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Phosphorus Compounds in Translocating Phloem

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Abstract. **Phosphate-^{32}P was introduced into a turnip leaf, and 3 hr later, the vascular bundles were stripped from the petiole and their phosphate ester pattern was studied. The pattern did not alter along their length and was like that of other tissues. Pumpkin leaves were painted with phosphate-^{32}P; and later, the petioles were cut, the sieve tube exudates were collected and their phosphate ester patterns were studied. Exudates collected after 10 min had a high proportion of their ^{32}P present in P_i and nucleoside triphosphates, while exudates collected after long translocation times (4–22 hr) had a lower proportion in these, and a higher proportion in hexose monophosphates and UDPglucose. In general, the ester patterns were like those of other tissues. The results indicate that sieve tubes are metabolically active, and that P_i is the primary form in which phosphorus moves in the phloem.**

When a compound moves from a leaf to the stem, it does so against the main flow of water in the plant, generally traveling through the specialized cells of the phloem. Carbohydrate, phosphorus and sulfur are all known to move in this tissue (1). Many studies have shown that carbohydrate moves as a single predominant compound, usually sucrose (33). In contrast, the form in which phosphorus is translocated is unknown. In the xylem stream, it moves mainly as P_i (22, 29, Bieleski, unpublished data); but phosphoryl choline and glycerophosphoryl choline can also be present, and it has been suggested that sieve tube exudate from spinach and cucumber may also contain these compounds (22). On the other hand, ATP has been clearly identified in phloem exudates from 17 tree species (20). Again, when isolated pieces of willow bark, which contained embedded aphid stylets, were irrigated for several hr with $^{32}P_i$, labeled glucose 1-P, glucose 6-P and fructose 1,6-diP were recovered from the phloem in the stylet sap (19).

Most other pertinent data relate to the nature of phosphorus compounds which can be extracted from isolated phloem tissues. UDPglucose was found in isolated *Heracleum* phloem (32). A more detailed analysis revealed that the general nucleotide pattern was very like that of xylem tissue (23). Also, the nucleotide pattern in sugar beet vascular bundles was very like that in the petiolar parenchyma and the leaf (24). Excised apple phloem and *Brassica* vascular bundles which had accumulated $^{32}P_i$, had phosphate ester patterns (6) very like those of other plant tissues (8, 10). In a more direct attack on the problem, Biddulph and Cory fed $^{32}P_i$ to a bean leaf, then identified the labeled compounds that appeared in the petiole (2): P_i, fructose 1,6-diP and glucose 6-P were the main ones found, and phosphoryl choline was not detected. The extraction procedure used would not have recovered all the phosphate esters (8, 11), and at the time of harvesting a large part of the ^{32}P had already moved from the phloem into the surrounding tissues (2), so there is no direct assurance that the ester pattern observed was that of the translocating cells.

Consequently, none of these studies directly answers the question, what is the form in which phosphorus is translocated in the intact phloem. Direct analysis of the phloem tissue is unsatisfactory, as the main translocating elements form only a small part of this metabolically active tissue. We must find a way of distinguishing between the esters that are present in whole phloem tissue, which appears to have a pattern like that of other tissues, and the compounds involved in translocation itself. The following study extends the approach used by Biddulph (2). The intact leaf has been fed $^{32}P_i$, but the subsequent analysis of ^{32}P-ester pattern in the petiole has been restricted to either vascular bundle tissue or to sieve tube exudate, instead of the whole petiole.

Methods

Ester Pattern in Conducting Vascular Bundles. A mature leaf of a turnip (*Brassica rapa* L.) was fed with ^{32}P as KH_2PO_4, 0.025 M, 1.5 mc in 0.12 ml, by the leaf-flap method (3) for 3 hr under field conditions. The petiole of the leaf, 26 cm long, was then taken; the vascular bundles were stripped from it, aligned, and cut into 5 cm lengths so as to yield 5 samples of vascular tissue that were originally located 5 to 10 to 25 to 30 cm distant from the point of injection of $^{32}P_i$. Each sample, weighing about 20 mg, was killed in 1 ml methanol:chloroform:formic acid:water, 12:5:1:2 v/v at −72°, and extracted, using a procedure (11) modified to suit the small amount of tissue available. A miniature filter stick

