THE FORMATION OF POLYPHOSPHATE IN *BANKSIA ORNATA*, AN AUSTRALIAN HEATH PLANT

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Summary

The Australian heath plant, *B. ornata* (Proteaceae), has been grown in water culture at a low phosphate level (0·001 p.p.m. phosphorus). Such plants, 22 weeks or more in age, have been used for 24-hr uptake experiments with ^{32}P-labelled orthophosphate. Characteristics of ^{32}P distribution are a low translocation from roots to tops, and the formation of a large fraction of trichloroacetic acid-insoluble phosphorus in the roots.

Components of this fraction are acid-labile, give a positive metachromatic activity with toluidine blue, show no mobility on paper chromatograms, and dialyse slowly. This evidence indicates the formation of long-chain polyphosphates. It is suggested that this phenomenon may be of ecological importance in a low-phosphorus environment.

I. INTRODUCTION

The papers of Specht (1957*a*, 1957*b*, 1963), Specht and Rayson (1957*a*, 1957*b*), Rayson (1957), and Specht, Rayson, and Jackman (1958) have given a clear picture of the environmental factors operating within a heath community in south-eastern Australia. The proteaceous shrub, *Banksia ornata* F. Muell., emerges from this study as an important and singular plant in its exploitation of an environment low in soil phosphorus.

Wet-ash analysis reveals a low level of phosphorus in all its organs, and, compared with other common species in the community, it exhibits least response, on a dry weight basis, to added superphosphate. On regenerating from seed after fire, *B. ornata* may become the major component of this heath at its climax, i.e. after 10–15 years.

Groves (1964), investigating the growth of *B. ornata* in water culture, discovered an apparently seasonal rhythm in the form of phosphorus within the plant. From December to March, the period of maximum growth, much of the phosphorus may be extracted with cold trichloroacetic acid (TCA). For the remainder of the year, TCA-insoluble phosphorus predominates.

The present investigation attempts to further this work at a more physiological level. Plants grown at a low level of added phosphorus have been placed in ^{32}P-labelled orthophosphate for short periods, with subsequent estimation of phosphorus fractions.

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II. General Methods and Materials

(a) *Culture Methods*

B. ornata seedlings, grown from seed collected from heath growing at Keith, S. Aust., were planted in Hoagland solution diluted 1 in 10, with phosphorus added at a concentration of 0·001 p.p.m. (Groves 1964). Polythene containers, 6 in. deep and holding 10 litres of solution, were employed. Twenty-four *B. ornata* seedlings, inserted through holes in four separate drilled and waxed Masonite covers, were grown in one container. The containers were surrounded with opaque paper, and the solutions aerated at regular daily intervals. The plants were grown in a greenhouse.

In the first experiments, sets of six plants 20 weeks old were used, secured by the original covers. When it became necessary, transplants were grown on under the conditions described, with either eight plants in 10 litres of solution, or two plants in cylindrical polythene containers of 2 litres capacity. Isotope experiments were started with plants 22 weeks old and subsequent experiments were performed with plants of the same sowing. All plants had a strong growth of proteoid roots (Purnell 1960).

(b) *Conditions of Isotope Uptake*

Polythene containers of 2 litres capacity were used in all uptake experiments. To a Hoagland nutrient solution, diluted 1 in 10 with sodium orthophosphate at the required concentration, 5μc/l ^{32}P, as sodium orthophosphate, was added. Continuous aeration was provided. The plants were illuminated at 27°C for 16 hr or for the whole 24-hr uptake period by a bank of white fluorescent tubes providing *c.* 1000 f.c. at the level of the leaves.

Uptake was terminated by transferring the plants to containers of water. Washing in tap water (2 × 20 min) and distilled water (2 × 10 min) preceded analysis. Tangled roots were then carefully separated and the tops severed just below the cotyledons. Four to six replicates were employed in most experiments.

III. Distribution of Phosphorus in Whole Plants

The plants were grown in the culture solution to which 0·001 p.p.m. phosphorus was added once. They were then placed in a culture solution containing either 10·0 or 1·0 p.p.m. ^{32}P-labelled orthophosphate. Root and top material was dried at 80°C for 72 hr, weighed, and wet-ashed by the "tri-acid" method of Piper (1942). Estimates of radioactivity were by liquid counting of the digests, with corrections for background and decay.

Analysis of the total endogenous phosphate was by the method of Strickland, Thompson, and Webster (1956), in which the acid phosphomolybdate complex was partitioned with an isobutanol–petroleum ether mixture.

