∝-amylase is progressively reduced. An increase from 30 to 100 µg/ml causes a greater reduction than any other increment.

Table 13.

Production of ∝-amylase by wheat aleurone tissue in the presence of GA (100 µg/ml) and different concentrations of Actinomycin D. The tissue was treated either immediately after preparation or following an equilibration period of 6 hours in water. Values in Starch-Iodine-Colour units per gm fresh weight of tissue.

(a) Treatments applied immediately after tissue isolation

	GA (100 µg/ml) + Act. D at:- (µg/ml).					
	Control	0	1	5	30	100	250
Tissue	0.1	13.3	16.7	12.5	13.9	0.9	0.4
Ambient	-	43.8	45.8	32.6	22.8	0.4	0.1
Total	0.1	57.1	62.5	45.1	36.7	1.3	0.5
Ratio of ambient to tissue	-	3.29	2.74	2.61	1.64	0.44	0.25

(b) Treatments applied after tissue equilibrated for 6 hours

Tissue	0.1	27.8	33.4	23.8	17.8	2.4	0.6
Ambient	-	61.6	57.1	42.1	23.2	1.9	0.6
Total	0.1	89.4	90.5	65.9	41.0	4.3	1.2
Ratio of ambient to tissue	-	2.22	1.71	1.77	1.30	0.79	1.00

The ratio of the amount of ∝-amylase in the ambient solution to that in the tissue falls consistently as the level of Actinomycin D increases. Since enzyme production by the tissue is only inhibited at relatively high concentrations, it follows that release in comparison with production of ∝-amylase, is more sensitive to Actinomycin D.

14. Incorporation of ^{32}P into the Free Nucleotides of Wheat Aleurone Tissue

Portions of aleurone tissue (4 gm) were weighed into 9 cm petri dishes, 10 ml of water added, and left to equilibrate for 6 hours. The tissue was then rinsed by removing 5 ml of the ambient solution, adding 10 ml of water to the petri dish and, after mixing the contents, removing 10 ml of the solution. The procedure was repeated with another 10 ml of water and then the volume of ambient solution made to 10 ml again by the addition of 5 ml aliquots of water of GA (200 µg/ml). After thorough mixing, the treatments were incubated for periods of 1 or 3 hours. ^{32}P was introduced 15 min. before the incubations were terminated, by rinsing the tissue again as described above and making the ambient solutions back to volume with 5 ml aliquots of water or GA (200 µg/ml) containing 50 µCi ^{32}P and 15 µg KH_2PO_4 as carrier.

The tissue was then washed in ice-cold 10^{-4} Molar KH_2PO_4, ice-cold water and transferred to the cold room for nucleotide extraction. Approximately 6 minutes elapsed

between the time of removal of the tissue from the isotope laboratory and its extraction, since the isotope laboratory was sited some distance from the cold room. Means of inhibiting enzyme activity during this period were examined. The tissue was either frozen in liquid nitrogen or placed in an ice-bath, the former being subsequently thawed in T.C.A. before extraction. Nucleotide levels were determined after each procedure and are shown in Table 14.

Table 14

The effects of freezing in liquid nitrogen or cooling in an ice bath on the amounts of nucleotides extracted from aleurone tissue. The period of incubation in the presence and absence of GA was 60 min., and the time the tissue was held frozen or on ice was 6 min. Nucleotide levels (nmoles per 4 gm fresh weight) were determined after thawing and extraction in 5% TCA at 4°C.

Nucleotide	Liquid Nitrogen -GA	Liquid Nitrogen +GA	Ice Bath -GA	Ice Bath +GA
NAD	34	42	34	41
AMP	45	22	-	-
ADP	77	71	20	19
ATP	64	109	212	210
CDP	18	12	-	-
CTP	33	31	87	81
GDP	15	15	-	-
GTP	10	17	35	41
UMP	4	-	-	-
UDP	34	18	-	-
UTP	36	61	135	139
UDPG	163	162	196	193

Since the nucleoside monophosphates were not normally detected in aleurone tissue, their presence in tissue frozen in liquid nitrogen indicated that extensive degradation had

occurred. Neither NAD nor UDPG were affected suggesting that the breakdown was due to the action of one or more phosphatases, possibly during the process of thawing. Similar evidence for degradation in ice-cooled tissue was not observed and it was assumed that adequate inhibition of enzyme activity was achieved by this procedure.

The final chromatograms of radioactive nucleotides were fastened to x-ray films for 6 hours. The films were developed, and the areas denoting the presence of radioactivity compared with the U-V absorbing areas on the chromatograms. Reference to the U-V photograph and the autoradiograph in Figs.3 and 7 respectively shows that radioactive areas and absorbing areas closely coincide. The absorbance of the compounds was measured, then portions of the eluates dried on to copper planchets and the amount of radioactivity estimated with the gas-flow counter. After making corrections for both decay (theoretical decay curve) and the daily variation in the sensitivity of the counter (standard ^{32}P planchet), the incorporation of ^{32}P was expressed as counts per min. per nmole. The actual data are shown in Appendix 2, and the means in Table 15.

Each section of Table 15 was statistically analysed as a 6 x 2 x 2 factorial with randomised complete-block design, after excluding certain values which were outside the limits of accurate detection. Thus, the values for UDP were omitted from the analysis of nucleotide levels, and those of UDP and NAD

Fig 7

Radioautograph of two-dimensional chromatogram, corresponding to U-V photograph in Fig. 3.

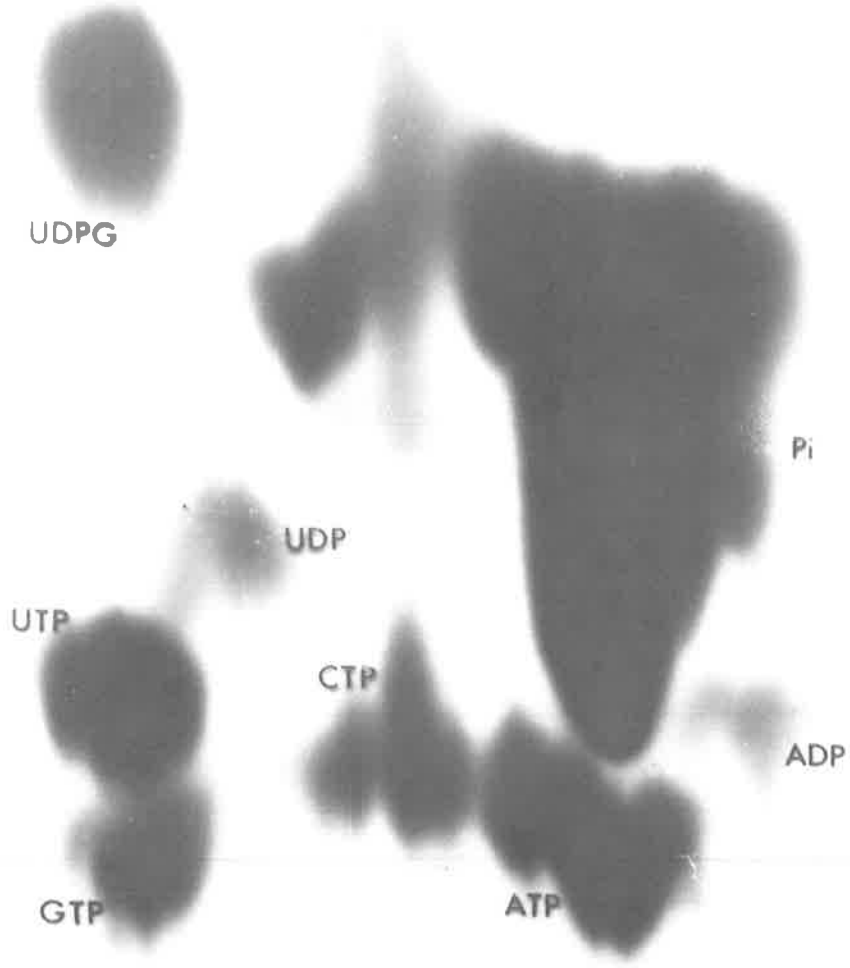

UDPG
Pi
UDP
UTP
CTP
ADP
GTP
ATP

Table 15

The incorporation of ^{32}P into the free nucleotides of wheat aleurone tissue. ^{32}P (50 μCi) was added to the ambient solutions for the final 15 min. of the indicated times of treatment. Nucleotide levels are given as nmoles per 4 gm fresh weight, specific activities as c.p.m./nmole. Values in parentheses are relative to the control as 100.

(a) Nucleotide levels

	1 Hour			3 Hours		
Nucleotide	-GA	+GA	Mean	-GA	+GA	Mean
NAD	42	39(93)	40	34	38(112)	36
ADP	21	18(86)	20	20	18(86)	19
ATP	227	225(99)	226	230	234(102)	232
CTP	53	50(94)	52	63	56(89)	60
GTP	36	27(75)	31	28	36(128)	32
UTP	129	121(94)	125	132	130(98)	131
UDP	5	3(60)	4	7	8(115)	8
UDPG	196	151(77)	174	179	192(107)	185
Mean *	100	90	95	98	100	99

NAD	ADP	ATP	CTP	GTP	UTP	UDP	UDPG
38	19	228	55	32	127	6	178

* UDP omitted from calculation of mean.

(b) Specific Activities

	1 Hour			3 Hours		
Nucleotide	-GA	+GA	Mean	-GA	+GA	Mean
NAD	9	6(67)	8	10	1(10)	6
ADP	343	455(133)	399	450	361(80)	405
ATP	776	896(115)	836	896	752(84)	824
CTP	631	850(135)	741	648	528(81)	588
GTP	566	703(124)	635	666	537(81)	602
UTP	816	1016(124)	916	1010	758(75)	884
UDP	1632	5314(326)	3473	2134	1558(73)	1846
UDPG	348	457(131)	402	377	281(74)	329
Mean *	580	730	655	674	536	605

NAD	ADP	ATP	CTP	GTP	UTP	UDP	UDPG
6	401	831	680	621	904	2660	373

* UDP and NAD omitted from calculation of mean.

Table 15 (Contd.)

(c) Total counts incorporated ($x10^3$)

Nucleotide	1 Hour -GA	1 Hour +GA	1 Hour Mean	3 Hours -GA	3 Hours +GA	3 Hours Mean
NAD	0.38	0.23(60)	0.30	0.34	0.04(12)	0.19
ADP	7.2	8.2(114)	7.7	8.6	6.6(77)	7.6
ATP	175.6	201.2(115)	188.4	205.2	174.0(85)	189.6
CTP	33.4	41.0(123)	37.2	40.8	30.1(74)	35.4
GTP	20.2	18.1(90)	19.1	19.0	19.1(100)	19.0
UTP	103.7	121.0(117)	112.3	132.7	98.7(74)	115.7
UDP	8.2	15.9(194)	12.0	14.9	12.5(84)	13.7
UDPG	68.6	67.8(99)	68.2	67.2	53.6(80)	60.4
Mean*	68.1	76.2	72.2	78.9	63.7	71.3

NAD	ADP	ATP	CTP	GTP	UTP	UDP	UDPG
0.24	7.7	188.9	36.5	19.1	113.7	12.8	65.1

* UDP and NAD omitted from calculation of mean.

omitted from the analyses of both specific activities and total counts incorporated.

The mean levels of all nucleotides in the presence and absence of GA were 94 and 99 nmoles respectively. This difference is significant at the 5% level (L.S.D.=4). After 1 hour, the mean nucleotide level was depressed by GA compared to both the control and the values with and without GA at 3 hours (significant at the 1% level). Moreover, during the first hour the level of UDPG was depressed by GA (significant at the 5% level). This effect was transient and could no longer be detected by 3 hours. There was no significant difference in the amount of UDPG either with or without GA at 3 hours, however, the

level in water-treated tissue was lower than the corresponding treatment at 1 hour (significant at the 5% level).

A further analysis of nucleotide levels was based on the null hypothesis of no difference between treatment means. After 1 hour, the levels of both ADP and UTP were depressed by GA, the effects being significant within 95% confidence limits. No other significant effects of treatment with GA were noted.

Factorial analysis of the specific activities showed that the mean value over all nucleotides was increased by GA after 1 hour (significant at the 1% level) and depressed after 3 hours (5% level) compared to the respective controls. Further, the mean value after 3 hours with GA was significantly lower than that following 1 hour with GA (1% level). For both periods of incubation, all nucleotides responded in the same way to the presence of the hormone. To reduce the variance, the data were transformed by making the control values equal to 100 and adjusting the other values accordingly. A factorial analysis carried out on the transformed data verified the absence of a specific effect of the GA on any one nucleotide. The mean specific activity for each nucleotide, in the presence and absence of GA, did not change significantly with time of treatment.

It is evident that the individual compounds exhibit different amounts of incorporation of ^{32}P. The least significant differences for the average values shown in

Table 14 are 117 (5%) and 158 (1%) c.p.m./nmole, and it can be seen that the nucleotides form 3 groups comprised of UTP and ATP, GTP and CTP, and UDPG and ADP. No differences occurred between the two members of a group, but each group was significantly different from the others.

The analysis of the total amount of counts incorporated showed that the mean value, after 3 hours in GA, was significantly lower than both the control and the values at 1 hour. No other differences were significant.

Thus, in aleurone tissue treated with GA for 1 hour, nucleotide levels are depressed, the specific activity is increased and there is no change in the total amount of counts incorporated compared to a water-treated control. On the other hand, after three hours treatment both the specific activity and the total counts incorporated are depressed in the presence of GA, but nucleotide levels are not altered. These results are consistent with an initial enhancement and subsequent depression of the rate of metabolism of the phosphate moiety of the free nucleotide pool by GA.

A comparison of the mean nucleotide levels obtained after incubation of aleurone for 1 hour in the absence of GA (total incubation time 7 hours) as shown in Table 15, with those obtained after incubation in water for 6 hours as given in Table 7, indicates certain differences. CTP and UTP

have increased in amount by 18% and 55% respectively, whilst the level of GTP has fallen by 14%. However, the variance of the individual values (Appendices 1 and 2) was sufficiently high to preclude these differences from being significant. The mean values of ADP, ATP and UDPG were almost identical in each case.

In some of the radioautographs prepared during these experiments (Fig.7) there were indications that inorganic phosphate and ATP were not completely separated on the final chromatogram. Where this occurred, the U.V absorbing area was compared with the radioautograph, and the portion not co-chromatographing with inorganic phosphate was cut from the paper, eluted, and the eluate used to determine the specific activity of ATP. It was assumed that the value so obtained was identical with all of the ATP extracted from the tissue. The remaining portion was eluted, the absorbance measured, and the total absorbance calculated from the two separate readings.

