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THE UTILIZATION OF PHYTATE BY GERMINATING WHEAT

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Abstract—In germinating wheat, when phytase was inactivated by macerating in dilute perchloric acid, no *myo*-inositol phosphate esters with fewer than six phosphate groups could be detected during the disappearance of phytate. This contrasts to the hydrolysis, in solution, of *myo*-inositol hexaphosphate by wheat phytase, when significant concentrations of lower esters are produced before hydrolysis is complete. In wheat, the inorganic phosphate content per fresh weight remained constant up to 14 days. Released *myo*-inositol did not accumulate and, at 3–4 days after initiation of germination, the distribution of label from *myo*-inositol-U-^{14}C into various fractions was similar to that of D-glucose-U-^{14}C. However, there was insignificant incorporation of label from D-glucose-U-^{14}C into the *myo*-inositol fragment of the lipid fraction which did incorporate label from *myo*-inositol-U-^{14}C. Label from D-glucose-U-^{14}C was not found in unbound *myo*-inositol.

INTRODUCTION

myo-Inositol hexaphosphate, as the mixed calcium, magnesium and potassium salt is regarded as a ballast material, preventing the accumulation of phosphate ion in developing seeds, or as a storage material, supplying inorganic phosphate on hydrolysis by phytase during germination. *myo*-Inositol phosphates with fewer than six phosphate groups have been reported in low amounts in seeds. Early work did not preclude their production by phytase during extraction. Small quantities have been found in stored rice but not in ripening rice,[1] and in immature but not in mature seeds of sunflowers, maize and vetch.[2] They have also been detected in barley[3] and crested wheat grass.[4] All the esters from mono to penta have been reported from germinating wheat when seedlings were dried in a hot-air stream before analysis.[5] Plant lipids contain a *myo*-inositol-1-monophosphate linkage.

Phytase is found in seeds wherever phytate occurs, although sometimes the activity is low until after germination. Phytase activity initially increases on germination[7] and this increase is influenced by gibberellin[8] and inorganic phosphate.[9] Solutions of sodium phytate and phytase produce *myo*-inositol, and its mono-, di-, tri- and tetraphosphates which have been separated by ion-exchange chromatography.[6]

In two previous studies on the release of free *myo*-inositol on germination an increase was found which was less than the theoretical release from hydrolysed phytate. In oats, substituted *myo*-inositol decreased by 15·9 μg per seed and free *myo*-inositol increased by 6·3 μg

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[3] I. A. PREECE, H. J. GRAV and A. T. WADHAM, *J. Inst. Brewing* **66**, 487 (1960).
[4] A. M. WILSON and G. A. HARRIS, *Plant Physiol.* **41**, 1416 (1966).
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[6] R. V. TOMLINSON and C. E. BALLOU, *Biochemistry* **1**, 166 (1962).
[7] F. G. PEERS, *Biochem. J.* **53**, 102 (1953).
[8] B. I. S. SRIVASTAVA, *Can. J. Botany* **42**, 1303 (1964).
[9] R. BIANCHETTI and M. L. SARTIRANA, *Biochim. biophys. Acta* **145**, 485 (1967).

per seed in a 10-day period[10] and in dwarf beans the decrease was 1227 μg per seed and the increase 480 μg per seed in a 12–14 day period.[11]

The aim of this work has been to quantitatively compare the *in vitro* release of *myo*-inositol phosphate esters containing fewer than six phosphate groups which are formed by phytase hydrolysis of sodium phytate, with the *in vivo* release in germinating wheat and also to follow the utilization of the second product of complete hydrolysis, *myo*-inositol.

RESULTS

Sodium phytate was incubated with wheat bran phytase in a ratio approximating that in germinating wheat and samples were taken when the inorganic phosphorus to total phosphorus ratios were 10, 19, 44 and 81 per cent. The phosphate fractions were separated by ion-exchange chromatography. They were characterized by the order of elution[6] and paper and thin-layer chromatography and by the production of *myo*-inositol only after complete hydrolysis. The hexa- and penta-phosphates were not separated by the techniques used. Comparisons between terminating the reaction with trichloracetic acid (final concentration of 5%) and with a strong cation exchange resin in the acidic form indicated that resin addition inactivated phytase. Inorganic phosphate content after both treatments was measured immediately, and after storing at $-15°$, 2° and room temperature for 45 hr. No differences were detected indicating there was no hydrolysis of phosphate ester after addition of the ion-exchange resin. Relatively high amounts of fractions with intermediate phosphate contents were produced during hydrolysis by phytase (Table 1). The recovery was quantitative as indicated in the Table. Higher phosphates were present in the early stages of hydrolysis and these were replaced by lower esters later.[6] The order of elution was monophosphate, inorganic phosphate, di-, tri-, tetra- and combined penta- and hexaphosphate.

