CXXXI. PHOSPHATE FRACTIONS IN BARLEY SEEDLINGS

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WORK on the acceleration of plant respiration by the addition of inorganic phosphates has never emerged from the unsatisfactory state which is revealed in Onslow's [1931] extensive summary. The present paper deals with the partition of phosphates in young barley seedlings, and the results of adding or withholding inorganic phosphate supplies. The bearing on seedling respiration is to be dealt with in a subsequent paper.

Methods

Method of cultivation. The paleae and glumes were first removed from the seeds, as it was found that a higher percentage germination could be obtained in this way, and that the seeds germinated more uniformly. The seeds were then soaked for 3 hr. in distilled water at 22.5°. After soaking, the seeds were washed in about a dozen rinsings of autoclaved water and finally in the appropriate culture solution, and placed on the surface of fine sand in a petri dish, the sand being saturated with culture solution. The petri dish and implanted seed were then enclosed in sterilized dark chambers [James & Norval, 1938], and the whole immersed in a thermostat at 22.5°. A continuous current of CO₂-free air was circulated through the chamber. Before entering the chamber, but after being freed from CO₂, the air stream was saturated with water vapour by passing it through a bubbling bottle immersed in the water bath. In this way evaporation from the seedlings and sand in the respiration chamber was cut down to the minimum. The air stream was also passed through a long cotton wool filter to remove spores.

The culture solution was that described by James [1933], and was made up freshly for each experiment from stock solutions which had been sterilized by autoclaving and kept in the dark until required for use; no precipitation occurred, at least until the experiment was actually in progress. In this paper, only "full" phosphate and "no" phosphate solutions are considered.

The sand used to grow the seedlings was purified from all traces of soluble salts by prolonged boiling in HCl followed by aqua regia. The leached sand was then washed repeatedly in distilled water and sterilized by autoclaving. The sterilization was repeated after each experiment, after thorough washing. The seed used in all experiments was "Plumage Archer" obtained from Messrs Sutton and Sons of Reading.

Sampling. While the seedlings were germinating in the respiration chamber, a continuous record of their CO₂ emission was kept. As soon as the chamber was opened up at the end of the germination period, the seedlings were prepared for analysis. They were first washed free from sand and culture solution, and the remainder of the husk and endosperm removed. They were graded at the same time into three grades according to size: grade I restricted to the longest individuals, grade II composed of individuals of length down to about two-thirds of

the maximum length and grade III including the remainder, i.e. all those seedlings under two-thirds of the maximum length. Grade III rarely contained more than 10% of the total number of seedlings, and these were always rejected in sampling for dry weight and analysis. This grading was carried out in order to eliminate, as far as possible, variations between the different samples caused by variation in size of the seedlings. All the samples in any one experiment were selected so that they contained the same proportion of seedlings from grades I and II.

It might be objected that the exclusion of the grade III seedlings from the samples made them incomparable with the whole set of seedlings whose respiration is measured. This procedure has been selected as the lesser of two evils for the following reasons. It has been observed in the present work, that not all the seedlings which are soaked at any one time will germinate simultaneously. A small percentage shows the first visible sign of germination (protrusion of the radicle) about two or three days after the rest. Seedlings formed from these late-germinating seeds are considerably shorter than those from seeds which germinate immediately after soaking, and their physiology may have a corresponding lag; they correspond to the grade III seedlings which are excluded from the samples. They will clearly contribute only a small percentage to the total CO_2 of respiration.

The size of the samples used for extraction varied at different stages. In the preliminary experiments of 1935, the samples contained only 20 seedlings each, two samples being taken from each experiment; the size of these samples was restricted by the size of the chambers in which they were grown. In later experiments, using larger chambers, a larger number of seedlings was grown in each experiment, and two samples each of 60 seedlings were analysed. This was done to reduce the experimental error, which seems largely due to differences between the samples, as will be shown later.

