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PHOSPHATE MOBILIZATION IN GRAINS OF *HORDEUM DISTICHON*

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Key Word Index—*Hordeum distichon*, Gramineae, barley, grain, aleurone, ash, phosphate, phytate, inositol phosphates, α-amylase

Abstract—When decorticated grains were germinated at 14 5°, inorganic substances moved from the endosperm, mainly the aleurone layer, to the embryo The level of Pi* rose in the embryo and endosperm, and the embryo appeared to accumulate Pi against a concentration gradient The level of organic-P declined in the endosperm, particularly in the aleurone layer Separated aleurone layers, incubated at 25° released only small amounts of organic or inorganic phosphate However, when incubated with gibberellic acid (GA_3), a massive release of Pi occurred at the expense of organic phosphates within the tissue This release followed a sigmoid pattern with time following a lag and was virtually complete in 6 days Allowing the aleurone layer to dry before incubating with GA_3 reduced or abolished the lag period of Pi release and only marginally depressed the total amount ultimately freed In contrast, α-amylase production was depressed by the longer periods of drying The major phosphate of the aleurone was phytate (*meso*-inositol hexaphosphate, IP_6), but traces of IP_4, IP_3, IP_2, IP_1, Pi and unidentified phosphates were detected During incubation with GA_3 the IP_6 content fell, and the lower esters of inositol rose slightly and then fell in a pattern indicating that the phosphate groups of each IP_6 molecule were being sequentially hydrolysed After 6 days incubation, the tissue phosphates were reduced to a very low level Attempts to isolate aleurone grains containing phytate were unsuccessful

INTRODUCTION

PHYTIC ACID (*meso*-inositol hexaphosphate), is a well-known constituent of cereal grains [1] In the barley grain phosphate comprises about 1% of the dry weight, of which *ca* 45% occurs at salts of phytic acid (phytin) [3] During malting the phytate content falls by about 30%, and the phytase activity increases about 8-fold [2,3] Enhanced levels of phytase and Pi are found in grains treated with gibberellic acid [4,5] Gibberellic acid is known to enhance the release of Pi from degermed barley grains[2,2] and separated aleurone layers [2,3] Detailed studies in barley are lacking, but in wheat some 70% of the minerals of the grain, and much of the phytate, occurs in the aleurone layer [6,7] During germination of wheat and oats[6–9] the level of phytate declines sharply and in wheat only phytate and inorganic phosphate

* The following abbreviations are used Pi, inorganic phosphate, IP_6, IP_4, etc phytate, *meso*-inositol tetraphosphate, etc Results are expressed as phosphate-P (phosphorus) Gibberellic acid, GA_3

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TABLE 1. PHOSPHATE AND ASH LEVELS IN DECORTICATED BARLEY

Days germination	Tissue	Tissue fr. wt (mg)	Tissue dry wt (mg)	Ash wt (mg)
0	Embryo	3.2 ± 0.6	1.2 ± 0.2	0.13 ± 0.05
	Whole endosperm	49.4 ± 3.9	30.8 ± 3.3	0.51 ± 0.26
1	Embryo	5.0 ± 0.6	1.3 ± 0.3	0.14 ± 0.08
	Whole endosperm	49.5 ± 5.2	28.0 ± 2.8	0.44 ± 0.19
2	Embryo	10.9 ± 1.2	2.1 ± 0.7	0.16 ± 0.06
	Whole endosperm	46.9 ± 5.0	27.4 ± 2.0	0.38 ± 0.07
	Aleurone layer	16.9 ± 1.7	9.9 ± 0.8	0.30 ± 0.08
	Starchy endosperm	25.8 ± 4.3	17.1 ± 1.0	0.09 ± 0.02
3	Embryo	21.3 ± 6.3	3.1 ± 1.3	0.16 ± 0.07
	Whole endosperm	47.9 ± 8.3	26.2 ± 3.7	0.28 ± 0.08
	Aleurone layer	12.4 ± 3.7	6.9 ± 1.7	0.22 ± 0.07
	Starchy endosperm	30.5 ± 4.7	18.2 ± 3.7	0.02 ± 0.01
4	Embryo	42.5 ± 9.1	4.8 ± 1.3	0.27 ± 0.07
	Whole endosperm	45.2 ± 11.7	22.8 ± 4.7	0.27 ± 0.08
	Aleurone layer	11.1 ± 4.3	5.2 ± 2.3	0.23 ± 0.07
	Starchy endosperm	30.7 ± 4.1	17.8 ± 2.0	0.03 ± 0.08
5	Embryo	60.2 ± 13.3	6.6 ± 2.3	0.29 ± 0.09
	Whole endosperm	41.8 ± 8.8	20.4 ± 4.7	0.24 ± 0.07
	Aleurone layer	11.3 ± 2.2	5.1 ± 2.7	0.20 ± 0.06
	Starchy endosperm	26.1 ± 5.0	14.3 ± 2.0	0.03 ± 0.02