was made by placing a small Willstaetter button in the bottom of a pasteur pipette. A disk of glass fiber paper was pressed down on top of the button, and acid-washed celite was added and tapped down to form a pad. The tissue was ground in a Duall conical glass homogenizer, the homogenate was transferred by pasteur pipette to the prepared filter stick, and the subsequent eluate was collected in a centrifuge tube. The residue in the filter stick was eluted with the remaining extractants. Final extracts were prepared in the usual way (11) and were separated by 2-dimensional thin-layer chromatography (5). Radioactive areas were marked out from autoradiographs, removed by a cellulose acetate peel technique (8), and measured for radioactivity.

Model Experiments on Inactivation of Phosphatase in Extracts. The second part of the study was to involve collection of sieve tube exudate for analysis. Phosphatases interfere with studies on phosphate ester patterns (4), and sieve tubes are known to be rich in phosphatase (21). Model experiments were used, in order to find procedures that minimized phosphatase action. The enzyme solution contained a plant phosphatase (Worthington) at 2.5 E.U./ml in pH 6.4 buffer, and the substrate was 20 mM *p*-nitrophenyl phosphate (Sigma). Each solution, 100 μl, was pipetted onto a square of parafilm that rested on ice. At zero time, the 2 droplets were mixed, and 20 μl was subjected to the appropriate sampling procedure (table II). After sampling, the amount of *p*-nitrophenol that had been formed during sampling was estimated.

Phosphate Esters in Exudate From Translocating Sieve Tubes. Four-week old pumpkin (*Cucurbita maxima* Duch.) plants were used. One leaf on each plant was selected, and petrolatum was smeared in a thin line across the top surface, just above the petiole, to act as a moisture barrier. The tracer solution contained 10^{-4} M $KH_2{}^{32}PO_4$, 20 mc/ml, plus 5 % Tween 80 as wetting agent. A droplet of 10 μl or 40 μl (short translocation times) was pipetted onto the top surface of the leaf, and spread out by stroking with the side of a thin glass rod. Absorption and translocation were allowed to occur for varying times, 10, 20, or 40 min, and 1, 2, 4, 16, or 22 hr, and then the leaf was held in absorbent paper while the petiole was cut cleanly with a razor blade at a point 3 to 5 cm from the junction of leaf and petiole, this being 6 to 10 cm from the patch of tracer on the leaf. The cut surface of the upper part of the petiole was immediately dabbed on filter paper to remove liquid from the cut cells, then the leaf and petiole were held for 5 to 7 sec while 0.5 to 5 μl sieve tube exudate collected on the cut surface of the phloem. The surface was then touched onto the origin of a cellulose thin-layer chromatography plate (5 μl 0.01 M EDTA had previously been spotted and dried on this origin), and the chromatography plate was immediately put into a tank which contained the first chromatography solvent for separating the phosphate esters, *n*-propanol:ammonia:water:EDTA, 6:3:1:0.01 v/v/v/wt (5). The total time required, from slicing the petiole to enclosing the thin-layer plate in the chromatography tank, was 9 to 13 sec. The plate was chromatographed in the normal way (5), and the radioactivity of each compound was measured.

Results

Phosphate Ester Distribution in Conducting Vascular Bundles. There was no variation in ^{32}P-phosphate ester pattern along the length of the translocating vascular bundles (table I). The pattern obtained was very similar to that for the leaf mesophyll tissue, or for excised vascular bundles which had accumulated $^{32}P_i$ from the surrounding medium (6), or for various other plant tissues (8).

Table I. *Radioactivity in Phosphate Esters From Vascular Bundles That Had Been Situated 5-10, 15-20, and 25-30 cm From the Point of Injection of $^{32}P_i$ Into the Leaf*

Total cpm per chromatogram: 5-10 cm sample, 3470; 15-20 cm sample, 3460; 25-30 cm sample, 3700.

