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A RAPID METHOD FOR THE EXTRACTION AND IDENTIFICATION OF RNA IN PLANT LEAF TISSUE*

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Abstract—A modified method featuring shorter exposure periods to alkali, column chromatography, together with acid–alcohol precipitation to remove interfering substances, has been used to extract and identify RNA from leaf tissue of deciduous, woody plants. This method differs from that proposed by Diener and Lasheen in that a 7-hr exposure time to 0·33 N KOH is adequate to hydrolyze the RNA into acid-soluble nucleotides. Although Dowex AG 1–8X will remove DNA and other interfering substances, it is possible to precipitate other unidentified substances by adjusting the pH of the alkaline hydrolysate to 5·0 and adding 1 vol. ethanol with 2 drops of 1·0 M $MgCl_2$. The supernatant is then passed over the resin without further pH adjustment. RNA is preferentially eluted with 0·12 N HCl.

INTRODUCTION

METHODS routinely used in extracting and identifying ribonucleic acid (RNA) constituents from animal tissues and bacteria usually give unsatisfactory results when applied to plant tissues. Consequently it has been necessary to develop specific modifications whenever leaf tissues of higher plants are examined for their nucleic acid components. Ingle[1] and Nieman and Poulsen[2] examined several plant species and concluded that the Schmidt–Thannhauser procedure[3] was the only satisfactory method for determining RNA in plant tissues. Zschiele and Murray[4] proposed a method using four extractions with 0·55 M NaCl. RNA was precipitated by the addition of an equal volume of ethanol and storage overnight at 2°. Diener and Lasheen[5] described a modification of the Schmidt–Thannhauser method which gives good results for leaf tissue, but the exposure time to alkali is unnecessarily long, and neutralization prior to passage over the column may be ignored.

Previous investigations[6] with apple leaf tissue indicated that 7 hr exposure to 0·33 N KOH was sufficient to hydrolyze the RNA into acid-soluble nucleotides. Other studies[7] showed that RNA was preferentially eluted with 0·12 N HCl from a mixture of nucleic acids adsorbed on Dowex AG 1–8X chloride-form resin. Present investigations were designed to determine if the Diener–Lasheen (D–L) method could be modified to combine the advantages of column separation and shortened hydrolysis time to give a more rapid method for determining RNA in plant leaf tissue.

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[1] J. INGLE, *Phytochem.* **2**, 353 (1963).
[2] R. H. NIEMAN and L. L. POULSEN, *Plant Physiol.* **31**, 38 (1963).
[3] G. SCHMIDT and S. J. THANNHAUSER, *J. Biol. Chem.* **161**, 83 (1945).
[4] F. P. ZSCHEILE and H. C. MURRAY, *Plant Physiol.* **38**, 133 (1963).
[5] T. O. DIENER and A. M. LASHEEN, *Proc. Am. Soc. Hort. Sci.* **75**, 195 (1960).
[6] D. F. MILLIKAN and E. E. PICKETT, *Nature*, **200**, 470 (1963).
[7] B. ZAWADZKA and D. F. MILLIKAN (unpublished data).

RESULTS

Separation of RNA by Dowex AG 1–8X

Early investigations in our laboratory indicated that mixtures of RNA and DNA are resolved by adsorption and elution from the column of Dowex AG 1–8X resin. RNA is eluted with 0·12 N HCl whereas DNA will not move unless more concentrated acid, such as 4·0 N HCl, is used. Repeatedly, complete recoveries of RNA were obtained both on mixtures containing RNA and leaf nucleic acids. Results of a typical experiment are shown in Fig. 1 wherein a mixture containing known amounts of RNA and DNA after column purification give the same absorbance at 260 mμ as that given by the same amount of RNA alone treated