15. Time-Course Study of the Incorporation of ^{32}P into Free Nucleotides in Response to GA.

Since it was apparent that GA caused changes in the rate of incorporation of ^{32}P into nucleotides, a more detailed investigation was undertaken. However, the conditions under which the tissue was incubated were modified from those used previously.

In an experiment designed to examine the effect of

the addition of inorganic phosphate to the incubation medium, it was observed that the nucleotides extracted from a sample of aleurone immediately after the equilibration period, had considerably lower specific activities than a similar sample incubated, in the absence of GA, for a further 60 min. Portions of tissue (4 gm) were weighed into 9 cm petri dishes and left for 6 hours in 10 ml of 0.25 mM KH_2PO_4. One sample was then rinsed by discarding 5 ml of the ambient solution, adding 10 ml of 0.25 mM KH_2PO_4 and, after mixing, removing 10 ml of the solution. The latter step was repeated and then the volume made to 10 ml again by the addition of 5 ml of 0.25 mM KH_2PO_4 containing 50 µCi ^{32}P. After 15 min. the tissue was rinsed and extracted for nucleotides. The same procedure, without the addition of ^{32}P, was carried out with another sample which was then incubated for 45 min., rinsed again as described above and treated with 50 µCi ^{32}P for 15 min. This was also extracted. The results are shown in Table 16. It is apparent that the specific activities have been considerably stimulated by the additional 60 min. of incubation.

To examine this effect further, similar batches of aleurone were incubated in conditions of constant agitation. The tissue was weighed into 250 ml flasks with 30 ml of mM KH_2PO_4 and incubated for 6 hours on a reciprocating water bath. The volume of solution was increased above that

used in the petri dishes to permit adequate mixing of the contents of the flasks. One of the samples was then drained, and the ambient solution replaced with 30 ml of mM KH_2PO_4 containing 50 μCi ^{32}P. After treatment for 15 min., the tissue was drained, rinsed and extracted for nucleotides. Portion (7.5 ml) of the ambient solution of a second sample was discarded and replaced with 7.5 ml of mM KH_2PO_4 for a further 45 min. The solution was then discarded, and another 30 ml of mM KH_2PO_4, containing 50 μCi ^{32}P, added for 15 min., after which this tissue was also drained, rinsed and extracted. From Table 16, it can be seen that this latter treatment has resulted in relatively little stimulation occurring after the additional 60 min. of incubation. The results thus suggested that the procedure of rinsing the tissue after equilibrating in petri dishes increased the rate of incorporation of ^{32}P. Since it was not known whether small variations in the efficiency of rinsing caused changes in the degree of stimulation, the technique of continuous shaking was adopted. α-amylase production, in response to GA, was compared for the two types of incubation and the results are shown in Table 17.

It is evident that the amount of α-amylase produced by both GA- and water-treated tissue is lower when the tissue is constantly agitated. However, the response to GA by the shaken treatment is 275:1 compared with 46:1 for the non-shaken, a six-fold increase.

Table 16

Aleurone tissue (4 gm) was equilibrated in petri dishes or on a shaking water bath. One sample from each was then treated with ^{32}P for 15 min. and the nucleotides extracted. Further samples were treated as described in the text and incubated for an additional 60 min. ^{32}P was introduced for the final 15 min. and the nucleotides estimated. Specific activities are expressed as c.p.m. per nmole. Values in parentheses are relative to the control as 100.

	Petri Dish*		Shaken	
Nucleotide	Control	60 min.	Control	60 min.
NAD	1	1 (100)	1	1 (100)
ADP	50	84 (168)	30	42 (140)
ATP	116	183 (158)	64	70 (109)
CTP	74	87 (118)	52	51 (98)
GTP	99	146 (147)	70	72 (103)
UTP	141	208 (148)	68	80 (118)
UDP	252	457 (181)	86	251 (292)
UDPG	42	76 (181)	26	31 (119)

*Average of two determinations.

Table 17

Batches of aleurone (4 gm in each) were held stationary in petri dishes or constantly agitated in 250 ml flasks. The volumes of ambient solution were 10 and 30 ml respectively. Both treatments were left for 6 hours after preparation of the tissue before the addition of GA. α-amylase was assayed after a further 22 hours. GA concentration: 100 µg/ml. Values are expressed in Starch-Iodine-Colour units per gm fresh weight of tissue (Average of duplicate experiments).

	Shaken		Not shaken (Petri dish)	
	-GA	+GA	-GA	+GA
Tissue	0.1	10.7	0.4	16.9
Ambient	-	16.8	0.6	29.0
Total	0.1	27.5	1.0	45.9

As described in the previous section, ATP and inorganic phosphate were sometimes not completely separated on the final chromatogram. It was found that the mobility of inorganic phosphate was greater when the pH of the final solvent was 3.8 than at 7.5 (P-L Biochemicals Circular OR-10). Comparison of the U-V photograph in Fig.8 and the radioautograph in Fig.9 prepared after adopting this modification, shows that the two substances were completely separated.

A time-course study of the effect of GA on the incorporation of ^{32}P into nucleotides was carried out in the following way. After isolation, the tissue was placed in 250 ml flasks (4 gm in each) with 30 ml of water, and the flasks were shaken on a reciprocating water bath maintained at 30°C. After 6 hours, 7.5 ml of solution were removed, replaced with 7.5 ml of either water or GA (400 µg/ml) and the flasks further shaken for periods up to 120 min. 15 min. before the incubations were terminated, the tissue was completely drained, and the ambient solutions were replaced with fresh solutions of water or GA, 0.05 mMolar with respect to KH_2PO_4 and containing 50 µCi ^{32}P. After 15 min. in ^{32}P, the tissue was drained, rinsed, placed in an ice bath and transferred to the cold room for nucleotide estimation. The means of two experiments are shown in Table 18 and the experimental data is given in Appendix 3. The specific activities are depicted graphically in Fig.10.

Each section of Table 18 was analysed as a 6 x 2 x 4

Fig 8.

U-V photograph of two-dimensional chromatogram.

Solvent 1 : iso-butyric acid : ammonia : water

(57 : 4 : 39 v/v/v)

Solvent 11: 95% ethanol : IM ammonium acetate

pH 3.8

(0.01 M in EDTA) (7 : 3 v/v).

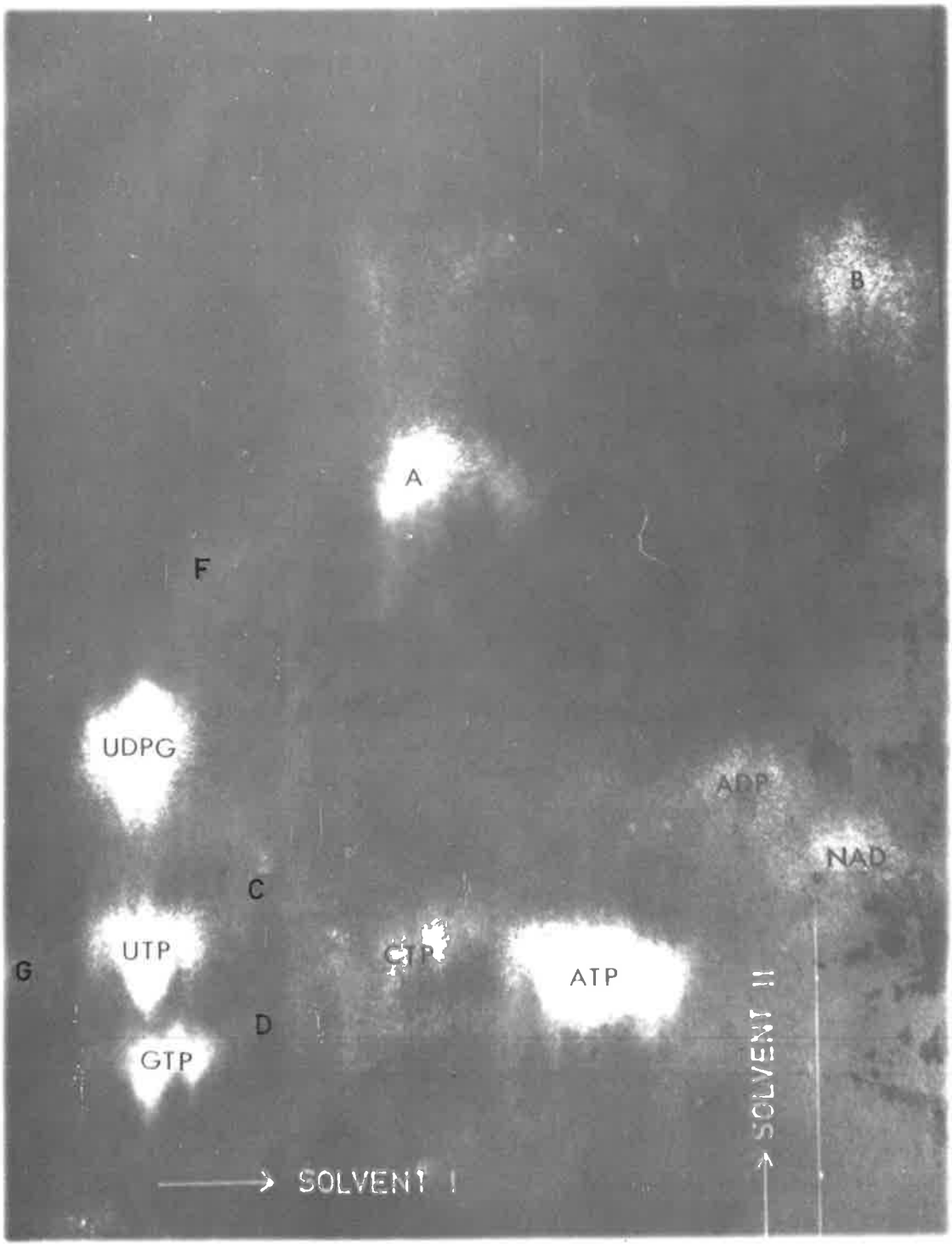

B
A
F
UDPG
ADP
NAD
C
UTP
GTP
ATP
G
D
GTP
SOLVENT II
SOLVENT I

Fig. 9

Radiograph of two-dimensional chromatogram corresponding to U-V photograph in Fig. 8.

FIG. 9

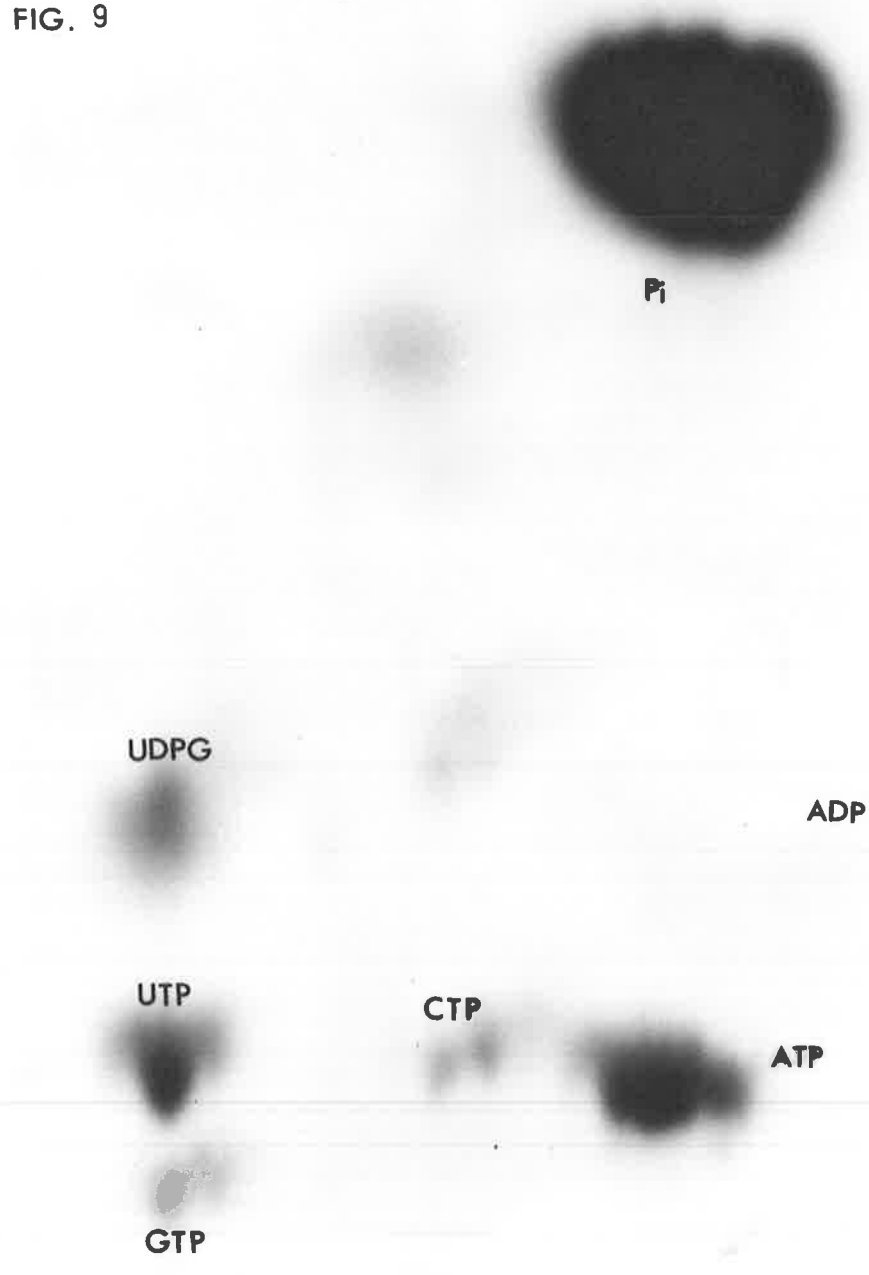


Table 18

Time-course study of the effect of GA on the incorporation of ^{32}P into the free nucleotides of wheat aleurone tissue. Aleurone tissue (4 gm) was incubated in flasks on a reciprocating water bath at 30°C. 50 µCi ^{32}P was added to the ambient solutions for the final 15 min. of the indicated times of treatment. (Average of two determinations.)