Compound	^{32}P Activity as % of total extract ^{32}P in:		
	5–10 cm sample	15–20 cm sample	25–30 cm sample
P_i	58.9	58.6	51.2
Glucose 6-P	16.6	16.0	17.4
Fructose 6-P	3.2	3.3	3.3
Mannose 6-P	2.0	2.4	1.7
ATP + UTP	6.1	6.1	10.4
ADP	1.9	1.8	3.5
UMP[1]	1.3	1.2	1.1
Hexose diphosphates	1.1	1.1	1.3
Phosphoglyceric acid	3.1	3.1	3.1
Phosphoryl ethanolamine	0.4	0.5	0.5
Phosphoryl choline	0.8	1.0	1.6
Other compounds	4.5	5.0	4.9

[1] Mostly derived from UDPglucose through breakdown.

Table II. *Formation of* p-*Nitrophenol During Various Procedures for Sampling Enzyme-substrate Mixture*

20 μl mixture was submitted to the sampling procedure, then the products were dissolved in 1.2 ml 0.1 M KOH, and the absorbance of the solution at 400 mμ was read.

Sampling method	OD
1. Capillary containing sample in paraffin, 95°/1 min	0.145
2. Sample mixed with 20 μl formic acid	0.085
3. Sample on thin-layer plate put into alkaline chromatography solvent	0.040
4. As (3), but substrate-enzyme mixed at 15°, not 0° (time from mixing till plate in solvent, 18 sec)	0.035
5. As (4), but time delay 63 sec	0.145
6. As (4), but time delay 183 sec	0.340

Model Experiments on Inactivation of Phosphatase. Various sampling procedures were tested. Some were found to be unsuitable for quickly collecting petiolar exudate, while others did not give an extract that could easily be chromatographed. Ultimately it was found that the simplest possible sampling procedure gave the least phosphatase action (table II). The model exudate was spotted on a thin-layer plate which was immediately put into a tank containing the first chromatographic solvent. Ammonia from the solvent inactivated the phosphatase (table II). The time delay, from first mixing enzyme and substrate to putting the chromatography plate in the tank, was 18 sec. With longer time delays, more enzyme action occurred. By measuring the rate of reaction in this way, it was shown that the actual inactivation of the phosphatase by the ammonia vapor took only 1 to 2 sec. This sampling procedure was used in subsequent experiments. Its effect on the chromatographic separation was then studied. Chromatography of the crude sieve tube exudate resulted in origin fixation of P_i and marked trailing of the esters. When EDTA (5 μl 0.01 M) was added to the origin to chelate divalent cations, interference was slight, and it was possible to separate and identify most of the major phosphate esters.

Phosphate Ester Pattern in Exudate From Conducting Sieve Tubes. In all, 35 leaves were sampled, from plants grown under various conditions, after various translocation times. Exudate samples contained from 150 to 4000 cpm ^{32}P. The resulting ^{32}P-phosphate ester patterns were all very similar to one another. A representative chromatogram is shown in Fig. 1, and the ester pattern for that sample in table III. There were several general features. Firstly, there was no sign of any unusual compound. Secondly, phosphoryl choline was present only in small amounts, and was not disproportionately labeled after short translocation times. Thirdly, the proportion of radioactivity in nucleotide triphosphate, after both short and long translocation times, was higher than is usual for tissues of other plants, while the proportion in nucleotide diphosphate was lower, so that the ratio of the 2, about 16/1, was markedly greater than in other tissues, typically 3/1 (8). The proportion of activity in hexose phosphates and, more notably, phosphoglyceric acid, was lower than usual. Fourthly, almost no radioactivity was recovered in phospholipid and relatively little in RNA, even after long translocation times.

Increasing the duration of translocation did not greatly change the ester pattern. The only consistent effects were an increase with time in the proportion of radioactivity in UDPglucose and in material fixed near the origin (mainly RNA), and a decrease with time in the proportion of radioactivity in nucleotide triphosphate and in P_i (table IV).

Table III. *Distribution of* ^{32}P *Between Phosphate Esters, in Sieve Tube Exudate From a Leaf That Had Been Translocating* ^{32}P *for 4 Hr, Expressed As a Percentage of Total Organic* ^{32}P

The chromatogram autoradiograph of this sample is shown in Fig. 1. Total cpm on chromatogram, 1020.

