The Isolation of Nucleic Acid Fractions from Plant Leaves and their Purine and Pyrimidine Composition

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Methods are readily available for the isolation of nucleic acids from animal and bacterial sources, where they often occur in relatively large amounts. In plants, the nucleic acid concentration is much lower and there is much interfering polysaccharide and other material. Consequently, apart from the particularly favourable case of wheat embryo nucleic acids, there is only fragmentary knowledge of the normal nucleic acids of plant tissues.

Nucleic acid, now known to be pentose nucleic acid (PNA), was first obtained from wheat embryo by Osborne & Campbell (1900). PNA was first isolated from barley roots and characterized by Takasugi (1943*a*, *b*, 1944). An improved procedure for the isolation of wheat embryo PNA was given by Lusena (1951). Deoxypentose nucleic acid (DNA) was isolated from the cell nuclei of rye embryos by Feulgen, Behrens & Mahdihassan (1937). Mirsky & Pollister (1946) selectively extracted pentose nucleoproteins from animal tissues with 0·14M sodium chloride and then deoxypentose nucleoproteins from the residue with 1·0M sodium chloride. With this procedure they were able to isolate fibrous DNA from wheat embryo. Laland, Overend & Webb (1950) described the isolation of pure, highly polymerized DNA from wheat embryo in more detail, but PNA and DNA were not selectively extracted from rye by sodium chloride solutions of different strength. Polymerized DNA was isolated from this source by a complicated fractionation procedure, but it still contained appreciable quantities of PNA and polysaccharide. The method of Mirsky & Pollister is not generally applicable to plant tissues.

Information on the purine and pyrimidine composition of normal plant nucleic acids is restricted to that on the PNA from the nucleoprotein comprising most of the fraction I protein of tobacco leaf cytoplasm (Eggman, Singer & Wildman, 1953), and the DNA of wheat embryo (Laland, Overend & Webb, 1952; Wyatt, 1951*b*).

In the present investigation, a method has been evolved for the isolation of nucleic acid fractions from various plant tissues, namely, bracken fern, clover and ryegrass whole leaves, sugar beet tops, marrow-stem kale, suitable for a comparative study of their purine and pyrimidine composition. For this purpose, it was not essential, nor indeed practicable, to preserve the macromolecular properties of the nucleic acids.

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EXPERIMENTAL

It was originally hoped to separate the nucleic acids by differential extraction of their protein complexes with NaCl solutions of varying strengths. Both fresh and dried leaf material were extracted with NaCl solutions over a range of concentrations, at pH 7. The extracted material was precipitated at pH 1 and dried. Upper limits for the PNA and DNA contents were estimated by means of the Schmidt & Thannhauser (1945) procedure. No significant separation of PNA and DNA, or concentration of the nucleic acids, was obtained, and no precipitates were obtained on diluting the extracts with water. Fractional precipitation of aqueous leaf extracts with NaCl was also unsuccessful.

Since the linkage between nucleic acids and proteins is generally supposed to be electrovalent, it was considered that the nucleoproteins might have been dissociated by very strong NaCl solution, the nucleic acid being extracted while the protein remained insoluble. Very little material was extracted by cold 5·0M or saturated NaCl. However, Williams (1945) described a method for estimating the total nucleic acid content of leaves by extracting them with boiling 10% (w/v) NaCl and precipitating the nucleic acids from the extract at pH 1, yielding products of high phosphorus content.

Extraction of leaves with boiling NaCl solutions over a range of concentrations showed an optimum extraction of acid-precipitable phosphorus compounds at 10% (w/v) NaCl. It appeared that extraction of the nucleic acids by the method of Williams, followed by their separation with the modified Schmidt & Thannhauser (1945) procedure, as used by Davidson & Smellie (1952) and Sherratt & Thomas (1953), would yield PNA and DNA fractions suitable for purine and pyrimidine determination.