(a) Nucleotide level (nmoles)

	Time of incubation (min.)								
	15		30		90		120		
	-GA	+GA	-GA	+GA	-GA	+GA	-GA	+GA	Mean
NAD	52	52	40	52	41	52	46	62	50
ADP	18	22	21	22	20	20	17	18	20
ATP	321	313	307	286	281	277	310	294	298
CTP	126	120	114	114	128	102	110	106	115
GTP	52	48	54	50	54	52	46	52	51
UTP	250	232	228	214	210	231	244	220	228
UDP	10	8	12	10	15	9	7	10	10
UDPG	214	193	190	194	166	168	208	190	191
Mean*	147	140	136	133	128	129	134	134	

Mean*	15	30	90	120	-GA	+GA
	144	135	129	137	138	134

* excluding UDP

(b) Specific activity (c.p.m./nmole)

	15		30		90		120		
	-GA	+GA	-GA	+GA	-GA	+GA	-GA	+GA	Mean
NAD	15	14	13	18	17	15	12	14	15
ADP	151	124	133	160	165	150	190	168	155
ATP	265	260	314	375	361	372	380	372	337
CTP	160	151	166	228	200	246	246	240	205
GTP	246	228	287	342	320	330	366	372	311
UTP	264	257	312	372	362	380	376	374	337
UDP	688	862	680	1682	609	857	770	712	857
UDPG	140	132	156	156	172	186	194	185	165
Mean*	204	192	228	272	264	277	292	285	

Mean*	15	30	90	120	-GA	+GA
	198	250	270	289	247	257

* excluding UDP and NAD

Table 18 (Contd.)

(c) Total counts incorporated (c.p.m. x 10^3)

Time of incubation (min.)

	15		30		90		120		
	-GA	+GA	-GA	+GA	-GA	+GA	-GA	+GA	Mean
NAD	0.78	0.73	0.52	0.94	0.70	0.78	0.55	0.87	0.73
ADP	2.7	2.7	2.8	3.4	3.2	2.9	3.1	3.2	3.0
ATP	85.4	81.0	96.3	105.8	100.7	102.2	119.8	111.4	100.3
CTP	19.9	18.2	18.5	25.4	25.0	24.4	27.2	25.5	23.0
GTP	12.8	10.6	15.5	16.9	17.8	17.2	17.0	18.4	15.8
UTP	66.3	59.3	71.4	77.4	74.4	86.0	93.2	84.9	76.6
UDP	6.9	6.9	8.2	16.8	9.1	7.7	5.4	7.1	8.5
UDPG	30.2	25.4	29.0	29.4	28.6	31.2	41.2	35.2	31.3
Mean*	36.2	32.9	38.9	43.0	41.6	44.0	50.2	46.4	

Mean*	15	30	90	120	-GA	+GA
	34.5	41.0	42.8	48.3	41.7	41.6

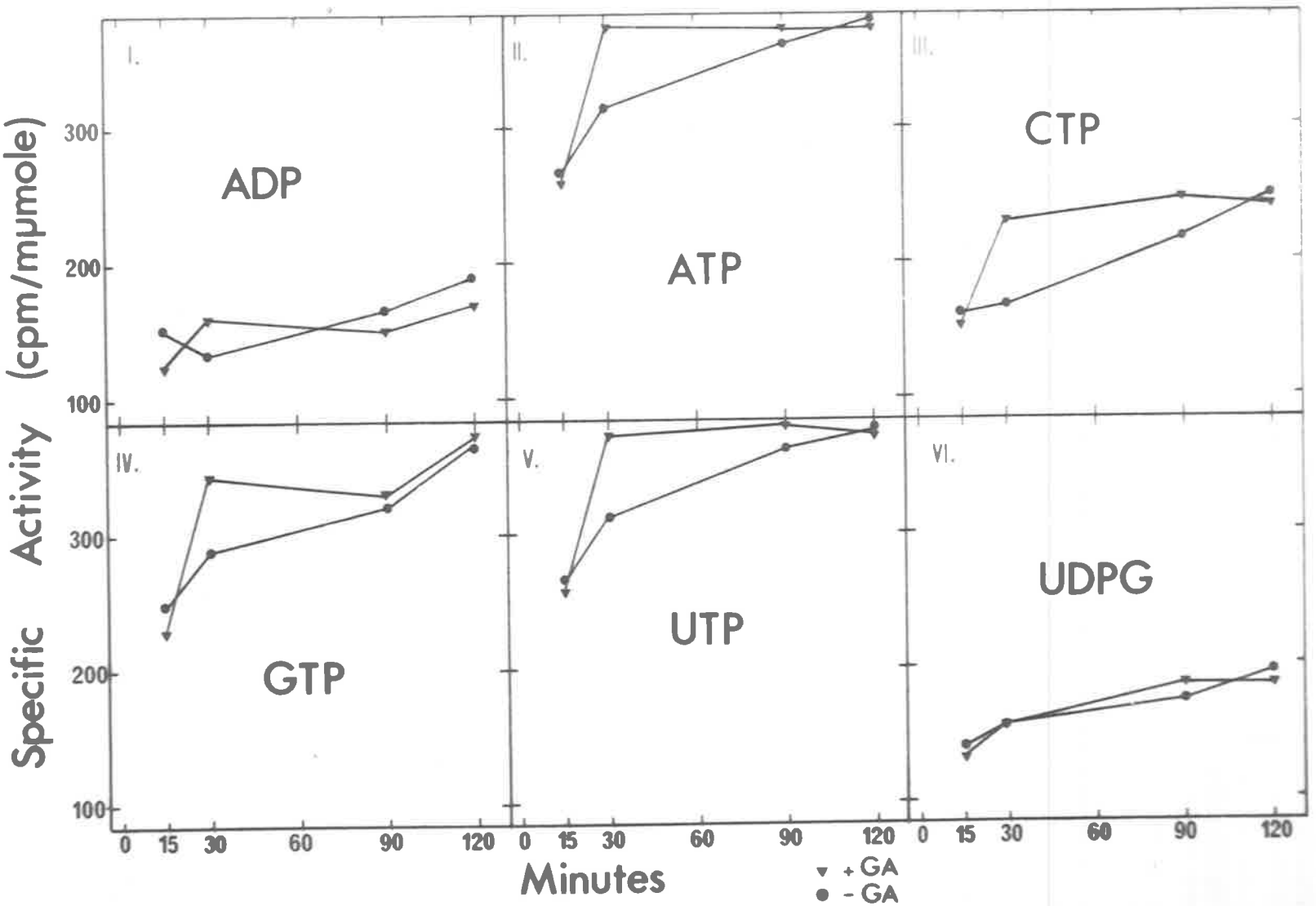
* excluding UDP and NAD

factorial with randomised complete-block design, after excluding the values for UDP from nucleotide levels, and those of UDP and NAD from both specific activities and total counts incorporated.

No significant differences occurred from the analysis of nucleotide levels. The mean value for the specific activity at 30 min. was 26% above that at 15 min. significant at the 5% level), 36% above after 90 min. (1%), and 46% higher by 120 min. (1%). Thus although the period allowed for the uptake of ^{32}P was the same in each case (15 min.), the average specific activity increased as the total time of incubation was extended. ATP, UTP and GTP were more highly radioactive

Fig 10

Time course of the effect of GA on the specific activities of free nucleotides.


I.
ADP
II.
ATP
III.
CTP
IV.
GTP
V.
UTP
VI.
UDPG
Specific Activity (cpm/mμmole)
300
200
100
0 15 30 60 90 120
Minutes
▼ + GA
● - GA

than CTP, UDPG and ADP (significant at the 1% level), and CTP more than ADP (5%).

In Table 19 for each time, the specific activities of the compounds are compared by expressing the values as a percentage of the value for the compound with the highest specific activity. As no significant hormonal effects were detected by the preliminary statistical analysis, the results in the Table are the averages of both treatments. It is clear that there is no difference between the specific activities of ATP and UTP and these two compounds are the most highly radioactive. The specific activity of GTP was only 8% lower than that of ATP and UTP, however, the amount in the tissue was some 80% less. Moreover, CTP was more than twice as abundant as GTP, but was 30% less radioactive. The relative uptake of ^{32}P by ADP and UDPG was almost identical, and that of NAD considerably lower than any of the other nucleotides.

Although the total counts incorporated increased with time, the statistical analysis indicated that a significant difference occurred only between the mean values at 15 and 120 min. (5% level). The mean value at 120 min. was 40% higher than at 15 min. ATP incorporated more counts than the other nucleotides (1% level). The uptake by UTP was 24% lower than that of ATP, but was significantly higher than the remaining four compounds (1% level). UDPG showed greater incorporation than GTP (5% level) and

Table 19

The incorporation of 32P by the free nucleotides of wheat aleurone tissue. The mean specific activity of each nucleotide was calculated for the specified incubation time, and is expressed relative to the highest value as 100.

	Time of incubation (min.)				
Nucleotide	15	30	90	120	Mean
UTP	99	99	100	100	100
ATP	100	100	99	100	100
GTP	90	91	88	98	92
CTP	59	57	60	64	61
UDPG	52	45	48	50	49
ADP	52	42	42	48	46
NAD	6	5	4	3	4

ADP (1% level), but was not significantly different from CTP. No difference was detected between CTP and GTP, however both were significantly higher than ADP (1% and 5% levels, respectively). Since the relative abundance of each nucleotide occurs in the same order, it is clear that the incorporation of ^{32}P is in direct proportion to the amount of the compound present.

Nucleotide levels were further analysed by treating each separately as a 4 x 2 factorial with randomised-complete block design. Both NAD and UDPG showed a significant variation with time (5% level) when the values in the presence and absence of GA were averaged. The least significant differences were 6 and 26 respectively. The level of NAD at 30 and 90 min. was depressed

below that at 15 min., whereas the drop in UDPG was not apparent until 90 min. After 120 min., the initial levels were regained in each case. In addition, the mean treatment effect independent of time, was significant for NAD and CTP (1% and 5% levels respectively), indicating that the level of NAD was stimulated in the presence of GA, whilst that of CTP was depressed. The interaction of time with treatment was not significant, thus no nucleotide was specifically responsive to GA at a particular time.

The specific activities at each time of incubation were analysed separately as 6 x 2 factorials with randomised complete-block design. After 15 min. the average value with GA was below that of the control. The means were 192 and 204 c.p.m./nmole respectively (least significant difference 12 at the 5% level). But after a further 15 min., the corresponding values were 272 and 228 (least significant difference 32 at the 5% level). Thus, in the presence of GA, the mean specific activity at first falls and then rises. No significant effects were noted after 90 and 120 min.

The null hypothesis of no difference between treatment means was applied to the change in value between 15 and 30 min., for each treatment. The rise of 24 c.p.m/nmole in the mean value of the control was not significant, whereas the difference of 80 c.p.m./nmole following GA treatment was significant within 90% confidence limits.

A further analysis was performed with the specific

activities for the four nucleoside triphosphates alone. The effect of GA, after 30 min., showed significance at the 1% level compared to 5% when all the nucleotides (except UDP and NAD) were analysed. Also, although there was no significant interaction of nucleotides with treatments, the data suggested that GA had a greater effect on one of the triphosphates than on the others. The variance of the values was therefore reduced by transforming the control levels to 100 and adjusting the other treatments accordingly. The transformed values are shown in Table 20. Statistical analysis of the transformed data was carried out as a 4 x 4 factorial of randomised complete-block design.

The least significant differences for time of incubation were 4 (5% level) and 6 (1% level). Thus after 15 min. GA has depressed the incorporation of ^{32}P into the triphosphates, after a further 15 min. greatly increased it, and over the next 90 min. the effect of GA disappears. The mean value for CTP was higher than the other three compounds (5% level).

Table 20

The specific activities of the nucleoside triphosphates shown in Table 19, are here expressed as the percent ratio of GA-treated to control.

	Time of incubation (min.)				
Nucleotide	15	30	90	120	Mean
ATP	98	124	105	98	106
CTP	95	138	127	98	114
GTP	93	126	106	100	106
UTP	97	124	106	98	106
Mean	96	128	111	99	

The interaction of nucleotide with time was also highly significant, the least significant differences being 12 (5% level) and 16 (1% level). In the presence of GA, the rise is the specific activity of CTP is clearly greater than for the other triphosphates after 30 min. (5% level) and 90 min. (1% level) but no different after 120 min. ATP, UTP and GTP also show a transient increase, with maxima at 30 min., but in each case return to the control level by 90 min.

As previously described, the specific activity of CTP is less than that of ATP, UTP and GTP. Also, the relative response to GA is greatest for CTP. The actual changes in specific activities are given in Table 21 where it can be seen that at 30 min., there is little difference in the effect of GA between the triphosphates, although the relative increase in CTP was about 13% greater at that time. At 90 min., however, both the relative and absolute response of CTP to GA is more evident. The results are therefore consistent with a general increase in phosphate metabolism of all the triphosphates, followed by a specific requirement for CTP. This specific effect is more apparent when the data of Table 18 are further examined. Whereas the increase in specific activity of ATP, UTP, GTP and CTP at 30 min. is due mainly to an increase in the total number of counts incorporated, the increase in specific activity of CTP at 90 min. results from a drop of 20% in the level of this nucleotide.

Table 21

Changes in specific activities of the nucleoside triphosphates. The difference between the values with and without GA, as shown in Table 17, are expressed below as c.p.m. per nmole. The relative effect compared to the control as 100 is shown in parentheses.

	Time of incubation				
Nucleotide	15	30	90	120	Total
ATP	-5(98)	61(124)	11(105)	-8(98)	59
CTP	-9(95)	62(138)	46(127)	-6(98)	93
GTP	-18(93)	55(126)	10(106)	6(100)	53
UTP	-7(97)	60(124)	18(106)	-2(98)	69
Total	-39	238	85	-10	274

Aliquots of the radioactive solutions prepared for the experiment were counted, the values adjusted to give the total c.p.m. added in each case, and the data analysed as a 4 x 2 factorial. The mean value was 26.7×10^6 c.p.m./30 ml of solution and the standard deviation of the mean was $\pm 1.2 \times 10^6$ c.p.m. ($P=0.99$). There were no significant differences between means and it was therefore concluded that variation in the amount of ^{32}P in which the tissue was incubated could not account for the differences recorded between times of incubation or for the effects of GA.

By comparing the mean nucleotide levels in Table 15 with those in Table 18 it is apparent that most of the compounds have increased in amount following continuous agitation of the tissue. The greatest effect has occurred with CTP which has increased by 92%. UTP has risen by

. 74%, GTP by 59%, NAD by 39% and ATP by 28%. ADP and UDPG were almost unchanged.

16. The effect of GA on the Uptake of ^{32}P by Wheat Aleurone Tissue

The finding that nucleotides were more highly radioactive in GA-treated aleurone could be accounted for by a greater uptake of ^{32}P in the presence of the hormone. Accordingly, the influx of ^{32}P into aleurone was investigated as a possible explanation of the observed changes in labelling of nucleotides after treatment with GA.