TABLE 1. *In vitro* HYDROLYSIS OF SODIUM PHYTATE BY WHEAT BRAN PHYTASE

	Phosphorus content of fraction (mg)			
	Degree of hydrolysis of solution $\left[\frac{\text{inorganic phosphate}}{\text{total phosphate}}\ \%\right]$			
Fraction	10	19	44	81
Total applied	14·9	14·7	14·5	14·8
myo-Inositol hexa-+pentaphosphate	11·5	8·2	—*	—
Tetraphosphate	0·9	3·4	6·0	—
Triphosphate	—	0·2	2·3	—
Diphosphate	—	—	0·4	1·5
Monophosphate	—	—	—	1·3
Inorganic phosphate	1·5	3·0	5·7	10·8
Water wash from column	0·0	0·5	0·2	0·3
Total recovery, %	93	104	101	94

* The limit of detection was 0·1 mg.

[10] A. DARBRE and F. W. NORRIS, *Biochem. J.* **64**, 441 (1956).
[11] L. N. GIBBONS and F. W. NORRIS, *Biochem. J.* **86**, 64 (1963).

When insoluble calcium phytate containing 15 mg of phosphorus was incubated with 18,800 units of phytase, 78 per cent hydrolysis was recorded in 24 hr. This was nearly as rapid as a mixture of the same amount of soluble sodium phytate with 5400 units of enzyme.

When the *myo*-inositol phosphates in germinating wheat were chromatographed, after inactivating phytase and phosphatase with 0·5 M perchloric acid,[12] the quantity of phytate was increased seven-fold. This was applied to a column four times the volume and the elution gradient used was steeper, to allow the collection of more concentrated fractions. 0·5 M Perchloric acid gave an extract with the same phosphorus contents as 0·5 M HCl.[21] The recovery of phosphorus fractions after precipitation of perchlorate with potassium was

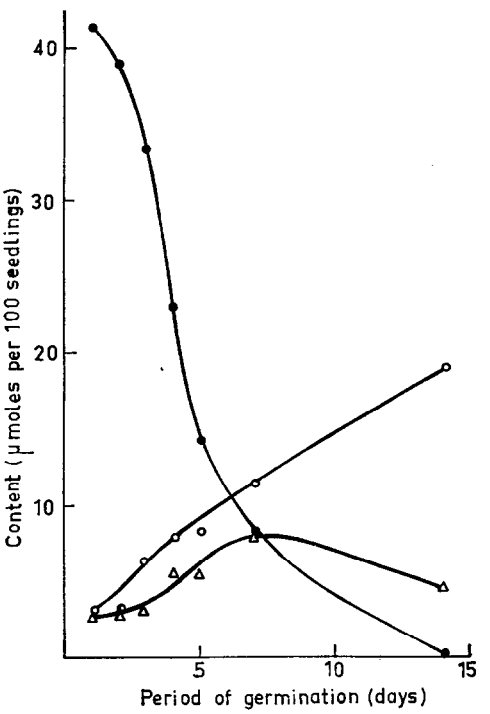

FIG. 1. PHYTATE, INORGANIC PHOSPHORUS AND *myo*-INOSITOL CONTENT OF WHEAT DURING GERMINATION.

● Phytate. o Inorganic phosphorus÷6. △ *myo*-Inositol.

quantitative. In the extracts prepared at 1, 2, 3, 7 and 14 days after the initiation of germination, no *myo*-inositol phosphate fraction other than phytate could be detected. The limit of detection was 0·05 per cent of total phosphorus. Any other phosphorus-containing peak did not correspond with the elution volume of any of the *myo*-inositol phosphates and no *myo*-inositol could be detected chromatographically after treatment of these peaks with phytase. Hydrolysis of phytate containing an equivalent amount of phosphorus gave detectable quantities of *myo*-inositol. These results indicate that lower *myo*-inositol phosphates do not appear in significant amounts in germinating wheat seedlings.