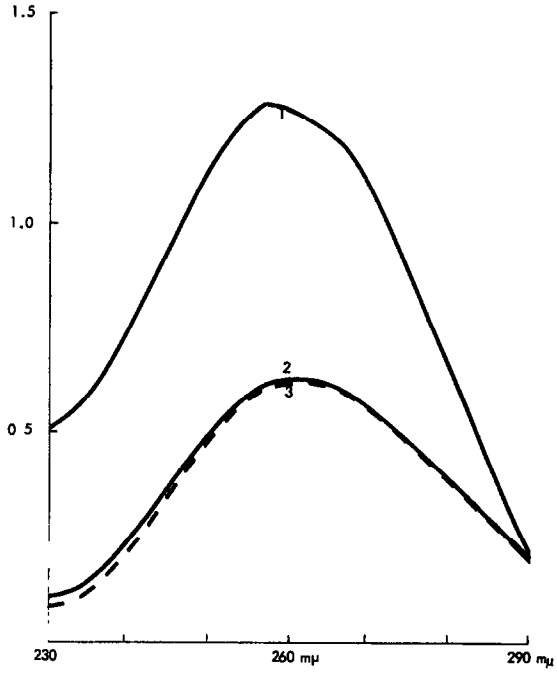

FIG. 1. EFFECTIVENESS OF DOWEX 1–8X IN SEPARATING RNA FROM MIXTURES OF NUCLEIC ACID.

Curve 1. Optical density of solution containing 26·7 μg/ml yeast RNA and 26·7 μg/ml sperm DNA before ion exchange separation.

Curve 2. Optical density of 0·12 N HCl eluates from resin on which above mixture had been adsorbed.

Curve 3. Optical density of 0·12 N HCl eluates from resin on which 26·7 mg/ml yeast RNA had been adsorbed.

similarly. Other experiments wherein solutions containing known amounts of DNA were added to leaf nucleic acid extracts and passed over the column gave curves identical with those containing the same amounts of leaf nucleic acid but no added DNA.

These data indicated that removal of DNA by acid precipitation is not necessary and therefore considerable time was spent attempting to separate RNA by column chromatography. In these studies the alkaline hydrolysate containing the nucleotides was neutralized and passed through the column. Eluates from these treatments always gave greater absorbancies than comparable eluates from duplicates treated according to Diener and Lasheen. However, the markedly lower maxima–minima ratios (approaching 1) found for the neutralized material indicated that the greater absorbancy at 260 mμ was due to substances other than

nucleic acid. Further studies on separating RNA from the neutralized alkaline hydrolysate were discontinued.

Effect of Hydrolysis Time on RNA Recovery

Previous work indicated that an alkali exposure period of 7 hr was adequate to hydrolyze the RNA into acid-soluble nucleotides. This work had been done on apple tissue and therefore was extended to *Vitis* (grape) and *Prunus* (cherry), plant materials presently under investigation in our laboratory. RNA determinations were made on both tissues at different time intervals following the D–L method. Triplicate sample of grape showed an average of 7551 μg/g dry wt. RNA for the 7 hr hydrolysate as contrasted to 7623 μg/g RNA for the 18-hr hydrolysate. Similarly, the RNA content for cherry leaf tissue was found to be 7·46 and 7·42 mg/g for the 7-hr and 18-hr hydrolysates, respectively. As had been found in apple, longer periods of hydrolysis failed to yield any additional RNA while shorter periods than 7-hr were inadequate.

Effect of pH on RNA Recovery

The absence of interfering substances in the eluates from the neutralized hydrolysate indicated that these substances were precipitated by the D–L method and, if not removed, were eluted with the RNA fraction. Extensive washing of the column with double distilled

TABLE 1. EFFECT OF pH UPON RECOVERY OF RNA FROM APPLE LEAF TISSUE
(7 hr exposure, 0·33 N KOH at 37°)

Adj. pH of Hydrolysate	RNA mg/g dry wt*	
	Expt. I	Expt. II
4·0†	6·97	7·01
4·0	6·89	
4·5	7·09	7·11
5·0	7·10	7·09
5·5	6·88	7·10

* Average of three determinations.
† pH adjusted to 7·0 prior to adsorption; in other cases pH not adjusted.

water after adsorption failed to remove these substances and weaker acid washes eluted some of the nucleotides. Since it appeared necessary to precipitate these interfering substances with an acid treatment, studies were made to determine the effective pH range. Yeast RNA was hydrolyzed and the pH adjusted to a range of 4·0 and 8·0, adsorbed on the column and quantitatively recovered by elution with 0·12 N HCl. This indicated that the pH of the solution containing RNA was not critical and the subsequent neutralization was not essential.