* Values are per single grain or part thereof and are means of 6 samples ± s.d. Results for whole endosperms inevitable losses in separating these tissues

(but no lower inositol phosphates) are detected when extractions are accompanied by enzyme inactivation.[7] Phytic acid and the tetra- and tri-phosphate esters are found in germinating barley.[3] Tetra-, tri-, di- and mono-phosphate esters of inositol are formed in autolysates of ground barley.[3] Cytokinins are reported to check the loss of inorganic phosphate from wheat bran and gibberellin enhances the loss at the expense of endogenous phytate.[10]

It has been surmised from electron micrographic work that phytate occurs as inclusions within aleurone grains and histochemical studies seem to support this.[11, 18] As yet undoubted aleurone grains have not been isolated from cereal aleurone layers. However, protein bodies which may have come partly from the aleurone as well as the starchy endosperm, have been separated from cereals.[19, 21] The homologies between these and "aleurone grains" isolated from dicotyledonous plants seem to be uncertain.

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GERMINATING ON WET FILTER-PAPER AT 14·5° IN THE DARK*

Organic phosphate–P (μg)	Inorganic phosphate–P (μg)	Total phosphate–P (μg)	Inorganic phosphate–P (μg/ml tissue H_2O)
14·6 ± 4·7	0·11 ± 0·05	14·7 ± 4·6	0·06
78·2 ± 13·9	7·7 ± 2·7	85·9 ± 14·3	0·42
16·1 ± 5·2	0·46 ± 0·31	16·6 ± 5·3	0·13
73·7 ± 12·3	8·1 ± 3·3	81·8 ± 14·1	0·38
19·0 ± 5·4	3·6 ± 2·0	22·6 ± 4·9	0·41
66·0 ± 8·8	6·8 ± 2·7	72·8 ± 9·3	0·35
57·1 ± 7·5	3·2 ± 1·7	60·3 ± 7·3	0·45
14·5 ± 5·1	0·6 ± 0·4	15·1 ± 5·3	0·07
28·5 ± 5·7	5·6 ± 2·2	34·1 ± 5·4	0·31
66·7 ± 9·2	7·1 ± 3·1	73·8 ± 8·9	0·33
47·8 ± 8·0	3·1 ± 1·2	50·9 ± 7·9	0·58
13·5 ± 4·1	2·0 ± 0·7	15·5 ± 4·7	0·16
39·3 ± 6·2	12·1 ± 3·4	51·4 ± 5·9	0·32
48·8 ± 7·7	13·5 ± 3·8	62·3 ± 8·2	0·60
35·3 ± 6·1	7·4 ± 2·0	42·2 ± 7·1	1·24
14·4 ± 3·9	1·9 ± 0·7	16·3 ± 3·8	0·15
26·9 ± 2·2	20·4 ± 3·6	47·3 ± 3·1	0·37
35·0 ± 2·3	15·4 ± 2·4	50·4 ± 4·1	0·72
24·0 ± 7·4	8·5 ± 3·6	32·5 ± 8·5	1·36
10·2 ± 2·6	2·5 ± 0·8	12·7 ± 2·8	0·21

were determined separately and do not necessarily equal aleurone layer + starchy endosperms, as there were

RESULTS

Ash and phosphate redistribution in barley germinating at 14·5°

The embryo increased in dry weight, ash, organic-P and Pi at the expense of the endosperm (Table 1) When it was possible to separate the endosperm into its parts (days 2–5) it was seen that, initially, the aleurone layer declined in weight more than the starchy endosperm Most of the minerals and phosphate originated in the aleurone layer during germination The organic phosphate of the aleurone layer was converted to Pi that moved into the starchy endosperm, and was taken up by the embryo where it was partly converted to organic phosphate In the embryo the concentration of Pi in solution (wt/ml tissue water) remained fairly constant from day 2 onwards, and as it exceeded the concentration in the starchy endosperm the embryo must have accumulated Pi against a concentration gradient