A second group of experiments was performed to determine the time course of phosphorus uptake and any redistribution after uptake. The ^{32}P-labelled phosphate in the roots was determined in both the TCA-soluble and the TCA-insoluble fraction.

In one experiment, uptake of phosphorus was determined after 8, 24, and 48 hr in 1·0 p.p.m. [^{32}P]orthophosphate. In a second experiment plants, having taken up

phosphorus from 1·0 p.p.m. [^{32}P]orthophosphate, were transferred to a nutrient unlabelled solution at the original low level (0·001 p.p.m.) of phosphorus. Analysis of tops for total phosphorus and of roots for TCA-soluble and TCA-insoluble phosphorus were carried out at 0, 4, 24, 50, and 168 hr after transfer.

After rinsing as previously described, roots were blotted with a number of changes of filter paper until surface moisture ceased to appear. Roots and tops were then weighed in aluminium foil envelopes and then frozen solid by placing the envelopes between slabs of dry ice for 10–15 min, and ground in a chilled porcelain mortar. The powdered material was allowed to thaw, and then extracted in 5% TCA at 4°C for 30 min. The supernatant was removed as the TCA-soluble fraction, and the residue digested by the "tri-acid" method of Piper (1942). The activity of both fractions was later determined.

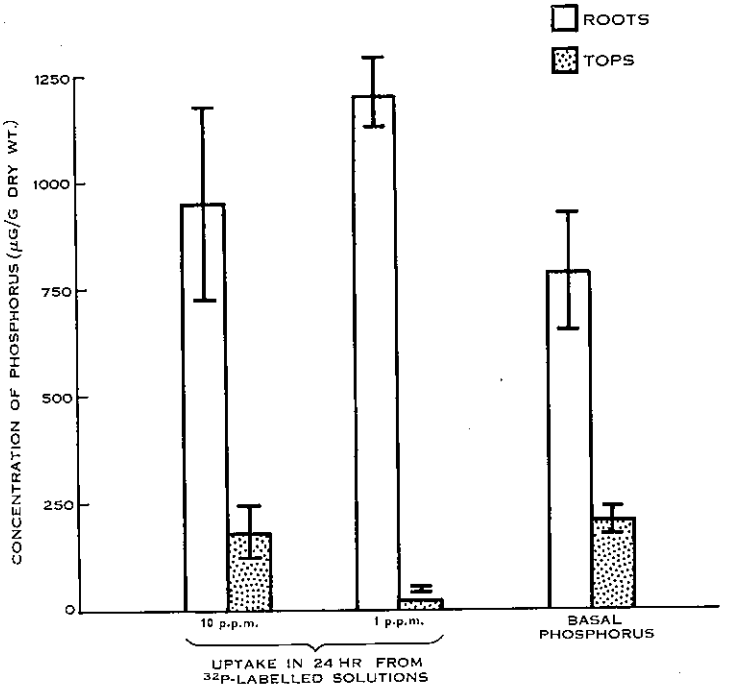

Fig. 1.—Distribution of newly absorbed phosphorus between root and top, and values for total phosphorus concentration before uptake (i.e. basal phosphorus). Twice the standard deviation of the mean is indicated for each value. Age of plants 22 weeks.

The low translocation from root to top is demonstrated in Figure 1. The transport indices (Russell and Martin 1953) of absorption from 1·0 p.p.m. and 10·0 p.p.m. orthophosphate solutions are 7% and 22% respectively. Comparable results for young barley (Russell and Martin 1953) would be about 70% for both concentrations. However, transport indices of 50% and 47% were obtained by Specht (unpublished data) in similar experiments with *B. ornata* seedlings 3 weeks old. The differences between these results may reflect a differing nutrient status of the

plants used or possibly storage in the cotyledons. A seasonal variation would explain the differences in transport index apparent in Figures 1 and 2. After 48 hr in a 1·0 p.p.m. orthophosphate solution, phosphorus taken up is substantially the same as at 24 hr. The experiments on the time course of uptake and redistribution, presented in a composite form in Figure 2, indicate a rapid uptake by roots, and a subsequent slow entry into the leaves.