Isolation of nucleic acid fractions

The fresh leaves were minced in a Hobart power mincer, sufficient water added to produce a mash and the whole adjusted to pH 8 with NaOH. The juice was pressed out and centrifuged to remove particulate material. The high-molecular weight material was precipitated from the juice by acidification to pH 3 and warming to 75°. The precipitate was collected, extracted with acetone and dried at 60°.

This powder (100 g.) was boiled under reflux with 500 ml. 10% (w/v) NaCl for 1 hr., the extract being maintained at pH 7 by the addition of dilute NaOH. The extract was squeezed through muslin and centrifuged. The clear supernatant solution was acidified to pH 1, when the nucleic acids, together with contaminating material, separated; they were centrifuged and dried with acetone and ether.

The mixed nucleic acids were incubated at 37° for 18 hr. with 0·3N-KOH. This treatment hydrolysed the PNA to mononucleotides, while the DNA remained acid-precipitable. The KOH concentration is critical: with solutions stronger than 0·3N, cytidylic acid is partially deaminated to uridylic acid (Davidson & Smellie, 1952), while if the concentration is much lower than 0·3N, the PNA is incompletely hydrolysed. Our plant extracts had considerable buffering capacity and it was necessary to adjust the pH to 9 to overcome this buffering action, and then to add KOH to a concentration of 0·3N.

The pH of the digest was then adjusted to 1 with $HClO_4$ and the precipitate, containing DNA and $KClO_4$, was centrifuged off. The acid supernatant was extracted several times with *iso*butanol which removed much colouring matter and material absorbing in the ultraviolet. The solution was then adjusted to pH 4 with KOH and allowed to stand at 0°. Insoluble $KClO_4$, which settled out, was filtered off and the solution concentrated *in vacuo* to a small volume (1–2 ml.) to give the PNA fraction.

The precipitate containing the DNA was dissolved in water at pH 7 and the solution saturated with NaCl at 100°. The incubation with KOH had modified the DNA so that it now remained in solution, while much of the interfering material was precipitated. Previously the DNA was precipitated with the interfering material by saturated NaCl. The DNA solution was dialysed for 48 hr. against running water and then concentrated *in vacuo*. The DNA was precipitated by acidifying to pH 1 and adding ethanol (1 vol.) and the precipitate was dried with acetone and ether to give the DNA fraction.

It was important throughout the procedures to keep the nucleic acid solutions as concentrated as possible in order to obtain good precipitation (at least 100 mg. of crude DNA/5 ml.).

METHODS

Total phosphorus. This was estimated according to Fiske & Subbarow (1925) after combustion with 60% (w/w) $HClO_4$.

Nucleic acid phosphorus. PNA-P and DNA-P were estimated according to Davidson, Leslie & Waymouth (1949) after Schmidt & Thannhauser (1945). This method gave the upper limit of nucleic acid content.

Identification of sugars. (i) Pentoses. The precipitated total nucleic acids (5 mg.) were hydrolysed with 2N-H_2SO_4 (1·0 ml.) for 2 hr. at 100° and the pH of the hydrolysate was adjusted to 4 with $Ba(OH)_2$. The supernatant and washings from the precipitated $BaSO_4$ were concentrated and chromatographed by descent on Whatman no. 1 paper with *n*-butanol:acetic acid:water (4:1:5, v/v/v) and with ethyl acetate:acetic acid:water (3:1:3, v/v/v). The spots were detected with the aniline hydrogen phthalate reagent (Partridge, 1949).

(ii) Deoxypentose. The DNA fraction (5 mg.) was extracted with 10% (w/v) trichloroacetic acid (1·0 ml.) at 90° for 30 min., and 2 vol. of the Dische reagent (pure diphenylamine, 2 g., H_2SO_4, 1·5 ml., and acetic acid, 100 ml.) were added. The mixture was heated at 100° for 20 min., a blue colour indicating deoxypentose (Deriaz, Stacey, Teece & Wiggins, 1949).

