The Fractionation and Enzymic Breakdown of some Phosphorus Compounds in Leaf Tissue

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In a recent paper Pirie (1950) has described the preparation and properties of a nucleoprotein, from the sap of healthy tobacco leaves, which has some similarity to plant viruses. The present paper is concerned with the fractionation of the phosphorus of leaf tissue to find how much of the phosphorus of the leaf is in the form of nucleic acid. The enzymic breakdown of some of the phosphorus-containing compounds has also been studied.

Although there is an extensive literature on the nature and fractionation of phosphorus compounds in animal tissues, plant tissues have received less attention. Until recently, work on plant tissues has been mainly confined to seeds. When leaves have been used the material has usually been dried as a preliminary. This will vitiate some of the results, for there would be changes due to enzyme action during drying, especially at room temperature. Knowles & Watkin (1932) investigated the phosphorus compounds in wheat at different stages of growth. They used air-dry material, which probably accounts for the very high figures for inorganic phosphorus. They claim to have found phytin in vegetative parts of the wheat plant, but other workers have been unable to find any phytin except in seeds. DeTurk, Holbert & Howk (1933) studied the chemical transformations of phosphorus in the growing corn plant and found that phytin disappeared rapidly on germination. They found differences in the phosphorus distribution between fractions when the material was slowly and rapidly dried.

Heard (1945) investigated the forms of phosphorus in a trichloroacetic acid extract of fresh barley seedlings. In a study of the nucleic acid content of green leaves, von Euler & Hahn (1947, 1948) extracted ribonucleic acid with sodium hydroxide as in the Schmidt & Thannhauser (1945) procedure for animal tissues. The nucleic acid was precipitated as the lanthanum salt and the pentose content determined by a phloroglucinol method. Pectic substances in the extract were precipitated with the nucleic acid, and estimation of the nucleic acid content based on the pentose figure gave values which were too high. Williams (1945) described a method for estimating nucleic acid phosphorus in plant material in which the dry fat-free tissue was heated with 12·5% (w/v) sodium chloride in the presence of triacetin. The nucleic acid in the extract was then precipitated at pH 1. In this method no distinction was made between ribo- and deoxyribonucleic acids. The method of Ogur & Rosen (1950) which, with modifications, was used in the present investigation for the differential extraction of ribo- and deoxyribo-nucleic acids was used by them on fresh corn root tips.

The enzymes in plant tissues concerned with the breakdown of phosphorus compounds have also been studied much less frequently than those of animal tissues. The presence of phosphatase activity in leaves is well known. Courtois & Khorsand (1950) have recently found that there are two phosphatases, one with an optimum pH at 5·0–5·2 and the other at about pH 4. Schlamowitz & Garner (1946) recognized a ribonuclease ('ribonucleinase') in sprouted soy beans. Pirie (1950) found that tobacco-leaf sap had ribonuclease activity. Hanahan & Chaikoff (1948) found a phospholipin-splitting enzyme in cabbage leaves which was able to attack only the nitrogenous base-phosphoric acid linkage of lecithin with the formation of choline and phosphatidic acid. Ducet (1949) found this enzyme and also lecithinase *B*, which splits lecithin to glycerophosphorylcholine and fatty acids, in numerous other plants such as potato, bean, pea and ryegrass.

MATERIAL AND METHODS

Leaves from glasshouse-grown tobacco plants (*Nicotiana tabacum* var. White Burley) were used for most of the work. Other species used are mentioned in the text. The midribs were cut out, the laminae minced in a domestic meat mincer and the sap squeezed out by hand through madapollam into a cooled receiver. The residue in the cloth, called 'fibre', was washed three times by suspending in a volume of distilled water about equal to that of the sap and squeezing out. If not used at once the fibre was stored at 4° with $CHCl_3$ added. The sap was centrifuged at 8000 rev./min. (6000*g*) for 15 min. and the deposit obtained called the 'chloroplast fraction'. This was washed twice by suspending in distilled water and recentrifuging at 8000 rev./min., then it was finally suspended in water and stored in the refrigerator.

For some experiments much of the starch was removed from the chloroplast fraction by scraping off the upper green layer from the white starch layer in the deposit obtained on centrifuging. The green material was suspended in water, centrifuged down again and separated from the starch.

This was repeated until there was no obvious starch layer.

Phosphorus. This was determined colorimetrically by a modification of the method of Kuttner & Lichtenstein (1932). Values for inorganic P were obtained by developing the colour in samples without incineration.

Dry matter of fibre and of aqueous fractions. This was determined by drying samples in an oven at 100° overnight. Ethanol-ether fractions were dried over H_2SO_4 in a vacuum desiccator.

Nitrogen. Total N was determined by a micro-Kjeldahl method using SeO_2:$CuSO_4$:K_2SO_4 (1:1:8) catalyst.

Carbohydrate. Total carbohydrate was determined by the orcinol method (Pirie, 1936), reducing sugar by the Hanes (1929) modification of the Hagedorn & Jensen method and uronic acid by the method of Tracey (1948).

Deoxyribonucleic acid (DNA). This was determined by the diphenylamine method (Dische, 1930) and the cysteine-sulphuric acid method (Stumpf, 1947), and a modification of the tryptophan-perchloric acid method of Cohen (1944).

Determination of phosphatase activity. The liberation of inorganic P from sodium β-glycerophosphate was used to measure phosphatase activity. For the tests a total volume of 5 ml. contained sodium β-glycerophosphate solution (pH 6) to give 100 μg. P/ml., sodium citrate buffer (pH 6) 0·04 M, enzyme solution and water to make up the volume. Samples were removed at intervals and pipetted into 5 ml. of 2 N-H_2SO_4 and inorganic P determined.