Phytase activity increased from 680 units per g of seeds at 0 days to 930 at 1 day, 1400 at 2 days and 2120 units per g at 3 days. At 0 days the acid-soluble phosphate was 109 mg per

[12] D. H. TURNER and J. F. TURNER, *Biochim. biophys. Acta* **51**, 591 (1961).

40 g seeds and most of this was phytate (99 mg). Phytate accounted for 95 per cent of the non-lipid *myo*-inositol and 72 per cent of the total phosphorus. Acid-labile phosphorus was 1 mg per 40 g of seeds and inorganic phosphorus 6 mg. After 3 days the acid-soluble phosphorus was 97 mg, made up of phytate (74 mg), acid-labile phosphorus (6 mg) and inorganic phosphorus (12 mg). A consequence of the absence of lower phosphate esters was that phytate could be determined by precipitation with iron without interference.

The decrease in phytate concentration from its maximum before germination to zero values required up to 14 days. At 3 days the coleoptile was 6–9 mm and the three roots 19–22 mm. At 7 days the shoots were 10–11 cm and roots up to 6 cm, and at 14 days the shoots, now including two true leaves, were 15 cm long. During this 14-day period inorganic phos-

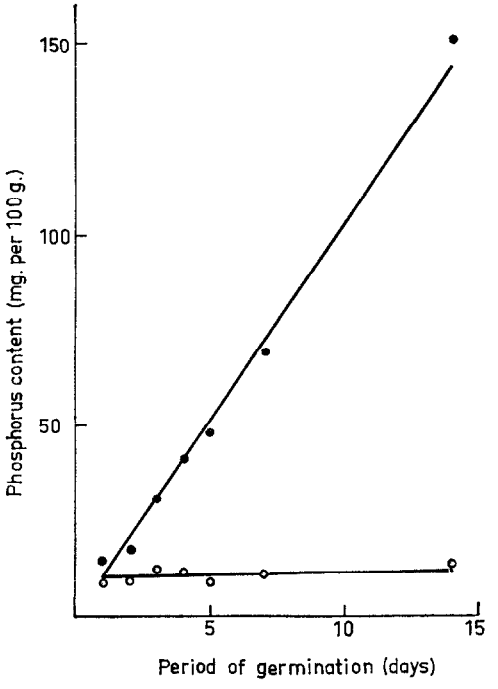

FIG. 2. INORGANIC PHOSPHORUS CONTENT OF WHEAT DURING GERMINATION.
● Per unit dry weight. o Per unit wet weight.

phate content per seedling increased but *myo*-inositol content, after a small increase, returned to its initial low value. Figure 1 illustrates these changes. The least significant difference at the 5 per cent level for the *myo*-inositol estimation was 1·5 μmoles. It was determined by transmission densitometry after paper chromatographic separation from a small residue of other material which stained with alkaline $AgNO_3$.[13] The bulk of the interfering compounds was removed by ion-exchange resins followed by hypoiodite oxidation of reducing sugars to aldonic acids and subsequent deionization. The $AgNO_3$ spray of Anet and Reynolds[13] was modified to dip in the first and second solutions. A proportional relationship was then found between the area under the recorder tracing of the absorbance and the concentration of *myo*-inositol in the range of 0 to 4·0 μg and this relationship was linear in the range 0·2–1·4 μg.

[13] E. F. L. J. ANET and T. M. REYNOLDS, *Nature* **174**, 930 (1954).

In Fig. 2 inorganic phosphate contents at various times of germination have been plotted against the fresh and dry weights of seedlings. Whereas there is a steady increase on a dry weight basis, the phosphate content per wet weight remains constant.

3-day-old seedlings were supplied with *myo*-inositol U-^{14}C and D-glucose U-^{14}C. The uptake of D-glucose by 3-day-old seedlings at the concentration used was effectively complete in 24 hr, but the uptake of *myo*-inositol was incomplete. However, the rate of uptake of *myo*-inositol had slowed considerably at this time. Table 2 shows the percentage incorporation of label into the various fractions prepared. The percentage of labelled D-glucose absorbed by the seedlings in 24 hr was 96 per cent and 95 per cent for 1 and 2 μc respectively. For *myo*-inositol the values were 59 per cent and 46 per cent. The CO_2 respired has been calculated by difference and is of the same order of magnitude as when respired $^{14}CO_2$ was collected as $Ba^{14}CO_3$.