Estimation of purines and pyrimidines. (i) Chromatography. The purines and pyrimidines of the PNA and DNA fractions were estimated by the methods of Wyatt (1951*a*, *b*).

(ii) Ionophoresis. The pH of the purine and pyrimidine nucleotide solution of the PNA fraction was adjusted to 9 with $Ba(OH)_2$, any precipitate being discarded. Three volumes of ethanol were added and the solution was cooled to 0°, when barium salts of the nucleotides separated and were centrifuged off. The pH was readjusted to 9 with $Ba(OH)_2$ and a second crop of barium salts of nucleotides collected. The barium nucleotides were redissolved in water and the solution was acidified to pH 4 with H_2SO_4, liberating the free nucleotides and producing insoluble $BaSO_4$ (Parker, 1952).

The solution of the mixed nucleotides was applied near one end of long strips (6 × 60 cm.) of Whatman no. 2 paper. The paper was soaked in 0·02M citric acid/trisodium citrate buffer, pH 3·5, and the paper run for 18 hr. with a potential difference of 500v. The paper was dried and the nucleotide bands were located by photography on reflex document paper in ultraviolet light. The source of ultraviolet light was an Osram ultraviolet lamp (General Electric Co. Ltd.) with the outer glass envelope removed. The voltage applied to the lamp was reduced by a 3000Ω resistance (mains voltage 230v a.c.). The resulting glow discharge yielded predominantly the mercury 2537Å line and was filtered through Chance filter OX7.

RESULTS

Pentose nucleic acid fractions. Satisfactory chromatograms of the component bases were obtained from the PNA fractions from all sources, after perchloric acid hydrolysis. The intact nucleotides were well resolved ionophoretically and their different mobilities corresponded exactly

with those obtained from an alkaline digest of yeast ribonucleic acid. The quantitative results obtained by ionophoresis were not well reproducible between different paper strips and will not be given.

Pentose sugars. Spots corresponding to a hexose, arabinose, and ribose were obtained on chromatograms of the hydrolysates of the mixed crude nucleic acids.

Deoxypentose nucleic acid fractions. The DNA preparations contained about 20–60% DNA, estimated by their phosphorus contents, assuming that DNA contains 10% P, and the bases accounted for most of this phosphorus. The best preparations were obtained from kale and clover. The preparations and their base composition appeared to be well reproducible. 5-Methylcytosine was present in all DNA fractions and its identity was confirmed by its ultraviolet absorption spectrum, which conformed exactly to that given by Wyatt (1951*a*) with a trough at 242 mμ. and peak at 283 mμ. The identities of all the separated bases and nucleotides were confirmed from the absorption curves of the eluates.

These fractions were suitable for the analytical investigation of the nucleic acids and might well be suitable for some physiological studies, such as those using isotopic tracers. The isolation of native, highly polymerized nucleic acids from leaves still presents a very difficult problem, which would appear to need a radically new approach.

The analytical results (Table 1) gave no evidence that the base compositions of the nucleic acid fractions had been altered by enzyme action during isolation, although ribonuclease activity has been found in tobacco leaves (Holden, 1952; Parker, 1952) and also some deoxyribonuclease activity (Holden, 1952).

Although the nucleic acids were not isolated in a pure state, our analysis of wheat embryo DNA compares quite closely with the analysis of Wyatt (1951*b*) of a pure preparation obtained by another method. This would appear to confirm the validity of the method in that the DNA obtained is representative of the DNA in the cell, with no fractionation having occurred such as was reported by Chargaff, Crampton & Lipshitz (1953) and by Brown & Watson (1953).

