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# ACID SOLUBLE AND ACID INSOLUBLE INORGANIC POLYPHOSPHATES IN *CUSCUTA REFLEXA*

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**Abstract**—Inorganic polyphosphates have been isolated in both the "soluble" and "insoluble" form from the fresh stem tissue of *Cuscuta reflexa* (Dodder). Together they constitute about 2 per cent of the total phosphate. The presence of phytin calls for special precautions in the identification and estimation of soluble polyphosphates in the cold trichloroacetic acid extracts.

## INTRODUCTION

INORGANIC polyphosphates find wide distribution in the lower organisms.[1] It has been the general opinion till recently that polyphosphates are not present in the higher plants.[2–4] In 1961 Miyachi[5] reported the isolation of two types of acid-insoluble polyphosphates from spinach leaves (*Spinacia oleracea* Linn.), one, extractable in very dilute potassium hydroxide pH 8–10) in the cold and, the other, in greater proportion, extracted by 2 N potassium hydroxide at 37° during a 20-hr period. The author did not examine the acid-soluble fraction for polyphosphates.

In the light of the increasing metabolic importance of polyphosphates, their possible presence in higher plants other than spinach calls for investigation. It is also of interest to show whether acid-soluble forms of polyphosphate are present along with the acid-insoluble forms. A previous report from this laboratory[6] indicated the presence of non-nucleic, acid-labile phosphate in the RNA fraction of Dodder (*Cuscuta reflexa*) obtained by extraction of the acid-insoluble lipid-free residue with 1 N sodium hydroxide. We are now reporting the isolation of both acid-soluble and acid-insoluble polyphosphates from the stem tissue of the parasite.

## RESULTS

### 1. *Analysis by Labile Phosphate Content*

(*a*) *Acid-soluble polyphosphate fractions.* Analytical values obtained for labile and stable phosphorus are reported in Table 1.

About 8 per cent of the phosphorus of the TCA extract passed into the "pH 2·5 fraction" and about 4 per cent into the "pH 4·5 fraction", together constituting about 8 per cent of the phosphorus of *Cuscuta* tissue. Orthophosphate was present in both the fractions, but in small

[1] A. KUHL, *Ergeb. Biol.* **23**, 144 (1960).
[2] K. KECK and H. STICH, *Ann. Botany* **21**, 611 (1957).
[3] A. N. BELOZERSKII, in *Nucleoproteins*, Ed. R. STOOPS, p. 199, Interscience Publishers, New York (1959).
[4] O. HOFFMANN-OSTENHOF, in *Acides Ribonucleiques et Polyphosphates*, p. 641, Editions du Centre National De La Recherche Scientifique, Paris (1962).
[5] S. MIYACHI, *J. Biochem.* **50**, 367 (1961).
[6] M. SINGH, K. K. TEWARI and P. S. KRISHNAN, *Indian J. Exptl. Biol.* **1**, 207 (1963).

proportion. The acid-labile phosphorus constituted only 16 per cent of the total phosphorus in the "pH 2·5 fraction" and 10 per cent in the "pH 4·5 fraction".

TABLE 1. ANALYSIS OF THE ACID-SOLUBLE FRACTIONS FOR LABILE AND STABLE PHOSPHATE

| Fraction | μg P/g dry *Cuscuta* tissue | As % of the total P: in the particular fraction | in the TCA extract | in *Cuscuta* tissue† |
|---|---|---|---|---|
| *"pH 2·5 fraction"* | | | | |
| $P_o$ | 8·5 | 6·5 | 0·5 | 0·36 |
| $P_{\Delta 10}$ | 20·7 | 15·8 | 1·2 | 0·88 |
| $P_{stable}$ | 102·0 | 77·7 | 6·0 | 4·36 |
| $P_{total}$ | 131·2 | (100) | 7·7 | 5·61 |
| *"pH 4·5 fraction"* | | | | |
| $P_o$ | 3·0 | 4·5 | 0·18 | 0·13 |
| $P_{\Delta 10}$ | 6·7 | 10·1 | 0·39 | 0·29 |
| $P_{stable}$ | 56·9 | 85·4 | 3·33 | 2·43 |
| $P_{total}$ | 66·6 | (100) | 3·90 | 2·85 |

P = Phosphorus, $P_o$ = Orthophosphorus, $P_{\Delta 10}$ = Orthophosphorus liberated in 10 min with 1 N sulphuric acid at 100°, $P_{stable} = P_{total} - (P_{\Delta 10} + P_o)$.