TABLE 2. DISTRIBUTION OF ^{14}C IN FRACTIONS FROM SEEDLINGS GIVEN *myo*-INOSITOL U-^{14}C AND D-GLUCOSE-U-^{14}C

Fraction		Seedlings given *myo*-inositol-U-^{14}C (% of total added label)		Seedlings given D-glucose-U-^{14}C (% of total added label)	
		1 μc	2 μc	1 μc	2 μc
Ethanol-insoluble fraction of the acid extract	I	10·8	13·4	3·1	6·5
Ethanol-soluble fraction of the acid extract	II	16·3	15·1	3·4	2·6
Hydrolysed, ethanol-soluble fraction of the acid extract	III	13·8	9·7	0·02	0·01
Lipid fraction	IV	3·2	2·8	2·1	1·4
Hydrolysed lipid fraction	V	0·6	0·9	0·0	0·03
"Pectin"	VI	5·5	5·7	2·7	3·0
"Hemi-cellulose–cellulose"	VII	4·6	5·3	3·5	4·7
Residue	VIII	0·3	0·3	1·3	1·8
Respired CO_2 (by difference)		59·3	57·4	83·9	80·0

Initial extraction of seedlings with chloroform–methanol (1:1) without prior aqueous extraction gave a lipid fraction contaminated with free *myo*-inositol which was detected by paper chromatography. When two cold acid extractions and a water extraction preceded the chloroform–methanol treatment, the lipid fraction was devoid of free *myo*-inositol. In fractions III (the hydrolysed ethanol-soluble fraction of the acid extract) and V (the hydrolysed lipid fraction) the method of preparation would remove all compounds except polyols. Paper chromatography in solvents A and C followed by spraying with alkaline $AgNO_3$[13] indicated only *myo*-inositol in fraction III and *myo*-inositol and a faster-moving compound (probably glycerol) in fraction V. There was negligible incorporation of label from D-glucose U-^{14}C into these fractions. When a small portion of fraction V from extracts of wheat supplied with *myo*-inositol U-^{14}C was chromatographed, and the areas due to *myo*-inositol and the faster-moving substance eluted and the label counted, the *myo*-inositol showed significant label but the faster-moving spot did not. There is an apparent difference in fraction II when plants take up *myo*-inositol U-^{14}C and D-glucose U-^{14}C. If a correction is made for labelled *myo*-inositol in the ethanol-soluble fraction from seedlings given *myo*-

inositol U-^{14}C, the percentages incorporated become 2·5 per cent for 1 μc and 5·4 per cent for 2 μc more in agreement with the values from seedlings given D-glucose-U-^{14}C.

DISCUSSION

In germinating wheat, when samples are ground in dilute perchloric acid, a procedure which rapidly inactivates phosphatases without breakdown of phosphate esters,[12] the hydrolysis of phytate by phytase does not give any intermediate in significant concentration between phytate and *myo*-inositol and phosphate ion. This is in contrast to the hydrolysis using comparable concentrations of soluble phytate and isolated phytase. The amount of *myo*-inositol monophosphate that would have been detected was less than 0·1 μmole per g of dry weight. Concentrations of this order have been found in seeds of crested wheat grass in the early stages of germination.[5] In these it was noted that the phytase activity was low as no reduction in phytate concentration was found 48 hr after imbibition. The interpretation of the significance of concentrations of lower *myo*-inositol phosphates below this level in wheat would be difficult as the speed of inactivation of phytase on crushing in a de-naturing medium will be finite. One reason for the lack of lower phosphate esters could be the slight solubility of the mixed salt of phytate, leading to a low solution concentration of phytate relative to phytase. However, the ease of hydrolysis of insoluble calcium phytate indicates a more complex situation exists in seeds, and probably involves the structure of the phytate globoids.

Assuming that intermediate phosphate esters are not utilized as such, then a consequence of their absence is that a continuous supply of *myo*-inositol and phosphate is available. The constant ratio of inorganic phosphate to fresh weight of seedlings indicates that its level is controlled. Inorganic phosphate has been shown to reduce the increase in phytase in isolated wheat embryos if applied early in germination, without affecting other processes.[9] Phosphate ions inhibit phytase preparations.[11, 14] Control of phytase would be simpler if only two products, *myo*-inositol and phosphate, were produced in significant concentrations.

