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THE MOBILIZATION OF MACRONUTRIENT ELEMENTS IN THE GERMINATING WHEAT GRAIN

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Abstract—During germination in wheat the macronutrient mineral elements (magnesium, calcium and potassium) are redistributed from the aleurone tissue to the developing seedling. The hydrolysis of phytin, the storage form of these minerals, by phytase appears to be initiated by hydration of the aleurone tissue. The hydrolysis and the subsequent mobilization of the mineral elements are promoted by a gibberellin from the embryo. The increased phytase levels that appear in the aleurone cells during germination are induced by certain nitrogen compounds, particularly glutamine. Indole acetic acid increases the efficacy of the glutamine. The induction process probably involves the activation of an inactive form of the enzyme.

INTRODUCTION

PHYTIN, the mixed potassium, magnesium, calcium salt of inositol hexaphosphate, is the major storage compound of the macronutrient mineral elements in the grain of cereals generally. In wheat, the aleurone tissue contains over 70% of the total mineral content of the grain[1] and almost all of the phytin.[2] The starchy endosperm, scutellum and embryo axis contain approximately 20%, 6% and 4% respectively of the total mineral content. There is no conclusive evidence for the presence of phytin in the starchy endosperm,[3] and the major phosphorus-containing compounds in this tissue are thought to be phospholipids, although some inorganic phosphate may be present.[4] Small quantities of phytin have been detected in the embryo axis, at least in oat.[5]

Early during germination a marked increase in phytase activity occurs[6] and this activity is associated with a hydrolysis of phytate to inorganic phosphate and myo-inositol.[7] The inorganic phosphate and its associated cations thus become available for redistribution within the germinating seed Although there is only limited information in the literature, it is generally understood that in the germinating wheat grain, as in other species, there is a general mobilization of mineral elements from the storage tissues to the developing seedling.

The purpose of the experiments described in this paper was to determine the precise patterns of redistribution of the mineral elements during germination in wheat, and to characterize some of the factors that initiate and control the redistribution.

RESULTS

Grain was germinated for periods up to 10 days. Quiescent grain and batches of germinated grain were dissected into their individual tissues and each tissue was analysed for its

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total content of phosphorus, potassium, magnesium and calcium. The inorganic phosphate and, in the case of the bran, the phytate–phosphate contents were also determined.

During the first 6 days of germination the increase in macronutrient content of the seedling and scutellum was accompanied by an almost stoichiometric decrease in that of the combined starchy endosperm and bran (Figs. 1–4). While the later results are distorted by the loss of phosphorus, potassium and magnesium, but not of calcium, to the filter paper on which the seeds were germinated, there was clearly a continued redistribution throughout

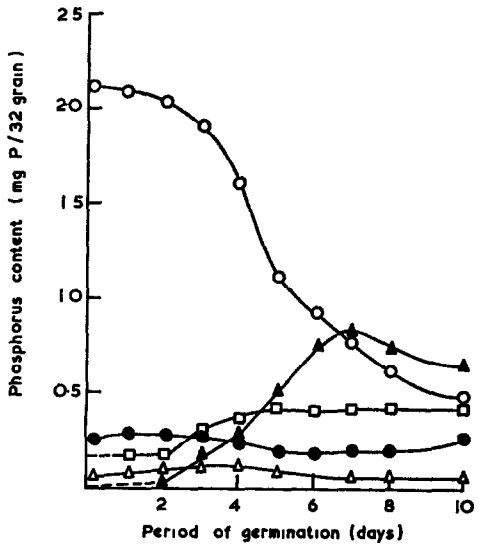

FIG. 1. TOTAL PHOSPHORUS LEVELS IN THE TISSUES OF GERMINATING WHEAT GRAIN.
○, Bran; ●, starchy endosperm; △, scutellum, ▲, shoot; □, root + hypocotyl.