Determination of nuclease activity. The liberation of P not precipitable by uranyl nitrate in trichloroacetic acid (TCA) (MacFadyen, 1934) from yeast nucleic acid was used to measure ribonuclease activity. For the tests a total volume of 5 ml. contained sufficient of the sodium salt of yeast ribonucleic acid to give 100 μg. P/ml., sodium citrate buffer 0·04 M (pH 6), enzyme solution and water to make up the volume. Samples were removed at intervals, made up to 1 ml., and 1 ml. uranyl nitrate solution 0·5% (w/v) in 2·5% (w/v) TCA was added. After standing the tubes on ice for 10 min. the precipitates were centrifuged down and total P determined in the supernatants. Deoxyribonuclease activity was determined similarly, using a solution of the sodium salt of deoxyribonucleic acid from thymus (British Drug Houses Ltd.) and either sodium diethylbarbiturate-sodium acetate buffer (pH 7), or an α-picoline-acetic acid buffer (pH 6) (Cecil, 1950).

Ultraviolet absorption spectra. These were obtained with a Hilger absorption spectrograph and with a Unicam quartz spectrophotometer.

pH *measurements.* These were made with a glass electrode.

RESULTS

Fractionation of phosphorus compounds in fibre, chloroplast and some sap fractions

Ogur & Rosen (1950) have recently described a method of fractionating P compounds in plant tissue involving the use of perchloric acid. This method has been modified, and forms the basis of the fractionation procedure used in the work described here.

Fibre

The P compounds of tobacco-leaf fibre have been fractionated into four groups: (1) P soluble in cold dilute acid; (2) P soluble in ethanol-ether; (3) P soluble in N-$HClO_4$ on soaking overnight at room temperature; (4) P soluble in N-$HClO_4$ on incubating at 37° overnight. The extraction of these removed over 98% of the fibre P. Table 1 shows the results of a typical experiment.

Table 1. *Fractionation of phosphorus in fresh and incubated fibre*

(3 g. lots of fibre (dry matter 0·617 g.) extracted successively as shown in the table. The method is described in the text, p. 434. The water extract from the incubated fibre was obtained by incubating the fibre twice in water at 37° overnight.)

	P in fresh fibre		P in incubated fibre	
Extractant	(mg./g. of dry fibre)	(% of total in fibre)	(mg./g. of dry fibre)	(% of total in fibre)
1. Water, 30 ml.	0·097	3·3	2·356	76·1
2. 0·2 N-$HClO_4$, 30 ml.	0·102	3·5	0·073	2·4
3. Ethanol-ether (3:1), 40 ml., i.e. lipid	0·64	21·8	0·082	2·6
4. N-$HClO_4$ at 16° overnight, 30 ml., i.e. RNAP*	1·61	54·8	0·194	6·3
5. Two extractions with N-$HClO_4$ at 37° overnight, 40 ml., i.e. mainly DNAP†	0·485	16·5	0·391	12·6
Totals	2·934	—	3·096	—

* RNAP, ribonucleic acid phosphorus.

† DNAP, deoxyribonucleic acid phosphorus.

As soon as possible after mincing and washing, two 3 g. lots of fibre were suspended in 20 ml. water. One lot was incubated twice, each time for about 24 hr. at 37° with $CHCl_3$ present. The other was squeezed out at once and re-washed with 10 ml. water. The fibre was then extracted with two lots of 0·2 N-$HClO_4$, 20 ml. and 10 ml., which were combined. The acid was not left in contact with the fibre for longer than 10 min. at room temperature. The fibre was then extracted with ethanol-ether (3:1) at room temperature until no more colour could be removed, as the removal of P paralleled the removal of pigments. The fibre was then suspended in 20 ml. N-$HClO_4$ and kept at room temperature overnight, i.e. about 16° for 18 hr. After removal of the extract the fibre was washed with 10 ml. N-$HClO_4$ and the N-$HClO_4$ extracts pooled. The fibre was then suspended in 20 ml. N-$HClO_4$ and incubated at 37° overnight, washed with 10 ml. N-$HClO_4$ and the extracts pooled. The extract from a second incubation at 37° overnight with 10 ml. N-$HClO_4$ was added to the first incubation extract.

The portion which was incubated at 37° in water was afterwards extracted with ethanol-ether and $HClO_4$ in exactly the same way as the other portion.

(1) *Cold dilute acid-soluble* P. For the extraction of this fraction 0·2 N-HCl, 0·2 N-$HClO_4$ and 5% (w/v) TCA were used and the results compared. There was no significant difference in the amount of P extracted by these acids. This fraction amounted to not more than 10% of the total P in well washed fresh fibre. From 30 to 50% of the P in this fraction was not in the form of PO_4^{---} and its nature has not been investigated. In fibre that had been stored in the refrigerator for 2–3 weeks as much as 30% of the total P of the fibre was in the acid-soluble fraction. The increased amount extractable was inorganic P due to enzymic breakdown of P compounds and could be removed with water alone, without acid.

(The small leaves were less than 10 cm. long and the large from 10 to 25 cm. long.) This shows that a much smaller proportion of the N was lost on incubation than of the P. The phospholipid-splitting enzyme investigated by Hanahan & Chaikoff (1948) caused only 5% loss of ether-soluble P compared with 35% loss of N when soy bean phospholipin was used as substrate. In the present investigation when cabbage-leaf fibre was incubated in water at 37° it behaved similarly to tobacco with the ethanol-ether soluble P being much reduced.

(3) *and* (4) P *soluble in* N-$HClO_4$. When fibre which had been washed with cold 5% (w/v) TCA was heated at 90° for 15 min. in 5% TCA (Schneider, 1945) or incubated at 37° for

Table 2. *Extraction of phospholipid from fibre*

(10 g. fibre (1·98 g. dry matter, 6·13 mg. P) extracted successively as shown in the table.)