Phosphate mobilization in isolated aleurone layers

It was of interest to confirm the nature of the phosphate reserve of the barley aleurone and to discover how its conversion to Pi was regulated Aleurone layers cannot be separated from dry grain Those freshly separated by our method contain *ca* 60 μg total phosphate-P, of which 10% is Pi–P When these preparations are incubated without GA_3, small amounts of phosphate are released into the medium, 2·3 μg Pi–P and 2·3 μg organic phosphate-P after 6 days, the amount increasing slowly to this level during incubation (Fig 1, Table 2) When GA_3 was included in the incubation medium a massive release of phos-

phate, mostly Pi occurred at the expense of organic phosphate following a sigmoid pattern with time (Fig 1 Table 2) By day 6 Pi release had almost ceased and the organic phosphate in the aleurone tissue was nearly depleted (Table 2) Thus in the intact aleurone phosphate mobilization is dependent on GA_3 Replacing the medium at 12 hr intervals appears to enhance the release of Pi Perhaps the continuing presence of Pi in the medium inhibits phytate hydrolysis Pi is known to repress phytase synthesis in wheat embryos [24 25]

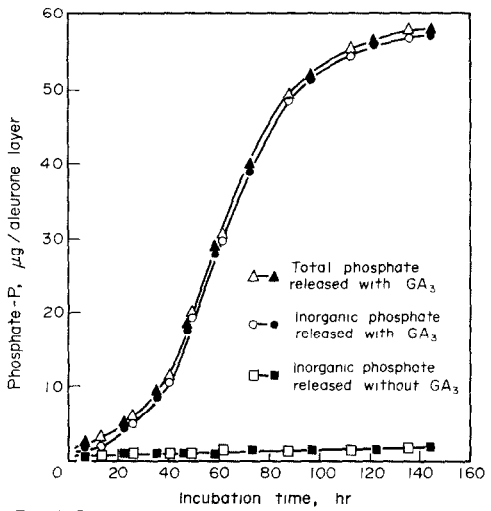

FIG 1 RELEASE OF TOTAL AND INORGANIC PHOSPHATE FROM ISOLATED ALEURONE LAYERS INCUBATED AT 25 0 WITH AND WITHOUT GA_3 (2 μg ml)
Open and closed symbols two sets of incubations started 6 hr apart Media were changed every 12 hr The curve for organic phosphate release without GA_3 is coincident with the inorganic curve and is not shown Results are the means of six determinations

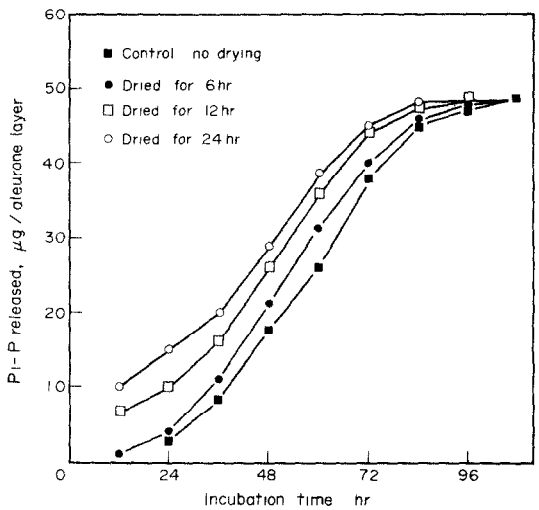

FIG 2 RELEASE OF INORGANIC PHOSPHATE FROM ALEURONE LAYERS INCUBATED AT 25 0 WITH GA_3 (2 μg ml) AFTER VARIOUS PERIODS OF 'DRYING'
The culture media were replaced every 12 hr Results are the means for six determinations

TABLE 2 ORGANIC AND INORGANIC PHOSPHATE LEVELS IN ALEURONE LAYERS AND CULTURE MEDIA AFTER INCUBATION AT 25 0 WITH AND WITHOUT GA_3 (2 μg ml) FOR 6 DAYS*