In each experiment, two other samples were taken for dry weight and total P determinations, both determinations being performed successively on each sample. These samples contained 10 seedlings each in the 1935 experiments, and 20 seedlings each in the subsequent experiments; they contained the same proportion of grade I and grade II seedlings as did the samples for extraction. All the results given in the present paper were obtained from the larger samples.

Extraction. Extraction was carried out with trichloroacetic acid, which coagulated all proteins; these could then be centrifuged down [Needham *et al.* 1932; Cori & Cori, 1932]. A somewhat similar technique has been used on plant material by Barrenscheen and co-workers [1927; 1929]. The seedlings were crushed in an ice-cold glass mortar with a little sand. They were then extracted in ice-cold 7.5% trichloroacetic acid for 30 min. in an ice bath, with frequent stirring to facilitate extraction.

A test experiment was performed to ensure that the extraction was complete. It was found that 60 ml. of trichloroacetic acid, followed by a washing in 10 ml. of the same solution, were sufficient for complete extraction, if the extraction lasted for 30 min. Prolonging the period of extraction for a further 30 min. did not result in any increase in the amount of phosphate extracted, but did lead to a slight amount of hydrolysis of esterified phosphate. The increase in inorganic phosphate during the second half hour was about 3%. The possibility of a more rapid breakdown of some of the esterified phosphate will be dealt with in the section on phosphagens which follows, in which it is shown that there is no breakdown of esterified phosphate during the first part of the extraction period. It can therefore be concluded that the slight variations which occurred in the length

of the extraction period in the various experiments to be reported later, are not responsible for the variation in the proportion of inorganic to esterified phosphate.

Fractionation. The trichloroacetic acid extract will contain the following groups of phosphorous compounds, if they are present in the seedlings: inorganic orthophosphate; "labile esters" such as adenylyl pyrophosphate; "resistant esters" such as hexosephosphates, triosephosphates etc; phosphagens such as arginine and creatine phosphates; phytin.

Phospholipins and phosphoproteins will not be extracted in the acid, and they are the main constituents of the "residual P" fraction which is estimated as the difference between the total acid-soluble P and the total P content of the seedlings estimated by incineration.

For simplicity, it will be convenient to state here some conclusions arrived at in the following sections, viz. that there is no phytin nor phosphagen present in the seedlings at this stage of growth. The separation of the remaining three classes soluble in acid is brought about by dividing the extract into aliquots. The total extract is neutralized with saturated NaOH until just alkaline to phenolphthalein. It is then made up to 101 ml. This allows 10 aliquots of 10 ml. each to be removed with a pipette, the extra 1 ml. allowing the last aliquot also to be removed by pipette.

The inorganic orthophosphate can be estimated directly on the extract, by the colorimetric method of Fiske & Subbarow [1925]. It was, however, found better to precipitate the inorganic phosphate by adding some 20 % CaCl_2 solution (saturated with CaO), to the neutralized aliquots, and to carry out the estimation of orthophosphate on the redissolved precipitate [Needham *et al.* 1932], because it was not always possible to obtain the crude extract quite clear, even with high centrifuge speeds, and the turbidity gave rise to a distinct yellow tinge in the phosphomolybdate blue, thus rendering comparison with the standard inaccurate. The inorganic orthophosphate determination was done on 4 aliquots. Two other aliquots were made up to N with HCl and placed in a boiling water bath for 7 min., being subsequently cooled and neutralized. This mild acid hydrolysis liberates orthophosphate from the "labile esters", and a Fiske & Subbarow determination on these hydrolysed aliquots, therefore, gives the sum of the preformed inorganic orthophosphate, plus the "labile ester" fraction. The "labile ester" fraction is then the difference between this estimate and the inorganic orthophosphate estimate previously obtained.