* Expressed as percentage of the total phosphorus in the "pH 2·5 fraction", or the "pH 4·5 fraction", as the case may be.

† Expressed as percentage of the total phosphate in the whole *Cuscuta* tissue, which includes all forms of phosphate in the starting material.

Data reported in Table 2 show that about 70 per cent of the total phytin in *Cuscuta* was precipitated in the "pH 2·5 fraction" and "pH 4·5 fraction", the greater part passing into the former fraction. As high a proportion as 60–70 per cent of the stable phosphorus of the two fractions was made up of phytin.

TABLE 2. ANALYSIS OF THE ACID-SOLUBLE FRACTIONS FOR PHYTIN PHOSPHORUS

| Fraction | μg P/g dry *Cuscuta* tissue | Phytin P as: % of stable P in the particular fraction | % of total P in the particular fraction | % of total P in the TCA extract | % of total phytin P of tissue |
|---|---|---|---|---|---|
| "pH 2·5 fraction" | 69·9 | 68·8 | 53·3 | 4·1 | 45·7 |
| "pH 4·5 fraction" | 33·4 | 58·7 | 50·2 | 1·96 | 21·8 |

(*b*) *Acid-insoluble polyphosphate fractions.* Phosphate partition values in the "pH 9·0 fraction" and "2 N potassium hydroxide fraction" are given in Table 3.

Slightly over 1 per cent of the total phosphorus of *Cuscuta* was precipitated as barium salts at pH 2·5 from the alkaline extracts of the tissue residue resulting on extraction with cold trichloroacetic acid, followed by phospholipid removal with organic solvents. In terms of the acid-insoluble tissue residue, the total phosphorus recovered in the polyphosphate fractions

represented about 3 per cent, the "pH 9·0 fraction" containing slightly more phosphorus than the "2 N potassium hydroxide fraction". As much as 98 per cent of the esterified phosphorus in the "2 N potassium hydroxide fraction" was acid labile in 10 min. The "pH 9·0 fraction" was more contaminated, in that only 75 per cent of the esterified phosphate was acid labile in 10 min; when the hydrolysis time was prolonged to 15 min, 90 per cent was liberated in the form of orthophosphate.

The values for phosphorus partition presented in the above table are from a typical experiment. The analyses of the acid-extractable fraction were carried out on six batches of dodder

TABLE 3. PHOSPHATE PARTITION IN "pH 9·0 FRACTION" AND "2 N POTASSIUM HYDROXIDE FRACTION"

| Fraction | μg P/g dry *Cuscuta* tissue | As % of the esterified phosphorus in the fraction* | As % of the total P: in the fraction | in acid insoluble lipid free plant tissue | in dry *Cuscuta* tissue |
|---|---|---|---|---|---|
| *"pH 9·0 fraction"* | | | | | |
| $P_o$ | 4·5 | | 29·6 | 0·48 | 0·19 |
| $P_{\Delta 10}$ | 8·0 | 74·8 | 52·6 | 0·85 | 0·34 |
| $P_{\Delta 15}$ | 9·6 | 89·7 | 63·2 | 1·02 | 0·41 |
| $P_{total}$ | 15·2 | | (100) | 1·62 | 0·65 |
| *"2 N KOH fraction"* | | | | | |
| $P_o$ | 2·4 | | 20·5 | 0·26 | 0·10 |
| $P_{\Delta 10}$ | 9·1 | 97·9 | 77·8 | 0·97 | 0·39 |
| $P_{total}$ | 11·7 | | (100) | 1·25 | 0·50 |

P = Phosphorus, $P_o$ = Orthophosphorus, $P_{\Delta 10}$ = Orthophosphorus liberated in 10 min with 1 N sulphuric acid at 100°, $P_{\Delta 15}$ = Orthophosphorus liberated in 15 min with 1 N sulphuric acid at 100°.

* Esterified phosphate is the difference between the total phosphate and the orthophosphate.

stem and those of the acid-insoluble fraction on three samples. The order of variation observed was less than 10 per cent in the different experiments.