Location		Levels of phosphates (μg P aleurone)	
		GA_3	No GA_3
Aleurone layer	Organic P	3 7 ± 3 0	42 1 ± 3 9
	Inorganic P	4 5 ± 1 2	6 0 ± 1 0
	Total P	8 2 ± 2 3	48 1 ± 4 0
Medium	Organic P	4 8 ± 1 6	2 3 ± 0 4
	Inorganic P	36 3 ± 3 4	2 3 ± 0 4
	Total P	41 0 ± 2 8	4 6 ± 0 8
Aleurone layer + medium	Total P	49 2 ± 4 3	52 7 ± 3 6

* Results are the mean of six determinations ± s d

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When aleurones were allowed to remain in a moist atmosphere, but not immersed in medium for specified periods and when then incubated with GA_3, they initially released phosphate more rapidly than did samples prepared in the usual way It was as though the lag period had been removed (Fig 2) At the end of the incubation period the levels of organic and inorganic phosphate remaining in the aleurone tissue were essentially the same in control and "dried" samples (Table 3)

TABLE 3 TOTAL PHOSPHATE RETAINED AND RELEASED BY ISOLATED ALEURONES INCUBATED AT 25 0° WITH GA_3 (2 μg/ml) AFTER PERIODS OF "DRYING OUT"

Location of phosphate	Total phosphate (μg P aleurone), after the following pre-incubation 'drying out" periods (hr)			
	Zero	6	12	24
Medium	53 3 ± 3 9	53 8 ± 6 5	51 1 ± 5 0	44 9 ± 8 2
Aleurone	3 8 ± 2 2	3 3 ± 1 7	5 3 ± 2 3	9 3 ± 2 7
Total	57 1 ± 5 3	57 1 ± 8 9	56 4 ± 9 4	54 2 ± 9 3

* Incubation time 108 hr The incubation media were replaced every 12 hr Results are the mean of six determinations ± s d

In view of the effect on phosphate release the effect of "drying" on the production of α-amylase and the temporal relationship between Pi released and α-amylase production were examined, to see if drying stimulated all the tissue functions A 6-hr drying period slightly enhanced the amount of α-amylase formed and released by the end of the incubation period, but 12 and 24 hr drying periods reduced enzyme formation and increased the amount of enzyme retained in the tissue (Table 4, Fig 3) Apparently the mechanisms for enzyme synthesis and release were both impaired Thus α-amylase release and the release of Pi are influenced independently by drying (Figs 2 and 3) In each case, in the controls, most rapid release occurred after about 60 hr incubation and had nearly ceased by the end of the incubation period (Figs 2 and 3)

TABLE 4 α-AMYLASE, RETAINED AND RELEASED BY ISOLATED ALEURONES, INCUBATED AT 25 0° WITH GA_3 (2 μg/ml) AFTER PERIODS OF "DRYING OUT"*

Location of α-amylase	α-Amylase (SIC units/aleurone), after the following pre-incubation "drying out" periods (hr)			
	Zero	6	12	24
Medium	60 3 ± 15 6	65 1 ± 7 9	43 9 ± 15 0	31 7 ± 14 2
Aleurone	3 7 ± 2 8	2 3 ± 1 1	5 5 ± 2 2	10 7 ± 3 5
Total	64 0 ± 13 1	67 4 ± 8 9	49 4 ± 13 7	42 4 ± 17 3

* The medium was replaced every 12 hr Total incubation time was 108 hr The results are the means of determination ± s d

The phosphates of the aleurone layer

Some 9 methods of extracting and recovering phytate and other phosphates from aleurone tissue were tested They were inadequate since the percentage of tissue phosphate extracted, or the recoveries from the extraction media were poor The method eventually used minimized sample transfers, and did not use protein precipitating agents or precipitation of phosphates with heavy metals since these were found to give irregular recoveries

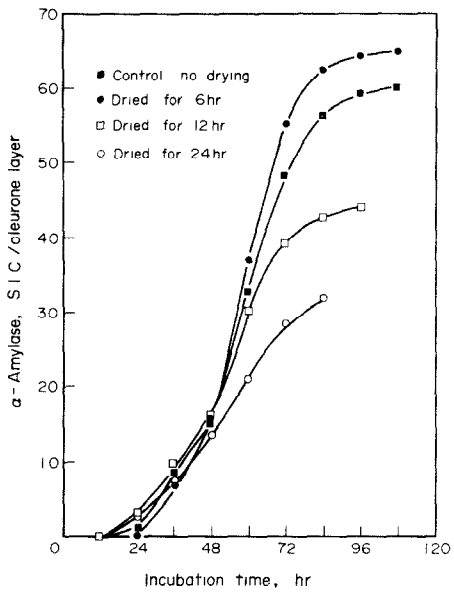

FIG 3 α-AMYLASE RELEASED BY ISOLATED ALEURONE LAYERS INCUBATED AT 25 0 WITH GA_3 (2 μg/ml) AFTER VARIOUS PERIODS OF "DRYING"
The media were changed every 12 hr Results are the means of six determinations