It was assumed that, after the addition of ^{32}P, the specific activity of inorganic phosphate in the tissue and ambient solution would rapidly reach equilibrium. Any hormonal effect would then be detected by a change in the time at which equilibrium was reached.

Batches of tissue (2 gm) were weighed into 9 cm petri dishes and allowed to equilibrate in 7 ml water. After 6 hours, the tissue was rinsed by removing 3.5 ml of ambient solution, adding 10 ml of water to the petri dish and, after mixing the contents, removing 10 ml of the solution. The procedure was repeated with another 10 ml of water, and then the volume made to 7 ml again by the addition of 3.5 ml aliquots of water or GA(200 µg/ml).

The solution and tissue were mixed and the incubation continued for a further 60 min. The rinsing described above was then repeated and the ambient solutions made back to 7 ml with 3.5 ml aliquots of water or GA(200 µg/ml) containing 30 µCi ^{32}P and 11 µg KH_2PO_4 as carrier.

Thereafter, at 5 min. intervals up to 20 min., the contents of each petri dish were transferred to a small nylon sieve and the ambient solution drained into a flask. The tissue, still in the sieve, was washed with two 10 ml aliquots of ice-cold water. It was then pressed gently with a spatula to remove much of the entrapped liquid, and the filtrate was collected with the ambient solution. The solution was placed in a refrigerator and the tissue, after further drainage for 15-20 seconds on absorbent tissue, was held in an ice-bath. When all the samples had been rinsed in this way, they were transferred to the cold room and assayed for inorganic phosphate by the method of Weil-Malherbe and Green (1951). Portions of the iso-butanol layer, containing the phosphomolybdate complex, were dried onto copper planchets and the radio-activity measured in the gas flow counter. The results are shown in section 1 of Table 22.

On another occasion a similar procedure was carried out with 1 gm portions of aleurone incubated in 5 ml solutions of water or GA. After adding 12.5 µCi ^{32}P, samples were taken at 5 min. and thereafter at intervals of 30 min. up to 90 min. In this instance, the tissue and ambient solutions

were assayed between samples. The results of this experiment are given in section 2 of Table 22.

Table 22

Wheat aleurone tissue (fresh weight specified) was equilibrated for 6 hours, then treated with either GA or water for 60 min. before the addition of ^{32}P. At intervals thereafter both issue and ambient solution were assayed for Pi.

Time (min)	P_i (µg/gm tissue) -GA	+GA	c.p.m.(x 10^3) per gm tissue -GA	+GA	Specific activity (c.p.m. x 10^3/µg P_i) -GA	+GA
1(a) Tissue (2gm)						
5	37	38	262.7	372.4	7.1	9.8
10	38	46	361.0	372.6	9.5	8.1
15	40	40	352.0	308.0	8.8	7.7
20	42	39	386.4	401.7	9.2	10.3
(b) Ambient solution						
5	20	20	8,332.	8,836	416.6	441.8
10	34	34	8,789.	9,115.	258.5	268.1
15	36	32	8,982.	8,941.	249.5	279.4
20	42	35	8,778.	9,366.	209.0	267.6
2(a) Tissue (1 gm)						
5	62	37	353.4	370.0	5.7	10.0
30	70	53	504.0	514.1	7.2	9.7
60	62	60	688.2	624.0	11.1	10.4
90	63	54	655.2	702.0	10.4	13.0
(b) Ambient solution						
5	14	15	6,889.	7,077.	492.1	471.8
30	19	20	6,561.	6,342.	345.3	317.1
60	22	26	5,980.	6,570.	271.8	252.7
90	23	35	6,079.	6,062.	264.3	173.2

It is clear that equilibrium does not occur rapidly since even after 90 min. there was a 10- to 20- fold difference between the specific activity of the phosphate in the tissue and that in the abient solution. Moreover, the decrease in the latter as the time of incubation was extended was due almost entirely to an efflux of inorganic

phosphate from the tissue. This efflux was presumed to arise by the dilution of inorganic phosphate in the ambient solution. Before and after the addition of ^{32}P the concentrations were 0.25 and 0.1 mMolar respectively.

The rate of accumulation of counts in the tissue is dependent upon the specific activity of the phosphate in the ambient solution. If the latter figure is divided into the counts taken up by the tissue, an estimate can be obtained of the net phosphate accumulated in the tissue. Between 5 and 60 min. there is an approximately linear rate of 0.032 µg per min. However, over the same period the net efflux is 0.173 µg/min or a 5.4- fold greater rate than uptake. There is therefore considerable control over the movement of phosphate into and out of the tissue.

Although GA has apparently increased the specific activity of inorganic phosphate in the tissue after 5 min., the results were considered to be spurious. In section 1 of Table 22, the value was due to a low incorporation of ^{32}P into control tissue, whilst in section 2 an abnormally low level of inorganic phosphate was recovered from hormone treated tissue.

Between 5 and 60 min. of incubation, the rate of uptake of ^{32}P was almost linear and was about 5×10^3 c.p.m./min. In the first 5 min., however. the rate was approximately 72×10^3 c.p.m./min. Uptake apparently occurred in two phases. This pattern of accumulation suggested that a large amount of ^{32}P was rapidly adsorbed on to the surface of the tissue.

If this postulate was correct, more of the ^{32}P should be removed from the tissue by rinsing it in non-radioactive phosphate as compared to water. Accordingly the following experiment was designed to test this hypothesis.

Batches of tissue (4 gm) were allowed to equilibrate for 6 hours in 10 ml of water, treated with GA or water for a further 60 min., then 100 μCi ^{32}P added to each for 10 min. 5 ml of solution were discarded and a small sample of tissue removed, mounted on a copper planchet and the radioactivity measured. 10 ml aliquots of water or mMolar KH_2PO_4 were added to the remainder of the tissue in the petri dishes, the contents mixed and 10 ml of the solution removed. Further samples of tissue were taken and counted, and this rinsing procedure repeated eight times in all. The samples were then dried in a forced-draught oven at 80°C, weighed, and the radioactivity calculated as c.p.m. per mg dry weight. The results, shown in Table 23, are expressed as moving point averages to reduce the variance. Each value is the mean of the count obtained for a particular rinse and that immediately preceding and following it. A graphical representation is shown in Fig.11, and the actual data are given in Appendix 4.

Two rinses in mMolar KH_2PO_4 removed about $^2/3$ of the radioactivity from the tissue, whereas three rinses in water were required to reduce it by the same amount. Thereafter both treatments removed approximately the same amount of ^{32}P,

Fig. 11

Removal of adsorbed ^{32}P from aleurone layers.

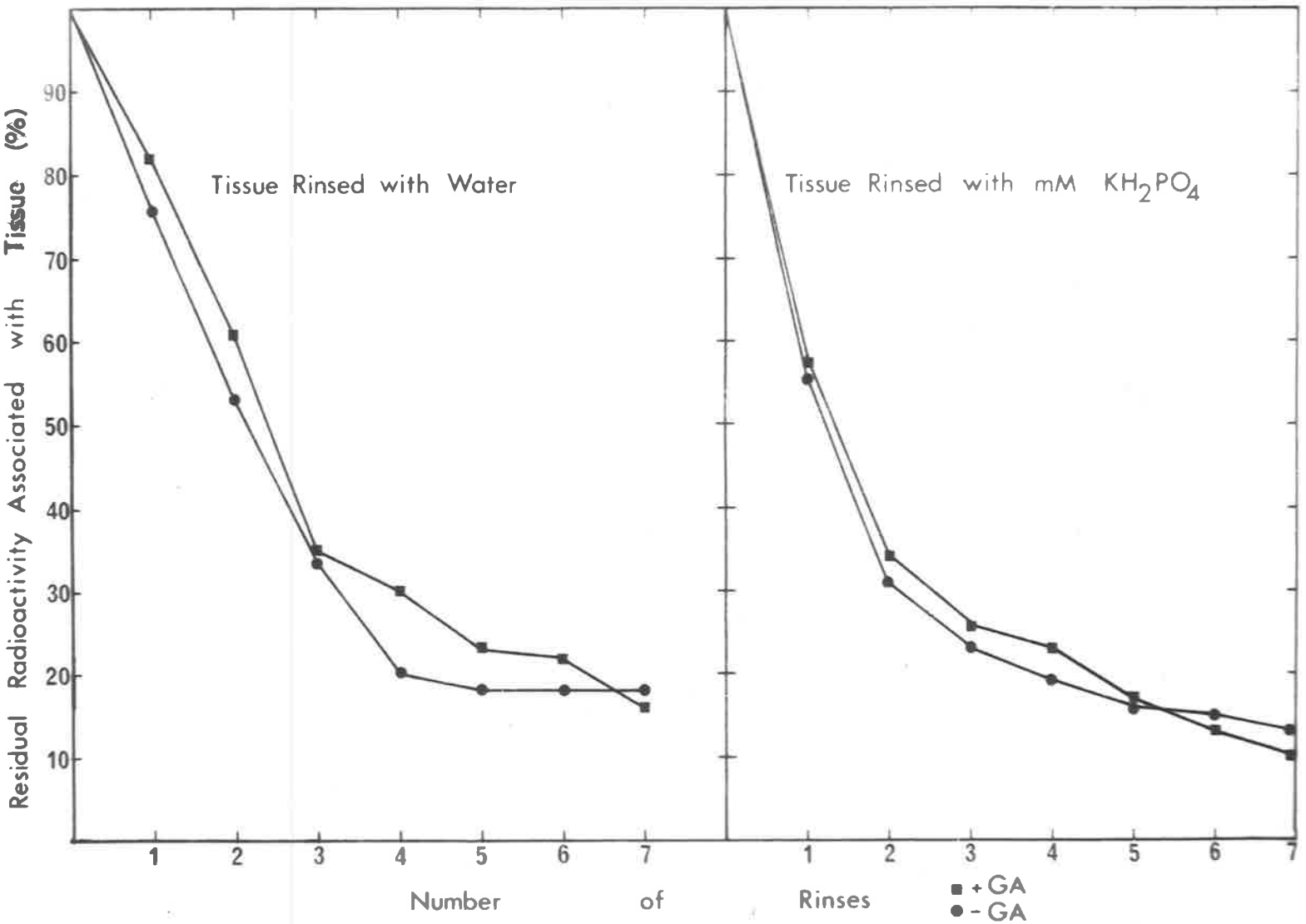

Residual Radioactivity Associated with Tissue (%)
90
80
70
60
50
40
30
20
10
Tissue Rinsed with Water
Tissue Rinsed with mM KH_2PO_4
1
2
3
4
5
6
7
Number of Rinses
■ + GA
● - GA

Table 23

Wheat aleurone tissue was treated with ^{32}P for 10 min. after incubating for 60 min. with either GA_3 or water. The radioactivity in the tissue was measured after successive rinses with water or mMolar KH_2OO_4. The results are expressed as moving point averages, each value being the mean of the radioactivity obtained for a particular rinse and that immediately preceding and following it (c.p.m. per mg dry tissue). Values in parentheses are the percentage of the initial radioactivity.

Number of rinses	Rinsed with water		Rinsed with mM KH_2PO_4	
	-GA.	+GA	-GA.	+GA
0	7274(100)	5431(100)	5796(100)	4908(100)
1	5568 (76)	4466 (82)	3250 (56)	2828 (58)
2	3871 (53)	3340 (61)	1818 (31)	1684 (34)
3	2402 (33)	1909 (35)	1346 (23)	1285 (26)
4	1462 (20)	1648 (30)	1082 (19)	1144 (23)
5	1303 (18)	1265 (23)	907 (16)	838 (17)
6	1327 (18)	1171 (22)	855 (15)	661 (13)
7	1314 (18)	866 (16)	732 (13)	504 (10)

(1100 c.p.m./mg dry weight). By comparing the values for GA- and water-treated tissue it also appears that the removal of ^{32}P is more variable when the rinsing is carried out with water.

It was therefore concluded that a large proportion of the radioactivity of the tissue shown in Table 22 could be attributed to adsorption.

The examination of whether GA influences the rate of uptake of ^{32}P was therefore repeated under conditions in which the tissue was more rigorously washed before assaying for inorganic phosphate. Batches of aleurone

(2 gm) were weighed into 9 cm petri dishes and equilibrated in 7 ml of water for 6 hours. It was possible that the observed efflux of inorganic phosphate from the tissue was initiated by renewing the ambient solution at this time. Any variation in the amount of efflux could be expected to cause variation in the specific activity of the phosphate in the ambient solution. To avoid such a source of error, GA was added without any prior rinsing of the tissue. 3.5 ml of each ambient solution, which contained an average of 0.44 mMolar inorganic phosphate (95% confidence interval 0.02 mMolar), was discarded and replaced with 3.5 ml aliquots of water or GA (200 µg/ml) containing 30 µCi ^{32}P and 11 µg KH_2PO_4 as carrier. At 5 min. intervals thereafter, the tissue was placed in a small porous holder, immersed 3 times in each of four 1 litre solutions of mMolar KH_2PO_4 and then 3 times in each of four 1 litre portions of water. Excess liquid was drained on absorbent paper and the sample of tissue placed in an ice-bath. After all the samples were collected, they were transferred to the cold room and assayed for inorganic phosphate by the method of Lipmann and Tuttle (1944). Aliquots of the acid-soluble extract were hydrolysed as described by Bartlett (1959) and also assayed for inorganic phosphate. The absorbance of the butanol layer containing the vanado-molybdophosphate complex was measured (Parvin and Smith, 1969) then portions of the butanol layer were dried onto copper planchets and radioactivity measured in the gas-flow counter. The results are shown in Table 24. As insufficient

Table 24

Wheat aleurone (2 gm) was treated with either water or GA in the presence of ^{32}P for the times indicated. After rigorous washing in both mMolar KH_2PO_4 and water, the tissue was assayed for inorganic and total acid-soluble phosphate (average of two separate experiments).

Time (min)	Level in tissue (µg/g fresh weight)		c.p.m.($\times 10^3$) per gm tissue		Specific activity (c.p.m./µg P)	
	-GA	+GA	-GA	+GA	-GA	+GA
(a)	Inorganic phosphorus					
5	52	54	304.2	306.8	5833	5632
10	50	57	312.6	344.8	6331	6112
15	40	40	319.8	318.0	7994	8054
20	38	45	336.0	359.5	8852	7951
(b)	Total acid-soluble phosphorus					
5	772	854	397.8	392.0	518	465
10	890	898	420.8	444.8	484	496
15	741	819	352.0	343.5	474	420
20	751	803	386.8	402.0	514	500

Table 25

α-amylase production, in response to GA, by the tissue used in obtaining the results of Table 24. Period of treatment was 24 hours. Values expressed as Starch-Iodine-Colour units per gm fresh weight. (Average of two determinations.)