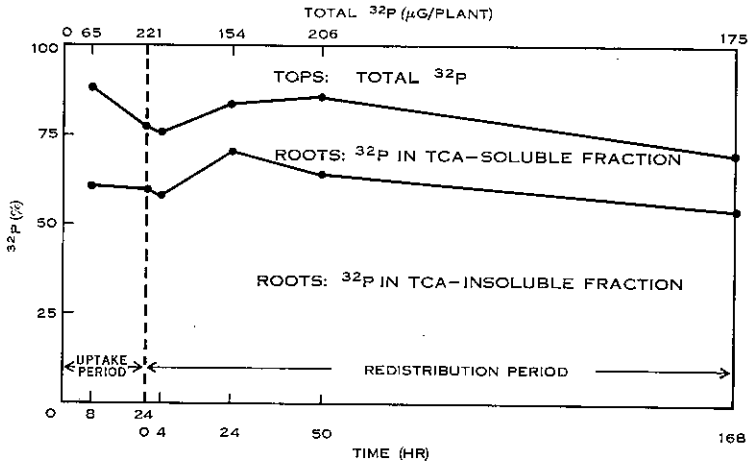

Fig. 2.—Composite graph showing time course for uptake over 24 hr from a 1·0 p.p.m. orthophosphate solution, and redistribution of ^{32}P in *B. ornata* seedlings. Values are also shown for the total ^{32}P-labelled phosphorus of the plants analysed. Plants 30 weeks old. Mean of two replicates plotted.

The occurrence of a TCA-insoluble fraction in the roots, accounting for 60% of the phosphorus absorbed, was of great interest, its relative size remaining virtually unchanged over a period of 7 days. The chemical nature of this phosphorus fraction is now considered in more detail.

IV. Fractionation of Phosphorus-containing Compounds Insoluble in Trichloroacetic Acid

A large TCA-insoluble phosphorus-containing fraction is an uncommon feature of higher plant extracts. As a minor component, this fraction could be regarded as containing nucleoprotein or phospholipid, but the size of the fraction in *B. ornata* indicated that polyphosphate was present. The chemistry of this type of phosphorus compound is discussed by Van Wazer (1958), and Kuhl (1960, 1962) has reviewed the role of polyphosphate in biological systems.

Polyphosphate is readily made acid-insoluble by complex formation with precipitating proteins (Katchman and Van Wazer 1954), and is also known to be extractable in association with nucleic acid (Correll and Tolbert 1962; Ebel *et al.* 1963). Accordingly, extraction methods devised for the extraction of polyphosphates were applied to *B. ornata* roots containing ^{32}P-labelled compounds. The method of Miyachi and Tamiya (1961) as applied to *Chlorella* and later to spinach leaves (Miyachi

1961) was tried with limited success. Another method, based on that of Langen and Liss (1958) and designed for use with yeast, was also investigated and eventually adopted with modifications.

(*a*) *Method of Extraction*

It was convenient to take the roots of four *B. ornata* plants, 40–60 weeks old, and divide them into six batches, each of 3–5 g fresh weight. Previously these plants had taken up ^{32}P-labelled orthophosphate from a 1·0 p.p.m. solution as described in Section II (*b*). The roots were frozen and ground, then extracted successively in 20-ml portions of the following solvents:

(1) 8% TCA at 4°C for 90 min.

(2) 95% ethanol at 25°C for 30 min.

(3) Ethanol–sulphuric ether (3 : 1 v/v) at 60°C, twice, for 30 and 15 min, respectively.

(4) Dilute (*c.* 0·001N) NaOH, pH 9–10, at 4°C for 40 min.

(5) 1·0N NaOH at 4°C for 90 min.

(6) 0·1N NaOH at room temperature (25°C) overnight.

The TCA extract was neutralized by adding 2·5N NaOH solution to reduce hydrolysis of any low molecular weight polyphosphates. The final residue was wet-ashed (Piper 1942).

(*b*) *Evaluation of Extracts*

The extracts were made up to volume, and their activity estimated by liquid counting of a portion, extracts (2) and (3) being combined. Results from a typical fractionation experiment are presented in Figure 3. These show the relative proportions of newly absorbed phosphorus extracted by each solvent from *B. ornata* roots. Results are expressed as micrograms of phosphorus per gram dry weight of root. The total labelled phosphorus in the roots amounted to between 114 and 140 μg/g dry weight, and the total for the tops (not fractionated) 46 μg/g dry weight. The acid lability, metachromatic activity, and dialysis rate of these extracts were then investigated, and attempts made to fractionate them further by descending chromatography on Whatman No. 1 paper.