High voltage electrophoresis separated pure phytic acid (IP_6) IP_4, IP_3 IP_2 IP_1 and Pi Electrophoresis of aleurone extracts revealed uncharacterized phosphate(s) at the baseline Pi and a large amount of material moving between pure standards of IP_6 and IP_4 The material separated from added IP_4, but added IP_6 was retarded relative to controls when mixed with the crude sample and moved with the unknown Following a preliminary treatment of the extract with Dowex-50 (H^+) ion exchange resin the unknown had an electrophoretic mobility equal to that of pure phytic acid Thus the "unknown was IP_6, with reduced mobility due to some contaminant However *ca* 30% of the total phosphate of the extract was lost during ion exchange so this step was omitted in quantitative studies

TABLE 5 PHOSPHATES IN ISOLATED ALEURONE LAYERS AND CULTURE MEDIA

Incubation time (hr)	Medium P_i P (μg)	Aleurone layer before electrophoresis: Organic phosphate P (μg)	Aleurone layer before electrophoresis: P_i P (μg)	Aleurone layer total phosphate–P of electrophoresis recovery (%)
0		53 2	8 1	79 1
10	2 1	47 7	6 4	93 5
22	6 8	47 3	7 1	88 2
46	29 7	26 9	6 6	94 4
70	50 3	15 8	5 4	95 6
94	54 9	5 4	1 5	87 1
142	63 6	2 6	1 5	60 7

* Results are expressed per aleurone layer from 1 grain Organic phosphates from the aleurone layer were separated by electrophoresis and are expressed as a percentage of the total phosphate-P recovered from the paper

The contaminant was suspected to be a heavy metal, but the addition of EDTA had no effect on the mobility of the spot

Alterations in the phosphates of aleurone layers incubated with GA_3

Aleurone layers were incubated for various periods with GA_3 The Pi released into the medium was determined and the tissue phosphates were extracted and separated by electrophoresis Recoveries varied between 61 and 96% of the phosphate applied when the zones on the electrophoretic strips were analysed (Table 5) During the incubation period IP_6 declined until little was left after 142 hr (Table 5) Assuming that the phosphates were recovered in proportion to the amounts present before electrophoresis, then during the incubation the level of IP_4 rose slightly but declined from 10 hr onwards, while the highest levels of IP_3 and IP_2 occurred at 46 hr The base-line phosphate, Pi and IP_1 levels remained relatively constant until 70 hr then declined sharply Thus in the aleurone layer IP_6 was degraded to the lower inositol phosphates, the Pi formed being released into the incubation medium Towards the end of the incubation period the "base-line" phosphates, possibly nucleic acids, were also degraded

Attempts to prepare aleurone grains from isolated aleurone layers

Attempts were made to separate aleurone grains from isolated aleurone layers, so as to test by direct analysis whether the inclusions seen by microscopic analysis are salts of IP_6 Particles, frequent in homogenates and of diameter 1 8–3 6 μm by scanning electron microscopy, were obtained in poor yield, they were contaminated by starch grains and probably cytoplasmic proteins, as they tended to adhere to each other However, organic phosphate, mostly IP_6, and increasing amounts of Pi were released into solution during the fractionation procedures Thus if phytate had been originally confined to the aleurone grains it was being lost during fractionation Analyses of grain-rich fractions gave a wide range of N P ratios At least some of the IP_6 was probably located in the grains

DISCUSSION

The results suggest that in barley, in response to gibberellins, minerals (ash) and phosphate move from the aleurone layer into the embryo, by way of the starchy endosperm, as occurs in wheat [6,7] However, unlike wheat bran,[6,10] separated barley aleurone layers do