	Dry matter		P		
Extractant	(mg.)	(% total in fibre)	(mg.)	(% total in fibre)	(mg./g. dry matter of fraction)
1. Ethanol-ether, 144 ml.	279	14·1	0·216	3·5	0·8
2. 2% (w/v) TCA, 84 ml.	—	—	0·337	5·4	—
3. Ethanol-ether, 98 ml.	85	4·3	1·120	18·1	13·2

Table 3. *Composition of ethanol-ether extracts from fresh and incubated fibre of large and small leaves*

(Two lots (10 g.) of fibre (3·20 g. dry matter, 7·5 mg. P) from large leaves (10–25 cm. long) and 2 lots (10 g.) of fibre (2·35 g. dry matter, 10·9 mg. P) from small leaves (<10 cm. long). The fresh fibres were extracted with 70 ml. 0·2 N-$HClO_4$ and then with 120 ml. ethanol-ether. The incubated fibres were first incubated at 37° overnight in water, then extracted with 0·2 N-$HClO_4$ and ethanol-ether in the same way as the fresh fibres.)

	Large leaves		Small leaves	
	Fresh	Incubated	Fresh	Incubated
Phosphorus:				
mg./g. dry matter of fibre	0·41	0·11	0·70	0·15
% total in fibre	17·4	4·8	15·1	3·3
Nitrogen:				
mg./g. dry matter of fibre	1·8	1·3	2·4	1·4
% total in fibre	3·9	2·8	4·2	2·5
Dry matter (% total in fibre)	15·3	12·4	19·8	14·4

(2) *Ethanol-ether soluble* P. Most of the pigments and about 80% of the lipid material were removed from fibre by extraction with neutral ethanol-ether at room temperature, or with hot solvents in a Soxhlet extractor, without removing more than a small fraction of the phospholipins. After extraction of the fibre with acid, i.e. below pH 2, 15–35% of the fibre P became soluble in cold ethanol-ether. Subsequent extraction with hot solvents showed that extraction in the cold was effective in removing lipid P. A phospholipin fraction with a P content of about 1·5% was obtained by first extracting the fibre with neutral ethanol-ether, then with dilute acid and again with ethanol-ether. Up to 20% of the fibre dry matter was soluble in ethanol-ether. Results are given in Table 2 showing the separation of phospholipin from other lipids.

Incubation of fibre in water decreased the amount of P soluble in ethanol-ether following acid extraction by as much as 90% (Table 1). The dry matter extracted was diminished by less than 20%. Table 3 compares the composition of the ethanol-ether fraction, following acid extraction, from fresh and incubated fibre of large and small leaves.

several hours in acid of the same strength all the P which was brought into solution was in organic form. Lipid P, if not already removed, was extracted in addition to nucleic acid and over 90% of the fibre P was brought out. Ogur & Rosen (1950) found that ribonucleic acid (RNA) could be extracted from root tips, without removing deoxyribonucleic acid (DNA), by soaking in N-$HClO_4$ at 4° for 18 hr. The DNA was then extracted by heating the residue with two lots of 0·5 N-$HClO_4$ for 20 min. at 70°. They used $HClO_4$ instead of TCA which had been used for much of the earlier work on P fractionation in animal tissues, because unlike TCA it has almost negligible absorption in the ultraviolet. With leaf tissues the RNA is not extracted so easily, and either a higher temperature or a higher concentration of acid has to be used. To remove all the RNA it is necessary to use conditions which will cause some of the DNA to become soluble. Fig. 1 shows the amount of P extracted from acid and ethanol-ether treated fibre with three concentrations of $HClO_4$ (3, 1·0 and 0·33 N) at 4° and 16° and also 1·0 N at 37°. DNA was determined in the extracts by the diphenylamine method. The amount of DNA was negligible except in the

3 N-$HClO_4$ extract at 16° after 2 hr. and in all the samples of N-$HClO_4$ at 37°. For the routine determination of RNA the fibre was extracted with N-$HClO_4$ at room temperature overnight. However, the amount of RNA extracted under these conditions does not represent the true total. A second extraction with N-$HClO_4$ at 16° will remove a small amount more RNA but some DNA then becomes soluble. In the chloroplast fraction (p. 438), where results are not complicated by the presence of DNA, it is clear that some of the RNA is not brought out with N-$HClO_4$ at 16°, but that a higher temperature had to be used to extract it. Much of the RNA of fibre is due to the presence of chloroplasts so the same is to be expected of the fibre RNA.

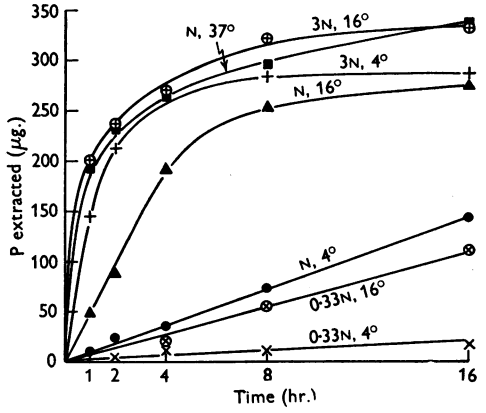

Fig. 1. Extraction of P from fibre with $HClO_4$. 0·5 g. lots of acid and ethanol-ether extracted fibre (dry matter, 141 mg.; P, 410 μg.) in 5 ml. $HClO_4$ (0·33 N, N, or 3 N) for the times and at temperatures shown.

Less than 5% of the total P in extracts made with N-$HClO_4$ at 16° was inorganic. The carbohydrate content was low, but when 2–3 vol. ethanol were added a precipitate appeared which was mainly 'soluble starch' and pectin. The precipitate contained up to 10% of the P of the extract. When saturated $Ba(OH)_2$ was added to the ethanolic solution to raise the pH to about 9 a precipitate formed containing 60–80% of the P originally present in the extract. The ultraviolet absorption of a N-$HClO_4$ extract had a maximum at about 260 mμ. and absorbed more strongly than an aqueous solution of yeast ribonucleic acid of the same P content. Ogur & Rosen (1950) found that treatment of nucleic acid samples with N-$HClO_4$ increased the absorption at 260 mμ. and this was confirmed using yeast ribonucleic acid.