## 2. *Analysis by Paper Chromatography*

(*a*) *Acid-soluble polyphosphate fractions.* The "pH 2·5 fraction" and "pH 4·5 fraction" were first concentrated by freeze drying and dissolved in a small volume of water. Using Ebel's[7] solvent, orthophosphate migrated, leaving the major portion of the phosphate on the base line. On elution of the latter spot and wet digestion for total phosphate, the amount was found to be almost equivalent to the sum of the acid labile phosphate and phytin phosphate in the sample applied on paper. Methanol–formic acid–water system, however, permitted a satisfactory separation on ascending paper chromatograms run for 8–10 hr at room temperature; phytate migrated ($R_f$ value 0·7) leaving a phosphorus fraction on the base line, which corresponded to 96 and 95 per cent respectively, of the 10-min labile phosphate of the "pH 2·5 fraction" and "pH 4·5 fraction". When the chromatogram was developed after

[7] J. P. EBEL, *Bull. Soc. Chim.* **20**, 991 (1953).

hydrolysing either fraction for 10 min with 1 N acid and subsequent neutralization with ammonia, no spot appeared on the starting line, but the orthophosphorus spot was intensified.

In other experiments the developed paper chromatograms were dried at 50° and sprayed with 0·1 per cent solution of toluidine blue in 0·04 N acetic acid. The material left on the starting line in the chromatograms of the "pH 2·5 fraction" and "pH 4·5 fraction" and the spot due to standard polyphosphate turned characteristically purple, and were acid fast, persisting on washing the paper with 2·0 per cent (v/v) acetic acid. The spot corresponding to phytate gave a light blue colour, which disappeared on acid washing.

(*b*) *Acid insoluble polyphosphate fractions.* Paper chromatography of the "pH 9·0 fraction" and "2 N potassium hydroxide fraction" using the solvent system of Ebel[7] showed that the major part of the material remained on the base line and was stained an acid-fast purple with 0·1 per cent solution of toluidine blue in 0·04 N acetic acid. Prior acid hydrolysis of the fractions resulted in the disappearance of the spots and considerable intensification of the spots due to orthophosphate.

3. *Analysis by Metachromasy*

The "pH 2·5 fraction", "pH 4·5 fraction", "pH 9·0 fraction" and the "2 N potassium hydroxide fraction", as well as the polyphosphate fractions isolated from the "pH 2·5 fraction" and "pH 4·5 fraction" after chromatographic separation on paper, responded metachromatically on mixing with toluidine blue solution (6 $\mu$g/ml in 0·04 N acetic acid), giving rise to a purple tinge characteristic of the reaction of synthetic polyphosphates with the dye. When the solutions were examined in a spectrophotometer, the absorption at 630 m$\mu$ was found to have decreased and that at 530 m$\mu$ to have increased.

## DISCUSSION

The experiments reported above unequivocally demonstrate the occurrence of polyphosphates in the stem tissue of *Cuscuta reflexa*. The identification was based on isolation as barium salts at low pH values, acid-lability, paper chromatography and metachromatic response with toluidine blue. If extractability in cold TCA be a rigorous criterion of classification, both acid-soluble and acid-insoluble polyphosphates are present. Our findings confirm Miyachi's[5] observation and extend it by demonstrating that the acid-soluble forms are present together with insoluble forms.

Assuming that polyphosphates are completely hydrolysed in 10 min with 1 N acid at the temperature of boiling water, the various fractions of polyphosphate isolated from *Cuscuta* together constitute about 2 per cent of the total phosphorus of tissue, the acid-soluble forms constituting a slightly higher proportion than the acid-insoluble forms. That the values for labile phosphate in the fractions constitute a reasonably correct estimate of polyphosphate follows from their close correspondence to the phosphate content of the base-line spots on chromatograms. Considering the manipulative details involved in the isolation of the four polyphosphate fractions, it is reasonable to assume that the actual content in the plant tissue may be considerably higher. The yield reported by us for total polyphosphate falls short of the labile phosphate in the *insoluble* polyphosphate fractions from spinach leaves.[5] Miyachi's[5] determinations were, however, carried out on crude alkaline extracts; it is likely that non-polyphosphate material might have contributed substantially to the labile phosphate. We find that only 6–7 per cent of the total phosphate of the crude alkaline extracts of *Cuscuta* passes into the purified fractions obtained through the barium salts. According to Winder and

Denneny[8] and Zaitseva, Belozerskii and Novozhilova,[9] as high a proportion as 23–25 per cent of RNA phosphate is split as orthophosphate in 7 min by N acid at 100°, subsequent to degradation by alkali.

