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# Uptake of Orthophosphate by the Pea Plant (*Pisum sativum*)

## II.* Identification of the Organic Phosphate Esters Formed

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The main phosphate esters formed in assimilation of orthophosphate in the pea plant are identified by two-dimensional paper chromatography using co-chromatography and radioautography. They are ** ATP, UTP, GTP, ADP, UDP, AMP, F6P, G6P, F1,6P, and an unknown compound marked No. 10. In the root several additional unidentified esters are formed, which are not found in the shoot extracts.

The general pattern of esterification of orthophosphate is similar in intact and detached pea root. No noticeable qualitative differences are found in the esterification pattern between pea, rye, oat, and barley plants, but some difference between these and the yeast *Torulopsis utilis* is noticeable.

In the first part [1] of this research project it was established that assimilation of orthophosphate by intact pea roots is a very rapid process. After 10 sec of assimilation, 2 organic phosphates — ATP and a diphosphate, probably ADP — showed labelling, and after 2 min, labelling was found in 4 triphosphates — ATP, GTP, UTP and an unidentified compound — and in 3 spots in the diphosphate area and 2 spots in the monophosphate area of the two-dimensional chromatogram. A dynamic equilibrium was reached in 5 min, after which time chromatograms from both roots and shoots were qualitatively very similar. The results with phosphorus-deficient pea plants (total P 30—50 % of normal) were not essentially different. The labelling obtained in the

* The first paper in this series is Ref.[1]

** The following abbreviations are used: AMP, ADP, ATP = adenosine-5'-mono-, -di- and -triphosphate, resp.; CMP, CDP, CTP = cytidine-5'-mono-, di- and -triphosphate, resp.; GMP, GDP, GTP = guanosine-5'-mono-, di- and -triphosphate, resp., and UMP, UDP, UTP = uridine-5'-mono-, di- and triphosphate, resp. 2-PGA = 2-phosphoglyceric acid, 3-PGA = 3-phosphoglyceric acid, PCHO = phosphorylcholine, G1P = glucose-1-phosphate, G6P = glucose-6-phosphate, F6P = fructose-6-phosphate, F1,6P = fructose-1,6-diphosphate, UDPG = uridine-diphosphoglucose, TCA = trichloroacetic acid.

short reaction times was relatively weak. Therefore identification of most of the spots was not achieved in the above study.

The main goal of the present study was the identification of the labelled phosphates formed. For this purpose somewhat longer assimilation times were used than in the above study to get stronger labelling in the spots. By the application of a new solvent combination [2] satisfactory two-dimensional paper chromatograms are obtained from the original extracts after removal of TCA, without any further purification, and this has made possible chromatographic identification of 10 of the labelled compounds formed. Though the time scale of the present experiment (1—30 min) extends far beyond the most interesting biokinetical interval of the primary phases of assimilation (1—60 sec), the activity of the separated spots was determined to give an idea of the quantitative changes taking place in longer times in the relative activities of the compounds formed. Stringent conclusions can hardly be drawn from figures like these, but the scale on which the experiment was carried out was too small to make determinations of specific activities possible. This will be tried later on and will require working on a larger scale and using different techniques, probably ion exchange columns, which may also provide conclusive identification of the isolated compounds. The present identifications by co-chromatography are only preliminary, although the probability that they are correct is relatively high, since a total of about one hundred two-dimensional co-chromatograms were made.

## METHODS

*Plant material.* The pea plants *(Pisum sativum, var.* Torsdag) were grown in a sterilized nutrient solution inoculated with an efficient strain of *Rhizobium*, the bottles being closed with cotton wool. The green parts were free in the greenhouse air. The nutrient solution was similar to that used in the previous study [1] for "high-phosphorus" plants. The plants were 23 days old when the experiments were made.

*Extraction and chromatography* were also made as mentioned previously [1] except for the first solvent in paper chromatography. All extracts were diluted with water to 12 ml after removal of the TCA and 0.5 ml of the diluted extract was used for each chromatogram. The solvent used in the present study for the first dimension was *t*-butanol:formic acid (98 %): water, 8:3:4 (Miettinen [1]). In the second dimension the mixture of Hanes, *n*-propanol:$NH_4OH$ (25 % $NH_3$):water, 6:3:1, was used.