INCUBATED AT 25 0° FOR VARIOUS PERIODS WITH GA_3 (2 μg/ml)*

	Aleurone layer phosphates (phosphate–P as % of the P recovered)					
Base-line	Pi	IP_1	IP_2	IP_3	IP_4	IP_6
—†	19 4	4 1	3 6	3 7	9 0	60 2
15 4	13 3	2 9	1 7	3 3	12 0	51 3
11 1	16 1	2 4	1 5	2 4	11 3	56 3
19 9	20 1	4 8	7 9	9 1	6 9	31 5
33 9	24 6	6 8	6 8	4 8	8 2	14 9
36 4	15 0	11 2	11 3	14 6	4 0	7 6
51 9	41 7	1 2	1 2	1 2	1 6	0 8

† Measurement not made

not require exogenous cytokinins to prevent the release of Pi Indeed Pi is only released in significant amounts to response to GA_3 The pattern of depletion of materials from the endosperm and the pH change that occur in the endosperm of germinating grains are consistent with the suggested movement of minerals [26] The marked fall in pH that occurs when isolated endosperms or aleurone layers are incubated with GA_3 [26,27] is probably partly due to the release of phosphoric acid The major reserve of phosphate in the aleurone layer is shown to be phytate, which probably resides in the aleurone grains As the lower inositol phosphates did not accumulate during phytate breakdown and indeed inositol pentaphosphate (IP_5) was not detected, these compounds were hydrolysed in preference to the phytate (IP_6) as seems to be the case with rice and wheat [27,28] Towards the end of the incubation period, when the aleurone cells were probably almost disorganized,[29] even the "base-line" phosphate (possibly nucleic acids) declined in amount as would be expected from electron-microscopic studies

The observation that "drying" aleurone layers reduces the lag period of Pi release when subsequently incubated with GA_3, although α-amylase production is adversely affected, is unexplained As the aleurones are prepared in well-aerated liquid it seems improbable that an enhanced supply of oxygen can be the cause Perhaps the phytate becomes more accessible to hydrolytic enzymes due to some damage to the structure of the aleurone grains, as appears to happen during attempts to separate the grain from aleurone homogenates It is of interest that phytate is rapidly degraded in autolysing whole, ground barley [3]

Numerous observations and preparations of "aleurone grains and 'protein bodies" have been made from monocotyledonous and dicotyledonous plants [11,21,30,37] As seen in the electron microscope these vary in appearance, and often do not resemble sections of aleurone grains seen *in situ*, so that their homologies are in doubt Cytochemical studies indicate that phytin is located in aleurone grains, but that under some conditions at least it readily "leaks out"[11] which is consistent with our findings

EXPERIMENTAL

Preparation of tissues Two-row barley, *Hordeum distichon* L var Proctor was graded, decorticated sterilized and faulty corns were rejected by the usual methods [38] Aleurone layers were prepared as before [27]

Germination of whole grain (14·5°) Grains (*ca* 1700) were germinated on moist filter paper (Whatman No 1 2 × 15 cm dia) in open Petri dishes at 14·5° in the dark and moistened (20 ml/dish) each day Samples of 15 corns were stored on dry ice and subsequently dissected into endosperm and embryo Where possible the endosperm was divided into the starchy endosperm and the aleurone layer Groups of six samples were weighed and analysed for Pi P, others were dried (16 hr, 120°) weighed and analysed for total phosphate-P The last groups were used to determine ash contents Ashing was carried out in porcelain crucibles at 800°

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Experiments with aleurone layers Separated half aleurone layers (10, equivalent to 5 barley corns) were incubated in an orbital shaker at 25° in the dark in 25 ml conical flasks containing autoclaved buffer (5 ml sodium succinate 20 mM, with $CaSO_4$, 15 mM, adusted to pH 5 3 with H_2SO_4) and with GA_3 (2 μg/ml) where necessary Solutions of GA_3 were sterilized by millipore filtration In specified samples media were replaced at intervals with fresh buffer containing GA_3 In certain samples the aleurones were added to the flasks and the buffer and GA_3 were added 6, 12 and 24 hr later Most aleurones remained moist during the initial "dry" period

Phosphate determinations (a) Pi–P was determined by the method of Wood[39] except that digestions were carried out in thick-walled test tubes (15 × 1 5 cm) (b) Determinations of total phosphate–P were made on digests of barley tissues standard preparations of inositol phosphates and segments of electrophoretograms when blank and "spots" of equal area were analysed Samples were heated with H_2SO_4 (0 2 ml, 18 M, 3 hr, 200) in thick walled test tubes in a heating block After 30 min several drops of perchloric acid (70%) were added to decolorize the digests Digestion continued for 2 5 hr Samples were cooled and the phosphate contents were determined after Bartlett[40]