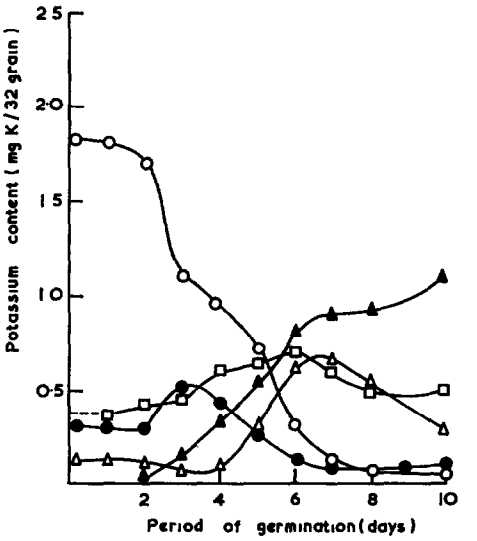

FIG. 2 POTASSIUM LEVELS IN THE TISSUE OF GERMINATING WHEAT GRAIN.
○, Bran; ●, starchy endosperm; △, scutellum, ▲, shoot, □, root + hypocotyl.

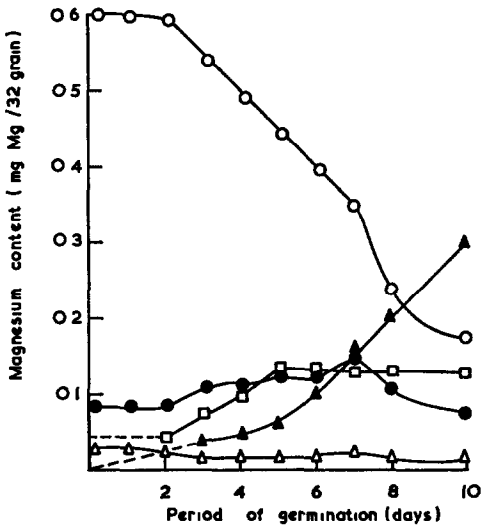

FIG 3 MAGNESIUM LEVELS IN THE TISSUES OF GERMINATING WHEAT GRAIN
○, Bran, ●, starchy endosperm; △, scutellum; ▲, shoot, □, root + hypocotyl.

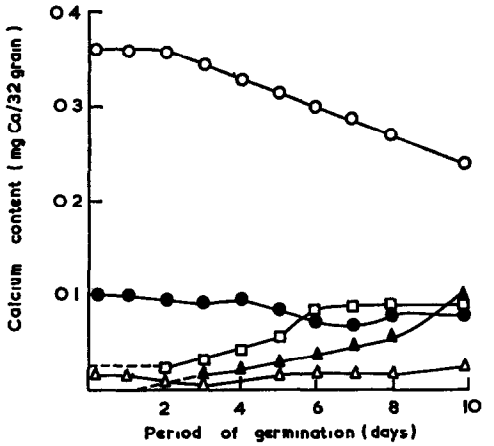

FIG 4 CALCIUM LEVELS IN THE TISSUES OF GERMINATING WHEAT GRAIN
○, Bran; ●, starchy endosperm, △, scutellum, ▲, shoot, □, root + hypocotyl

the experimental period. The elements passing to the seedling originated in the bran and there was only an intermittent tendency towards accumulation in the starchy endosperm. Potassium alone showed a tendency to accumulate in the scutellum. During the early stages of germination the root was predominant over the shoot in accumulating mineral elements. Thereafter, the major accumulation was confined to the shoot. Of the cations potassium passed most quickly and completely to the seedling. In contrast the movements of magnesium, and especially of calcium, were slower, and were incomplete even after 10 days. Total phosphorus redistribution followed a similar pattern to that of potassium.