*Performance of the experiment.* About 10 mC of carrier-free orthophosphate (PBS 1, Radiochemical Centre, Amersham, England), neutralized to pH 7.0, was added to 150 ml of phosphate-free but otherwise complete nutrient solution [1].

This solution was then evenly divided into 5 small beakers. Each sample consisted of a bunch of 5 plants, the roots of which were dipped into the isotope solution for the proper time (1, 5, 15 or 30 min), at the end of which the roots were rapidly washed in cold water, separated from the shoot and crushed in 8 % TCA. A fifth sample (30 min) was taken to study the effect of detachment (of the roots from the shoot) on the uptake rate of the phosphate ion. The detachment was made just before dipping. Of the roots all except the cutting place was dipped, of the shoot about 1 cm of the lower end only. The detached roots were dipped into the same solution as the 1 min sample of the intact plants. The detached shoots were dipped into a separate solution.

During the experiment the plants were, in addition to diffuse daylight, illuminated with one "Photolita" SM lamp at 70 cm distance.

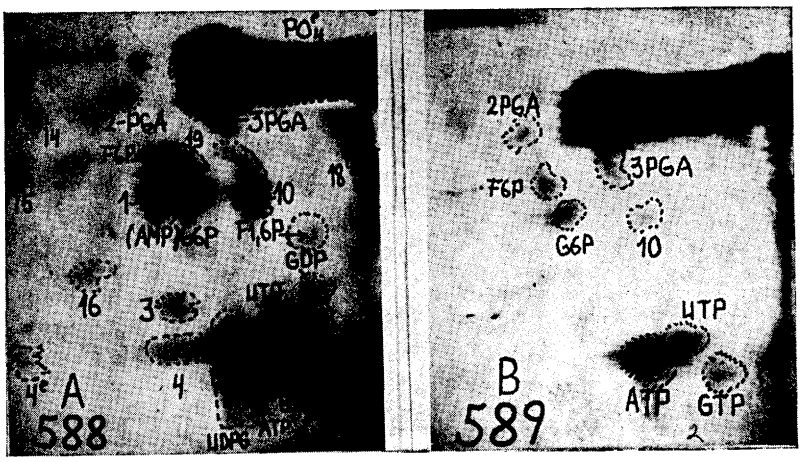


*Fig. 1.* Radioautograms of two-dimensional paper chromatograms of soluble phosphates present in intact (A) and detached (B) pea roots after 1 min uptake of radioactive orthophosphate.

## RESULTS

The following compounds were found to be labelled *in the roots of intact plants* after 1 min assimilation: ATP, UTP, GTP, UDPG, AMP(?), G6P (?), F6P, 2-PGA, 3-PGA, unknowns Nos 1, 3, 4, 10, 13—16, 18, 19, and traces of F1,6P and GDP (Fig. 1, A). The unknown No. 1 may be identical with G1P, but this is still uncertain because the coloured spot of added authentic compound did not exactly match the radioactive spot in one case. In the *roots detached from the shoot* (Fig. 1, B) only weak labelling was found in ATP, UTP and GTP and in traces of extremely faint spots of the other compounds. Total activity of the root extracts was: intact roots $2.0 \times 10^7$, detached roots $0.77 \times 10^7$ c.p.m. Because of their low activity the individual spots of these chromatograms were not counted.

In the 5 — 30 min samples PCHO and the nucleotide diphosphates ADP, UDP and GDP became labelled in addition to the compounds mentioned above. Chromatograms of the root (A) and shoot (B) extracts of the 15 min sample are presented in Fig. 2. The same spots are found labelled in both cases except for Nos 1, 3, 4, 18 and 19, which do not appear in the shoot (Fig. 2, B). The total activities of the extracts and relative activities of some individual compounds are reported in Table 1. The total activity of the shoots is *ca.* 2—3 % of the activity of the roots, but the relative activity of the organic phosphates, compared with the inorganic spot, is much higher in the shoot.