Table 1. *Purine and pyrimidine composition of PNA and DNA fractions from plants. Molar ratios calculated to a total of* 100·0

	Beet	Bracken	Clover	Kale	Rye	Wheat germ	Wheat germ (Wyatt)
			DNA				
Adenine	27·7	28·3	29·8	27·5	27·5	25·4	26·4
Guanine	25·5	19·8	20·9	24·8	19·9	22·8	22·5
Cytosine	12·3	18·2	15·5	16·6	20·1	18·2	17·3
5-Methylcytosine	4·4	5·6	4·8	3·6	10·0	5·6	5·75
Thymine	30·1	28·4	28·5	27·5	24·5	28·2	27·0
% P accounted for by bases	79	78	88	82	82	90	—
No. of preparations	1	4	3	1	3	3	—
			PNA				
Adenine	—	25·5	26·4	—	24·2	21·5	—
Guanine	—	26·4	26·0	—	29·0	26·7	—
Cytosine	—	25·4	18·5	—	20·6	24·4	—
Uracil	—	22·6	29·2	—	26·0	27·4	—
% P accounted for by bases	—	58	90	—	62	80	—
No. of preparations	—	4	3	—	3	3	—

Deoxypentoses. A characteristic blue colour was obtained with the Dische test for deoxypentoses with the DNA preparations from clover, kale and wheat embryo. Bracken, beet and rye grass yielded an equivocal purple-brown colour, but when 3 vol. of ethanol were added, this was resolved into a pale blue solution and a brown precipitate.

DISCUSSION

With the procedure developed, it was possible to obtain both PNA and DNA fractions from the leaves of a wide range of plants, so wide a range that it might seem reasonable to suppose that the procedure is generally applicable to plant tissues.

The base compositions of the different PNA fractions show no remarkable features, not differing greatly from the PNA from other sources (e.g. Davidson & Smellie, 1952) or from the PNA of some plant viruses (Dorner & Knight, 1953; Markham & Smith, 1951).

The DNA preparations are all characterized by a relatively high content of 5-methylcytosine. Previously, the highest reported content of this base in a DNA preparation was from wheat embryo (Laland *et al.* 1952; Wyatt, 1951*b*).

The molecular equality between adenine and thymine and between guanine and cytosine plus 5-methylcytosine, required by the Watson & Crick (1953) theory of DNA structure, involving specific

pairing of bases, is shown generally by our results. The base composition of all the DNA preparations correspond to the excess adenine–thymine (AT) class and show surprisingly little variation over a wide range of plant types. In this respect the plant DNA preparations resemble the AT pattern of the DNA from many animal tissues (Laland *et al.* 1952; Wyatt, 1951*a*) except for their consistently higher 5-methylcytosine content.

In contrast, DNA preparations from bacterial sources exhibit a wide range of compositions, from the extreme AT types to the extreme guanine–cytosine (GC) class with the apparent absence of 5-methylcytosine (Laland *et al.* 1952; Sherratt & Thomas, 1953; Smith & Wyatt, 1951).

SUMMARY

1. The mixed nucleic acids were extracted from five species of leaves by 10% (w/v) NaCl at 100°. After incubation with alkali, PNA and DNA fractions were isolated.

2. Quantitative analyses for purines and pyrimidines of the nucleic acid fractions were made. The PNA fractions had no unusual features. The DNA fractions were all in the excess adenine–thymine class and had a relatively high content of 5-methylcytosine.

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The Amino Acid Content of the Proteins of Barley Grains

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In his classical researches on the cereal proteins, Osborne (1895*a*) found that barley grains contained four distinct classes of protein. Three of these, albumin, globulin and prolamin (hordein), he separated from the grain and showed by elementary analyses to be distinct and reproducible fractions of the protein reserves. The fourth type of protein, glutelin, was only partially soluble in dilute acids and alkalis and Osborne was unable to obtain reasonably pure and reproducible preparations of it. Subsequently, Larmour (1927) and Csonka & Jones (1929) described preparations of barley glutelin (hordenin), but in neither case was the reproducibility of the fraction fully demonstrated.

More recent studies by means of the ultracentrifuge and by electrophoresis have extended our knowledge of the homogeneity of some of the protein fractions. For example, Quensel & Svedberg (1938) found that, while hordein behaved