The remaining four aliquots were incinerated (without calcium precipitation) with H_2SO_4 and H_2O_2 , and the total P content estimated as orthophosphate by the Fiske & Subbarow method. This gives an estimate of the total acid-soluble P. The "resistant ester" content can be determined as the difference between this estimate and the estimate from mild acid hydrolysis, which includes both the other fractions. This will be referred to as "the difference method". The "resistant ester" content can also be determined more directly on the solution left over after precipitation of orthophosphate and pyrophosphate as Ca salts ("solubility method"). The Ca remaining in the solution is first precipitated as oxalate in the presence of acetic acid which keeps other phosphates in solution. The solution is then oxidized with H_2SO_4 and H_2O_2 , and the phosphate content estimated by the Fiske & Subbarow method. It has been found that the same values are obtained whether the "labile esters" have been hydrolysed before the Ca precipitation or not. It follows that the labile esters must be fully precipitated from solution by the Ca precipitation technique. Since the labile esters are precipitated, the method of determining the resistant ester content (by oxidation of the centrifugate after Ca precipitation), is sound, and may be

expected to give reliable results. Table I shows the values for resistant esters obtained by the two methods in two independent experiments.

Table I

	mg. per seedling	
	(1)	(2)
Total acid-soluble phosphate less phosphate after mild hydrolysis	0.0336	0.0214
Phosphate not precipitated by Ca	0.0327	0.0210

RESULTS

In Table II the results of two successive experiments carried out on Dec. 9 and Dec. 16, 1936 are given in full detail. The groups of four estimations represent the four determinations on four aliquots from the same extract. The two groups of four for each experiment represent the two separate extractions carried out on the two comparable samples from the set of seedlings grown in any one experiment.

Table II. *Mg. phosphate per seedling*

	Inorganic	Labile esters	Total acid-soluble	Total phosphate
Dec. 9, 1936				
Extract 1	0.0632	0.0134	0.1004	
	0.0659	0.0119	0.1049	—
	0.0703	—	0.1044	—
	0.0715	—	0.1037	—
Mean	0.0677	0.0123	0.1033	0.1431
Extract 2	0.0629	0.0159	0.1099	—
	0.0627	0.0144	0.1004	—
	0.0616	—	0.1004	—
	0.0618	—	0.1047	—
Mean	0.0622	0.0151	0.1038	0.1441
Dec. 16, 1936				
Extract 1	0.0812	0.0029	0.1140	—
	0.0808	0.0029	0.1147	—
	0.0812	—	0.1141	—
	0.0800	—	—	—
Mean	0.0808	0.0029	0.1143	0.1517
Extract 2	0.0818	0.0081	0.1114	—
	0.0812	0.0059	0.1110	—
	0.0808	—	0.1110	—
	0.0800	—	0.1113	—
Mean	0.0809	0.0070	0.1112	—

In both experiments the "no-phosphate" solution was used, so that there is no difference in treatment between the two experiments. It is obvious by inspection of these figures that the errors incurred in the estimation of inorganic "resistant ester" and total phosphate are small compared with the large differences which occur between seedlings from different, but comparable, experiments. Only in the estimation of the labile esters do the errors of estimation become anything like commensurate with the differences between experiments. It has, all along, been found that the estimation of labile esters involves considerable experimental error; this is largely because they are determined by difference between fractions which are about ten times larger than the labile ester fraction itself.

Another point which is shown by the above table, and is confirmed by the details of other similar experimental results, is that the difference between the mean values obtained from the two separate extractions in any one experiment

is much smaller than the difference between the mean values for different experiments; thus showing that the variation between experiments is not due merely to sampling errors. It is therefore considered that the analytical method adopted is in general adequate, although the method of estimating labile esters needs improvement.

Phytin

The occurrence of phytin in green plants has not been investigated very thoroughly, apart from the Gramineae, in which group its presence seems to be fairly general. Moutuori [1934] has recently demonstrated its presence in the seeds of several tropical cereals, and Goldfiem [1934] found it in a number of tropical African food plants, many of them not belonging to the order Gramineae. Wiaddrowska [1936] demonstrated the presence of phytin in carrots, beans and cabbage plants, and found that treatment with MgSO_4 increased the uptake of phosphorus in these plants, and increased the amount of phytin present in the carrot roots. Marimpietri [1933] found that, in various cereals, after the first period of germination (which roughly corresponds to the period under consideration in the present research), the proportion of phytin-P/total P increased. Kolobkova [1936], in investigating the phytase of wheat flour, found that, in the ungerminated seed, 55 % of the total P is present in the form of phytin, but that, on germination, the phytin content falls considerably and other P compounds, especially phosphoproteins and lipins, accumulate.