Electrophoresis Electrophoretogram paper (Whatman No 1, 42 × 57 cm 24 sheets) were washed in a chromatographic tank with HCO_2H (2 M, 8 l), $MeCO_2H$ (1 M, 8 l), H_2O (40 l), EDTA (10 mM) in $NaHCO_3$ (100 mM, 3 l, pH 9 0), and finally H_2O (50 l) The paper was dried (70°) and divided into strips (12 × 57 cm) Samples (200 μl) and authentic standards (10 μl) were applied as spots (0 5 cm dia), 3 cm from the negative end of the strip Strips were wetted with buffer ($MeCO_2H$–HCO_2H 4 1 pH 2 0) from the ends so concentrating the spots Excess buffer was blotted away and strips, mounted on a polythene rack were immersed in a tank of cooled petroleum spirit with the ends dipping into the buffer reservoirs Electrophoresis was carried out at 4000 V, 15 mA-strip for 30 min[41–46] The strips were dried at 70° Compounds were detected with the acid phosphomolybdate spray Pi initially appeared yellow, turning blue after heating and exposure to sunlight Inositol phosphates turned blue The Pi spots were distorted by the presence of IP_1 Inositol monophosphate only appeared after exposure to sunlight for 3 hr The treated paper disintegrated in *ca* 3 days With aleurone extracts usually only IP_6, IP_4, Pi and the base-line phosphates were visible, but IP_3, IP_2 and IP_1 were found when strips were analysed for phosphate

Preparation of authentic inositol phosphate esters A mixture of inositol phosphates was prepared according to Preece *et al*[3] The esters were purified using ion exchange[45] IP_6 was purified after Johnston and Tate[43] IP_1 was separated from contaminating Pi by the method of Hagihara and Lardy[47] For inositol determinations a sample of each ester (20 mg) was hydrolysed in a sealed Carius tube with HCl (0 8 ml, 10 M, 24 hr, 140°)[48] Mannitol and erythritol were added as internal standards Aliquot samples were dried, dissolved in pyridine (70 μl) with hexamethyldisilazane (70 μl) and trimethylchlorosilane (10 μl) The trimethylsilyl ethers of inositol and the standards were analysed by GLC (Pye 104 double column GLC, with FID, column 1 5 m of 10% SE 30 on silanized Supasorb (A R), 60–80 mesh, inositol R_f 20 5 min), the relative amounts present being determined from the peak areas The inositol to phosphate ratios (means of duplicates) were IP_6, 1 5 90, IP_4, 1 4 09, IP_3, 1 2 96, IP_2, 1 2 01, IP_1, 1 1 03

Extraction of phosphates from aleurone tissue Half aleurone layers (10) were homogenized in HCl (5 ml, 0 5 M) and with washings (5 ml, 0 5 M HCl), were refluxed (30 min) then filtered hot through HCl washed Whatman No 50 paper The residue and lower half of the filter paper were re-extracted under reflux (10 ml 0 5 M HCl, 30 min) and filtered hot The combined filtrates were dried at 70° on a rotary evaporator H_2O was added and evaporation was repeated until the HCl had been removed (bromophenol red indicator in the receiver) The residue was extracted (H_2O, 0 3 ml, followed by NaOH, 0 5 ml, 2 3 mg/ml) The solution was stored at 4° before electrophoresis

Separation of impure aleurone grains Aleurone layers (200) were homogenized in sucrose (10 ml, 30% w/w) The homogenate was layered on sucrose (5 ml, 30%) in a narrow glass centrifuge tube, and centrifuged (50 *g*, 5 min) The creamy supernatant and brown suspension was removed The remaining, unbroken cells were re-homogenized The supernatant and suspensions were combined, diluted to 20 ml and centrifuged (400 *g*, 10 min) Attempts were made to fractionate the crude preparation further by centrifugation in sucrose density gradients and by filtration through graded sintered glass filters Appearance of aleurone grain preparations were monitored using light and scanning electron microscopy[27] Preparations were analysed for total –N and total P

Estimation of α-amylase was by our usual method[49]

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Estimation of total nitrogen The method used was a scaled-down version of that of Williams [50] Samples were digested in thick-walled test tubes (15 × 1 5 cm) in a heating block at 320 Analysis of the digest was by direct Nesslerization

[50] WILLIAMS, P C (1964) *Analyst* **89**, 276