Compound	% Total esters ^{32}P
P_i	187
Glucose 6-P	21.2
Fructose 6-P	6.5
Mannose 6-P	8.0
Glucose 1-P	2.4
ATP	17.3
UTP	15.1
ADP	1.0
UDPglucose	3.7
UDPX[1]	2.8
Hexose diphosphates	3.2
Phosphoglyceric acid	5.4
Phosphoryl ethanolamine	2.5
Phosphoryl choline	1.7
Triose-P[1]	4.5
X[1]	2.5
RNA	2.2

[1] UDPX is an unknown UDPglucose-like compound (8); may be breakdown product of NAD (8); the identification of triose phosphate is tentative.

Discussion

Phosphate Esters in Conducting Vascular Bundles. The experiment which studied ^{32}P ester pattern in conducting vascular bundles was very similar to that of Biddulph and Cory (2). The improvements which were made—study of vascular tissue alone rather than the whole petiole, use of better extraction and chromatographic procedures, and a search for a gradient in ester pattern along the petiole—were expected to yield more definitive information, and so the results are rather disappointing. There was a complete absence of any gradient in phosphate ester distribution down the length of the petiole, and the pattern was very similar to that given by the leaf

Table IV. *Distribution of ^{32}P in Phosphate Esters From Sieve Tube Exudates After Short and Long Times of Translocation*

Five leaves were harvested after 10 min of translocation, and 5 leaves after 22 hr, and sieve tube exudates were taken. Phosphate esters were separated and their radioactivity measured as described in the text. Results are expressed as means of the percentage of total organic ^{32}P present in that compound or group of compounds.

Compound	10 Min sample Mean	SE	22 Hr sample Mean	SE
P_i	451	± 91	165	± 25
Hexose phosphates	30.6	± 8.2	33.6	± 3.8
Nucleoside triphosphates	54.0	± 9.2	36.0	± 7.4
Nucleoside diphosphates	1.8	± 1.2	2.6	± 0.8
UDPglucose	1.8	± 0.7	7.6	± 2.7
Phosphoglyceric acid	5.0	± 0.6	1.8	± 1.0
Phosphoryl choline	1.8	± 1.0	2.2	± 1.0
RNA	0.6	± 0.8	12.4	± 1.8

blade itself, and by vascular bundles and several other plant tissues after accumulating $^{32}P_i$ from an external solution (see 6, 8, 10). This similarity of patterns could arise in at least 3 ways: phosphate esters translocating in the phloem could indeed conform to this pattern; or the experiment could have been carried on so long that any front-running wave of a special phosphate ester had already passed into the stem and root; or the true pattern of translocating esters in the sieve tubes could be hidden by the overshadowing presence of esters that had come from surrounding phloem parenchyma. These explanations are not mutually exclusive. Other preliminary experiments then suggested that ^{32}P moving down the sieve tubes rapidly moved into the surrounding cells and that any study of the whole vascular bundle, no matter for how short a translocation time, would lead to ambiguous results. A sample of the translocation stream itself was needed. In the apparent absence of a suitable species of aphid in this country, use of sieve tube exudate from *Cucurbita* appeared to be the only suitable method.

Aspects of the Method Used to Study Sieve Tube Contents in Cucurbita. Initial experiments were aimed at obtaining as true a sample of the sieve tube contents as possible. Two precautions which were needed were the minimizing of phosphatase action so that the ester pattern was not altered during sampling (4), and sampling of the phloem exudate in such a way that contents from cells other than sieve tubes were not collected. Model experiments showed that the selected sampling procedure resulted in complete inactivation of the phosphatase within 10 to 14 sec of cutting the petiole. The enzyme action that would have occurred in this time at the prevailing temperature (12 sec at 16°) was considerably less than the action which occurs when slices of ordinary plant tissue are killed in boiling methanol [action equivalent to that obtained in 110 sec at 16° (4)], and only slightly more than occurs with a special procedure designed to minimize phosphatase action [equivalent to 3 sec at 16° (4)].