Phytin is only present in very small quantities in the seedlings used in the experiments to be considered here.

Tests were carried out on a solution of phytin containing 2 mg. per 100 ml. of water. This is about the concentration in which phytin would be present in the trichloroacetic acid extract if all the "resistant ester" phosphate proved to be phytin phosphate. Aliquots of this solution, neutralized till just alkaline to phenolphthalein, were then treated with the CaCl_2 solution used for precipitating phosphates. It was found that the solution left over contained absolutely no phosphate, either free or combined, while the precipitate contained the amount of phosphate which would be expected if all the phytin had been precipitated by the CaCl_2 . Phytin is therefore completely precipitated under the conditions of the routine estimations. If, therefore, phytin is present in the seedlings, it will all be precipitated along with the inorganic phosphate and labile esters. It will not be estimated with either of these fractions, however, since phytin is stable to the comparatively mild hydrolysis which is used to break down the labile esters. Similarly, it will not be estimated as resistant esters after oxidation of the solution left over after Ca precipitation, since it is itself precipitated; but it will be estimated in the total acid-soluble determinations in which the original acid extract is oxidized.

Thus, if phytin is present, we should expect to find a discrepancy between the resistant ester figures obtained by the "difference method" and the "solubility method". Instead of resistant esters = total acid-soluble - (inorg. PO_4 + labile esters) the relation in the presence of phytin would be resistant esters = total acid-soluble - (inorg. PO_4 + labile esters + phytin). Table I gives results in which both estimates of resistant esters are available, and no such discrepancy occurs; so it can safely be concluded that phytin is absent from the seedlings used in the present experiments.

Confirmation was sought by carrying out some phytin estimations on the extract from seedlings, using the iron titration method of Hubner & Stadler [1914]. The difficulty in obtaining a satisfactory end-point, which has been experienced by other workers, renders this method of little value for the precise

estimation of small quantities of phytin. Nevertheless, the results obtained indicate that only minute quantities of phytin could be present in the extracts, viz. not more than 0.0069 mg. per seedling.

Examination of the remains of the endosperm and pericarp removed from seedlings after 5 days' germination, showed that little phytin was present in them after germination of the embryo; 39 of these endosperms and pericarps were ground up and extracted in 20 ml. of 0.3 *N* HCl for 1 hr.; the extract was then filtered, and diluted to 0.16 *N* with water. Titration with FeCl₃ indicated the presence of 0.227 mg. of phytin phosphate (=0.0058 mg./seedling).

The paleae and glumes removed from the seeds before they were germinated were also shown to contain very little phytin. The figures in Table III were each obtained on separate extracts; the husks from about 80 seeds were used for each extract. As a check on the results, two sets of husks were incinerated for total P; the agreement between these figures, and those obtained by titration, indicates that all the P present is estimated by the titration method, and is therefore probably present in the form of phytin.

The total P content of the seeds is about 0.45 mg. of phosphate-P per seed. Thus the husk contains less than 2% of the total P present in the seed.

Table III. *Content in mg. of phosphate per grain*

	Phytin-P (by titration)	Total P (by incineration)
Paleae and glumes (removed before germination)	0.0168	0.0070
	0.0069	0.0068
	0.0049	—
	0.0053	—
	0.0085	0.0069
Endosperm and pericarp (after 5 days' germination)	0.0058	—
Seedlings without endosperm (after 7 days' germination)	0.0069	—

Phosphagens. Two phosphagens are of common occurrence in animal tissues, creatine phosphate and arginine phosphate, and the wide range of distribution of these two compounds has been shown by the work of Needham *et al.* [1932]. An extensive search of the literature has revealed no mention of the occurrence of phosphagens in green plants. It was, however, necessary to determine whether phosphagens were present or not in the seedlings used in the present work, in order that the correct significance might be attached to the various phosphate fractions estimated. Experiments were therefore performed to test whether there were any labile phosphate-forming compounds of the phosphagen type present in the trichloroacetic extract.

