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THE HORMONAL CONTROL OF INORGANIC ION RELEASE FROM WHEAT ALEURONE TISSUE

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Abstract—During germination the aleurone cells of wheat undergo extensive changes in their ion release capacities. Within 12 hr of imbibition a cytokinin-like hormone from the endosperm induces an increase in the ability of the cells to retain their reserves of potassium, phosphorus, magnesium and calcium. The induction process appears to be dependent upon both protein synthesis and oxidative energy metabolism. The maintenance of the induced state is dependent upon oxidative energy metabolism. Later during germination, a gibberellin from the embryo induces the release of the retained ions. This induction process appears to be independent of protein synthesis but is inhibited by abscisic acid.

INTRODUCTION

IN THE quiescent wheat grain the major reserves of the macronutrient elements are located in the aleurone cells.[1] Most of these reserves are confined as phytin in the aleurone grains.[2] This phytin is hydrolysed by phytase activity during germination and the inorganic products are redistributed to the developing seedling.[3] In the accompanying paper[3] the precise patterns of redistribution have been defined and some of the controlling factors have been identified. The studies using endosperm halves indicated, in particular, that the gibberellins play an important part in stimulating mineral mobilization from the aleurone cells. In view of the known effects of gibberellic acid[4] and of the cytokins[5] in regulating the metabolisms of several organic storage materials in cereal grain, we have sought similar evidence with regard to the mobilization of the mineral reserves. To this end, the ion release capacities of excised bran were studied to determine the roles that other tissues of the grain, and the possible roles that the phytohormones play in controlling these functions.

RESULTS

In order to avoid congestion, the results for calcium have been omitted from most of the figures. The changes in release rates of this element under the various experimental conditions were qualitatively similar to those of magnesium.

Bran quarters excised from the quiescent grain released their macronutrient mineral reserves rapidly into aqueous media. Within 5 hr all of the available reserves of potassium, magnesium and calcium had passed into the medium and within 24 hr the release of phosphate was complete. The minerals were not reabsorbed by the bran during incubation for a further 48 hr. In contrast, bran excised from grain that had germinated for 24 hr released its inorganic ions at rates less than half of those for the quiescent grain. Furthermore, some

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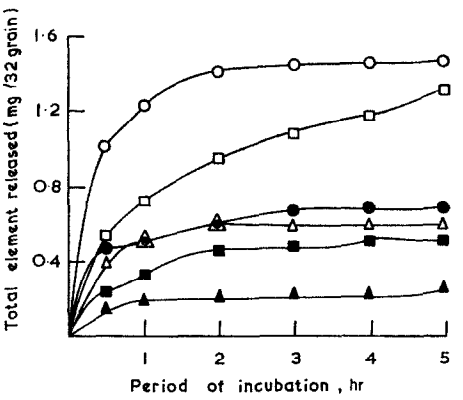

FIG. 1. ION RELEASE FROM THE BRAN OF QUIESCENT GRAIN AND THE BRAN OF GRAIN GERMINATED FOR 24 hr.

○, Potassium release from bran of quiescent grain. ●, Potassium release from bran of grain germinated 24 hr. □, Phosphate–phosphorus release from bran of quiescent grain. ■, Phosphate–phosphorus release from bran grain germinated 24 hr. △, Magnesium release from bran of quiescent grain. ▲, Magnesium release from bran of grain germinated 24 hr.

50 per cent of the tissue minerals were now unavailable for release (Fig. 1). Bran that had been excised from quiescent grain and then maintained in a moist atmosphere for 24 hr behaved in exactly the same way as the bran from the quiescent grain. The use of moist conditions, where water availability to the bran was limited, ensured that minerals did not move from the aleurone cells during the 24 hr incubation. When bran was studied over a longer period of germination, the rates of release of all of the elements increased again up to the fifth day (Fig. 2).