The levels of the different fractions of phosphorus within the bran during germination are presnted in Fig. 5. The extensive loss of phytin-phosphate and the much smaller increase in inorganic phosphate in this tissue illustrate the overall picture of hydrolysis of phytin

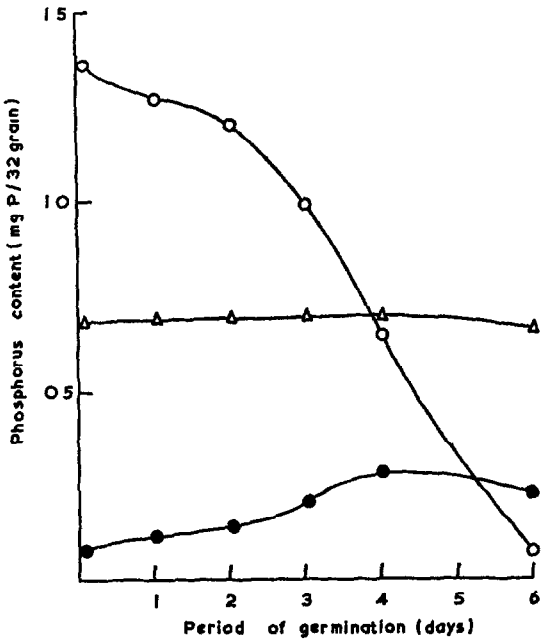

FIG. 5. PHOSPHORUS COMPOUNDS IN THE BRAN OF GERMINATING WHEAT GRAIN. ○, Phytate phosphorus; ●, inorganic phosphorus; △, other phosphorus compounds (by difference).

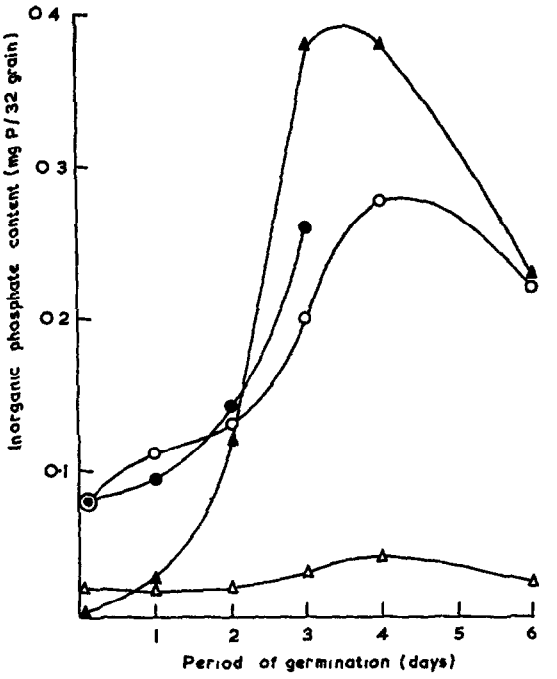

FIG. 6. INORGANIC PHOSPHATE LEVELS IN THE TISSUES OF GERMINATING WHEAT GRAIN. ○, Bran; ●, starchy endosperm, △, scutellum, ▲, seedling.

followed by the translocation of inorganic phosphate from the tissue. It is interesting that the other phosphorus fraction, which is presumably composed of organic phosphorus compounds, does not represent an important reserve of phosphorus during germination. This does not, of course, rule out the possibility of turnover between different groups of phosphorus compounds. These overall patterns of phosphorus metabolism in the bran are essentially similar to those derived from whole grain analyses by Matheson and Strother.[7] The analysis of inorganic phosphate contents of other tissues during germination (Fig. 6) also confirm and extend Matheson and Strother's observations. The increase in the inorganic phosphate content observed by these workers occurs in the bran, starchy endosperm and seedling, but less significantly in the scutellum. The increase in the inorganic phosphate content of the whole grain does not correlate quantitatively with the loss of phytin phosphate from the bran. This indicates that part of the inorganic phosphate taken up by the developing seedling is there incorporated into organic forms.