By making only one extraction in trichloroacetic acid, and omitting the second washing of the residue, an estimate of the inorganic phosphate present after only 15 min. from the first crushing can be obtained. This does not give time for phosphagens such as creatine and arginine phosphates to break down to any considerable extent, especially as the extraction was performed in an ice-bath. If the solution, with the blue colour developed, is left for about 30 min. after the first reading, the major portion of the phosphagen should have broken down; especially since the Fiske & Subbarow reagents act as catalysts of the hydrolysis. But in the experiments performed along these lines, no increase in the blue intensity, over and above that found in the standard phosphate solution also, was found to occur.

In another type of experiment the extract was divided into aliquots and the inorganic phosphate was precipitated with CaCl_2 at the end of 15 and 60 min. from crushing. No increase in phosphate could be detected after the longer standing. Hence, it seems certain that the seedlings contain no labile phosphagens of the kind found in animal tissues.

Labile and resistant esters. Recent work has demonstrated the highly important role played in yeast fermentation and muscle glycolysis by the compounds of adenosine with phosphoric acid. The P of this group of compounds is estimated by conversion into inorganic orthophosphate by mild acid hydrolysis with N HCl at 100° for 7 min. The P estimated in this way has usually been designated pyrophosphate, since it was originally thought to contain only inorganic pyrophosphate and adenylyl pyrophosphate.

It has now been found that this fraction may also contain adenylic acid, in which the phosphate has no pyrophosphate bond, and so more recent publications refer to the whole group as "easily hydrolysable esters". By contrast, the hexose- and triose-phosphoric esters, together with phosphoglyceric and phosphopyruvic acids, become the "difficultly hydrolysable esters".

In the present paper these two fractions are called "labile esters" and "resistant esters" respectively.

The difficulties attending the estimation of the labile esters have already been stated; because of this difficulty they have not been estimated in every experiment, but only in a selection of experiments representative of the various treatments and ages for which results are available. Table IV shows that a low but definite fraction of the acid-soluble P is present in the labile ester form.

Table IV. *Mg. phosphate per seedling*

	Inorganic phosphate	Labile esters	Total esters	Resistant esters	
				A	B
5-day seedlings on no phosphate solution	0-088	0-0086	0-0364	0-0278	—
	{0-0677	0-0122	0-0356	—	0-0234
	{0-0622	0-0155	0-0416	—	0-0216
	{0-0808	0-0029	0-0335	—	0-0306
	{0-0809	0-0070	0-0303	—	0-0233
	{0-0703	0-0041	0-0372	—	0-0331
	{0-0673	0-0073	0-0337	—	0-0264
	Mean	0-0739	0-0082	0-0355	—
5-day seedlings on full phosphate solution	{0-1064	0-0070	0-0374	—	0-0304
	{0-1056	0-0069	0-0371	—	0-0302
	0-1188	0-0032	0-0359	0-0336	0-0327
	0-1109	0-0048	0-0268	0-0214	0-0210
	Mean	0-1104	0-0055	0-0333	—
7-day seedlings on full phosphate solution	{0-1450	0-0104	0-0150	—	0-0046
	{0-1546	0-0126	—	—	—
	{0-1312	0-0160	0-0318	—	0-0158
	{0-1359	—	0-0259	—	—
	0-1361	0-0100	0-0263	—	0-0163
	Mean	0-1406	0-0123	0-0234	—

Bracketed series were obtained from independent samples from a single crop.

Under resistant esters, column B gives results obtained by the "difference method", and column A those obtained by the "solubility method".

Analysis of variance shows significant variation in all the phosphate fractions. There are significant differences ($P < 0.05$) between each of the inorganic phosphate means. The difference between full and no phosphate is not significant among resistant esters. All other comparisons of means give significant differences.

Stable esters are also present, and usually contain a much larger part of the phosphate, the exception being in the 7-day-old seedlings.