Figure 3 illustrates the ion release capacity of bran from incubated endosperm halves and of bran excised from quiescent grain and incubated alone in a moist atmosphere. The removal of the embryo clearly does not affect the decrease in release capacity during the

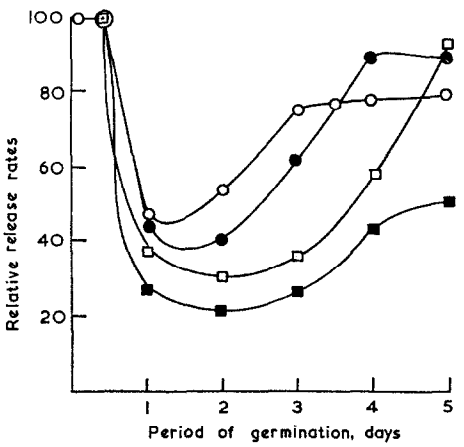

FIG. 2. ION RELEASE CAPACITIES OF THE BRAN EXCISED FROM GERMINATING GRAIN.

○, Potassium; ●, Phosphate–phosphorus; □, Magnesium; ■, Calcium.

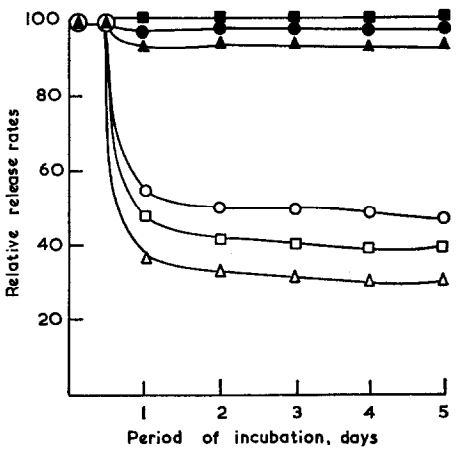

FIG 3. ION RELEASE CAPACITIES OF THE BRAN EXCISED FROM INCUBATING ENDOSPERM HALVES AND OF QUIESCENT BRAN INCUBATED ALONE.

○, Potassium release from endosperm half bran. ●, Potassium release from incubated bran. □, Phosphate–phosphorus release from endosperm half bran. ■, Phosphate–phosphorus release from incubated bran. △, Magnesium release from endosperm half bran. ▲, Magnesium release from incubated bran.

first day of germination, but it nullifies the subsequent increase. The early removal of the starchy endosperm prevents the decrease in ion release capacity.

The results obtained so far suggest that factors from both the embryo and the starchy endosperm are instrumental in controlling ion release from the aleurone cells. Bran from quiescent grain was, therefore, incubated for 48 hr with extracts of starchy endosperm and with solutions of authentic phytohormones. The ion release capacities of the bran were then

TABLE 1. THE INDUCTION OF REDUCED ION RELEASE CAPACITY IN THE BRAN FROM QUIESCENT GRAIN

	Initial ion release rate (mg/hr/32 grain)			
	P	K	Mg	Ca
Bran from quiescent grain (high release control)	1·0	2·0	0·80	0·40
Bran from grain germinated 48 hr (low release control)	0·36	1·0	0·24	0·08
Quiescent bran incubated 48 hr with:				
0·06 M Mannitol	1·0	2·0	0·80	0·40
Endosperm extract	0·47	1·3	0·37	0·17
10 nM Kinetin	0·58	1·5	0·48	0·24
10 μM GA_3	1·0	2·0	0·82	0·42
1 μM 1AA	0·95	2·0	0·74	0·38
10 μM ABA	1·0	2·0	0·78	0·47

GA_3, gibberellic acid.
1AA, indole acetic acid.
ABA, abscisic acid.

determined. Either a starchy endosperm extract or kinetin was able to produce a considerable reduction in both the release rates and the total release of ions from the bran. The extract was more effective than the authentic hormone, but neither was able to induce a change equal to that occurring in incubating endosperm halves or in germinating grain (Table 1). Kinetin was most effective at 10 nM concentration. Neither indole acetic acid, gibberellic acid nor abscisic acid had any effect. The ion release capacities of bran from quiescent grain were unaffected by any of the hormones, including kinetin, when the hormones were included in the medium used for the assay of the ion release capacity.