	Tissue	Ambient	Total
-GA	0.9	0.6	1.5
+GA	37.9	80.1	118.0

tissue could be isolated at one time the experiment was carried out in two stages, the values for 5 and 10 min., and those of 15 and 20 min. being obtained separately.

Small portions of tissue (0.5 gm) were used to test the production of α-amylase in response to GA. The results are given in Table 25.

It is clear that even after washing thoroughly in non-radioactive phosphate, the specific activity after 5 min. had already risen to 70% of the value at 20 min. The data obtained for the amounts of orthophosphate in the tissue, the total counts incorporated and the specific activity were analysed separately as 4 x 2 factorials with randomised complete-block design. In no case was the presence of GA associated with a significant effect. Since no significant changes occurred during a period when it has been shown that significant effects of GA occur on free nucleotides, it was concluded that the presence of the hormone was not associated with changes in the rate of uptake of ^{32}P.

17. Hormone-induced changes in the Levels and Specific Activities of Free Nucleotides after Incubation of Aleurone Tissue with Different Concentrations of Inorganic Phosphate

The efflux of inorganic phosphate which occurred after replacement of the ambient solution with water (Table 22) suggested that some site within the tissue was sensitive to modifications in the size of the external pool of inorganic phosphate.

The effect of adding different concentrations of KH_2PO_4 to the ambient solution was examined in a number of different experiments. In one experiment, carried out in petri dishes, the concentration of KH_2PO_4 was kept at 0.25 mMolar throughout the entire incubation period of 7 hours, including 6 hours for equilibration. In another, the tissue was continuously agitated in solutions maintained mMolar with respect to KH_2PO_4. In both cases GA was added for the last 60 min. and ^{32}P for the final 15 min. The results are shown in Table 26 and in Appendices 7 and 8 respectively. As GA had no significant effect on nucleotide levels, the values for each treatment were averaged. The specific activities are presented as the ratio of GA treated to control. For comparison, the corresponding values for tissue incubated without added KH_2PO_4 (except for a small amount of carrier with the ^{32}P) have been calculated from those presented in Tables 15 and 18, the latter being the mean of the values obtained after 30 and 90 min. of incubation.

Since the results are derived from different experiments, they may simply reflect differences in batches of tissue. However, it is apparent that in experiments where inorganic phosphorus was added to the external medium higher levels of free nucleotides are measured. Moreover, the greatest differences are evident among the nucleoside triphosphates. The average level of these four compounds, extracted from tissue incubated in petri dishes

Table 26

Wheat aleurone tissue (4 gm) was equilibrated for 6 hours and then incubated in water or GA for 60 min. The concentrations of KH_2PO_4 specified below were maintained throughout. Where petri dishes are indicated, the volume of ambient solution was 10 ml. For continuous shaking, a volume of 30 ml was used. ^{32}P was added for the final 15 min. of incubation in each case. The level of each nucleotide, relative to the smallest value as 100, is given in parentheses.

Nucleotide	Petri dish		Continuous agitation	
	No KH_2PO_4***	0.25mM KH_2PO_4**	No KH_2PO_4***	mM KH_2PO_4*
(a) Nucleotide levels (nMoles)				
NAD	40 (100)	48 (120)	46 (115)	44(110)
ADP	20 (100)	21 (105)	20 (100)	21(105)
ATP	226 (100)	290 (128)	288 (127)	327(145)
CTP	52 (100)	68 (131)	114 (219)	134(258)
GTP	32 (100)	47 (147)	52 (162)	62(194)
UTP	125 (100)	192 (154)	221 (177)	232(186)
UDPG	174 (100)	194 (111)	180 (103)	200(115)
Total	617 (100)	860 (139)	921 (149)	1020(165)
(b) Specific activity - Ratio of GA treated to control)				
NAD	0.67	0.82	1.13	0.92
ADP	1.33	0.88	1.04	0.81
ATP	1.15	1.00	1.11	1.01
CTP	1.35	1.49	1.30	1.18
GTP	1.24	1.22	1.11	1.11
UTP	1.24	1.01	1.12	0.95
UDPG	1.31	1.01	1.04	0.97
Mean	1.19	1.06	1.12	0.99

*Values obtained from one experiment.

**Values obtained from two experiments.

***Values obtained from three experiments.

in the presence of 0.25 mMolar KH_2PO_4, is 40% greater than for incubation in water. The effect has been most prounounced for UTP and GTP.

With continuous agitation of the tissue, the total level of free nucleotides is again greater in the presence of added mMolar KH_2PO_4. In this case, however, the difference is smaller, about 14% for the nucleoside triphosphates.

Compared with incubation in petri dishes, the amounts of nucleotides extracted from continuously agitated tissue are considerably greater: in water the amount of CTP is more than double, and is 2.6 times greater in mMolar KH_2PO_4. However, there are relatively small differences between experiments, in the amounts of NAD and ADP.

With the exception of the experiment in which tissue was shaken in mMolar KH_2PO_4, GA appears to have increased the incorporation of ^{32}P into nucleotides, when the mean values are considered. However, closer inspection of the data in Table 26 reveals that for several of the nucleotides the results are inconsistent. For example, in three cases out of four, GA depressed the uptake of ^{32}P by NAD, and a similar result is seen in two cases for ADP. In only one treatment is there a marked effect of GA on UDPG, and in only two treatments is there much effect on ATP and UTP. The most consistent effects of GA are evident in the values for CTP and GTP, and of these the larger response is seen in CTP.

18. The Uptake of ^{32}P, in Response to GA, by Free Nucleotides. Changes in Composition of the Incubating Medium.

The results of several unrelated experiments with tissue incubated in petri dishes, suggested that the relative effect of GA on the incorporation of ^{32}P by nucleotides was influenced by the composition of the ambient solution. The experimental details of these experiments are collated and shown in Table 27, and α-amylase production in Table 28.

It can be seen that the effect of GA is more obvious in experiments where the ambient solution was completely replaced with fresh water or solutions of GA. The value for GTP in the treatment where the ambient solution was returned almost unchanged was not significantly lower than the other values. In the experiment in which half the ambient solution was replaced, only the value for CTP is significantly greater than unity (5% level of significance).

It could be suggested that the differences in the effects of GA on the incorporation of ^{32}P into nucleotides, which have been attributed to differences in the composition of the ambient solution, could equally well be due to variation between batches of tissue. To distinguish between these two possibilities, two of the treatments were repeated on a single batch of tissue. In one treatment, the tissue was rinsed with two 10 ml aliquots of water prior to both the addition of GA or water, and ^{32}P.

Table 27

Wheat aleurone tissue (4 gm) was equilibrated for 6 hours in 9 cm petri dishes containing 10 ml water.

In one series of experiments. 5 ml of ambient solution was then discarded and replaced with 5 ml of either water or GA (200 µg/ml). After 15 min., another 5 ml was discarded and the tissue rinsed twice with 10 ml aliquots of water. The volume was again made to 10 ml by the addition of 5 ml aliquots of water or GA (both containing 50 µCi ^{32}P and 15.5 µg KH_2PO_4 as carrier) and left for a further 15 min. ("Half replaced") (Appendix 6).

In the other series, 5 ml of ambient solution was also removed after the first six hours. However, after adding 0.5 ml water or GA (2 mg/ml) to these, they were returned to the tissue. After 15 min., 5 ml was again removed, 50 µCi ^{32}P added with a micro-syringe, and the solutions returned to the tissue for a further 15 min. ("unchanged") (Appendix 5).

The tissue from both series was assayed for free nucleotides after the 15 min. treatment with ^{32}P.

The results are presented below as the ratio of the specific activities of GA treated to control. Values from Table 18 are shown for comparison. The experimental conditions in this instance were that the tissue was constantly shaken in 30 ml. water and all of the ambient solution was decanted, and replaced with fresh solutions containing ^{32}P. The total period of incubation was again 30 min. ("completely replaced".). (Appendix 3)

	Ambient solution** completely replaced	Half the ambient* solution replaced	Ambient solution** returned unchanged
NAD	1.38	1.00	0.83
ADP	1.20	1.05	1.12
ATP	1.19	1.04	0.91
CTP	1.37	1.20	0.94
GTP	1.19	1.02	0.79
UTP	1.19	1.06	0.90
UDPG	1.00	0.97	0.90

*Average of 2 experiments

**Average of three experiments

Table 28

α-amylase production by wheat aleurone tissue in response to GA . Experimental details shown in Table ~~32~~ 27. Enzyme assay after 24 hours incubation. Values in Starch-Iodine-Colour units per gm fresh weight.

	Ambient solution completely replaced		Half ambient solution replaced		Ambient solution unchanged	
	-GA	+GA	-GA	+GA	-GA	+GA
Tissue	0.1	10.7	1.9	29.5	1.4	25.4
Ambient	-	16.8	1.0	66.8	3.5	45.2
Total	0.1	27.5	2.9	96.3	4.9	70.6

In the other, the ambient solution was kept almost unaltered throughout. An α-amylase assay was carried out on the latter treatment only. Nucleotide levels and ^{32}P incorporation are shown in Table 29, and α-amylase production in Table 30.

With the exception of NAD, GA stimulated the incorporation of ^{32}P into nucleotides when the tissue was suspended in fresh, but not in unchanged solutions. The effect was due entirely to an enhanced uptake of label, since the effect of GA on nucleotide levels was not appreciably different between the two treatments. There was a consistent increase, due to GA, in the specific activity of CTP and this nucleotide had the lowest specific activity of all the nucleoside triphosphates.

Table 29

Two of the treatments, carried out on different batches of tissue as described in Table 27, were repeated on a single batch of tissue. They were "completely replaced" and "unchanged".

	Ambient solution completely replaced		Ratio +GA to	Ambient solution returned unchanged		Ratio +GA to
	-GA	+GA	-GA	-GA	+GA	-GA
(a)	Nucleotide level (nMoles/4 gm fresh weight)					
NAD	38	30	0.79	34	34	1.00
ADP	14	10	0.71	16	14	0.88
ATP	182	177	0.97	196	191	0.97
CTP	60	48	0.80	59	50	0.85
GTP	34	32	0.94	37	35	0.94
UTP	112	118	1.05	110	105	0.95
UDPG	125	116	0.93	160	137	0.86
Mean	81	76	0.94	87	81	0.93
(b)	Counts incorporated ($\times 10^3$)					
NAD	3.5	1.9	0.54	0.8	0.5	0.63
ADP	14.1	13.1	0.93	3.1	2.3	0.74
ATP	422.4	493.9	1.17	86.3	79.5	0.92
CTP	80.3	84.4	1.05	15.7	15.2	0.97
GTP	70.8	77.8	1.10	14.2	13.2	0.93
UTP	295.2	359.6	1.22	53.2	47.4	0.89
UDPG	130.4	136.6	1.05	31.7	25.1	0.79
Mean	145.2	166.8	1.15	29.3	26.2	0.89
(c)	Specific activity (c.p.m/nMole)					
NAD	91	62	0.68	23	16	0.70
ADP	1011	1310	1.30	194	162	0.84
ATP	2321	2790	1.20	440	416	0.94
CTP	1338	1759	1.31	267	303	1.13
GTP	2084	2432	1.17	384	377	0.98
UTP	2635	3048	1.16	484	451	0.93
UDPG	1044	1178	1.13	198	183	0.92
Mean	1503	1797	1.20	284	273	0.96

Table 30

∝-amylase production, in response to GA, by the treatment in Table 29 in which the ambient solution was returned almost unchanged in composition. Period of incubation with GA was 18 hours. Values in Starch-Iodine-Colour units per gm fresh weight.

	Tissue	Ambient	Total
-GA	2.9	2.3	5.2
+GA	18.2	25.0	43.2

There was less incorporation of label in the treatment where the ambient solution was returned unchanged. This was probably due to the dilution of ^{32}P by the presence of a greater amount of non-radioactive phosphorus in the solution. The amount of inorganic phosphorus in the ambient solution had generally reached a concentration of about 0.4 mMolar at the end of the 6 hour equilibration period. Since only water was added initially, this phosphorus is secreted by the tissue.

19. The Production of ∝-amylase by, and Incorporation of ^{32}P into Free Nucleotides of, Tissue Extensively Damaged During Preparation

Both ∝-amylase production and the incorporation of ^{32}P into free nucleotides have been shown to be enhanced in wheat aleurone layers by the presence of GA. No evidence, however, has so far been obtained to indicate whether the two responses are related. An opportunity to test the existence of a relation between the two phenomena occurred when the polypropylene bottle used for

tissue preparation developed a fault which resulted in extensive shredding of the aleurone tissue during rolling. The production of α-amylase in response to GA under these conditions fell markedly as shown by the results in Table 31.

To examine the incorporation of ^{32}P into free nucleotides, batches of tissue (4 gm), prepared with the faulty bottle, were incubated in 30 ml water on a reciprocating water bath. After 6 hours, 7.5 ml of solution were discarded and replaced with aliquots of 7.5 ml of GA, at different concentrations, or water. The incubations were resumed, and after 15 min. the solutions were decanted and replaced with fresh solutions of water or GA, 0.05 mMolar with respect to KH_2PO_4 and containing 50 μCi ^{32}P. After a further 15 min., the treatments were terminated and the tissue assayed for free nucleotides. The results are shown in Table 32.

Table 31

The production of α-amylase in response to GA by wheat aleurone tissue extensively damaged by a fault which developed in the polypropylene bottle used during isolation. Enzyme production after the bottle was repaired is shown for comparison. Time of incubation with GA (100 μg/ml): 18 hours. Values in Starch-Iodine-Colour units per gm fresh weight.

	Damaged tissue		Normal tissue	
	-GA	+GA	-GA	+GA
Tissue	-	6.9	0.2	13.8
Ambient	-	7.2	0.3	22.9
	-	14.1	0.5	36.7

Table 32

Wheat aleurone tissue, extensively damaged during preparation, was incubated with GA or water for 30 min. after equilibrating for 6 hours in water. ^{32}P was added for the final 15 min. of incubation, after which the tissue was extracted for free nucleotides. (Average of two determinations).