In order to determine the role of the embryo, and the possible role of gibberellins from the embryo, in controlling the mobilization of mineral reserves from the bran, de-embryoed grain was incubated for 4 days either in the absence or in the presence of authentic hormone. The macronutrient distribution between the bran and the starchy endosperm was determined (Table 1). On the assumption that during germination the gibberellin from the embryo

TABLE 1. MINERAL REDISTRIBUTION IN DE-EMBRYOED GRAIN

Element (mg/32 grain)	De-embryoed grain (Control) day 0	day 4	De-embryoed grain +10 μM. GA_3 day 4	Germinated grain (Control) day 5
Bran				
Potassium	1 79	1·48	1 00	0 70
Magnesium	0 62	0 61	0 50	0 45
Calcium	0 36	0 36	0 31	0·32
Total phosphorus	2 10	1 98	1 42	1 08
Inorganic phosphate	0 076	0·13	0 19	0 24
Starchy Endosperm				
Potassium	0 24	0 54	0 90	0 24
Magnesium	0 07	0 08	0 13	0 10
Calcium	0 10	0 10	0·13	0 08
Total phosphorus	0 15	0 22	0 65	0 16
Inorganic phosphate	0 177	0 10	0 23	—

GA_3: gibberellic acid

arrives at the bran some 24 hr after initial water uptake, an *in vivo* control is included in the table. This consists of analyses from grain that had germinated for 5 days. In the absence of the embryo the level of inorganic phosphate in the de-embryoed grain increased upon incubation. It follows that, as well as inorganic phosphate, there will be a liberation of potassium, magnesium and calcium from the phytin molecule, and yet under these experimental conditions only phosphate and potassium left the bran. In the presence either of the embryo or of gibberellic acid not only did the level of inorganic phosphate increase above that of the control, but all of the macronutrients now moved from the bran; in increased quantities in the cases of inorganic phosphate and potassium. The nutrients released from the bran by gibberellic acid accumulated in the starchy endosperm. Maximal response was

produced by 10 μM gibberellic acid. Compared with the *in vivo* control, there was a significantly greater retention of total phosphorus, potassium and magnesium, but not of calcium, in the bran of the de-embryoed grain treated with gibberellic acid.

The primary importance of phytase in the initial hydrolysis of stored phytin makes it of interest to correlate the observed breakdown of phytin with the development of phytase activity. To this end phytase levels in the germinating grain and in incubated, de-embryoed grain were studied. During germination phytase levels in bran increased from 36 units/32 grain at day 0 to 147 units/32 grain by day 4. The levels of phytase in the other tissues were low and increased only slightly during germination. In contrast, there was no increase in phytase in the bran of the incubated, de-embryoed grain. Gibberellic acid, over a wide range of concentrations, was without effect on these levels, although it promoted increased acid

TABLE 2. THE EFFECTS OF PHYTOHORMONES ON PHYTASE AND ACID PHOSPHATASE LEVELS IN THE BRAN OF DE-EMBRYOED GRAIN

		Phytase (units/32 grain)	Acid phosphatase (units/32 grain)
Water (control)		36	13
Gibberellic acid	(1 μM)	37	22
	(10 nM)	36	16
Indole acetic acid	(10 μM)	38	13
	(1 μM)	36	13
	(10 nM)	36	—
Kinetin	(1 μM)	38	—
Indole acetic acid + Gibberellic acid	(10 μM) (10 μM)	33	32
Indole acetic acid + Gibberellic acid	(10 μM) (10 nM)	36	19

The de-embryoed grain were incubated for 4 days with the specified hormone solutions The enzyme levels in the bran were then determined

phosphatase (Table 2) Furthermore, the other known phytohormones had no significant effect on the levels of either enzyme. Aqueous diffusates from embryos of grain that had germinated for 24 hr induced an increase of some 50% in phytase levels. In contrast to the phytohormones, a series of nitrogen compounds, at substrate concentrations, were very effective in inducing an increase in phytase levels (Table 3) Sodium nitrite, ammonium chloride and glutamine were particularly effective and the action of glutamine was enhanced by indole acetic acid but not by gibberellic acid. A range of purine and pyrimidine nucleotides were either ineffective or produced only small responses. Although the control de-embryoed grain in these experiments showed no increase in phytase content even after several days of incubation, crude cell-free systems from the bran showed a progressive increase in their phytase activities when incubated alone at 30°. This increase, from 32 units/32 grain at the beginning of the incubation period to 60 units/32 grain after 5 hr, continued linearly for several hours When the bran alone was incubated with glutamine, only a very small increase in its phytase content resulted.