To determine the precise time-scale of the movement of the endosperm factor to the aleurone cells, batches of grain were germinated for periods up to 24 hr. At 2 hr intervals, batches were dissected and their bran was incubated for the remainder of the 24 hr period in a moist atmosphere. The ion release capacities of the bran were then determined. Changes were observable within 5 hr of imbibition and they were complete within 12 hr. The active factor is therefore mobile very early during germination.

In order to identify the effector of the increase in ion release capacity that occurs later during germination, bran excised from grain that had germinated for 24 hr was used. Gibberellic acid, when added directly to the assay medium, increased the release of each macronutrient element from the tissue (Table 2). Indole acetic acid increased and abscisic acid inhibited the action of gibberellic acid, although these hormones and kinetin were without effect on their own. Gibberellic acid at the optimum concentration of 10 μM induced an increase in ion release rates after a lag period of only 2–3 hr (Fig. 4).

A number of anti-metabolites inhibited the kinetin-induction of reduced release capacity in incubated endosperm halves (Table 3). Inhibitors of RNA and protein synthesis showed a variable ability to block the induction process. Actinomycin D was notably without effect.

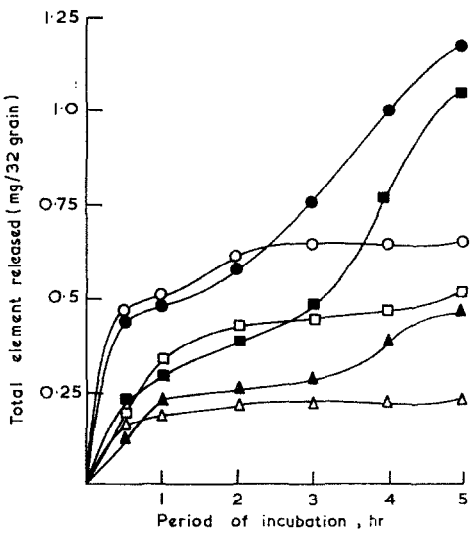

FIG. 4. THE EFFECT OF GIBBERELLIC ACID ON ION RELEASE FROM THE BRAN OF GRAIN GERMINATED FOR 24 hr.

○, Potassium release from bran in buffer. ●, Potassium release from bran in buffer + 10 μM.GA_3. □, Phosphate–phosphorus release from bran in buffer. ■, Phosphate–phosphorus release from bran in buffer + 10 μM.GA_3. △, Magnesium release from bran in buffer. ▲, Magnesium release from bran in buffer + 10 μM.GA_3. GA_3 = gibberellic acid.

TABLE 2. THE INDUCTION OF INCREASED ION RELEASE CAPACITY IN THE BRAN FROM GERMINATED GRAIN

Addition to assay medium	Total ion release after 5 hr (mg/32 grain)			
	P	K	Mg	Ca
None (control)	0·48	0·65	0·21	0·11
10 μM GA_3	1·15	1·27	0·47	0·29
10 μM 1AA	0·49	0·68	0·21	0·11
10 nM 1AA	0·51	0·70	0·23	0·12
1 nM 1AA	0·48	0·68	0·21	0·10
10 μM Kinetin	0·50	0·69	0·22	0·12
10 nM Kinetin	0·48	0·71	0·21	0·13
1 nM Kinetin	0·49	0·68	0·22	0·11
10 μM ABA	0·48	0·69	0·21	0·10
10 μM GA_3 + 10 nM 1AA	1·25	1·47	0·60	0·32
10 μM GA_3 + 10 nM Kinetin	1·17	1·30	0·49	0·30
10 μM GA_3 + 10 μM ABA	0·50	0·70	0·23	0·14
10 μM GA_3 + 10 μM ABA + 10 nM Kinetin	0·50	0·71	0·22	0·14

GA_3, gibberellic acid.
1AA, indole acetic acid.
ABA, abscisic acid.