The composition of the extracts of the intact and the detached shoots after 30 min assimilation is illustrated in Fig. 3. Again, both extracts are qualitatively similar but the labelling of all compounds is very much stronger in the detached shoot which was able to absorb radioactive nutrient solution without the "sieving" effect of the roots. As the only unknowns in both of

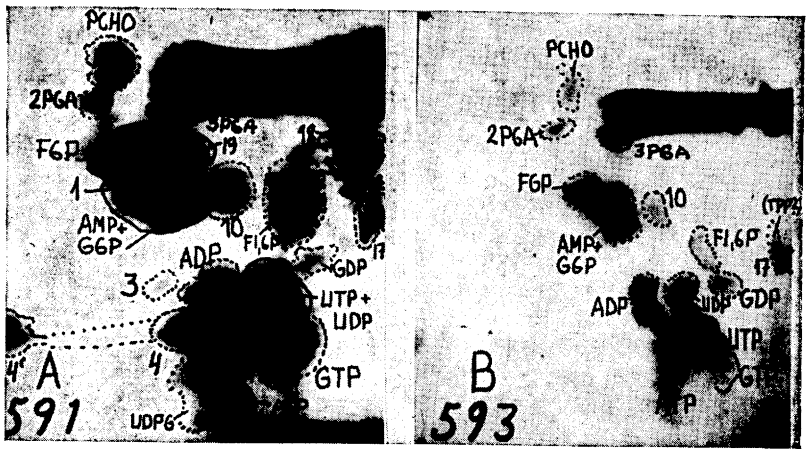


*Fig. 2.* Radioautograms of two-dimensional paper chromatograms of soluble phosphates present in the root (A) and the shoot (B) of intact pea plants after 15 min uptake of radioactive orthophosphate.

these extracts, spots Nos 10, 17 and 18 show labelling, of which the two latter ones well match with inorganic pyrophosphate ($P_2O_7$) and thiamine pyrophosphate (TPP). The identity of these two spots is not yet fully established, however.

## DISCUSSION

The main purpose of this study was the identification of the acid-soluble phosphates formed during phosphorus assimilation in the pea plant. A number of acid soluble phosphates have been chromatographically identified by earlier workers in the pea and in other higher plants. Rowan [3] identified in pea seed-

*Table 1.* Total acid soluble radioactivity and relative activities of orthophosphate and the main phosphate esters, separated by paperchromatography.

| Sample | Total acid soluble activity c/min in millions | Relative activity, % | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ATP | ADP | AMP + G6P | UTP | UDP | GTP | F1,6P | F6P | 10 | $PO_4$ |
| Root | | | | | | | | | | | |
| 5 min. | 23.0 | 0.40 | 0.11 | 0.16 | 0.15 | 0.05 | 0.07 | 0.26 | 0.05 | 0.06 | 98.6 |
| 15 » | 32.0 | 1.97 | 0.30 | 2.80 | 0.17 | 0.35 | 0.08 | 0.53 | 0.60 | 0.18 | 93.0 |
| 30 » | 27.2 | 0.50 | 0.83 | 1.00 | 0.33 | 0.19 | 0.13 | 0.04 | 0.22 | 0.06 | 96.8 |
| Shoot | | | | | | | | | | | |
| 15 min. | 0.58 | 3.84 | 1.60 | 2.78 | (1.17) | 1.40 | — | 0.87 | 1.83 | 0.44 | 86.1 |
| 30 » | 0.88 | 2.91 | 2.86 | 4.12 | 0.97 | 3.24 | 0.32 | 0.89 | 2.10 | 0.32 | 82.5 |
| 30 min. detached | 4.56 | 1.49 | 1.79 | 4.27 | 0.58 | 1.55 | 0.45 | 0.79 | 1.17 | 0.29 | 87.5 |