Similar studies were extended to apple leaf tissue. After a 7-hr exposure to 0·33 N KOH, one volume of ethanol and 2 drops of 1 M $MgCl_2$ were added to the supernatant and the pH adjusted as desired. Following storage in the cold for at least 2 hr, the supernatants were collected and adjusted to pH 7·0 or left unadjusted. These data are given in Table 1.

These data indicated that a single pH adjustment with perchloric acid and precipitation encouraged by the addition of ethanol and $MgCl_2$ resulted in recoveries of RNA equal to

treatments following the D–L method. Neutralization prior to adsorption on the column is not necessary and, although there is considerable latitude in the initial pH, more consistent results are obtained by adjustment of pH to around 5·0.

Similar data were obtained using grape and cherry tissue. In a typical experiment, triplicate samples of grape tissue treated according to Diener and Lasheen resulted in recoveries of 7·41 mg/g dry weight RNA. Companion samples hydrolyzed simultaneously, but adjusted to pH 5·1 and otherwise treated identically to those by the D–L method resulted in recoveries averaging 7·36 mg/g RNA. For cherry leaf tissue the D–L method gave recoveries of 8·23 mg/g RNA as contrasted to 8·33 mg/g for pH 5·0 treatment.

DISCUSSION

The present modification of the alkaline hydrolysis method for solubilizing RNA to spectrophotometrically identifiable nucleotides uses a shortened hydrolysis time and Dowex AG 1–8X resin is used to remove DNA from a mixture of nucleic acids. In addition, since the resin effectively removes all DNA, it is not necessary to precipitate this nucleic acid by lowering the pH to 4·0. It is, however, necessary to add one volume of ethanol and lower the pH to about 5·0 to remove substances in woody plant tissues that absorb in the same region of the u.v. as RNA. Neutralization of this solution containing the nucleic acids is not necessary since RNA will adsorb from solutions with pH values ranging from 4·0 to 8·0. RNA is eluted with three 5-ml aliquots of 0·12 N HCl.

EXPERIMENTAL

Preparation of Leaf Tissue

Methods of preparing leaf tissue have been described previously.[8] In general, we have found it convenient to use leaf tissue lyophilized in a manner similar to that used by Levitt[9] for potato tissue (frozen in liquid air, dried over alumina and ground in a Wiley mill using 60 mesh screen). Dried leaf tissue (500 mg) is then thoroughly washed in a 50-ml centrifuge tube with the following mixed solvents in a stepwise procedure (25-ml aliquots of each) acetone: water: methanol (80:16:4) 2 X; 0·05 M formic acid in methanol; 5% perchloric acid in 80% ethanol; ethanol: ether (1:1); and ethyl ether. This is allowed to dry in air before use.

Extraction of Nucleic Acids

Fifty mg of air-dried extracted leaf tissue is weighed into 50-ml centrifuge tubes. This is wetted with about ½ ml ethanol and 5 ml 0·33 N KOH added. Tubes then are incubated for 7 hr at 37°, centrifuged, and the supernatant drawn off. The precipitate is washed with 5 ml water made to pH 8·5 with NH_4OH and again centrifuged. The supernatants are combined, the pH adjusted to 5·0 and transferred to a 50-ml centrifuge tube along with 1 vol. 95% ethanol and 2 drops of 1 M $MgCl_2$. The tubes are left overnight in a refrigerator (at least 2 hr), centrifuged and the supernatants decanted off. The precipitates are washed with a few ml of 50% ethanol and centrifuged. The supernatants are then combined and made to 25 ml vol.

Dowes resin is treated with 0·5 N NaOH, water, 1·0 N HCl and then with water until neutral. One gram of this resin is used in a small glass column. After removal of air bubbles and drainage of water, 10 ml of the nucleic acid sample solution is added to the column. After this has drained the resin is washed with 5 ml double distilled water and allowed to drain. RNA is then eluted with three 5-ml aliquots of 0·12 N HCl.

[8] R. W. BROWN, B. ZAWADZKA and D. F. MILLIKAN, *Phytochem.* **2**, 221 (1963).
[9] J. LEVITT, *Physiol. Plant.* **5**, 470 (1952).