In view of the heavy contamination with phytin, mere analysis of the total phosphorus of the barium salts formed at low pH values also does not yield reliable data on the acid-soluble polyphosphate content of tissue. The claim of Klein[10] to have found soluble metaphosphate in tomato is, therefore, not well-founded. The use of chromatography with Ebel's solvent system and estimation of the total phosphate of the material eluted from the start line may also lead to erroneous conclusion as to the occurrence and quantity of acid-soluble polyphosphate, since phytin has very low $R_f$ values in this system.

According to Hoffmann-Ostenhof,[4] polyphosphate and phytate are mutually exclusive. The present data disprove this hypothesis, since both are present in *Cuscuta*. The exact significance of the occurrence of polyphosphates in *Cuscuta reflexa* is uncertain. It is not known whether these compounds are elaborated *de novo* by the parasite, or are derived from the tissues of the host plants through the haustoria. The recent demonstration by Lynn and Brown[11] that (insoluble) polyphosphate is formed during the oxidation of substrates by liver mitochondria is indicative of a more central role for polyphosphates than hitherto recognized.

## MATERIALS AND METHODS

### *Sources of Plant Material*

The tender stem of *Cuscuta reflexa* Roxb. was harvested from hedges of *Pithecolobium dulce* Benth. growing on the University campus.

### *Extraction and Isolation of Acid-Soluble Polyphosphates*

Fresh *Cuscuta* stem (75 g) was extracted exhaustively employing 10 ml of 20% followed by 30 ml of 10% cold trichloroacetic acid for the first extraction, and 150 ml of 5% cold trichloroacetic acid in three subsequent extractions. The trichloroacetic acid was removed by shaking with ether. The resulting aqueous extract had a pH of about 3. Small samples of the extract were used for the estimation of orthophosphorus ($P_o$), 10 min acid labile phosphorus ($P\Delta_{10}$), 10 min acid stable phosphorus ($P_{stable}$), total phosphorus ($P_{total}$) and phytin phosphorus. The bulk of the extract (210 ml) was adjusted to pH 2·5 with acetic acid and mixed with 3–4 ml saturated solution (4·6 N) of barium acetate previously adjusted to pH 2·5 with glacial acetic acid. After storage overnight at about 2°, the precipitate was collected by centrifugation in the cold and washed once with 1·0% solution of barium acetate. The supernatant was raised to pH 4·5 with NaOH, whereupon additional material separated and after adding 1 ml (4·6 N) barium acetate at pH 4·5 the suspension was left for 10 hr in the cold. The precipitate was collected as above. The two precipitates were separately treated with Amberlite IR–120 ($Na^+$ form) to replace barium with sodium by contact exchange, giving rise to the "pH 2·5 fraction" and "pH 4·5 fraction".

### *Extraction and Isolation of Acid-insoluble Polyphosphates*

The extraction procedure was based on that of Miyachi[5] and the compounds isolated as their barium salts.

[8] F. G. Winder and J. M. Denneny, *J. Gen. Microbiol.* 15, 1 (1956).
[9] G. N. Zaitseva, A. N. Belozerskii and L. P. Novozhilova, *Biokhimiya* 24, 971 (1959) (in English translation by Consultants Bureau Enterprises, Inc.).
[10] R. M. Klein, *Plant Physiol.* 27, 335 (1952) New York.
[11] W. S. Lynn and R. H. Brown, *Biochem. Biophys. Res. Comm.* 11, 367 (1963).

The residue left after extraction of acid-soluble material was washed free of TCA with water, dried at 60–70° and powdered. Phospholipids were removed by the method of Ergle and Guinn,[12] using petroleum ether (b.p. 40–60°), followed by ethanol-benzene azeotrope (32·4:67·6% by wt., b.p. 68·2°) in a Soxhlet extractor.

The acid-insoluble, lipid-free material was extracted at pH 9·0 in the cold, by suspending in water and adding a few drops of dilute potassium hydroxide (final concentration approximately 0·0004 N). The extract was separated by centrifugation and treated for isolation of polyphosphate as described below. The residue was treated with 2 N potassium hydroxide at 37°. The extract so obtained was freed from DNA and undegraded protein by acidification with 5 N perchloric acid. The resulting clear solution also was treated for the isolation of polyphosphate. The two solutions were brought to pH 2·5, using 1 N hydrochloric acid for the former and 1 N sodium hydroxide for the latter. They were now mixed with a saturated solution (4·6 N) of barium acetate (adjusted to pH 2·5 with acetic acid) and the two suspensions were left overnight in the cold. The precipitates were collected by centrifugation in the cold. (The supernatant solutions were raised to pH 4·5 with 1 N sodium hydroxide, but no further precipitate appeared even on adding more barium acetate.) The precipitates were separately treated with Amberlite IR–120 ($Na^+$ form) to replace barium with sodium by contact exchange, yielding the "pH 9·0 fraction" and "2 N potassium hydroxide fraction".