	Concentration of GA (µg/ml).				
	0	10^{-4}	10^{-1}	100	Ratio of GA-treated (100 µg/ml) to control
(a)	Nucleotide level (nmoles).				
NAD	42	40	46	40	0.95
ADP	21	22	19	19	0.90
ATP	330	310	320	320	0.97
CTP	113	120	118	116	1.03
GTP	52	50	52	49	0.94
UTP	244	232	234	228	0.93
UDP	12	10	12	10	0.83
UDPG	240	224	226	230	0.96
(b)	Specific activity (c.p.m./nmole)				
NAD	33	20	22	29	0.90
ADP	156	163	155	155	0.99
ATP	328	335	329	328	1.00
CTP	215	191	203	204	0.95
GTP	292	292	292	294	1.01
UTP	328	318	322	315	0.96
UDP	512	469	485	465	0.91
UDPG	158	159	156	158	1.00

Since α-amylase production by the damaged tissue was not entirely inhibited, it would be expected that some increase in the labelling of nucleotides would occur in response to GA. The absence of the latter suggests that the two processes may not be directly related. On the other hand, however, less than 40% of α-amylase was produced

by damaged, compared to normal aleurone. From Table 18, it can be seen that the mean increase in specific activity of nucleotides was 19% after treatment for 30 min. with GA. A reduction in the incorporation of ^{32}P by damaged tissue of the same magnitude as the reduction in α-amylase, would therefore mean an increase of less than 8% in specific activity. A response of this size may not have been detected by the methods employed.

Further comparison between the data in Tables 18 and 37 reveals that the level of UDPG is 20% higher in damaged tissue, whilst that of NAD is 16% lower. In addition, the specific activity of NAD is 73% greater.

DISCUSSION

The production of α-amylase by aleurone layers isolated from mature wheat grain was greatly enhanced after the administration of gibberellic acid. Enzyme activity was linearly related to the logarithm of the concentration of GA applied (Table 1). Similar results for wheat (Phillips, 1968), barley (Varner and Johri, 1967), and rice (Ogawa, 1966) support this finding. At concentrations of GA higher than 10-100 µg/ml (2.89×10^{-5} - 2.89×10^{-4} M), production of α-amylase decreased.

The ratio of the amount of α-amylase in the ambient solution to that in the tissue was also responsive to GA. The relation was sigmoidal up to 10 µg/ml, but thereafter was less clearly defined. This effect has been shown to occur in wheat aleurone layers by Phillips, (1968).

There was a lag phase of approximately 8-10 hours between the application of GA and the appearance of α-amylase (Table 12), and this is comparable to the interval found for barley (Chrispeels and Varner, 1967a). However, Phillips (1968), working with aleurone isolated from wheat, found a lag phase of 16 hours. Since the incubation procedures she used were identical to those used in the present study, it seems probable that the difference in the time of detection of the enzyme was due to the smaller amounts of tissue assayed by Phillips (1968).

Postponement of the addition of GA until 6 hours

after the tissue was isolated decreased the period of enzyme induction by 2 hours (Figure 6.). Other workers have observed similar effects with barley aleurone layers (MacLeod, Duffus and Horsfall, 1966; Yung and Mann, 1967). However the increase in the activity of α-amylase which occurred after the induction period was considerably greater when the tissue was pre-incubated in water for 6 hours.

The effect of GA on the production of α-amylase was almost completely prevented by the addition of Actinomycin D at a concentration of 100/µg/ml (Table 13). Varner and Chandra (1964) reported that barley aleurone responded similarly to Actinomycin D. Postponement of the addition of both hormone and inhibitor until 6 hours after wheat aleurone layers were prepared did not greatly alter the degree of inhibition (Table 13).

It is apparent that the aleurone layers of wheat and barley respond in a similar fashion to the application of GA and that therefore many of the findings reported elsewhere for the response in barley may be assumed to be pertinent for wheat.

Isolated aleurone tissue may thus be regarded as a homogeneous collection of cells which react in a reproducible manner to the addition of small amounts of a specific compound classed as a hormone. The response becomes greater as the concentration of the hormone is

increased, reaches a maximum, and then apparently declines.

During the first phase of this response, each added increment of GA causes a relatively greater increase in the amount of α-amylase secreted by the tissue compared to that detected in the tissue. This result could be explained by assuming that release of α-amylase occurs only when a critical concentration of the enzyme has been synthesised in the tissue. Secretion would then become proportionately greater as total enzyme production was increased. On the other hand, separate mechanisms may exist for synthesis and release of α-amylase. In this case, either the cell wall could become more permeable to the passage of α-amylase, or secretion itself might be influenced by the hormone, higher concentrations of GA causing release earlier than lower levels.

There was a lag phase of some 6 - 10 hours between the addition of GA and the first detection of α-amylase. Several alternative hypotheses can be put forward to explain this phenomenon-

(a) isolated aleurone does not respond to the presence of GA before a time interval of 6 - 10 hours has elapsed. However the inhibitory effect of Actinomycin D on α-amylase production by barley aleurone is greatest when it is applied at the same time as GA. The effect becomes smaller as the addition of Actinomycin D is delayed,

and no effect is detected when the interval between adding GA and Actinomycin D is 7 hours (Chrispeels and Varner 1967B). Thus, it is unlikely that the lag phase is due to an unresponsive state of the tissue.

(b) the level of α-amylase which can be detected by the methods used is not reached in aleurone tissue for the first 6 - 10 hours. It can be seen from Table 12 that α-amylase can be detected in tissue treated with water alone. Further, the level of α-amylase within tissue incubated in water is sometimes greater than the amounts measured there in the presence of GA. It is apparent therefore that the sensitivity of the enzyme assay is adequate to preclude this hypothesis.

(c) certain biochemical events preceding the appearance of α-amylase require 6 - 10 hours for completion. This hypothesis is supported by evidence that, in barley aleurone, all of the α-amylase induced by GA arises from de novo synthesis (Filner and Varner, 1967). Thus, by implication, both transcription and translation could be expected to precede the detection of α-amylase in wheat aleurone.

(d) a short period of rapid synthesis may be followed by a long period of activation, (e.g. organisation of secondary or tertiary structure), lasting several hours. This infers that α-amylase synthesised at 10 hours would not be detected until, for example, 16 hours. Inhibitors

of protein synthesis such as cycloheximide, if added between 10 and 16 hours, would then be expected to have no effect on the amount of α-amylase measured at 16 hours. Chrispeels and Varner (1967b) have shown however that the addition of cycloheximide at intervals after the addition of GA causes an immediate cessation in the production of α-amylase. Moreover, if tissue is treated with GA for 7 hours and then incubated in its absence, less α-amylase is produced after 13 hours than if GA had been present continuously. However, when such tissue is again treated with GA at 13 hours, the rate of synthesis of α-amylase almost immediately rises to that observed in continuously treated tissue (Chrispeels and Varner, 1967b). It may be concluded therefore that activation does not require a significant portion of the lag phase.

The biological property of Actinomycin D is considered to arise from its binding to the guanine moieties of DNA and consequently preventing transcription (Reich and Goldberg, 1964). As discussed in the Literature Review, the use of this compound by several workers studying its effects on the production of α-amylase by barley aleurone treated with GA, has produced conflicting results. In the present study, considerable inhibition was produced, and, assuming that Actinomycin D has no effect other than the blockage of transcription, it can be concluded that at some stage(s) in the response sequence, from the initial action

of GA to the production of α-amylase, there is a requirement in this tissue, prepared in this way, for RNA produced from DNA.

It was found that if the amount of GA was kept constant and the level of Actinomycin D was increased, the amount of α-amylase found in the ambient solution was inhibited to a greater extent than that extracted from the tissue (Table 13). Up to a concentration of 30 µg/ml, the decline in total α-amylase production was accounted for almost entirely by the decrease in the amount of α-amylase released, but thereafter enzyme activity in both tissue and ambient solution dropped sharply. These results may indicate that as the concentration of Actinomycin D is increased there is an increasingly longer interval before GA produces any effect. They may also be interpreted as indicating that release does not occur until enzyme concentration inside the tissue reaches a critical level. Again, as with the effect of increasing concentrations of GA, a third alternative is that both synthesis and release of enzyme are controlled by different mechanisms. Chrispeels and Varner (1967a) concluded that their results, on the effect of Actinomycin D on the GA-induced synthesis of α-amylase in barley aleurone, supported the latter hypothesis. Several studies with tissues derived from animals indicate that r-RNA production is inhibited at low levels of Actinomycin D before any significant effect is detected on m-RNA synthesis (Roberts

and Newman, 1966; Penman, Vesco and Penman, 1968; Cheevers and Sheinin, 1970). There does not appear to be any evidence to show that this occurs in plant tissues. However, low concentrations of GA are reported to enhance the incorporation of ^{14}C-uridine into r-RNA in barley aleurone layers to a greater extent than into other forms of RNA, (Chandra and Duynstee, 1968). As the level of GA is increased, the amount of radioactivity associated with these other forms of RNA also increases.

It was found that the interval between the addition of GA and detection of α-amylase could be shortened by subjecting the tissue to a period of pre-incubation in water. Six hours pre-incubation reduced by only 2 hours the time at which the response was first detectable. One possible explanation for this result is that the permeability of the aleurone cells to GA increases after a time of incubation in water and that GA is able to act more quickly. A second explanation is that the method used to isolate the aleurone from the starchy endosperm may make freshly prepared aleurone unresponsive to the presence of GA for the first 2 - 3 hours. A third alternative is that the sensitivity of the aleurone to GA increases after a period of pre-incubation in water due to the accumulation of a factor(s) necessary for the action of GA. Studies by Marcus and Feeley (1964, 1965) indicate that both the activity of ribosomal preparations, as measured by the incorporation of amino acids into proteins, and

the formation of polyribosomes, increase during imbibition in peanut cotyledons and wheat embryos. A further hypothesis can be advanced suggesting that the series of events which precede α-amylase synthesis take place in both GA- and Water-treated tissue, but that all of the events are greatly accelerated in the presence of GA. According to this hypothesis the addition of GA to aleurone after a period of pre-incubation of about 30 hours should result in an almost immediate production of α-amylase. However, no evidence was obtained to show whether an interval of pre-incubation longer than 6 hours would reduce by more than 2 hours, the time at which α-amylase appears.

From the foregoing it is clear that GA elicits a hormonal response in wheat aleurone which is detectable, after a lag period, by an increase in the activity of α-amylase in both tissue and ambient solution. By analogy with results published for barley aleurone it can be inferred that the enzyme arises by de novo synthesis, and further, that this process requires some DNA-directed RNA synthesis. As discussed in the Literature Review, increases in the incorporation of radioactive precursors into the RNA of GA-treated barley aleurone have been reported by some workers to occur under specific conditions. The results of others do not support this finding. Evidence indicating that protein synthesis precedes nucleic acid metabolism in germinating wheat embryos has been reported (Marcus and

Feeley, 1966; Chen, Sarid and Katchalski, 1968), suggesting that some or all of the mechanism for transcription may be present in an inactive form.

This is substantiated by the results of Chen and Osborne (1970), which show that whilst wheat embryos were capable of incorporating labelled amino acids into acid-insoluble compounds immediately after germination, there was no detectable incorporation of ^{14}C-uridine into RNA until 12 hours later. Moreover, the incorporation of labelled amino acids was enhanced by GA after 6 hours. Although these results strongly suggest that an effect of GA on translation precedes an effect on transcription, and thus infer the presence of an inactive species of m-RNA, the authors point out that there may have been synthesis of a small RNA component not detectable by the methods used. No comparable studies have been carried out on the cells of the aleurone layer.

In view of the conflicting results obtained by different workers on the events preceding the GA-induced increase in protein synthesis, it was apparent that some type of metabolism other than that of nucleic acids warranted investigation. It was highly probable that some or all of the biochemical manifestations of hormonal action would involve free nucleotides as cofactors. In addition a stimulation of RNA synthesis, if it did occur after GA treatment, would probably require an increased supply of

nucleotide precursors. A study of these compounds in the aleurone of wheat, shortly after the administration of GA, would probably reveal facets of the mode of action of GA not detectable by other means so far studied.

A technique was developed for the extraction and separation of free nucleotides from isolated aleurone layers of wheat, recovery of these compounds being greater than 80%. The method was based on extraction with cold trichloroacetic acid followed by separation with ion-exchange and paper chromatography, and measurement of extinction at 260 nm. The nucleotides identified were NAD (50 nmoles/gm dry weight), ADP (20), ATP (218), CTP (34), GTP (34), UTP (72) and UDPG (204). The latter compound yielded glucose, fructose, galactose and xylose after mild acid hydrolysis, and was presumed to be a mixture of the four UDP-sugars. However, confirmation of this suggestion was not obtained as no clear separation took place in the solvent systems used by Carminetti et al (1965). Measurements of "UDPG" therefore probably represent the total absorbance of four different compounds.

Derivatives of adenine and uracil together accounted for almost 90% of the nucleotides measured. This finding is similar to data obtained for wheat plants (Bergkvist, 1956), corn roots (Cole and Ross, 1966), yeast (Oura and Suomalainen, 1967), Spirodela oligorrhiza (Bieleski, 1968), immature wheat grain (Jenner, 1968), phloem exudate (Bieleski, 1969),

and Chlorella pyrenoidosa (Sanwal and Preiss, 1969).

Individual nucleotide levels were somewhat different to those found in intact immature wheat grain by Jenner (1968). Twenty days after anthesis, the amounts of ATP, UTP, and UDPG in immature grain were 2.8-, 5.2-, and 6.8- fold higher respectively than those found in mature aleurone (per gm fresh weight), while derivatives of cytosine and guanine in the former were either absent or too low to be measured. In addition, the developing grain contained significant quantities of ADPG, which was not detected in either the aleurone or endosperm of mature grain.

Moreover, the nucleotide content of the endosperm of the developing grain was higher than that of the testa-pericarp fraction (which included aleurone and embryo). This is in contrast to the mature grain, the endosperm of which was found to contain no free nucleotides. (Table 5).

The absence of detectable levels of AMP, and a constant ratio of ATP to ADP (approximately 11:1) indicated the presence of the enzyme, adenylate kinase. This enzyme controls the reaction -

$$(AMP) + (ATP) = 2(ADP).$$

Bomsel and Pradet (1968) suggested that a measure of the degree of activity of adenylate kinase can be obtained by estimating the ratio e/E,

where - $e = 2(ATP) + (ADP)$, and

$$E = (ATP) + (ADP) + (AMP).$$

For isolated aleurone, where (ATP) and (ADP) are 218 and 20 nmoles respectively, this value is approximately 1.9. Using data from several sources, Bomsel and Pradet (1968) showed that a value of this order corresponds to an equilibrium constant, K, of about 2.5, where -

$$K = \frac{(ATP)\ (AMP)}{(ADP)^2}$$

Thus, the concentration of AMP can be estimated as 3 nmoles, an amount too low to be detected by the techniques and amounts of tissue used in these experiments.