For extraction of DNA the fibre residue was incubated at 37° with N-$HClO_4$ overnight. One extraction removed the greater part of the P remaining in the fibre, while a second extraction lowered the P content of the residue to a level where determinations became uncertain. A longer extraction at a lower temperature was used instead of the conditions of Ogur & Rosen. This was to minimize degradation of pectic substances because of the interference of galacturonic acid in colorimetric methods of determining DNA (Holden, unpublished). Determinations of DNA by the diphenylamine and tryptophan-$HClO_4$ methods usually agreed fairly well. The results obtained by the cysteine-sulphuric acid method were always lower than by the other two methods. The observed P content of the extract was usually higher than the P to be expected from the colour intensity given by the extract compared with thymus nucleic acid standards of known P content. This is not unexpected for some RNA is present. When agreement between the observed P and the value calculated from deoxyribose determinations was very close it was probably fortuitous, due to interfering substances increasing the absorption at the wavelengths used for colorimetric determinations of deoxyribose.

Extracts made with N-$HClO_4$ at 37° contained more carbohydrate than those made at room temperature, but this was mainly non-reducing. Much of the carbohydrate was precipitated in a bulky gelatinous form, with up to 20% of the P of the extract in it, when 2–3 vol. ethanol were added. From 50 to 70% of the P originally present in the extract was precipitated on addition of saturated $Ba(OH)_2$ to the ethanolic solution.

Nitrogen determinations on N-$HClO_4$ extracts were done after precipitation of $KClO_4$ at 4°, as the presence of large amounts of $HClO_4$ is known to cause loss of N during incineration (Weeks & Friminger, 1942). During the fractionation procedure about 25% of the total N of the fibre was removed, of which about 5% was in the ethanol-ether fraction. There was a greater loss of N when fibre was incubated before fractionation, as up to 15% of the fibre N appeared in the incubation extract. With the usual ratio of fibre to acid each N-$HClO_4$ extract contained about 7% of the fibre N, or 3 mg./g. of the fibre dry matter, whether the fibre had been previously incubated or not.

Incubating fibre in water at 37° diminished the amount of P in the ribonucleic acid fraction by as much as 90%. This is due to the action of ribonuclease present in the fibre (p. 441). There was less diminution in the amount of P in the deoxyribonucleic acid fraction, a loss of from 15 to 35% was found with different batches of fibre. Incubating the fibre in the presence of Mg salts did not cause a greater decrease in the amount of P in this fraction.

Phosphorus fractions in the fibre of leaves of different ages

Pirie (1950) found that the greatest yield of nucleoprotein was from the sap of small young leaves. Table 4 shows the results obtained in one experiment on the fractionation of P in the fibre of leaves of different ages. The leaves were from tall plants, about to flower, and the leaves were grouped according to size and position on the plants. The leaves in the group 'young small' were under 10 cm. long, those in the group 'young large' were 10–20 cm. long and those in the group 'old large' were over 20 cm. long. Some of the leaves in the latter group were slightly yellowed but no completely yellow or withered leaves were included. The total P/g. of the dry matter was highest in the youngest leaves. The acid-soluble P as a percentage of the total P was slightly higher in large leaves than in small but per g. of the dry matter was lower. The inorganic P in this fraction remained fairly constant per g. of the dry matter, but the P in organic form was lower in the large leaves. The phospholipin P/g. of the dry matter was about the same in the different aged leaves, but was a much higher percentage of the total P in the large leaves. Both RNAP and DNAP formed a lower percentage of the total P in the large leaves than in the small and the amount per g. of dry matter was much lower. In small young leaves the RNA content was of the order of 35 mg./g. of the dry matter.

In another experiment the total amount of P in the various fractions in two sizes of leaves was compared. The twelve leaves in each group were chosen to be as uniform in size as possible. In one group, 'small', the leaves were $14 \pm 0{\cdot}5$ cm. long, $6{\cdot}5 \pm 0{\cdot}5$ cm. broad at the widest point and weighed 17·3 g. In the other group, 'large', the leaves were 27 ± 1 cm. long, $13{\cdot}5 \pm 1$ cm. broad and weighed 74·8 g. The leaves were minced without the midribs being removed and approx. 58 sq.cm. and of the large leaf 240 sq.cm. There were 4·3 times as many cells per unit area in the small leaf as in the large, so that the number of cells was approximately the same. The results for dry matter, N and P values are given in Table 5. The amount of P in the large leaves was greater by only 60%, whereas there was about three times as much dry matter and N. In the fibre the amount of phospholipin P had doubled but the DNAP and RNAP remained the same.

Table 4. *Fractionation of phosphorus in fibre of leaves of different ages*

(3 g. lots of each fibre (known dry matter contents) extracted successively as shown in the table.)

Extractant	P in old, large leaves (mg./g. of dry matter)	P in old, large leaves (% of total)	P in young, large leaves (mg./g. of dry matter)	P in young, large leaves (% of total)	P in young, small leaves (mg./g. of dry matter)	P in young, small leaves (% of total)
1. 0·2 N-$HClO_4$, 30 ml.: inorg. P	0·269	13·1	0·219	11·4	0·247	10·4
org. P	0·115		0·266		0·453	
2. Ethanol-ether, 40 ml. i.e. lipid P	1·02	34·9	0·965	22·7	1·24	18·9
3. N-$HClO_4$ at 16° overnight, 30 ml. i.e. RNAP	1·045	35·6	1·99	46·6	3·16	48·3
4. Two extractions with N-$HClO_4$ at 37° overnight, 40 ml. i.e. mainly DNAP	0·475	16·4	0·811	19·1	1·46	22·4
Totals	2·904	—	4·251	—	6·560	—

Table 5. *Fractionation of phosphorus in leaves of two sizes*

	Twelve large leaves, 74·8 g. wet wt. P (mg.)	N (mg.)	Dry matter (g.)	Twelve small leaves, 17·3 g. wet wt. P (mg.)	N (mg.)	Dry matter (g.)
Sap + fibre washings	6·75	69·5	2·615	3·88	20·0	0·720
Chloroplast fraction	1·87	39·2	0·853	1·06	8·4	0·236
Fibre: Soluble in 0·2 N-$HClO_4$	0·76			0·32		
Soluble in ethanol-ether	1·89			0·88		
Soluble in N-$HClO_4$ at 16°	1·82	101·0	3·34	1·79	42·7	1·175
Soluble in N-$HClO_4$ at 37°	1·13			1·10		
Unextracted	0·22			0·12		
Totals	14·44 = 2·12 mg./g. of dry matter	209·7 = 30·8 mg./g. of dry matter	6·808	9·15 = 4·3 mg./g. of dry matter	71·1 = 33·4 mg./g. of dry matter	2·131

Table 6. *Fractionation of phosphorus in fresh and incubated chloroplast fraction*

(165 mg. lots of a suspension of the chloroplast fraction (3·20 mg. P/g. dry matter) extracted successively as shown in the table. The water extract from the incubated portion was obtained by incubating in water at 37° overnight.)