Inorganic phosphate always accounts for the bulk of the acid-soluble phosphate, even in those seedlings which received no external supply. Phosphate feeding results in considerable increases of the inorganic phosphate within the seedling, but this is not followed by a corresponding increase in the esters of either class. The natural inference is that, in these early seedling stages, the phosphate supplied by the reserve tissues is itself sufficient to maintain all the available "phosphate acceptor" in the esterified form. Between the fifth and seventh days the labile esters, unlike the resistant esters, increase. This may be supposed to have been made possible by further synthesis or absorption from the endosperm of phosphate acceptor.

Comparison of root and shoot contents

In a further set of analyses, the seedlings were divided into "root" and "shoot" portions and the phosphate of these contents determined separately. The seedlings were cut across just above the scutellum into two portions, one containing the coleoptile, first leaves and shoot rudiment, and the other containing the roots and scutellum. In each experiment about 80 seedlings, grown on full phosphate solution, were used; and a further 40 seedlings were similarly divided and used for determinations of fresh and dry weight and of total P.

The results of these experiments are given in Table V. The relative bulks of the root and shoot portions, as indicated by fresh weight, are not very different, the root portion being somewhat less than the shoot.

Table V. (a) *mg. phosphate per seedling*; (b) *mg. phosphate in 1 g. fresh wt.*

	Root		Shoot	
	Exp. 27	Exp. 28	Exp. 27	Exp. 28
Fresh wt. (g. per seedling)	0.0810	0.0783	0.1042	0.1053
Inorganic PO ₄	(a) 0.0548 (b) 0.6760	0.0500 0.6383	0.0641 0.6150	0.0609 0.5783
Labile esters	(a) 0.0017 (b) 0.0207	0.0038 0.0485	0.0015 0.0144	0.0010 0.0095
Resistant esters	(a) 0.0116 (b) 0.1427	0.0067 0.0855	0.0216 0.2072	0.0150 0.1424
Total esters	(a) 0.0123 (b) 0.1512	0.0110 0.1402	0.0237 0.2274	0.0158 0.1500
Residual PO ₄	(a) 0.0022 (b) 0.0284	0.0042 0.0536	0.0258 0.2475	0.0258 0.2450
Total PO ₄	(a) 0.0693 (b) 0.8556	0.0652 0.8321	0.1136 1.0899	0.1025 0.9733

All the fractions determined were present in both portions of the plant, the shoot containing rather more phosphate than the root, in the aggregate.

The distribution of phosphate is expressed in Table VI as fractions of the total phosphate.

Table VI

	Root	Shoot
Inorganic phosphate	0.77	0.58
Labile esters	0.04	0.02
Resistant esters	0.14	0.17
Residual phosphate	0.05	0.23
Total phosphate	1.00	1.00

The proportions allotted to labile and resistant esters do not differ greatly between "root" and "shoot"; but a much larger proportion of the inorganic phosphate is transferred to residual phosphate in the "shoot" than in the "root". This is probably related to the bulkier meristem, since the residual phosphate consists mainly of phosphatides and nucleoproteins.

SUMMARY

1. A method is described for the estimation of the various classes of phosphorus compounds present in plant material, and results are given for seedlings of Plumage Archer barley grown for 5 and 7 days in the absence of light on solutions with and without phosphate.

2. Phytin was only present in very small quantities in the husks (paleae and glumes), endosperms or seedlings proper.

3. Phosphagens of the type of creatine and arginine phosphate could not be detected in the seedlings.

4. The inorganic phosphate content of the seedlings was increased by growing the seedlings on culture solution containing phosphate, but this did not result in an increase of either labile or resistant esters.

5. Inorganic phosphate, labile esters and resistant esters all vary with the stage of development.

6. The contents of the root and shoot portions of the seedlings have been determined and compared. There seems to be little difference between the two portions in regard to labile esters and resistant esters. The shoot has a much larger proportion of lipid and protein phosphorus than the root. This is assumed to be associated with the greater proportion of meristematic tissue present in the shoot portion.

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