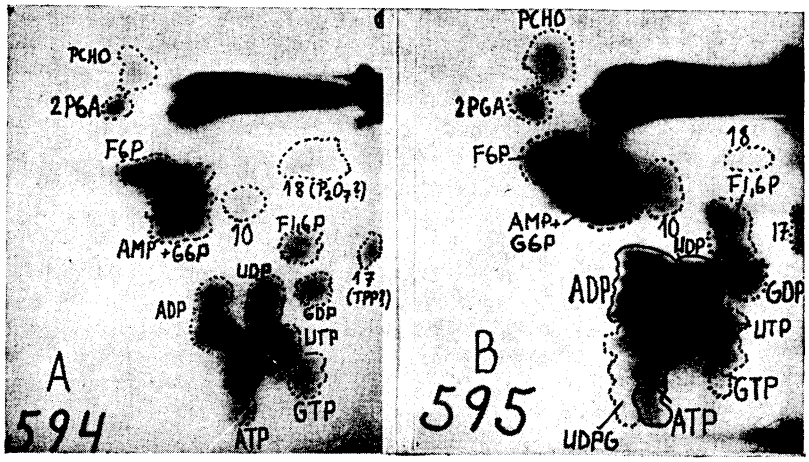


*Fig. 3.* Radioautograms of two-dimensional paper chromatograms of soluble phosphates present in intact (A) and detached (B) pea shoots after 30 min uptake of radioactive orthophosphate.

lings ATP, ADP, UTP, UDP, UMP and a dinucleotide containing both adenosine and guanosine. In another paper [4] he identified G1P, G6P and F1,6P. Loughman and Scott Russell [5] found in barley roots 5 nucleotides, of which they identified ATP and ADP. Of sugar phosphates they mention G6P, F6P and F1,6P. Ginsburg, Stumpf and Hassid [6] isolated UDPG from mung bean seedlings. According to Burma and Mortimer [7] UDPG is synthetized in the leaves of sugar beet from G1P and UTP. Maizel, Benson and Tolbert [8] have found PCHO to be the main organic transport form of phosphate in higher plants. Lindeman [9] identified in *Chlorella* ATP, ADP, UTP, UDPG, AMP, F6P, G6P, F1,6P and 3PGA. We have found these same compounds among several other ones labelled in the pea plant in the present study.

Aronoff [10], when studing phosphate assimilation in the roots of soybean, concluded that the first compound to become labelled was F1,6P. Mitsui, Aso and Ishizuka [11], on the other hand, consider G6P and F6P to be the primary labelled phosphate esters in wheat seedlings. From these the labelling is transferred to nucleotides. Contrary to the above results we found in one of our earlier investigations [1] labelling earliest in nucleotides, primarily in ATP. Nearly simultaneously a weaker labelling appears, however, in three sugar phosphates, G6P, F6P, and F1,6P [12]. ATP is without doubt a primary assimilation product, but any of the other ones mentioned above may also be formed independently of and simultaneously with ATP [12].

All identifications in the present study are based on several tests by cochromatography. The nucleotide nature of spots so identified was further confirmed in an unpublished experiment in which the acid soluble phosphates were fractionated by means of Norite active charcoal. All spots identified as nucleotides and the unknowns 3 and 4 were adsorbed by the charcoal (and re-

eluted by pyridine), whereas the spots identified as sugar phosphates passed through the column unabsorbed.

In another unpublished experiment we compared acid soluble phosphates of pea, rye, oat and barley plants after 10 min's orthophosphate assimilation. The same and only the same labelled compounds were found in all these plants. However, comparison of pea and yeast [2] shows slight differences, *i.e.* first, the yeast does not show labelling in 2-PGA and second, two compounds in yeast, which have been preliminarily identified as phosphoserine and phosphothreonine, do not show labelling in the roots of higher plants.

In yeast extracts one of the strongest labelled phosphates is the unknown No. 3, which is absorbed by Norite like a nucleotide and is located on the two-dimensional chromatograms in the region of nucleotide triphosphates. It becoms faintly labelled after about 1 min's assimilation, but after 10—30 min it becomes one of the strongest spots. We are presently working on the identification of this compound.

An interesting difference between the root and shoot of the pea plant is, that the root extracts contain several unidentified phosphate esters which are not found in the shoot.

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