Extractant	Fresh P (mg./g. dry matter)	Fresh P (% total)	Incubated P (mg./g. dry matter)	Incubated P (% total)
1. Water	—	—	2·25	70·3
2. 0·2 N-$HClO_4$, 11 ml.	0·38	11·9	0·18	5·6
3. Ethanol-ether, 17 ml.	0·93	29·1	0·25	7·8
4. N-$HClO_4$ for 18 hr. at 16°, 13 ml.	1·21	37·9	0·29	9·0
5. N-$HClO_4$ for 18 hr. at 37°, 10 ml.	0·51	16·0	0·12	3·8
Totals	3·03	—	3·09	—

the sap, chloroplast and fibre fractions separated as usual, the washings of the fibre being included with the sap. A count of the number of cells per unit area was made on a representative leaf of each group by the method described by Morton & Watson (1948). The area of the small leaf was

Chloroplast fraction

Table 6 gives the results of a typical experiment on fractionation of P in the chloroplast fraction following the same general scheme as for fibre. The P content per g. of the

dry matter was very variable, depending partly on the age of the leaf and on how much starch was present. The amount of acid-soluble P was about 10% of the total present which is of the same order as that in fibre. The ethanol-ether soluble P was 25–45% of the total in the fraction, which is a higher range than in fibre. As with fibre, most of the phospholipid P was not extractable until after acid treatment.

Extraction with N-$HClO_4$ at room temperature removed about 70% of the P still present after extraction of lipid P; a higher temperature was needed to extract the remainder. One incubation with N-$HClO_4$ at 37° overnight removed all but traces. The acid extracts at room temperature and 37° had ultraviolet absorption spectra similar to that of a yeast ribonucleic acid solution of the same P content in N-$HClO_4$. DNA could not be detected unequivocally by any of the three methods used. The carbohydrate content of the acid extracts was lower than in the corresponding ones from fibre as they contained only starch and no pectin. Nucleotide preparations made by precipitation of the Ba-ethanol insoluble P also contained much less polysaccharide than similar preparations from fibre. The RNA content of the chloroplast fraction from which much of the starch had been removed by differential centrifugation was 30–40 mg./g. of the dry matter.

In the chloroplast fraction which had been incubated in water at 37° the amount of P in the various fractions was greatly diminished owing to the action of ribonuclease and the phospholipid-splitting enzyme. The ribonucleic acid which is not extracted by N-$HClO_4$ at room temperature and which appears to be more firmly bound than the rest, is, however, susceptible to the action of ribonuclease.

Table 7. *Phosphorus fractionation in some sap precipitates*

(200 ml. sap centrifuged at 8000 rev./min. (6000 *g*) to sediment the chloroplast fraction and the successive precipitates formed after standing for 1 and 3 days at 4°. The chloroplast fraction, with much of the starch removed, and the precipitates were washed and then suspended in water. The P fractions were obtained by extracting successively with 0·2 N-$HClO_4$, ethanol-ether and N-$HClO_4$ at 37°.)

	Chloroplast fraction	Ppt. after 1 day	Ppt. after 3 days
Dry matter (g.)	1·62	0·52	0·48
P (mg./g. dry matter)	2·1	2·7	2·8
N (mg./g. dry matter)	46·7	94·3	135
P % of total P in fraction:			
Soluble in 0·2 N-$HClO_4$	16·1	10·4	6·5
Lipid P	47·0	23·8	3·0
Nucleic acid P	36·9	65·8	90·5

Sap fractions

The P was also fractionated by the $HClO_4$ procedure in the following materials prepared from sap: (1) the precipitate obtained by adding $HClO_4$, to a concn. of 0·2 N, to sap after removal of the chloroplast fraction; (2) the precipitates which separated out from sap (after removal of the chloroplast fraction) on standing at 4°; (3) material which sedimented at 8000 rev./min. from the resuspended sediment from sap which had previously been spun at 40 000 rev./min. No deoxypentose was detected in any of these fractions. The $HClO_4$ precipitate of sap had a low lipid-P content, 5–10% of the total P. One extraction with N-$HClO_4$ at room temperature removed the greater part of the remaining P, and it was not necessary to incubate at 37° to free the residue of P, a second extraction at room temperature was effective in doing this. Table 7 compares the results of the P fractionation in the chloroplast fraction and the precipitates which appeared in the sap after 1 and 3 days' standing. The lipid P as a percentage of the total P of the fraction decreased in the successive precipitates while the nucleic acid P increased. The material not resuspending after ultracentrifugation had a high P content (over 10 mg./g. of the dry matter), about half of which was nucleic acid P and 40% lipid P. No attempt has been made to fractionate the P in the sap which is not precipitable with acid.