The value of the equilibrium constant of the adenylate control system appears to change with the stage of maturity of the wheat grain. From data supplied by Jenner (1968) it can be calculated that, at 10 and 20 days after anthesis, the values for K are 0.58 and 0.49 respectively. Thus, the free adenyl nucleotides of the fully imbibed mature aleurone layer are clearly at a high energy potential.

Considerable quantities of AMP were extracted from aleurone after thawing tissue previously frozen in liquid nitrogen. Other nucleoside mono- and diphosphates also appeared as a result of this treatment or, if already present, were increased in amount. This occurred at the expense of the nucleoside triphosphates which were extensively degraded. Since NAD and UDPG were unaffected, it was apparent that phosphatases of high activity were present and were capable of action at 4°C in the few minutes

before TCA could completely penetrate the thawing tissue. Poor recoveries of ATP were reported by Williams (1970) after thawing fractions of wheat grain, previously frozen in liquid nitrogen, in perchloric acid. This extremely rapid hydrolysis, by enzymes capable of working at low temperatures, has also been described by Bieleski (1964).

The amount of inorganic phosphorus in the nucleotides represented less than 2% of the total acid-soluble phosphate. The level of acid-soluble organic phosphorus was 16-fold higher than the level of inorganic phosphate, and this finding is in agreement with the results of Jennings and Morton (1963), and Williams (1970). Up to 20-30 days after anthesis, inorganic phosphorus accounts for almost all of the total acid-soluble phosphorus, but thereafter non-labile organic phosphorus (such as phytic acid) increases substantially. By day 35, nearly all of the phosphate in the aleurone layer occurs as phytic acid.

The addition of dilute solutions of KH_2PO_4 to the incubation media induced increases in the levels of nucleotides, especially those of ATP, UTP, CTP and GTP. Since the level of inorganic phosphate in mature aleurone is relatively low, it may be the limiting factor in phosphate-requiring metabolic reactions. Bieleski (1968) showed that the imposition of a phosphate deficiency on the aquatic plant, Spirodela, caused a concomitant drop in the phosphate ester content. However, the relative proportions of the

esters remained constant and this is in contrast to the results obtained with aleurone tissue. In the presence of 1mM KH_2PO_4, the level of ATP rose by about half, whilst that of CTP increased 2.6-fold. It has been observed that the level of ATP in red blood cells is increased significantly when additions of inorganic phosphate are made to normal blood (Lichtman and Miller, 1970). The activity of phosphorylases can be stimulated by increases in the physiological concentrations of inorganic phosphate. Degradation of polysaccharides, phospholipids, and nucleic acids may then be catalysed and the high energy phosphate bonds conserved in phosphorylated intermediates such as free nucleotides (Kornberg, 1962).

Samples of aleurone tissue were incubated in the presence and absence of GA and the nucleotides measured at intervals up to 24 hours (Table 7). It was clear from the results that while only small changes were related to the presence of the hormone, relatively large fluctuations were recorded in both water- and GA-treated tissue. These reached a maximum after about 2 hours, when the levels of ATP and UDPG were depressed and those of ADP and UTP were increased. Except for UTP, the effects disappeared over the next 2-4 hours. UDPG was especially affected. After an initial drop of 33%, there was a net accumulation between 2 and 6 hours of 121 nmoles or almost 60% of the original amount.

Further examination of the effect revealed that it was closely associated with the method used to isolate the tissue (Table 9). Compared to most other plant parts, the cell walls of the aleurone are exceptionally thick. Nevertheless, damage occurs during preparation, and it was concluded that this damage provided the stimulus for the major modifications in nucleotide levels. Ambient solutions were examined for evidence of leakage of either nucleotides or bases, but no significant amounts of U.V-absorbing compounds were found. However, this does not preclude the possibility of extrusion of purine and pyrimidine degrading enzymes by damaged aleurone which would be capable of transforming the bases of any nucleotides in the ambient solution into non U.V. -absorbing products. This possibility was not investigated.

Studies with the Saguaro cactus (Steelink, Yeung and Caldwell, 1967), indicate that mechanical wounding leads to a marked stimulation of the reactions leading to shikimic acid, and thence to flavenoids via aromatic amino acids. In living cells, flavenoids are converted to the more stable glycosides by glycosyl transfer from sugar nucleotides such as UDPG (Barber, 1962).

In addition, rapid cell differentiation and major changes in carbohydrate metabolism follow wounding, (Steelink, Riser and Onore 1968) and this may account for some of the changes observed in UDPG.

Bagi and Farkas (1967) demonstrated that ribonuclease

activity in tobacco leaves increased after the leaves were mechanically damaged. A similar increase has been reported to occur in barley aleurone layers after separation from the starchy endosperm (Chrispeels and Varner, 1967a). It seems highly probably therefore that some of the changes which occurred in free nucleotide levels during the 6 hours after preparation of wheat aleurone may have resulted from degradation and resynthesis of part or all of the RNA complement.

During the total incubation period of 24 hours, two distinct effects of GA were discerned. The levels of ATP and UDPG, depressed after 2 hours in the absence of GA (Table 7), were depressed still further when the hormone was present. The effect disappeared after a further 60 min. A second phase occurred at 15 hours, after the production of α-amylase had commenced, when the levels of UDPG and ADP rose, and then at 24 hours, similar trends were observed in UTP and ATP. The first effect, manifest at 2 hours, was generally not observed if the tissue was left to equilibrate for 6 hours in water before GA was administered. It seems likely, therefore, that some part of the wound response is sensitive to the presence of GA. Since only the short-term effects of GA were of major interest, the changes taking place from 15 hours onwards were not investigated further.

To obviate the influence caused by damage during isolation, the practice of allowing the tissue to equilibrate in water for 6 hours was adopted. Subsequently, treatment

with GA, in the presence of ^{32}P, showed clearly that the specific activities of the nucleotides were altered by the hormone. The mean incorporation of label was 6% less than the control treatment after 15 min. and 19% greater after 30 min., both effects being significant. By 90 min. the increase had fallen to 5%, and after 120 min. there was a slight (3%) but non-significant depression (Table 17). These results were supported by those of another experiment in which GA caused an increase in specific activity of 26% after 60 min. and a depression of 20% after 3 hours (Table 14).

Only slight effects were found to occur in nucleotide levels. When the values for UDPG in the presence and absence of GA were averaged for each time of incubation, it was found that the level at 90 min. was depressed significantly below the levels at 15 and 120 min. Similarly, the values for NAD at 30 and 90 min. were significantly lower than those at 15 and 120 min. The average effect of GA on NAD was found to be significant, this value being 12% higher than the control. In contrast to the earlier experiments, carried out without a 6 hour recovery period, GA had no effect on the levels of UDPG or ATP. Levels of UTP and GTP were also unaffected by GA, and, as with ATP, were not affected by time of incubation. However the amount of CTP was significantly depressed overall by 8% by GA.

An increase in the uptake of ^{32}P by GA could be expected to lead to the increases in specific activities of

nucleotides observed at 30 and 60 min. The incorporation of ^{32}P into the inorganic and total acid-soluble phosphate pools of water- and GA-treated aleurone was examined. It was found that uptake of ^{32}P is a relatively slow process, since even after 90 min. the specific activity of the phosphate in the ambient solution was 10- to 20-fold greater than that in the tissue. Moreover, uptake occurred in two phases, the first and most rapid being attributed to adsorption. When precautions were taken to ensure that this component of the total counts was the same for all treatments, it was found that GA had no effect on the uptake of ^{32}P up to 20 min. after administration. Thus, this factor did not contribute to the effect of GA on the radioactivity of the nucleotides. It was also apparent that movement of inorganic phosphate into and out of the tissue is under stringent control. Efflux was estimated to be 5.4-fold greater than uptake when both were measured over the same period of time.

The specific activities of ATP, UTP and GTP increased by approximately 25% after treatment for 30 min. with GA, whereas that for CTP increased by 38%. However, initially the specific activity of CTP was significantly lower than the other triphosphates, and the absolute increase in specific activity for each of the four compounds was almost identical. By 90 min. the level of radioactivity in ATP, UTP, and GTP had fallen to the same value as the control, but that in CTP remained significantly higher than the control. It then fell to the control level by 120 min. Thus, of the

four triphosphates, CTP appeared to respond uniquely to GA. This was further shown by the fact that whereas the increase in the specific activity of each of the triphosphates at 30 min. was due almost entirely to an increase in the total counts incorporated, the increase in the specific activity of CTP at 90 min. was ascribable to a drop of 20% in the level of that compound.

Although ^{32}P was administered for only the final 15 min. of incubation in each case, the total counts incorporated by the control increased as the incubation period was extended indicating a general increase in the rate of phosphorus metabolism.

As discussed earlier, very few changes occurred in the actual levels of nucleotides. However it is apparent that, in some cases, specific activities are increased by at least 25% by GA. These results suggest either a stringent control on nucleotide levels, or the participation of only the phosphate moieties of the nucleotides. It is unlikely that UTP, GTP and CTP would be involved in reactions requiring only phosphate donation to the same extent as ATP. Thus, the most reasonable explanation is that of stringent control.

If the ambient solution around tissue incubated in petri dishes during the 6 hour equilibration period, was diluted before further incubation of the tissue for 60 min. in water alone, the uptake of ^{32}P by nucleotides was stimulated

1.5-fold (Table 16). This result could be explained by either a decrease in the concentration of some inhibitory end-product(s) of of metabolism, or a lower level of Pi in the ambient solution when ^{32}P was added after the additional 60 min. of incubation. Moreover, a reduction in the extent to which nucleotides increased in specific activity in response to GA could be brought about by the same treatment (Table 32). When a portion of an ambient solution was removed, small quantities of concentrated test solutions added, and the portion of ambient returned to the tissue for a further 30 min., a depression in specific activity of nucleotides was observed in the treatment which received GA. A similar depression in ^{32}P uptake was noted after 15 min. under conditions in which the tissue was kept constantly agitated. It seems probable, therefore, that the response to GA can be slightly retarded by maintaining the ambient solution and its concentration relatively unchanged. Slight effects were also observed on the production of α-amylase.

When aleurone layers were extensively damaged during preparation and, as a consequence, production of α-amylase was severely restricted, no effects were observed on the specific activity of nucleotides after treatment with GA (Table 37). A relationship between nucleotide metabolism and enzyme production may none the less exist, however more direct evidence for this was not obtained here. Changes in nucleic acid metabolism, induced by hormones, have been

reported for a number of tissues. For example, the addition of indolyl-3-acetic acid to sub-apical sections of Avena coleoptile is reported to increase, by about 50%, the incorporation of ^{3}H-uridine into all classes of coleoptile RNA after 8 hours (Hamilton et al., 1965). Chandra and Duynstee (1967) found that the incorporation of ^{32}P into RNA of barley aleurone cells was increased by 88% after treatment with GA for 30 min. However, these authors treated embryo-less half seeds and then removed the aleurone layers by hand peeling before estimation of RNA. As discussed earlier the removal of aleurone layers from the endosperm rapidly initiates both a wound response and an increase in ribonuclease activity in the tissue. Moreover, it was found that, in wheat aleurone, part of the wound response is enhanded by GA. Thus at least part of the effect of GA on RNA metabolism in barley aleurone reported by Chandra and Duynstee (1967) may be attributed to the same phenomenon. Other transient increases in the incorporation of precursors into RNA have been reported for specific RNA fractions obtained from rat kidney after treatment with aldosterone (Forte and Landon, 1968), and for both acid-soluble and acid-insoluble compounds extracted from rat uterine tissue exposed to oestradiol (Billing, Barbiroli and Smellic, 1969). In addition the initial significant depression in the specific activity of nucleotides after treatment of aleurone for 15 min. with GA maybe analogous to the slight but reproducible depression in nuclear RNA synthesis (incorporation of ^{32}P)

found by Kenny and Kull (1963) to occur in the liver of adrenalectomised rats 15 min. after the administration of hydrocortisone.

Although both free nucleotides and RNA metabolism appear to be involved in the hormonal responses of various animal and plant systems, the relation between the former two seems to be obscure. Thus, Varner and Chandra (1964) concluded there was no qualitative change in the incorporation of ^{32}P into acid-soluble compounds of barley aleurone after treatment with GA, although the amount of RNA was found to increase. Other reports with different tissues show that both RNA and acid-soluble components are affected by hormones, but differ in the time sequence at which each is affected (Wicks and Kenney, 1964; Billing, Barbiroli and Smellie, 1969; Liew and Gornall, 1969; Peck and Messinger, 1970).

As already mentioned, the increase in specific activity of ATP, GTP, UTP, and CTP after treatment of aleurone for 30 min. with GA, was almost identical in each case, although the relative increase in CTP was greater. If nucleic acid synthesis alone was stimulated by GA at 30 min. then the increases in specific activity would be expected to be in proportion to the amount of each triphosphate present (assuming approximately equal incorporation into RNA). As this was not found, the results cannot be interpreted as reflecting exclusively the synthesis of new RNA. Also contrary to the evidence that many hormones can bring about

changes in RNA metabolism, the relatively small effect of GA observed on nucleotide metabolism in wheat aleurone argues against a major alteration in nucleic acid synthesis in this tissue. The data appear to be more consistent with the presence of m-RNA in a masked or inactive form which has the potential to support the greatly enhanced enzyme synthesis induced by GA.

It is apparent that in wheat aleurone CTP exhibited a unique response in relation to the effect of GA on the metabolism of free nucleotides. Additional evidence for this response is given in Tables 33 and 34. In both cases the results of 10 experiments, which include a total of 20 observations, have been pooled. These observations cover incubation periods varying from 15 to 180 min. Only the specific activities of the nucleoside triphosphates have been considered, and these have been transformed to the percentage ratio of GA-treated to control. The data collated in the Tables is detailed in Appendices 2 to 8. The mean percentage effect of GA is shown in Table 33 together with the values of t for the analysis of paired observations.

Table 33

Test of significance of the percent effect of GA on specific activities of nucleoside triphosphates. Analysis of 20 paired observations pooled from 10 experiments.

Nucleotide	% effect of GA	Value of t.	Level of Significance
ATP	101.5	0.383	N.S.
CTP	112.6	2.099	5%
GTP	103.6	0.723	N.S.
UTP	102.0	0.485	N.S.