(i) *Acid Lability*—Polyphosphates are characteristically acid labile (Van Wazer 1958). The proportion of acid-labile phosphate in each extract was determined by heating a neutralized sample with an equal volume of 2N HCl for 10 min at 100°C. Orthophosphate thus released was determined by a modification of the method of Strickland, Thompson, and Webster (1956), by estimating the radioactivity of the acidic phosphomolybdate complex which was dissolved in isobutanol. Orthophosphate originally in the extract was similarly estimated. The separation of the fractions into components is derived from these hydrolysis results (Fig. 3). The TCA-soluble fraction was not hydrolysed, but the orthophosphate was determined. The ethanol–ether fraction contained a considerable acid-labile component (which also gave a positive metachromatic reaction), the magnitude of which varied considerably [see

Section IV (*b*) (ii)] and because of these factors, extraction with organic solvents was omitted in subsequent experiments. The components of this fraction were then distributed throughout the other three fractions and the residue. The acid-labile, TCA-insoluble phosphorus is taken to be an approximate measure of the polyphosphate component, and comprises between 30 and 35% of the total root phosphorus. The indeterminate phosphorus in the three alkaline extractions is the difference between the total and acid-labile phosphorus plus orthophosphate.

(ii) *Metachromatic Activity*—Polyphosphates, in common with other polyanions, exhibit metachromatic activity with certain cationic dyes; e.g. in the presence of toluidine blue, a shift in absorption at 630 mμ to one at 530 mμ occurs, usually visible as a change in colour from blue to pink (Wiame 1947; Damle and Krishnan 1954).

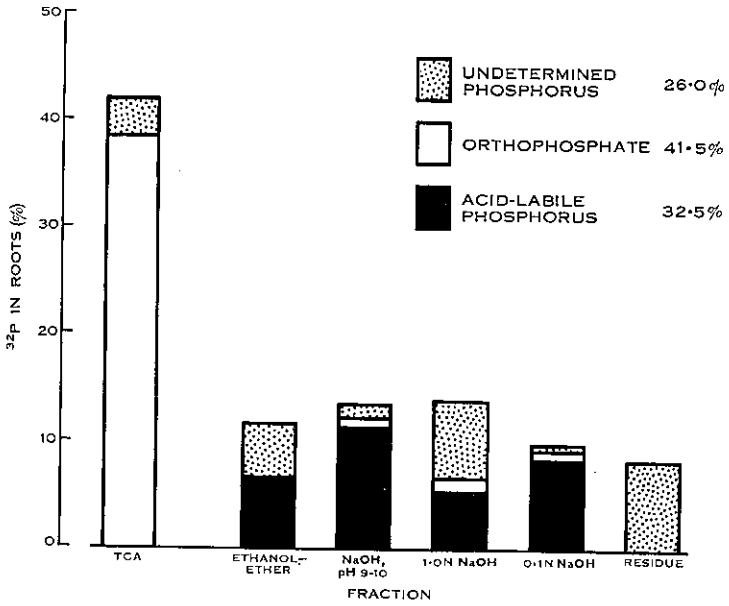

Fig. 3.—Distribution of ^{32}P in various fractions of *B. ornata* roots. Phosphorus taken up from 1·0 p.p.m. solution in 24 hr. Data from a typical experiment. Age of plants: *c.* 60 weeks.

The magnitude of this change in absorption, expressed as the ratio $E_{530\,m\mu}/E_{630\,m\mu}$, is a function of both polymer length and concentration. The method used was to purify equal volumes of extracts (1), (4), (5), and (6), by precipitating with saturated barium acetate (buffered to pH 4) and allowing to stand at 4°C overnight. The precipitate, resuspended in distilled water, was shaken gently with Amberlite-120 (H^+ form) resin for 4 hr at 25°C. The barium-free solution was made to 10 ml with distilled water, and 1 ml of this solution was mixed with 1 ml 10^{-1}M aqueous toluidine blue (Gurr Toluidine Blue Vital), and the volume made up to 5 ml with distilled water. The extinctions at 530 mμ and 630 mμ were read within 20 min. These results are presented in Table 1. The strong metachromatic activity of the 0·1N NaOH extract

indicates that it contains the highest polymer, and the dilute NaOH the next highest. This is in accordance with the findings of Langen and Liss (1958).

TABLE 1

COMPARISON OF ABSORPTIONS AT TWO WAVELENGTHS BY VARIOUS B. ORNATA ROOT FRACTIONS IN PRESENCE OF TOLUIDINE BLUE

Fraction Extracted with:	$E_{530\ m\mu}$	$E_{630\ m\mu}$	$E_{530\ m\mu}/E_{630\ m\mu}$
Distilled water	0·067	0·296	0·231
Trichloroacetic acid	0·090	0·223	0·403
NaOH, pH 9–10	0·132	0·234	0·564
1N NaOH	0·116	0·245	0·473
0·1N NaOH	0·101	0·150	0·673

(iii) *Dialysis*—To obtain a further estimate of the order of molecular weight, the time of escape of extracts from Visking 8/32 dialysis tubing was investigated. Portions (1 ml) of extracts (4) and (5) [see Section IV (*a*)] purified by barium precipitation both gave 63% recovery (three replicates) after 24 hr dialysis against 8 litres of distilled water at 4°C. Extract (6) was dialysed for 30 hr in a similar, but stirred, system. Results of this dialysis, expressed as percentage recovery, are presented in Figure 4. The final slope denotes a half-time of escape of approximately 80 hr, indicating a high molecular weight component.