In a final series of experiments the possible effects of a number of inhibitors of DNA-dependent RNA synthesis and RNA-directed protein synthesis were investigated. Neither

TABLE 3. THE EFFECTS OF NITROGEN COMPOUNDS ON PHYTASE LEVELS IN THE BRAN OF DE-EMBRYOED GRAIN

	Phytase (units/32 grain)
Water (control)	36
Sodium nitrate (1 mM)	48
Sodium nitrite (1 mM)	73
Ammonium chloride (1 mM)	77
Hydroxylamine hydrochloride (2 mM)	60
Glutamine (1 mM)	73
Glutamic acid (1 mM)	43
Asparagine (1 mM)	45
Aspartic acid (1 mM)	40
Glutamine (1 mM) + Indole acetic acid (1 μM)	78
Glutamine (1 mM) + Gibberellic acid (10 μM)	72
Casein hydrolysate (1 mg/ml)	57
Embryo diffusate after extraction by ethyl acetate	58

The de-embryoed grain were incubated for 4 days with the specified solution at pH 7 0. The levels of phytase in the bran were then determined

actinomycin-D in the presence of potassium bromate, cycloheximide, puromycin nor L-azetidine-2-carboxylic acid had any effect on the induction of increased phytase levels in de-embryoed grain by a combination of glutamine and indole acetic acid. Azaserine, a structural analogue of glutamine, was similarly without effect.

DISCUSSION

During germination in wheat the macronutrient mineral elements accumulate in the developing seedling at the expense of the storage tissue, agreeing with the limited evidence available from several other species of seed.[8–10] The developing seedling itself plays an important role in controlling this mobilization and several control mechanisms appear to be involved. The seedling undoubtedly acts as a sink to remove, either actively or passively, those nutrient elements released from the aleurone cells. Thus, the lower efflux rates that were observed in the bran of incubated, de-embryoed grain could have resulted from a simple mass action effect or from the inhibition of phytase by inorganic phosphate.[11] Probably more important than these feed-back mechanisms is the role of a gibberellin-like hormone emanating from the embryo. It is well established that gibberellins move early during germination of cereals from the embryo to the aleurone tissue and there induce the *de novo* synthesis of new protein.[12] The present experiments with de-embryoed grain show clearly that gibberellic acid is able to partly substitute for the embryo in stimulating the release of mineral elements from phytin and increasing their efflux rates from the aleurone

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cells. These results raise an apparent anomaly because gibberellic acid does not increase the level of phytase in this system.

Since there is no tendency in the whole, germinating grain for minerals to accumulate in the starchy endosperm, it seems most probable that the embryo axis takes up these nutrients at almost the same rate as they are released from the aluerone cells. Thus, it would appear that the overall rates of redistribution, and the differences between the redistribution rates of the different ionic species, are established in the aluerone tissue. Because it is a dead tissue, movement through the starchy endosperm is almost certainly by diffusion. It is unlikely that an electrochemical gradient in this tissue would move one ionic species at the expense of another especially as the diffusion coefficients of monovalent cations are reduced nearer to those of divalent cations in hetero-ionic heterogenous environments.[13]