The *myo*-inositol released on hydrolysis is extensively utilized, as the loss by leaching is small.[15] The distribution of label from *myo*-inositol U-^{14}C shows an incorporation into the pectin (fractions VI and I) and hemicellulose–cellulose fractions as has been described by Loewus.[16] The label from D-glucose U-^{14}C is also incorporated. *myo*-Inositol is extensively converted to CO_2 and incorporated into other fractions in a similar way to D-glucose, indicating that it can be readily utilized by wheat seedlings, probably after conversion to pentose.[17]

The distribution of label from *myo*-inositol and D-glucose differed in two fractions, the lipid after hydrolysis and removal of all products except *myo*-inositol and glycerol and the ethanol-soluble fraction after removal of all but *myo*-inositol. The enzyme that cyclizes D-glucose 6-phosphate[18] has been isolated from two plants, *Phaseolus vulgaris* and *Sinapis alba*.[19] The synthesis of phosphoinositide in animals involves reaction of free *myo*-inositol with nucleotide-diglyceride. Wheat seedlings at 3–4 days do not convert D-glucose to *myo*-inositol. This may be due to lack of D-glucose 6-phosphate cyclase or its inhibition and suggests one possible role for *myo*-inositol, one of the products of phytase breakdown of phytate, in germinating wheat.

[14] M. SUGIURA and Y. SUNOBE, *Botan. Mag. (Tokyo)* **75**, 63 (1962).
[15] K. E. RICHARDSON and B. AXELROD, *Plant Physiol.* **32**, 334 (1957).
[16] R. M. ROBERTS, J. DESHUSSES and F. LOEWUS, *Plant Physiol.* **43**, 979 (1968).
[17] F. A. LOEWUS, B. J. FINKLE and R. JANG, *Biochim. biophys. Acta* **30**, 629 (1958).
[18] I. W. CHEN and F. C. CHARALAMPOUS, *J. Biol. Chem.* **241**, 2194 (1966).
[19] H. RUIS, E. MOLINARI and O. HOFFMANN-OSTENHOF, *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 1705 (1967).

EXPERIMENTAL

Plant Material

Grain of the wheat cultivar, Mendos, was stored at 2°. Wholemeal was prepared immediately before use by grinding through a sieve of 1 mm dia. Seeds were germinated on filter paper for up to 3 days, at 25° after 5 min treatment with NaClO solution (2% available Cl) followed by washing with distilled water. For longer periods (up to 14 days), washed, moistened sand was used as the support.

Enzyme Preparations

Pectinase (Sigma Chemical Co.) was assayed before use with apple pectin.

Phytase for *in vitro* hydrolyses was prepared from wheat bran (60 g) which was extracted overnight at 2° with water (300 ml). The supernatant from low-speed centrifugation (4000 × *g*, 10 min) was centrifuged at 30,000 × *g* for 30 min. The supernatant from this was saturated with $(NH_4)_2SO_4$, the precipitate collected (30,000 × *g*, 15 min) and suspended in water (60 ml). The solution was dialysed against water overnight. On standing, a precipitate formed which showed no phytase activity. The solution was centrifuged before use and contained 700–900 units of activity per ml. A unit is defined as that amount of phytase that releases 1 μg of phosphorus as inorganic phosphate in 1 hr from a 1·6 mM phytate solution (10 ml) in acetate buffer (0·1 M, pH 5·15) and $MgSO_4$ (2 mM).

Phytase activity in seedlings was estimated by a modification of Peers' method.[7] Seedlings (40 g) were extracted overnight after maceration in distilled water (230 ml) at 2°, centrifuged (30,000 × *g*, 30 min) and the supernatant poured into acetone at −15°. The precipitate was washed with ether and dried. It was extracted in water (100 ml) at 2° for 2 hr, centrifuged (30,000 × *g*, 15 min) and the supernatant saturated with $(NH_4)_2SO_4$. The precipitate was collected (30,000 × *g*, 15 min), suspended in water (15 ml) and dialysed against water.

Radioactivity Labelled Solutions

myo-Inositol-U-^{14}C, of specific activity 160 mc/mmole, was diluted with unlabelled *myo*-inositol to produce a solution 0·025 M with respect to *myo*-inositol with an activity of 5 μc/ml. D-Glucose-U-^{14}C, of specific activity 3·9 mc/mmole, was diluted with water to form a solution with an activity of 25 μc/ml.