Comparison of methods for estimation of nucleic acids

The method of fractionating RNAP and DNAP used by Schmidt & Thannhauser (1945) on animal tissues and by von Euler & Hahn (1947, 1948) on leaf tissue was tried on several batches of tobacco-leaf fibre and the results compared with the modified Ogur & Rosen method. Incubation of fibre with N-NaOH at 37°, after removal of acid and ethanol-ether soluble P, brought out all the P of the fibre in organic form. However, when fibre was incubated with alkali, even after thorough treatment with solvents to remove pigments, the extract was yellow or brown and unsuitable for colorimetric estimations of inorganic P or of DNA. An added disadvantage was the high N and carbohydrate content of the extracts. The amount of RNAP as a percentage of the total P was higher in the Schmidt & Thannhauser method than in the $HClO_4$ method, while the DNAP was correspondingly lower. This is to be expected because of the DNAP fraction in the $HClO_4$ method containing some RNAP.

Factors affecting the enzymic breakdown of phosphorus compounds in fibre, chloroplast and some sap fractions

The release of phosphorus on incubation in water and salt solutions has been observed with fractions from the leaves of a number of species, including tomato (*Lycopersicum esculentum* Mill.), potato (*Solanum tuberosum* L.), bean (*Phaseolus vulgaris* L.), bryony (*Bryonia dioica* Jacq.), comfrey (*Symphytum officinale* L.), groundnut (*Arachis hypogaea* L.), and cabbage (*Brassica oleracea* L.). Most experiments were done with tobacco leaves.

Conditions of incubation

Effect of temperature. At 4° there was a gradual increase in the amount of phosphorus extractable with water. At room temperature the rate of release was increased, and at 37° the action was rapid and substantially complete in a few hours. The greater part of the phosphorus in the extracts was inorganic phosphorus. Fig. 2 shows the effect of temperature on the liberation of P from fibre.

Effect of pH. The optimum pH for the liberation of phosphorus is between 5 and 6. Fig. 3 shows the results of an experiment in which portions of a chloroplast suspension were incubated for 4 hr. at

pH values between 4 and 8. At pH values on the acid side of the optimum the inorganic and total phosphorus in the extracts was of the same order and the amount liberated fell off steeply as the pH became lower. On the alkaline side of the optimum there was a less steep fall in the amount released and the inorganic phosphorus was only half of the total phosphorus in the extracts.

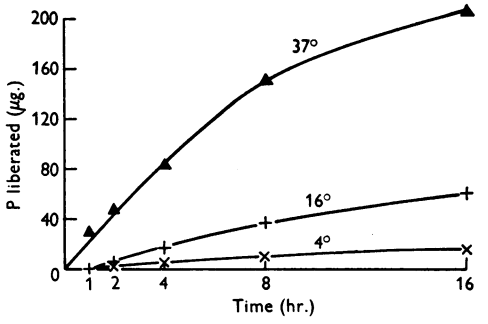

Fig. 2. Effect of temperature on enzymic release of P from fibre. 0·5 g. lots of fibre (dry matter, 100 mg.; P 300 μg.) in 8 ml. 0·025 N-NaCl incubated at 4, 16 and 37° for times shown.

Effect of salts. Sodium chloride, sodium citrate and sodium azide increase the rate of liberation of phosphorus. Table 8 shows the effect of various concentrations of sodium chloride and sodium citrate on the release of phosphorus from fibre. Total and inorganic phosphorus liberation were activated by both salts, but a citrate concentration of only about one-twenty-fifth that of the sodium chloride was needed to achieve the same result. Fig. 4 shows the results for three concentrations of sodium azide, compared with sodium chloride, on liberation of total phosphorus from the chloroplast fraction.

The effects due to the activity of ribonuclease and the phospholipin-splitting enzyme can be separated from each other as the phosphorus is split from the RNA much more rapidly than from the phospholipin, particularly in the presence of citrate. The results of an experiment using fibre are given in Table 9. When the phosphorus fractionation in fresh fibre and in fibre incubated for 1 hr. at 37° in

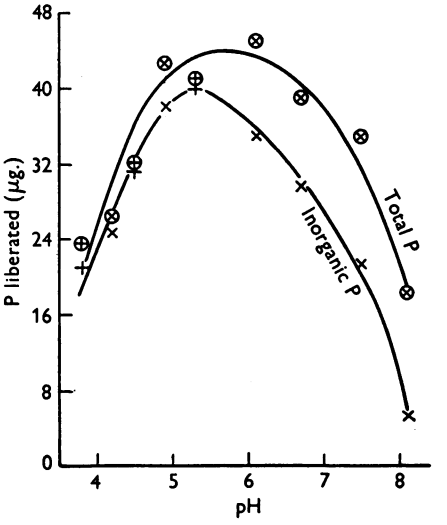

Fig. 3. Effect of pH on enzymic liberation of P from the chloroplast fraction. 1 ml. lots of a suspension of the chloroplast fraction (dry matter, 49 mg.; P, 100 μg.) + 1 ml. buffer solution. The final concentration of the acetate buffers was 0·05 M, and the Michaelis sodium diethylbarbiturate-acetate buffers were a 1 in 10 dilution of the stock solution. Incubated for 4 hr. at 37° and 2 ml. 10% (w/v) TCA added before being centrifuged. Inorganic and total P determined in the supernatants. The P values given by a portion of suspension which was not incubated and to which TCA was added were subtracted from the results. ⊗ and ×, Michaelis buffers; ⊕ and +, acetate buffers.

0·05 M-sodium citrate buffer (pH 6) is compared (*A* and *B* in the table) it is clear that most of the phosphorus in the incubation extract is from the fraction soluble in N-perchloric acid at room

Table 8. *The effect of various concentrations of sodium chloride and sodium citrate on the liberation of phosphorus from fibre*

(0·5 g. lots of fibre (dry matter, 146 mg.; P, 220 μg.) incubated with 5 ml. water or NaCl solution or sodium citrate solution (pH 6) of concn. shown for 2, 4 and 18 hr. at 25°.)