Phytase obviously plays an essential part in the hydrolysis of phytin during germination. The activity of the enzyme does not, however, appear to play a central role in the control of macronutrient mobilization. The phytase in the bran of the quiescent grain is more than adequate to account for the observed rates of phytin hydrolysis *in vivo* during germination. Hydration of the aleurone tissues would appear to be the only prerequisite for the initiation of the hydrolysis. It seems, therefore, that the four-fold increase in phytase during germination is a superfluous production. The induction mechanism that is responsible for this increase is, nevertheless, important in the context of control mechanisms generally. In contrast to the majority of hydrolytic enzymes in germinating cereals,[14] and to phytase itself in germinating barley,[15] the increased phytase level in wheat aleurone tissue is not induced by any of the plant hormones. Instead certain nitrogen compounds, perhaps acting synergistically with indole acetic acid, are able to induce the increase. This situation is similar to that described for lipase in the same tissue.[16] Interactions between the phytohormones and organic nitrogen compounds in the control of cellular processes are, in fact, well known.[17] In other biological systems also, nitrogen compounds including amino acids have been found to affect the amount and activities of enzymes and the activities of hormones.[18] It is very relevant that glutamine is synthesized in large quantities during germination of wheat and many other species.[19]

The induction of increased phytase levels during germination differs from that of lipase in that the former process does not appear to involve a *de novo* synthesis of the protein. The complete lack of action by several inhibitors of RNA and protein synthesis, together with the observation of increasing phytase activity in incubated cell-free systems, strongly suggests that the increase is the result of the activation of an inactive form of the enzyme. The present experiments do not allow any speculation concerning the nature of the activation process. Further unanswered questions relate to the problem of why glutamine and indole acetic acid, when administered to de-embryoed grain, induced an increase in phytase population that was only half that reached in the germinating grain, and why glutamine has little effect on the enzyme level in excised, incubated bran. Other inducing factors appear to be involved.

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EXPERIMENTAL

Sterilization and Incubation of Plant Material

Grain of the soft winter variety Cappelle Desprez were used in batches of 2 g equivalent to 32 grain. The grain were sterilized by vacuum infiltration of dil. NaOCl and washed with several changes of sterile H_2O [20] Subsequent dissections and incubations were carried out under aseptic conditions.

Sterilized grain were germinated in the dark at 25° on ashless filter paper in a 9 cm covered dish in 5 ml deionized H_2O. For long periods of germination a further 2 ml deionized H_2O were added after 4 days and subsequently at 2 day intervals

De-embryoed grain were prepared by soaking sterilized grain for 1 hr in deionized H_2O and then cutting around the embryo groove and lifting the embryo axis and scutellum from the grain The de-embryoed grain were incubated in the same way as the whole grain.

Bran was isolated by quartering the de-embryoed grain longitudinally and placing the endosperm quarters with their starchy endosperm resting downwards on ashless filter paper moistened with 5 ml 0·06 M mannitol. After incubating in this manner for 3 hr, the starchy endosperm could be scraped from the bran with the back of a scalpel blade This method of preparation minimized the loss of mineral elements from the bran In the case of those grain and de-embryoed grain that had taken up H_2O for at least 48 hrs, the starchy endosperm was soft enough to be removed from the bran without prior soaking.

Solutions of diffusates and compounds were sterilized by filtration through a Sietz 5/3 millipore filter immediately before application to incubating tissues.

Preparation of Embryo Diffusate

The embryo axes and scutella were excised from 10 g grain that had germinated for 24 hr. The excised tissues were incubated for 6 hr at 25° in 150 ml sterile, de-ionized H_2O. At the end of the incubation, the medium was acidified to pH 3·5 (HCl) and extracted 3 × EtOAc The extracted medium was reduced to dryness at 40° and the residue taken up in de-ionized H_2O.

Determination of Total Potassium, Phosphorus, Calcium and Magnesium

Tissue samples were placed in pre-weighed porcelain crucibles in which they were dried at 80° and ashed for 24 hr at 450° The cooled ashes were treated with 3 ml conc. H_2SO_4 and taken to dryness. This converts P_2O_7'' to PO_4''' and usually completes the oxidation process.