It can be seen that over a large range of treatments and incubation times, GA has a specific and reproducible effect on the specific activity of CTP. This is not so in the case of ATP, UTP and GTP. An increase in specific activity can be brought about by either a decrease in nucleotide level or an increase in total counts incorporated, or both. Increases in the specific activity of CTP were found to be due to different combinations of both factors, and this can be seen from the data in the Appendices from which Table 33 has been compiled.

In Table 34, the percent effect of GA on the triphosphates has been compared for pairs of compounds and the value of t for the mean difference computed. In addition, the correlation coefficients between the percent effect of GA on pairs of nucleotides have been calculated.

Table 34.

Test of significance of the difference in percent effect of GA between pairs of nucleotides, and the correlation coefficient between the response of pairs of nucleotides to GA.

Nucleotide Pairs	Mean Difference (%)	t value	Correlation Coefficient (r)	Confidence Interval (95%)	Coefficient of Determination (r^2)
ATP-GTP	2.2	0.873	0.859	0.673-0.943	73.8
GTP-UTP	1.9	0.795	0.770	0.498-0.904	59.3
ATP-UTP	0.5	0.388	0.947	0.871-0.979[a]	89.7
ATP-CTP	11.0	2.258*	0.591	0.202-0.819[b]	34.9
GTP-CTP	9.3	2.104*	0.717	0.390-0.884	51.4
UTP-CTP	10.7	2.351*	0.654	0.298-0.850[b]	42.8

* Significant at the 5% level

a Significantly different from b.

It is apparent from the mean difference of the effect of GA on different nucleotides that CTP responds in a significantly different manner to that of ATP, UTP and GTP. It is also clear that both ATP and UTP respond in an almost identical fashion to the presence of GA. Furthermore, the correlations between the effect of GA on ATP and CTP, and on UTP and CTP, are significantly lower than between ATP and UTP. The co-efficient of determination indicates that proportion of the sum of squares of the correlation coefficient which can be attributed to the interdependence of the sample populations. Thus, ATP and CTP respond in a similar fashion to GA on only 35% of the occasions investigated, whereas ATP and UTP show similar effects on 90% of occasions.

The initial work of Kennedy and Weiss (1956) outlined the relation between the cytidine nucleotides and lipid synthesis, and further studies have confirmed that, in addition to its role as a nucleic acid precursor, this is the major function of CTP in cellular metabolism at present known (Kennedy, 1956; Weiss et al, 1958; Kiyasu et al, 1963; Carter, 1968; Sumida and Mudd, 1968). The importance of ribosomes, and membranes of the endoplasmic reticulum, in protein synthesis has been discussed by Tata (1967, 1970) and it is clear that there is a close correlation between the rates of RNA accumulation, membrane phospholipid synthesis, and protein synthesis. In addition, Kerkof and Tata (1969) have shown that the incorporation of ^{32}P-labelled phospholipids

into microsomes of rat parotid slices is greatly enhanced in the presence of thyrotrophic hormone. Some evidence is also available for the selective effect of hormones on the incorporation of cytidine nucleotides into RNA, although again this is derived from work on mammalian tissues (Talwar, Gupta and Gros, 1964; Bashirelahi and Villee, 1970). On the other hand, Johri and Varner (1968) have reported a decrease in the number of uracil-cytosine pairs in RNA extracted from dwarf pea seedlings after treatment with GA.

It is clear that the most consistent effect of GA on the free nucleotides of wheat aleurone is an increase in the specific activity of CTP. In addition, according to present knowledge, most of the metabolism of the cytidine nucleotides can be accounted for by either nucleic acid metabolism or the synthesis of phospholipids. However, as has been discussed earlier, the results obtained do not support the conclusion that major changes in nucleic acid metabolism occur, nor is there any evidence to suggest that cytidine-rich nucleic acids are induced by hormones in plant tissues. Thus, the most reasonable explanation for the observed response is an enhanced synthesis of phospholipids.

Chen and Osborne (1970) found that ribosomes extracted from wheat embryos which had been incubated with GA had a greater capacity to incorporate labelled amino acids into acid-insoluble material than the controls. However, there was no effect of the hormone on the formation of

polysomes. Moreover, the addition of GA to a cell-free system of ribosomes from wheat embryos actually evoked an inhibition in ribosomal activity. They suggested therefore that a cofactor, which could be associated with the membranes, was necessary for the in vivo function of GA.

Tata (1970) has described the effect of hormones on the incorporation of various precursors into the phospholipids of the endoplasmic reticulum obtained from different tissues, and concludes that the formation of membranes is tightly co-ordinated with an increase in protein synthesis.

It is suggested therefore that one of the earliest effects of GA on wheat aleurone tissue is manifest in an increase in membrane synthesis, and that the new membranes so formed act as a participant in a system already provided with sufficient m-RNA, albeit inactive, and ribosomal material to support active protein synthesis.

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APPENDIX 1

Levels of free nucleotides in wheat aleurone tissue (nmoles per 4 gm fresh weight).

Time (Hrs)	Treatment	ATP			ADP			UDPG		UTP				GTP			CTP		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	C	222	192	214	21	21	19	230	190	236	70	93	78	31	26	32	29	29	25
	-GA	227	198	216	20	23	19	184	177	188	99	98	103	38	30	38	36	25	50
	+GA	197	171	218	20*	21	18	172*	160	183	86	86	116	35	23	38	36	23	47
2	C	237	268	232	21	19	15	242	274	245	89	96	95	38	36	17	40	40	24
	-GA	237	218	220	31	22	28	176	171	186	123	110	131	35	32	24	41	35	36
	+GA	208	216	216	26	21	29	156	164	169	126	131	130	32	29	26	43	40	37
3	C	236	202	188	16	17	47	208	109	227	66	62	63	48	35	19	27	32	28
	-GA	222	195	159	26	19	50	204	102	165	87	79	81	37	38	14	30	31	28
	+GA	222	180	163	31	11	51	189	100	153	94	75	87	41	37	19	36	37	30
6	C	209	267	204	12	23	15	156	194	168	54	73	51	38	44	44	44	37	42
	-GA	205	248	235	13	25	23	209	216	174	77	93	80	44	43	38	43	45	47
	+GA	214	246	230	15	29	25	217	235	191	96	103	93	42	50	46	43	59	37
15	C	206	200	209	19*	19	19	188	187	234	63	50	67	33	34	33	32	33	33
	-GA	222	202	208	18*	18	17	202	195	222	82	88	89	28	32	33	52	35	56
	+GA	218	222	235	21*	20	22	229	247	284	89	83	93	31	37	45	50	29	55
24	C	202	236	203	19	31	12	169	250	168	62	81	83	36	39	24	39	35	37*
	-GA	199	216	210	16	24	13	165	254	234	65	70	63	34	35	41	47	61	54*
	+GA	244	274	230	16	22	14	196	272	240	116	133	109	43	39	39	51	67	59

* Estimated missing values.
C = Control at zero time

-GA = Treated with water
+GA = Treated with GA

APPENDIX 2

Nucleotide levels and specific activities following incubation of aleurone tissue with ^{32}P in the presence and absence of GA

		nmoles/4 gm fresh weight				c.p.m. per nmole			
		1 hr		3 hrs		1 hr		3 hrs	
		-GA	+GA	-GA	+GA	-GA	+GA	-GA	+GA
NAD	1	49	48	35	39	9	12	-	-
	2	39	36	33	36	-	-	10	1
	3	36	33			-	1		
ADP	1	22	18	22	23	359	532	393	342
	2	20	18	17	14	370	464	506	380
	3	21	18			299	368		
ATP	1	218	223	234	226	868	1159	837	956
	2	225	221	225	241	801	840	954	547
	3	239	232			659	688		
CTP	1	48	50	62	60	763*	982*	684	599
	2	59	41	64	53	619	1039	612	458
	3	53	59			512	530		
GTP	1	38	27	22	36	636	864	668	640
	2	32	18	35	35	607	745	664	434
	3	37	35			456	500		
UTP	1	119	111	133	128	948	1347	917	877
	2	121	116	130	133	838	939	1103	640
	3	146	137			663	763		
UDP	1	1	1	8	10	1578	2062	2012	1440
	2	6	1	7	6	2254	11752	2256	1675
	3	9	6			1066	2129		
UDPG	1	206	156	203	188	426	553	362	339
	2	194	129	155	195	348	524	392	223
	3	188	168			269	295		

*Estimated missing values.

APPENDIX 3

Time-course of levels and specific activities of nucleotides after treatment of aleurone with GA.

Time (min.)	Treatment	NAD 1	NAD 2	ADP 1	ADP 2	ATP 1	ATP 2	CTP 1	CTP 2	GTP 1	GTP 2	UTP 1	UTP 2	UDP 1	UDP 2	UDPG 1	UDPG 2
(a) nmoles																	
15	-GA	53	51	17	19	286	356	107	144	49	55	211	288	11	8	198	230
	+GA	55	49	24	20	326	300	113	128	58	37	249	214	8	8	207	179
30	-GA	38	43	23	19	304	310	102	127	55	52	230	226	18	6	175	206
	+GA	52	52	20	23	263	308	103	125	48	51	179	249	8	11	180	208
90	-GA	38	44	19	20	271	291	117	138	62	45	188	231	19	11	167	166
	+GA	47	57	22	17	258	296	88	115	51	53	194	268	5	13	163	174
120	-GA	46	47	20	14	280	340	107	113	42	50	213	274	6	8	190	227
	+GA	62	61	16	21	268	319	103	109	62	42	178	263	6	13	185	194
(b) Specific activity (c.p.m/nmole)																	
15	-GA	13	17	154	148	256	274	170	150	230	261	256	273	448	927	132	149
	+GA	10	19	108	141	241	278	148	154	204	253	245	269	862	408	122	143
30	-GA	13	13	146	120	402	227	209	124	374	200	402	222	737	623	198	113
	+GA	18	18	176	145	431	319	280	175	391	294	435	309	852	2512	222	91
90	-GA	-	17	189	141	434	288	257	144	394	247	434	291	443	775	202	142
	+GA	17	14	136	165	410	333	287	204	368	292	426	334	1175	539	209	162
120	-GA	12	-	150	229	314	446	207	285	312	420	328	425	906	633	160	229
	+GA	12	15	130	207	295	450	216	264	276	467	316	432	838	585	146	224

APPENDIX 4

Residual radioactivity of wheat aleurone tissue after incubation with ^{32}P and subsequent rinsing with indicated solutions (c.p.m. per mg dry weight).

Number of rinses	Rinsed with water		Rinsed with mM KH_2PO_4	
	-GA	+GA	-GA	+GA
0	7274	5431	5796	4908
1	5562	5362	2200	2152
2	3869	2605	1755	1424
3	2183	2052	1499	1476
4	1164	1070	785	955
5	1050	1823	963	1000
6	1705	903	973	559
7	1225	787	628	424
8	1012	909	595	528

APPENDIX 5

Ambient solution "unaltered". Incubated 30 min.

	nmoles		Counts/min.(x 10^3)		Specific	
1.	-GA	+GA	-GA	+GA	-GA	
NAD	38	51	0.2	0.1	7	2
ADP	11	20	0.5	0.5	42	24
ATP	167	230	12.5	11.6	75	51
CTP	61	83	2.9	2.6	48	31
GTP	40	41	3.5	2.2	87	53
UTP	80	134	6.8	7.8	85	59
UDPG	143	180	7.1	6.2	49	35
2.						
NAD	22	23	0.4	0.1	17	6
ADP	23	20	1.4	1.3	61	67
ATP	201	186	32.7	30.4	162	163
CTP	64	79	7.9	7.7	123	97
GTP	37	33	5.1	4.7	139	143
UTP	122	103	20.9	16.9	171	164
UDPG	183	175	14.4	14.0	79	80
3.						
NAD	-	18	-	0.4	-	22
ADP	-	15	-	2.0	-	132
ATP	198	183	61.3	52.9	310	289
CTP	76	55	10.5	9.0	138	164
GTP	37	43	9.1	7.8	246	182
UTP	102	97	31.1	27.9	305	287
UDPG	166	155	25.6	22.5	154	145

APPENDIX 6

Ambient solution "half replaced". Incubated 30 min.

	nmoles		Counts/min(x 10^3)		Specific A	
1.	-GA	+GA	-GA	+GA	-GA	
NAD	43	43	0.4	0.4	8	8
ADP	16	12	0.9	0.7	57	62
ATP	224	218	27.2	28.2	122	129
CTP	63	54	5.1	5.1	80	94
GTP	38	39	4.5	4.7	118	121
UTP	143	136	18.9	18.6	132	137
UDPG	168	168	10.6	10.5	63	63
2.						
NAD	34	41	0.6	0.3	17	7
ADP	20	19	2.1	2.0	103	104
ATP	212	210	63.8	65.3	301	311
CTP	87	81	14.4	16.6	166	204
GTP	-	41	-	10.8	-	264
UTP	135	139	44.8	49.5	332	356
UDPG	196	193	26.8	24.9	137	129

APPENDIX 7

Entire incubation carried out in mM KH_2PO_4.
Treated with and without GA for 60 min.

	nmoles		Counts/min(x 10^3)		Specific Activ.	
	-GA	+GA	-GA	+GA	-GA	+GA
NAD	32	55	0.04	0.07	1.3	1.2
ADP	18	24	0.8	0.8	42	34
ATP	328	326	23.1	23.1	70	71
CTP	132	137	6.7	8.2	51	60
GTP	58	67	4.2	5.4	72	80
UTP	209	255	16.8	19.3	80	76
UDPG	199	200	6.2	6.1	31	31

APPENDIX 8

Entire incubation carried out in 0.25 mM KH_2PO_4.
Treated with and without GA for 60 min.

	nmoles		Counts/min(x 10^3)		Specific Activi.	
1.	-GA	+GA	-GA	+GA	-GA	+GA
NAD	45	52	0.1	0.1	2	2
ADP	22	26	2.0	2.2	89	83
ATP	288	299	57.5	60.2	200	201
CTP	66	..	-	-	-	-
GTP	48	49	7.7	9.3	160	190
UTP	191	201	42.6	46.7	223	230
UDPG	200	200	16.5	17.6	83	88
2.						
NAD	43	43	0.02	0.01	0.4	0.3
ADP	15	18	1.2	1.2	79	66
ATP	279	260	46.6	43.0	167	166
CTP	75	73	6.5	9.5	87	130
GTP	43	44	5.7	7.2	133	165
UTP	187	185	35.9	34.9	192	189
UDPG	189	161	13.2	10.6	70	66