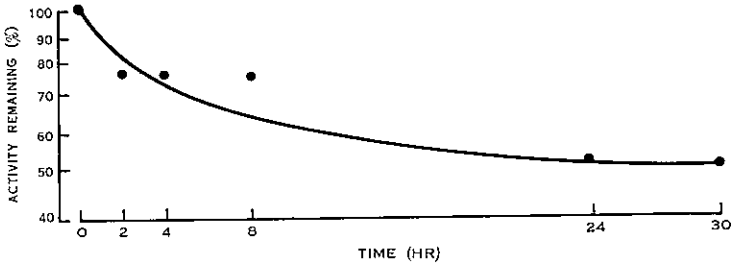

Fig. 4.—Loss of activity with time on dialysing 1·0N NaOH-soluble extract against distilled water.

(iv) *Chromatography*.—Aqueous solutions of barium-precipitated extracts were applied to acid-washed Whatman No. 1 filter paper and chromatographed in a descending system for 20 hr at 25°C. The acidic solvent of Ebel and Volmar (1951) was used and the positions of any spots determined by scanning with an end-window Geiger tube. All TCA-insoluble extracts failed to leave the origin, whereas the TCA-soluble extract gave a single spot corresponding exactly with orthophosphate. Sodium orthophosphate, pyrophosphate, tripolyphosphate, and hexametaphosphate, which were run as markers, gave R_F values slightly exceeding those quoted by Ebel and Volmar (1951). This indicates that the shortest chains are longer than 11 $-PO_3$ units (or the molecular weight is more than 1000) as it is theoretically possible to separate

by this method phosphate polymers having a chain length of up to 11 $-PO_3$ units with this solvent (Grunze and Thilo 1953).

(c) *Conclusion*

These results indicate that the TCA-insoluble fraction of newly absorbed phosphorus in *B. ornata* roots contains a considerable proportion of polyphosphate, with a component having a molecular weight possibly of the order of several thousand.

Microscopic examination of fresh frozen sections of roots and of the external root surface was carried out in conjunction with one uptake experiment. No evidence was found of microbial contamination that could account for the level of polyphosphate synthesis demonstrated. It is considered that this is a product of *B. ornata* root metabolism under these experimental conditions.

V. Discussion

This investigation is being undertaken in an attempt to explain the capacity of *B. ornata* to survive and successfully compete under low nutrient conditions. The preliminary work affords a link with the field work of Specht and co-workers and the experiments of Groves (1964).

The general hypothetical picture of phosphate relations in *B. ornata* is of an ability to tolerate a low internal turnover of phosphorus. Also, any transient rise in available soil phosphorus may be utilized by an "opportunist" uptake and stored as polyphosphate. Internal availability of phosphorus on demand in the growing season may be indicated by Groves' (1964) work in showing seasonal changes in acid-soluble phosphorus within the plant.

On the community scale, an analogous situation has been described by Lund (1950) and Mackereth (1953) who experimented with the alga *Asterionella formosa* from the English Lakes, another environment with low phosphorus levels. This organism can accumulate phosphorus when growth is stationary, for example during winter, when temperature and light may be limiting. During the phase of active growth, cell phosphorus diminishes with successive cell divisions as the accumulated element is distributed through the numerically expanding community. The chemical nature of this reserve has not been investigated for *Asterionella*.

There are two records of the occurrence of polyphosphates in higher plants, e.g. Hardin (1892) and Miyachi (1961), who extracted their products from cotton seed meal and spinach leaves, respectively. Thus the distribution of polyphosphates within the higher plants may be of interest. At the present stage, two hypotheses suggest themselves. Firstly, polyphosphates may be present in all higher plants, in quantities varying from minute amounts in well-manured agricultural crops to massive amounts in phosphate-deficient plants suddenly presented with an adequate supply. Detection of small quantities, especially if unexpected, would be unlikely, since acid reagents and even moderate temperatures would lead to their hydrolysis to orthophosphate. A second hypothesis is that polyphosphate synthesis is a feature common to plants adapted to long-term survival in a phosphate-deficient medium. This is suggested by the present work, and by the ability of microorganisms to synthesize polyphosphate.

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