Time of incubation	P in extract (μg.)					
	2 hr.		4 hr.		18 hr.	
	Inorganic	Total	Inorganic	Total	Inorganic	Total
Water	15	22	30	30	54	55
NaCl: 0·25 M	42	58	65	63	109	117
0·05 M	25	27	45	49	76	88
0·01 M	20	22	28	38	60	75
Sodium citrate: 0·05 M	80	120	108	127	151	160
0·01 M	39	53	65	72	135	141
0·002 M	24	24	40	49	67	69

Table 9. *Separation of the effects due to ribonuclease and the phospholipin-splitting enzyme during incubation of fibre*

(3 g. lots of fibre (dry matter 30·2%, P 2·5 mg./g. of dry matter) (*A*) P fractionated without previous incubation. (*B*) P fractionated after 1 hr. incubation in 0·05 M-sodium citrate buffer solution (pH 6). (*C*) Incubated for 1 hr. as (*B*), extract removed and fibre reincubated for 18 hr. in fresh solution. P fractionated in fibre residue.)

	(*A*)		(*B*)		(*C*)	
	P (mg./g. of dry matter)	P (% total)	P (mg./g. of dry matter)	P (% total)	P (mg./g. of dry matter)	P (% total)
Incubation for 1 hr. at 37°	—	—	1·240	49·5	1·195	48·0
Incubation for 18 hr. at 37°	—	—	—	—	0·508	20·4
0·2 N-$HClO_4$, 25 ml.	0·262	10·5	0·083	3·3	0·083	3·3
Ethanol-ether, 35 ml., i.e. lipid P	0·655	26·2	0·591	23·7	0·161	6·5
N-$HClO_4$, 16°, 25 ml., i.e. RNAP	0·985	39·4	0·117	4·7	0·055	2·2
N-$HClO_4$, 37°, 25 ml., i.e. mainly DNAP	0·497	19·9	0·423	16·9	0·387	15·5

temperature, i.e. RNAP. The lipid phosphorus and DNAP fractions have not been much diminished. When *B* and *C* in the table are compared it is seen that a second incubation for 18 hr., of another portion of fibre, leads to a further diminution in the RNAP fraction, but that the greater part of the phosphorus in the incubation extract is now from the lipid fraction.

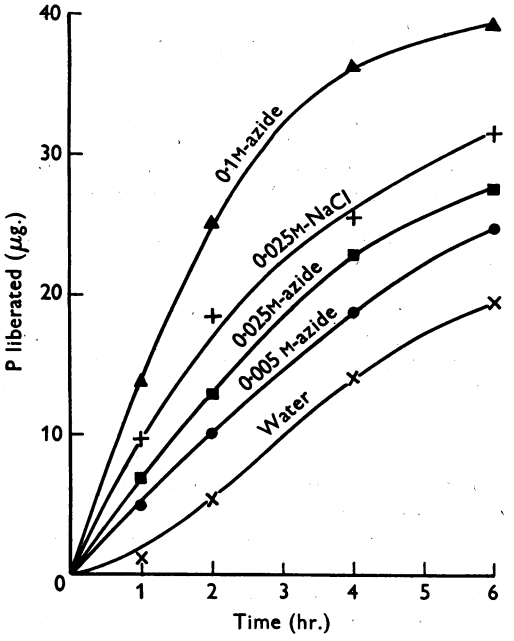

Fig. 4. Effect of sodium azide on enzymic liberation of P from the chloroplast fraction. 1 ml. lots of a suspension of the chloroplast fraction (dry matter, 47 mg.; P, 90 μg.) + 1 ml. water, NaCl or sodium azide solution (with pH previously adjusted to 6·0). Incubated at 37° for times shown and 2 ml. 10% (w/v) TCA added before being centrifuged. Total P determined in the supernatants. The P value given by a portion of suspension which was not incubated and to which TCA was added was subtracted from the results.

Pretreatments

Milling. Grinding the fibre in a triple roller mill (Bawden & Pirie, 1944) did not increase the rate of liberation of phosphorus or the total amount of phosphorus released when the fibre was subsequently incubated in salt solution at 37°.

Boiling. Fibre which had been boiled did not liberate inorganic phosphorus when it was incubated at pH 6, but some phosphorus in organic form was released. Lipid phosphorus did not decrease in amount when boiled fibre was incubated. The addition of dialysed sap or a dialysed extract from fresh fibre caused inorganic phosphorus to be liberated and the total amount of phosphorus brought out was increased. Table 10 shows the effect of adding a dialysed extract to boiled fibre.

Table 10. *Effect of adding a dialysed fibre extract to boiled fibre*

(0·5 g. lots of fibre (dry matter, 29·3%; P, 2 mg./g. dry matter) + 4 ml. sodium citrate buffer, 0·02 M (pH 6) + 1 ml. water or 1 ml. dialysed extract of fresh fibre. Incubated at 37° for times given. Total and inorganic P determined in the extracts.)

	P in extract (mg./g. dry fibre)			
	Boiled fibre		Boiled fibre + dialysed extract of fresh fibre	
Time (hr.)	Inorganic	Total	Inorganic	Total
1	<0·02	0·146	0·068	0·247
3	—	0·218	0·102	0·314
6	—	0·287	0·222	0·564
22	<0·02	0·486	0·324	0·966

Acid extraction and ethanol-ether extraction. Phosphorus in organic form, but no inorganic phosphorus, was liberated from fibre which had been extracted with dilute acid or with ethanol-ether when it was subsequently incubated at pH 6.

Enzyme activities of fibre extracts

Extracts made by soaking fibre in sodium chloride or sodium citrate buffer (pH 6), followed by dialysis, had ribonuclease and phosphatase activity. Inorganic phosphorus was also split from nucleotide preparations made from fibre and chloroplast fractions. Citrate did not activate the liberation of organic phosphorus from yeast ribonucleic acid by leaf ribonuclease (Pirie, 1951), nor the enzymic liberation of inorganic phosphorus from sodium β-glycerophosphate and nucleotide preparations. Phosphatase and nucleotidase activity was lost by bringing the enzyme solution to the boil, but the ribonuclease is more heat stable and only about half the activity was destroyed by this treatment. Deoxyribonuclease activity was detectable, but with 24 hr. incubation at 37° only 5% of the phosphorus from thymus deoxyribonucleic acid was released compared with 75% from yeast ribonucleic acid. The action of a fibre extract on either lecithin or a phospholipid preparation from leaves has not been tested. The properties of the phospholipid-splitting enzyme are being investigated further.