Samples were dried on planchettes under i.r. lamps and counted with a Geiger–Müller tube. Counting efficiencies were determined by mixing labelled glucose of known decomposition rate with unlabelled fractions prepared from germinating wheat and plating. The efficiency was reproducible within replicate platings of the same fraction and varied from 2·0 to 5·0 per cent for the different extraction fractions.

Chromatography

The solvents used were: A, the upper phase of *n*-butanol–benzene–pyridine–water (5:1:3:3); B, *iso*-propanol–18 M ammonia–water (5:4:1); C, acetone–water (4:1).

Estimation of Total Phosphorus, Inorganic Phosphate and Acid-labile Phosphate

Total phosphorus was determined by the Allen method as modified by Turner.[20] For acid-labile phosphate an aliquot of dilute $HClO_4$ extract (5·0 ml) was adjusted to pH 7 with 0·5 M NaOH and diluted to 10 ml. A portion (1·0 ml) was heated in a boiling water bath with 2 M $HClO_4$ (1·0 ml) for 7 min. Inorganic phosphate was determined in a sample of suitable volume.

Estimation of Phytate

Seedlings were ground in a mortar at 2° and wholemeal extracted overnight at 2° in 0·5 M HCl and the supernatant from centrifugation (30,000 × *g*, 30 min) filtered. Samples (20 ml) were adjusted to pH 2·5 with 25% NaOH and diluted to 50 ml with water. $FeCl_3$ solution (4 ml—containing 1 mg Fe/ml in M HCl) was added to an aliquot (20 ml) which was then heated in a boiling water bath for 15 min. After centrifuging (4000 × *g*, 10 min), the precipitate was washed with M/6 HCl (5·0 ml), then suspended in water (2·0 ml), heated, NaOH added and the mixture heated in a boiling water bath for 15 min. The solution was filtered and made up to 25 ml. Total phosphate was determined on an aliquot of suitable size.

Separation and Estimation of myo-*Inositol Phosphates formed during Phytase Hydrolysis of Sodium Phytate*

An aliquot (25 ml), containing initially sodium phytate (15 mg total phosphorus) in 0·2 M NaOAc (4 mM with respect to $MgSO_4$) and phytase (6000 units), was added to Dowex 50 H^+ resin (12·5 g) producing a pH of 1·8 and inactivating phytase. The slurry was filtered, diluted to 50 ml and centrifuged (37,000 × *g*, 10 min). The supernatant was chromatographed on Dowex 1–X8 formate (200–400 mesh) (53 cm^3), eluting with water (50 ml) and a gradient of ammonium formate. A portion of each fraction was incubated with phytase

[20] J. F. Turner, *Biochem. J.* **67**, 450 (1957).
[21] R. A. McCance and E. M. Widdowson, *Biochem. J.* **29**, 1694 (1935).

for 2 hr and inorganic phosphate estimated. Total phosphorus was determined in an aliquot from the pooled tubes of each peak. Ammonium formate at the maximum concentration used was shown not to interfere with phosphorus determination.

Separation and Estimation of myo-*Inositol Phosphates from Germinating Wheat*

Wheat was germinated for 1, 2, 3, 7 and 14 days. Samples with original weights of 40 g were used for 1, 2 and 3 days and 80 g for 7 and 14 days. There was 98 mg of phosphorus combined as phytate in 40 g of grain. The samples were ground in a mortar with 0·5 M $HClO_4$ at 2° and then extracted overnight by shaking. After centrifuging (30,000 × *g*, 30 min), the supernatant was made 20 mM with respect to EDTA and adjusted to pH 7 with 10% KOH. After several hours at 2° the precipitated $KClO_4$ was removed by centrifuging. The phosphates were chromatographed on a column of Dowex 1–X8 formate (230 cm^3).