Precautions for maintaining the purity of the sample of sieve tube contents were influenced by the way in which sieve tube exudates appear to form. The sieve tubes are plasmolysable (16), accumulate solutes (7), and are probably delimited by semipermeable membranes (7, 26). As they contain a high concentration of sugars (16, 33), osmotic phenomena result in their contents being under considerable turgor pressure (33), forcing against the surrounding cellulose walls and tissues. These cellulose walls, unlike those of most cells, have large gaps at the sieve plates (26). Thus when the turgor in any 1 sieve tube is released (either by inserting an aphid stylet or by making a cut) the pressure in the adjacent cell is no longer balanced at the weakest point, the sieve plate, and the semipermeable membrane there can rupture (28). Release of turgor is followed by an osmotically powered inrush of water from the surrounding cells, so that the diluting contents of the sieve tubes continue to flow out through the rupture (33). In this way, by a ninepins effect, the contents of a long line of sieve tubes can pass out through the 1 puncture or cut (30). In the early stages, this exudate should therefore be a true sample of the sieve tube contents (33). After a time, the contents of these injured cells may become degraded, or materials other than water may pass from the surrounding phloem parenchyma cells into the resulting lumen (25), and the identity of the exudate will be less certain.

Thus, in the sampling procedure used, the cut petiolar surface was immediately blotted, to remove solutes released from destroyed parenchyma cells. Then sieve tube exudation was allowed to proceed, but only for a limited time so that there was the greatest chance of obtaining sieve tube contents alone. Finally the exudate was taken, phosphatase was inactivated, and the esters were chromatographed.

One last requirement of the experiment, as already discussed, was to have the duration of translocation as short as possible in 1 set of samples. The speed of travel of phosphorus in phloem has been calculated as about 50 cm/hr (14); and thus a point 7 cm from the site of ^{32}P application should receive a front of ^{32}P approximately 8 min after application of tracer to the leaf. This expectation was confirmed with *Cucurbita*, as 10 min was the shortest translocation time that would give an exudate with sufficient radioactivity for subsequent analysis.

Metabolism of Sieve Tubes. The data show that the sieve tubes must be an integral part of a metabolically active system. As noted above, the exudate collected 10 min after supplying $^{32}P_i$ to the leaf came from phloem which had been in contact with ^{32}P for much less time, probably 2 min or so. The labeling pattern at this time can be compared with that of other tissues which have accumulated $^{32}P_i$ for 2 min; and the 22 hr pattern can be compared with that in

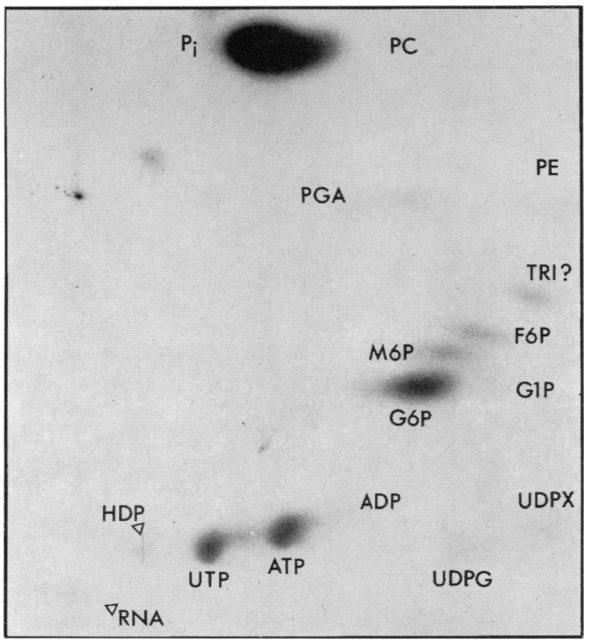

FIG. 1. Autoradiograph of ^{32}P-containing compounds in sieve tube exudate, after their separation by 2-dimensional thin layer chromatography. Abbreviations: Standard abbreviations for nucleotides, and PC, phosphoryl choline; PE, phosphoryl ethanolamine; PGA, P-glyceric acid; TRI, triose-P (?); F6P, fructose 6-P; M6P, mannose 6-P; G6P, glucose 6-P; G1P, glucose 1-P; UDPG, UDPglucose; X and UDPX, unknown compounds; HDP, hexose diphosphates.