The ashes were taken up in warm, redistilled 0 6 M HCl The solutions were centrifuged to remove any remaining C particles, which were then washed with 3 × 0 6 M HCl. The bulked extracts were made up to a standard volume with 0 6 M acid An aliquot of the solution was removed for the analysis of phosphate and the remaining solution was made to 20% (v/v) with spectroscopic EtOH[21] and analysed by flame spectrophotometric methods for K, Mg and Ca

$PO^{4'''}$ was determined by the colourimetric method of Dryer, Tammes and Routh [22] K was determined with a Unicam SP90 flame spectrophotometer operated in the emission mode with an air/acetylene flame; Mg was determined with the spectrophotometer operated in the absorption mode with an air/acetylene flame; Ca was determined using the absorption mode with a nitrous oxide/acetylene flame K markedly increases Ca absorption readings in this flame system and excess (to 80 ppm) KCl was added to both the unknown and standard solutions prior to Ca determinations

Determination of Inorganic Phosphate and Phytate-phosphate

Tissue samples were extracted 3 × at low temperature with a mixture of MeOH, $CHCl_3$, HCOOH and H_2O [23] The bulked extracts were made up to a standard volume An aliquot of this solution was made biphasic by the addition of $CHCl_3$ and H_2O followed by centrifugation The upper aqueous phase was removed and analysed for inorganic and phytate phosphate

Inorganic phosphate was determined by a procedure based on that of Berenblum and Chain.[24] 2 ml of 2 5% (w/v) ammonium molybdate in 1 5 M H_2SO_4 were added to 5 ml of the upper phase This was followed by 3 ml 0 5 M citrate buffer, pH 7 0 to complex excess molybdate, thus preventing the molybdate-catalysed hydrolysis of phosphate esters [25] 1 ml p-semidine reagent and 3 ml isoBuOH were then added; the mixture was shaken and allowed to settle The lower aqueous phase was re-extracted with a further volume of isoBuOH and the bulked extracts made up to 10 ml, after 1 hr the absorbance at 750 nm[22] was read against an isobutanol blank.

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The lower aqueous phase from the determination of inorganic phosphate was brought to pH 8·0 by the addition of a few drops of dil. NaOH solution. Phytin was then precipitated as Fe^{+++} phytate by the addition of 0·25% (w/v) $FeCl_3$ in 1 M HCl (0·2 ml $FeCl_3$/1 ml test solution). The precipitate was washed twice with cold 0·2 M HCl and then taken up in warm 0·2 M NaOH. $Fe(OH)_3$ was removed by centrifugation. The phytin was then hydrolysed by digestion for 4 hr with conc. H_2SO_4 (1 ml/1 ml phytate solution) and a few drops of 30 vol. H_2O_2. Excess H_2O_2 was destroyed by the addition of a few drops of 0·01 M $KMnO_4$ and PO_4''' determined by the routine method.[22]

Enzyme Assays

Phytase was assayed by a method similar to that of Peers.[6] In contrast to the results of previous studies the crude phytate from our wheat gave maximal activity pH 5·8 rather than pH 5·0–5·2. Therefore, tissues were homogenized in 0·05 M citrate buffer, pH 5·8 at 4° (10 ml buffer/g fr. wt tissue). The homogenate was filtered through two layers of cheese-cloth. The filtrate was made up to a standard volume and assayed immediately. Incubations were carried out at 30° and with 2 mM sodium phytate as substrate, so that in our results 1 unit of phytase activity is the amount of enzyme that releases 1 μg of ortho-phosphate in 1 hr from 2 mM sodium phytate at pH 5·8 and at 30°; cf. Peers [6] Phosphate was assayed by the routine method.

For the assay of acid phosphatase activity, the crude tissue extracts were adjusted to pH 5·0, the optimum for that enzyme. A simple assay procedure utilizing the chromogenic substrate *p*-nitro phenyl phosphate[26] was used.

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