Estimation of myo-*Inositol in Germinating Wheat*

A sample of 100 seedlings was ground with 0·5 M $HClO_4$ (20 ml) and centrifuged (30,000 × *g*, 30 min). The supernatant was made 20 mM to EDTA and to pH 7 with 10% KOH. After several hours at 2° the precipitated $KClO_4$ was removed by centrifuging. The supernatant was de-ionized (IRA 400—OH^- and Dowex 50—H^+) and H_2SO_4 added to a final concentration of 0·75 M and the solution refluxed for 5 hr, cooled and neutralized with $BaCO_3$. The precipitate was centrifuged and washed twice with water (2 × 70 ml). Supernatant and washings were filtered and evaporated to dryness below 40° under reduced pressure. The residue was dissolved in water (40 ml) and centrifuged (37,000 × *g*, 10 min). An aliquot (0·5 ml) and phosphate buffer (5·0 ml—pH 11·3) and 0·05 M I_2 (3·0 ml) were stored at 25° in the dark. After 1 hr the solution was deionized and concentrated. The residue was dissolved in water (1·0 ml) and the concentration of *myo*-inositol determined after separation by paper chromatography from small quantities of interfering compounds. The *myo*-inositol was developed with alkaline $AgNO_3$,[13] and compared with a series of standards on the same paper using transmission densitometry. Papers were irrigated in solvent A and were fixed by dipping in thiosulphate solution for 10 min. Each estimation was done in quadruplicate. A Photovolt densitometer with Light Source Unit 52-C, fitted with automatic scanning TLC stage, multiplier photometer 520-A and Varicord response recorder Model 42B, was used. The tube used (28-C) was sensitive in the range 300–600 nm and was used with a slit 1 × 17 mm and a filter transmitting at 420 nm. The recovery of samples of *myo*-inositol was 97 per cent.

Fractionation of Seedlings Supplied with myo-*Inositol* U-^{14}C *and* D-*Glucose*-U-^{14}C

Ten seedlings which had germinated 72 hr were incubated with *myo*-inositol-U-^{14}C or with D-glucose-U-^{14}C at two levels, 1 μc and 2 μc plus 5 μmoles of the unlabelled compound for 24 hr at 25°. They were ground in a mortar with 0·5 M $HClO_4$ (40 ml) at 2° and then in a Tenbroeck all-glass homogenizer. After centrifuging (30,000 × *g*, 10 min) and washing with 0·5 M $HClO_4$ (30 ml) and water (30 ml), the combined supernatants were adjusted to pH 7 with 10% KOH. After concentrating to 20 ml, the solution was centrifuged (4000 × *g*, 5 min). Ethanol (100 ml) was added and the solution stored at 2° overnight. The precipitate was centrifuged (30,000 × *g*, 30 min) and dissolved in water to give *the ethanol-insoluble fraction of the acid extract* (*fraction I*). The supernatant was evaporated to dryness under reduced pressure and dissolved in water to give the *ethanol-soluble fraction of the acid extract* (*fraction II*). An aliquot of this was refluxed in 6 M HCl for 40 hr, filtered and concentrated to small volume under reduced pressure and deionized, giving the *hydrolysed ethanol-soluble fraction of the acid extract* (*fraction III*).

The residue from the acid extraction was dried and extracted with $CHCl_3$–methanol (1:1) (40 ml) by grinding in a mortar and then in a glass Tenbroeck homogenizer. The mixture was centrifuged (30,000 × *g*, 30 min) and extracted with a further two portions of solvent (30 ml). The supernatants were combined and divided. One half gave the *lipid fraction* (*fraction IV*). The second half was evaporated and refluxed for 40 hr in 6 M HCl, filtered, concentrated to small volume and deionized. This gave the *hydrolysed lipid fraction* (*fraction V*). The dried residue, after lipid extraction, was suspended in 0·1% EDTA and the pH adjusted to 4·0 with acetic acid. Pectinase (100 mg) was added and the pH readjusted to 4·0 and incubated at 35° with shaking. After centrifuging (30,000 × *g*, 30 min) and washing the precipitate with water (2 × 40 ml), the supernatants were passed through a column of Dowex 50 H^+. The eluate gave the "*pectin*" *fraction* (*fraction VI*). Cold 72% (w/w) H_2SO_4 (3·0 ml) was added to the residue and mixed occasionally. After 2 hr, cold water (27 ml) was added and the mixture refluxed for 2 hr. Centrifuging (37,000 × *g*, 20 min) gave a precipitate of the *final residue* (*fraction VIII*) and a supernatant which was neutralized with solid $BaCO_3$ and centrifuged again (4000 × *g*, 10 min). The precipitate was washed with water (20 ml) and the combined supernatants gave the "*hemi-cellulose and cellulose*" *fraction* (*fraction VII*).

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