Composition of incubation extracts

Table 11 gives the composition of a typical incubation extract of fibre. Part of the nitrogen and carbohydrate is present as nucleosides and the remainder as breakdown products of starch and protein due to the activity of the leaf enzymes. No deoxypentose could be determined with certainty in the extracts. The extracts absorbed strongly in the ultraviolet with a maximum at 260 mμ. Adenine, guanine, cytosine and uracil were detected by paper-partition chromatography in the extracts after hydrolysis.

Table 11. *Composition of an incubation extract of fibre*

(6 g. fibre (1·80 g. dry matter, 58 mg. N, 5·4 mg. P) incubated at 37° in 30 ml. water for 18 hr.)

	Total in extract (mg.)
Dry matter	234
Total P	3·33
Inorganic P	2·64
Nitrogen	9·9
Total carbohydrate	102
Reducing sugar	72

DISCUSSION

The observation that most of the phospholipid of leaf fibre and chloroplast fractions is not extracted with ethanol-ether until after treatment with acid is in agreement with results obtained with animal and some other plant tissues (Lovern, 1942). In animal tissues there is evidence that lipids are bound because of the formation of lipoprotein complexes but in plants combination with carbohydrates has also been suggested (Jamieson, 1938). Frey-Wyssling (1949) was of the opinion that lipoproteins were present in intact chloroplasts, but on isolation and preparation the lipids dissociated from the protein. It is clear, however, that it is a stable linkage between the phospholipid and protein or other substance to which it is attached.

Hanahan & Chaikoff (1948) found that an enzyme preparation from cabbage leaves acting on soy bean lecithin caused a loss of only 5% of the ether-soluble phosphorus, when 35% of the nitrogen was removed. They suggested that the activity of this enzyme might explain why Chibnall & Channon (1927) isolated a phosphatidic acid from fresh cabbage leaves. In tobacco leaves, and in others including cabbage, phospholipin-splitting enzymes capable of attacking other linkages are also present. The loss of ethanol-ether soluble P from fibre and chloroplast fractions was much greater than the loss of nitrogen on incubation. The different results obtained by Hanahan & Chaikoff might be due to the different substrate used or to a difference in the stability of the enzymes concerned with phospholipin breakdown.

Sodium chloride, sodium citrate and sodium azide have an activating effect on the liberation of phosphorus from fibre and chloroplast fractions similar to that found with the fission of nucleoprotein (Pirie, 1950). Sodium azide appears to act like any other salt. As ribonuclease activity is not much affected by citrate, the effect of citrate on phosphorus liberation may be due to increased accessibility of the nucleic acid. The apparent activation by citrate of nucleotidase action in fibre, which is not observed with nucleotide preparations, is probably due to more substrate being available.

When fibre which had been boiled, or extracted with dilute acid or with ethanol-ether, was subsequently incubated at pH 6 some phosphorus in organic form but no inorganic phosphorus was brought into solution. The release of organic phosphorus was probably due to ribonuclease activity still present in the fibre. The ribonuclease is relatively heat stable but the phosphatase is readily inactivated. Parker (1951) found that TCA precipitates of sap had about 4% of the ribonuclease activity of the original sap, whereas the phosphatase was almost completely destroyed.

The deoxyribonuclease activity of dialysed sap and fibre extracts was so low that it is not unexpected that the DNAP was affected much less than the RNAP during incubation of fibre. The decrease in the DNAP fraction is probably due mainly to the RNAP present in the fraction being removed by ribonuclease action.

During expansion of the leaf there is a large increase in the total amount of phospholipin present. The dry matter has increased in amount so that phospholipid expressed on a dry matter basis remains the same or increases. The total amount of phosphorus in the nucleic acid fractions from fibre remains about the same, so that it shows a decrease per g. of the dry matter. If the weight of DNA per cell is constant in amount as has been found for various animal tissues (Vendrely & Vendrely, 1948) and bacteria (Mitchell & Moyle, 1951), it would not be expected that DNA would vary during expansion of the leaf as cell division is not taking place.

The perchloric acid method of fractionating phosphorus compounds, although not giving a perfect separation of ribo- and deoxyribo-nucleic acids in leaf tissue, is useful in giving an RNA fraction substantially free from DNA, though the DNA fraction contains RNA. The fact that some of the RNA is not extracted by perchloric acid at room temperature, but requires a higher temperature to bring it out, suggests that it might be bound in a different way from the more easily extracted fraction.

Of the total P in the leaf about 30 % is in the form of ribonucleic acid phosphorus, two-thirds of which is in the fibre. Deoxyribonucleic acid phosphorus accounts for about 7 % and lipid phosphorus a further 15 % of the total phosphorus of the leaf.

SUMMARY

1. The phosphorus of tobacco-leaf fibre and chloroplast fractions has been fractionated into acid-soluble phosphorus, lipid phosphorus, ribonucleic acid phosphorus and deoxyribonucleic acid phosphorus. The distribution in leaves of different ages has been compared.

2. Incubation of fibre and chloroplast fractions in water and salt solutions leads to the liberation of up to 80 % of the total phosphorus present, owing to the activity of ribonuclease and a phospholipin-splitting enzyme.

3. The optimum pH for the enzymic liberation of phosphorus is between 5·5 and 6.

4. Sodium chloride, sodium azide and sodium citrate activate the enzymic release of phosphorus.

5. Milling the fibre does not increase the rate of liberation or the total amount of phosphorus released by enzymic action.

6. Boiled fibre, acid-extracted fibre and ethanol-ether extracted fibre on incubation at pH 6 release phosphorus in organic form but no inorganic phosphorus. When incubated in the presence of dialysed sap or a fibre extract, which have ribonuclease and phosphatase activity, inorganic phosphorus is released and the total phosphorus liberated is increased.

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