tissues which have accumulated $^{32}P_i$ for a day or so (8, 9). The same esters are present in the sieve tube exudates as in other tissues, their proportions are similar, and their course of labeling is similar. The proportion of radioactivity in ATP and UTP is very high after short translocation times, and falls after longer translocation times. The proportion of radioactivity in nucleoside diphosphates and UDPglucose rises fairly rapidly, and the proportion in RNA rises only slowly. These changes in nucleotide radioactivity in the sieve tube system occur as rapidly as in other tissues [compare data in tables III, IV with Fig. 1, reference (10) and Fig. 4, reference (9)], and so the turnover times must be similar. However, the amount of nucleoside triphosphate present is likely to be much greater in the sieve tube exudate, as exudates from a variety of plants have been shown to have ATP concentrations, 70 to 590 mμmoles/ml (20), higher than those of other tissues, 10 to 100 mμmoles/g fresh wt (8). In the present experiments, the proportion of nucleoside triphosphate was also higher than usual. This combination of a normally rapid turnover of nucleoside triphosphates, coupled with an unusually high proportion and amount, must mean that the sieve tube system is supporting an unusually active metabolism. This appears to be the clearest indication so far that sieve tubes might be actively metabolizing, functional and competent cells, even though some evidence has already pointed this way. Thaine has reported seeing active protoplasmic streaming in sieve tubes, which would require a supply of metabolic energy (27); and I have claimed that sieve tubes present in excised pieces of phloem can actively accumulate solutes from surrounding solutions—this process would also require energy, as well as the presence of functional cell membranes (7). [The ability of sieve tubes to accumulate solutes is shown clearly in some data obtained by Ford and Peel (18), though the authors appear to have overlooked the point: ^{14}C-glucose and ^{14}C-sucrose at a concentration of 2 μc/ml initially and less than 0.05 μc/ml finally, were actively accumulated into excised strips of bark, and a sampling of the sieve tube contents by aphid stylets then showed that the 2 sugars were then present in the sieve tubes at concentrations of 0.9 and 0.7 μc/ml respectively—a concentration at least 15 times higher than was present at that stage in the external solution.]

Not yet clear is the site of this ATP synthesis. Most earlier morphological studies reported that sieve tubes were devoid of mitochondria, but it seems that the cell contents must have become lost during fixation through the disruptive osmotic process already described. Later studies agree in reporting the presence of mitochondrion-like bodies in the sieve tube cell (12, 17, 26), though these bodies do not always have a completely typical mitochondrial structure (17, 26), and it has been suggested that they may be rather degenerate (17). There is general agreement that the companion cells are notably endowed with mitochondria (31). One possibility is that these cells supply ATP to the sieve tubes as if to their own cytoplasm (7, 31). Alternatively the sieve tubes could be fully functional, independent cells. Ribosomes are not clearly apparent in electron microscope photographs of sieve tubes, but RNA-containing particles have been demonstrated in sieve tube exudates (13). The present results show that RNA within the sieve tube exudate incorporates ^{32}P, and is therefore turning over, so it appears that ribosomes too may be present and functioning.

Nature of the Translocating Compound. All of the organic compounds that have been implicated at one time or another in the translocation of phosphorus—phosphoryl choline, glucose 6-P, fructose 1,6-diP, glucose 1-P, UDPglucose, and ATP—were present in the sieve tube exudate and labeled with ^{32}P. None satisfied the requirements for a possible primary translocation form. None was present as the sole labeled compound and none was disproportionately labeled after short translocation times. The only compound to fit this pattern was P_i itself; for after the shortest translocation time, over 80 % of ^{32}P was in P_i, but after longer times the proportion was much less. This suggests that P_i is the primary translocation product of phosphorus in the phloem. Phosphate esters probably became radioactively labeled in a secondary way. Incorporation of $^{32}P_i$

into esters is a very rapid process, even in tissues much less metabolically active than phloem tissues (6, 10).

P_i is, like sucrose, notable as a storage material in the normal parenchyma cell. It appears to be located in a segregated non-metabolic pool, probably the vacuole (9, 10). When a parenchyma tissue accumulates $^{32}P_i$, the ratio $^{32}P_i$/ester-^{32}P is low in the beginning, and rises as the ^{32}P in the metabolic pool equilibrates with the P_i in the non-metabolic pool. In sieve tube exudates, this ratio changed in the opposite way, from 5 to 1.8. One simple explanation would be that P_i is moving in a vacuole-like space in the sieve tube, and is equilibrating as it goes with a cytoplasmic phase. One such interpretation has already been put forward to explain aspects of carbohydrate movement (15).

Acknowledgments

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