TABLE 3. THE EFFECTS OF METABOLIC INHIBITORS ON THE INDUCTION OF REDUCED ION RELEASE CAPACITY

	Initial ion release rate (mg/hr/32 grain)		
	P	K	Mg
Bran from quiescent grain (high release control)	1·1	2·2	0·84
Bran from incubated endosperm halves (low release control)	0·42	0·90	0·30
Inhibitors of RNA and protein synthesis:			
Actinomycin D, 50 μg/ml + 1 mM potassium bromate	0·50	1·1	0·34
Acridine orange, 20 μg/ml	0·90	1·5	0·58
Chloramphenicol, 20 μg/ml	0·50	0·94	0·32
Mikamycin, 50 μg/ml	0·70	1·2	0·38
Cycloheximide, 5 μg/ml	0·68	1·4	0·40
Puromycin, 50 μg/ml	0·70	1·4	0·36
Inhibitors of energy metabolism:			
1 mM sodium cyanide	0·98	1·8	0·90
1 mM sodium azide	1·0	1·6	0·92
Antimycin A, 30 μg/ml	0·54	1·1	0·54
100 μM, 2,4-dinitrophenol	0·74	1·4	0·68
Chloramphenicol, 500 μg/ml	1·1	2·0	0·74
1 mM sodium arsenate	1·0	1·6	0·78
Oligomycin, 10 μg/ml	0·90	1·9	0·90
Other metabolic inhibitors:			
1 mM sodium monofluoroacetate	0·46	0·98	0·36
1 mM sodium malonate	0·44	0·96	0·32
1 mM sodium fluoride	0·60	1·4	0·74
Endosperm halves incubated at 4°	0·90	1·8	0·70

Endosperm halves were soaked for 1 hr in the specified inhibitor solution and then incubated for 48 hr in the presence of the inhibitor.

Several inhibitors of energy metabolism were, however, much more effective. Incubation of the endosperm halves at 4° also strongly inhibited the induction. In these experiments it was necessary to confirm that the inhibitors were affecting the induction process and not merely the subsequent assay of ion release capacity. None of the inhibitors of RNA or protein synthesis had any significant direct effect on ion release from the bran of either the quiescent grain or the incubated endosperm halves. Several inhibitors of energy metabolism, on the other hand, stimulated ion release from both of these tissues (Table 4). This observation can partly, but not entirely, explain the action of these inhibitors on the induction process. It is notable in this respect that fluoride specifically stimulated the release of potassium from both control tissues, although it inhibited the induction process with respect to all the macronutrient elements.

TABLE 4. THE EFFECTS OF METABOLIC INHIBITORS ON ION RELEASE CAPACITY

Inhibitor	Initial ion release rates (mg/hr/32 grain)		
	P	K	Mg
Bran from quiescent grain			
None	1·1	2·2	0·88
1 mM sodium cyanide	1·2	2·4	1·0
1 mM sodium azide	1·1	2·3	0·98
1 mM 2,4-dinitrophenol	1·1	2·3	0·92
Chloramphenicol, 550 μg/ml	1·4	3·2	1·3
1 mM sodium arsenate	1·2	2·4	1·1
Oligomycin, 10 μg/ml	1·7	3·2	1·3
1 mM sodium fluoride	1·2	2·7	0·88
Anaerobic incubation	1·4	2·9	1·0
Bran from incubated endosperm halves			
None	0·42	0·90	0·30
1 mM sodium cyanide	0·80	1·6	0·58
1 mM sodium azide	0·80	1·5	0·48
Chloramphenicol, 500 μg/ml	0·58	1·0	0·48
1 mM sodium arsenate	0·76	1·4	0·52
Oligomycin, 10 μg/ml	0·76	1·2	0·58
1 mM sodium fluoride	0·44	1·2	0·32

Sodium ions at the concentrations of the sodium salts used do not influence the ion release capacities of bran.[6]

The induction of increased ion release capacity by gibberellic acid showed a quite different pattern of sensitivity to the metabolic inhibitors (Table 5). Actinomycin D, chloramphenicol, puromycin and several amino-acid analogues were entirely without effect, although most of them inhibited the gibberellic acid-induction of α-amylase in the same tissue. Only cyclohexamide blocked the induction and this inhibition was absolute.