*Estimation of Phytin-Phosphorus*

5 ml trichloroacetic acid extract was adjusted to phenolphthalein red with 1 N sodium hydroxide and made acidic by the addition of 1 ml of 0·1 N HCl. 2 ml acid ferric chloride reagent was added and the suspension heated in a boiling-water bath for 15 min with occasional stirring. After cooling in cold-water bath for 20 min the precipitated ferric phytate was collected by centrifugation and washed thrice with dilute hydrochloric acid (0·6%). The supernatant and washings were discarded. The ferric phytate was suspended in 4 ml of hot distilled water and 2 ml of 1 N sodium hydroxide added. This suspension was heated in a boiling-water bath for 15 min with occasional stirring and centrifuged. The residue (ferric hydroxide) was washed thrice with 5 ml of hot distilled water each time and the washings mixed with the original supernatant to give a solution of sodium phytate. Aliquots of this solution were digested for total phosphorus.

*Colorimetric Estimations*

Orthophosphorus was estimated by the method of Fiske and Subbarow[13] and phytin by the method of Pons, Stansbury and Hoffpauir.[14]

*Paper Chromatography*

The following two solvent systems were used for ascending chromatograms on Whatman No. 1 filter paper:

$$MeOH:50\%\ v/v\ HCOOH:H_2O,\ 16:3:1\ v/v,$$

following Bandurski and Axelrod[15] and Kerr and Kfloury[16] and

$$IsoPrOH:0{\cdot}8\ N\ CCl_3COOH:20\%\ NH_3,\ 70:30:0{\cdot}4\ v/v,$$

of Ebel.[7]

[12] D. R. ERGLE and G. GUINN, *Plant Physiol.* 34, 476 (1959).
[13] C. H. FISKE and Y. SUBBAROW, *J. Biol. Chem.* 66, 375 (1925).
[14] W. A. PONS, M. F. STANSBURY and C. L. HOFFPAUIR, *J. Assoc. Offic. Agr. Chemists* 36, 492 (1953).
[15] R. S. BUNDURSKI and B. AXELROD, *J. Biol. Chem.* 193, 405 (1951) New York.
[16] S. E. KERR and G. A. KFLOURY, *Arch. Biochem. Biophys.* 96, 347 (1962).

Detection of spots was by the method of Runeckles and Krotkov.[17] We have used toluidine blue for the selective staining of polyphosphates on paper chromatograms.[18] In some experiments the areas on the start line were cut out subsequent to development, but without spraying and digested with concentrated sulphuric acid for total phosphorus determination. In some other experiments solutions of "pH 2·5 fraction" and "pH 4·5 fraction" were applied in bands on Whatman No. 1 sheets and the chromatograms developed in methanol–formic acid–water system for 8 hr. The material left on the starting line was eluted with water and lyophilized. The residue was taken up in a small volume of water, the material centrifuged and the supernatant solution analysed.

*Metachromasy*

Polyphosphates are known to react with toluidine blue metachromatically, that is, there is a hypsochromic effect and colour shift in the dye which is evident to the naked human eye. Absorption spectrum studies reveal that the absorption peak of toluidine blue at 630 $m\mu$ decreases and a new peak appears at 530 $m\mu$ on the addition of polyphosphate. Under controlled conditions this reaction has been shown to be specific.[19–21]

Metachromasy was measured according to Damle and Krishnan.[19] To 1-ml portions of toluidine blue solution (30 mg/l) 1 ml of 0·2 N acetic acid was added, followed by solutions of polyphosphate from the different fractions equivalent to 10–30 $\mu$g of labile phosphate. The volume was made up to 5 ml in each with water and measurements of the decrease in absorption at 630 $m\mu$ and increase at 530 $m\mu$ made using Unicam spectrophotometer.

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[20] P. S. Krishnan, S. P. Damle and V. Bajaj, *Arch. Biochem. Biophys.* **67**, 35 (1957).
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