DISCUSSION

The results allow a number of conclusions to be drawn. During the early stages of germination a barrier to the release of macronutrient elements arises in the aleurone cells. The development of the barrier is induced by a factor from the starchy endosperm and it is a

[6] D. EASTWOOD, Ph.D. Thesis, University of Wales (1969).

TABLE 5. THE EFFECTS OF METABOLIC INHIBITORS ON THE INDUCTION OF INCREASED ION RELEASE CAPACITY

	Initial ion release rate (mg/hr/32 grain)			α-Amylase activity (mg starch degraded/ 15 min/32 grain)
	P	K	Mg	
Endosperm halves incubated alone (low release control)	0·38	0·80	0·26	24
Endosperm halves incubated with 10 μM GA_3 (high release control)	0·76	1·2	0·48	250
Inhibitors of RNA and protein synthesis:				
Actinomycin D, 50 μg/ml + 1 mM potassium bromate	0·76	1·2	0·48	21
Chloramphenicol, 20 μg/ml	0·76	1·2	0·46	200
Puromycin, 25 μg/ml	0·72	1·2	0·46	101
Cycloheximide, 5 μg/ml	0·40	0·80	0·30	38
Mikamycin, 50 μg/ml	0·74	1·2	0·50	—
Amino acid analogues:				
1 mM L-azetidine-2-carboxylic acid	0·72	1·2	0·44	130
D,L-Selenomethionine, 10 μg/ml	0·68	1·2	0·46	—
1 mM *p*-fluorophenylalamine	0·74	1·2	0·50	144
100 μM azaserine	0·70	1·2	0·44	—
100 nM abscisic acid	0·38	0·80	0·26	80

GA_3, gibberellic acid.
Endosperm halves were incubated for 24 hr. They were then soaked for 1 hr in the specified inhibitor solution and incubated for a further 3 days in the presence of the inhibitor and 10 μM gibberellic acid.

necessary pre-requisite for the later action of a factor from the embryo in stimulating the release of the retained macronutrients. The experiments with authentic phytohormones suggest strongly that the endosperm factor is cytokinin. Significantly, cytokinins have been isolated from the endosperm of maize[7] and detected in germinating barley.[8] The present results, in the context of the established functions of the gibberellins in germinating cereals[4] make it almost certain that the embryo factor is a gibberellin. The synergism between gibberellic acid and indole acetic acid observed in our experiments may also occur *in vivo*, since indole acetic acid has been implicated in other induction processes in germinating wheat.[9] The sequential action of a cytokinin and gibberellic acid in this system is analogous to their action in inducing the synthesis of α-amylase in the same tissue.[10]

While the development of the barrier to ion release is obviously important in regulating mineral mobilization during germination, it cannot, in itself, explain all aspects of the phenomenon. It does not, for example, explain why potassium and phosphate, but not magnesium and calcium ions, are released from the bran of incubated, de-embryoed grain.[3] Factors in addition to the cytokinins appear to be involved.

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The observed changes in salt relationships of the aleurone cells resemble those of other plant storage tissues. Tissue discs prepared from such tissues release considerable quantities of inorganic ions into incubation media. Unlike aleurone cells, however, they later reabsorb and retain these ions.[11,12] In beetroot tuber discs, for example, the phenomenon of reaccumulation has been attributed to the reorganization of endoplasmic reticulum, which becomes disorganised after the preparation of the discs.[13] Electron micrographs of aleurone cells show that they undergo an extensive development of their endoplasmic reticulum and mitochondria during germination.[14] It is therefore possible that this development is associated with the development of the barrier to rapid ion release. Although the two situations are not exactly the same, the terminology applied to the beet discs may appropriately be applied to the aleurone tissue. Thus, the aleurone cells of the quiescent grain may be said to have membranes with a 'loose' configuration and the tissue that has developed the barrier to rapid release may be said to have membranes with a 'tight' configuration.

Inhibitor studies suggest that the development of the tight configuration is dependent upon both protein synthesis and aerobic energy metabolism. The fact that actinomycin D does not inhibit the development suggests further that a long-lived RNA, possibly synthesized during grain development, is available for translation as a result of the cytokinin action. This situation is similar to those described for the substrate-induction of nitrate reductase[15] and for ubiquinone synthesis[16] in the aleurone tissue. Since both chloramphenicol at low concentrations and cycloheximide inhibit the induction, both cytoplasmic and mitochondrial protein synthesis may be involved.[17] These conclusions must however be treated with caution since acridine orange, which inhibits DNA-dependent RNA synthesis, was inhibitory in our system and it has been reported that cycloheximide can also interfere with energetic processes in mitochondria.[18] Nevertheless, as has already been pointed out, the extensive developments of both mitochondrial and cytoplasmic membrane systems occur during germination in these cells.[14] Since both of these structures have the capacity to retain ions,[13,19] they may both constitute sites of the 'barrier' to ion release. The present results show that the maintenance of the tight configuration is partly dependent upon oxidative energy metabolism but not on protein synthesis.

The ineffectiveness of several inhibitors in blocking the gibberellic acid loosening of the tight configuration demands the conclusion that this function of the hormone does not act via the promotion of protein synthesis. The inhibitory actions of cycloheximide and abscisic acid nevertheless suggest that ribonucleic acid function is concerned with the hormone response.[20,21] An RNA of unknown species has been implicated in ion transport in roots,[22,23] and both gibberellic acid and indole acetic acid alter membrane diffusion potentials in several plant tissues.[24] It may thus be speculated that, in the aleurone cells,

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gibberellic acid influences ion release via its action on a ribonucleoprotein. Inhibitor studies of this kind must, however, be interpreted cautiously, a point that is continually expressed in the literature.

EXPERIMENTAL

Sterilization and Incubation of Plant Material

Grain of the soft winter variety Cappelle Desprez were sterilized by our routine procedure.[3]

Endosperm halves were prepared from dry grain by removing the embryo-containing end of the grain with a transverse cut just behind the scutellum. Small amounts of bran and endosperm are unavoidably removed with the embryo; Cf. the preparation of de-embryoed grain in the accompanying paper.[3] The endosperm halves were sterilized and incubated by the routine procedure.

Bran was prepared from sterilized endosperm halves in the presence of 0·06 M mannitol to minimize mineral loss.[3] The excised bran was incubated under sterile conditions at 25° on nylon mesh suspended in a water-saturated atmosphere in a small glass tank.

Solutions of extracts and compounds were sterilized by filtration through a Sietz 5/3 millipore filter immediately before application to incubating tissue. For application to incubating bran, the extracts and compounds were made up in a small volume of 0·06 M mannitol and sprayed on to the bran.

Preparation of Starchy Endosperm Extract

The starchy endosperm from 5 g quiescent grain was extracted three times at 4° with 25 ml 1:1 (v/v) propan-2-ol–$CHCl_3$. The bulked extracts were taken to dryness at 40° and the residue extracted with de-ionized H_2O. This aqueous extract contained the hormone activity.

Determination of the Ion Release Capacity of Bran

The excised bran samples were incubated under sterile conditions at 25° in 50 ml 0·05 M tartaric acid–ethylamine buffer, pH 5·0. The solutions were shaken continuously to ensure aeration of the medium. At regular intervals up to 5 hr, 1-ml aliquots of media were removed for analysis. The aliquots were made up to 10 ml with deionized H_2O and analysed for phosphate, K, Mg and Ca by our routine procedure.[3] In most experiments the initial release rates of ions were recorded.

α-Amylase assay. Bran and starchy endosperm were extracted in 3 times 0·05 M citrate buffer, pH 6·0 containing 20 mM $CaCl_2$ to stabilize α-amylase and 10 μM *p*-chloromercuribenzoate to inhibit β-amylase.[25] The bulked extracts were made up to a standard volume and assayed for α-amylase.[26]

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