Phytochemistry, 1964, Vol. 3, pp. 693 to 699. Pergamon Press Ltd. Printed in England

STUDIES ON THE NUCLEIC ACIDS OF *OENOTHERA ORGANENSIS* AND *ZEA MAYS**

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(*Received* 29 *August* 1963)

Abstract—Methods are described for the isolation and characterization of the nucleic acids of *Oenothera organensis* pollen. Alkaline hydrolysis, followed by paper chromatography served to characterize the ribonucleic acid. However, the low concentration of deoxyribonucleic acid and the large amounts of interfering polysaccharide material required application of special techniques and column chromatographic methods. In addition, a method is described for the isolation of "native" deoxyribonucleic acid from root tips of *Zea mays* and *Oenothera*.

INTRODUCTION

Oenothera organensis has been extensively used in genetic studies and in research related to the nature of the pollen-style incompatability reaction.[1-4] Reports on the nucleic acids of Oenothera are however totally lacking. Further, there has been only a single report of the characterization of the nucleic acids of plant pollen[5] and the method reported required large quantities of pollen. In the present study, methods are described for the isolation and characterization of the nucleic acids of Oenothera pollen and for the preparation of a "native" DNA fraction from the roots of Oenothera and *Zea mays*.

RESULTS

Analysis of Standard Samples

Table 1 presents data on the recovery and base ratios of the hydrolysis products obtained from 10 mg yeast RNA and 4 mg salmon sperm DNA. The molar ratio of the four nitrogenous constituents of RNA as determined by the methods here described compares well with those previously reported for yeast RNA.[6] The base ratios for salmon sperm RNA are also in agreement with those reported by Chargaff *et al.*[7] Over-all base recoveries are approximately 80–90%.

Analysis of Plant Samples

As can be seen in Fig. 1, separation of the purines and pyrimidines of pollen DNA was obtained by column chromatography on Dowex 50-X2. The low base-line between peaks

* This work was supported, in part, by the Michigan Agricultural Experiment Station, Journal Article 3329, and by grants from the U.S. Public Health Service (AM 05916) and the National Science Foundation (G 22069) to Robert S. Bandurski.

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TABLE 1. RECOVERY OF BASE COMPONENTS FROM YEAST RNA BY PAPER CHROMATOGRAPHY AND FROM SALMON SPERM DNA BY COLUMN CHROMATOGRAPHY

	Moles per 100 g atom phosphorus	
	RNA	DNA
G	24·1	18·4
A	20·9	24·1
C	16·8	15·7
U* or T†	20·2*	23·9†
% Recovery	82·0	82·0

G—guanine, A—adenine, C—cytosine, U—uracil, and T—thymine.

and the symmetry of the peaks indicates that interference by u.v. absorbing substances has been eliminated. The absence of a uracil peak, which normally emerges before thymine, indicates the absence of RNA contamination of the DNA fraction. The small unlabeled peak at about 25 ml has a non-typical purine spectrum and has not been further studied. Data on the base composition of the RNA and DNA, together with the previously reported values of Wan-

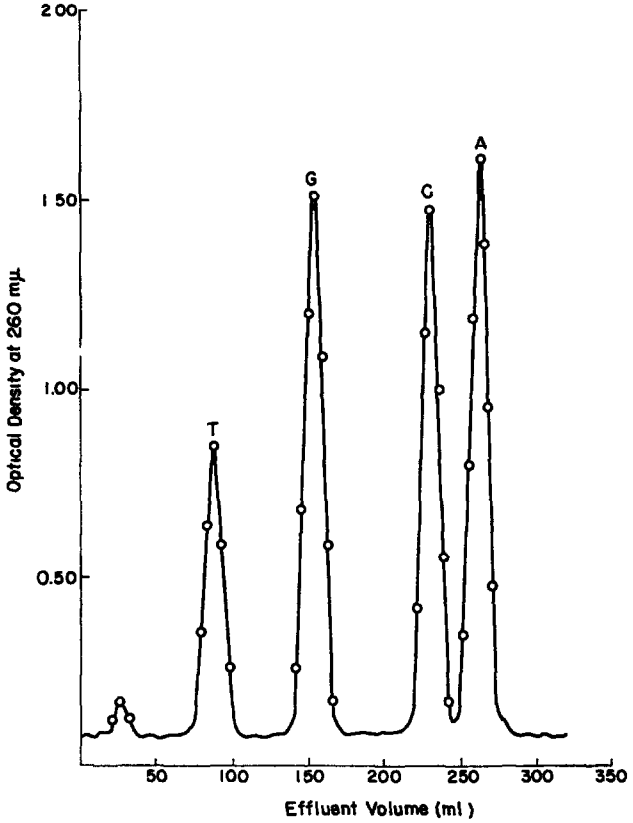

FIG. 1. COLUMN CHROMATOGRAPHIC SEPARATION OF PURINE AND PYRIMIDINE BASES ISOLATED FROM THE DNA FRACTION OF 4·5 g OF OENOTHERA POLLEN.
Explanation in text.

juschin *et al.*[5] are given in Table 2. In the case of RNA, where the absence of polysaccharide impurities permitted use of paper chromatography, the values obtained by column chromatography (G = 4·8, A = 4·3, C = 3·2, and U = 3·4 mμmoles/mg dry wt. pollen) agree with those obtained by paper chromatography. The ratios, A/T, Pu–Py, and G/C of Oenothera pollen DNA were found to be 0·91, 1·0, and 1·1, respectively. Whether the deviations from unity[8] are significant is not certain. The A/T and G/C ratios for Oenothera pollen DNA are closer to unity than those for Corylus pollen.[5]

TABLE 2. NUCLEIC ACID CONTENT OF OENOTHERA AND CORYLUS POLLEN

		G	A	C	U	T
Oenothera RNA	mμmoles/mg dry wt.	4·4	3·9	3·0	3·1	
	molar ratios	30·6	27·1	20·8	21·5	
Oenothera DNA	mμmoles/mg dry wt.	0·31	0·21	0·29		0·23
	molar ratios	29·8	20·2	27·9		22·1
Corylus DNA[5]	molar ratios	31·5	26·5	17·3		24·7

The percentage of RNA and DNA in Oenothera pollen on a dry weight basis were found to be 0·64 and 0·04, respectively. It was interesting to compare the present data with those previously reported by Ogur and Rosen[9] (4·7% RNA and 0·74% DNA in corn root-tips), Chang[10] (4·2% RNA and 0·71% DNA in barley embryo), and Monselise[11] (0·61% RNA and 0·13% DNA in orange leaf).

On a dry weight basis, the contents of RNA and DNA progressively decrease in the order: root, embryo, leaf, and pollen. Our value of 0·6% for pollen RNA (dry weight basis) is within the range of 0·6–1·2% reported.[5]

Properties and Yield of a "Native" DNA from Root Tissues

As can be seen in Fig. 2, the DNA preparation showed maximum and minimum u.v. absorption at 260 and 230 mμ, respectively. The ratio of maximum to minimum was 2·1. The material here described was from corn root-tips, but the preparation from Oenothera root-tips was similar.

The E(P) value of this DNA was 6100 in 10^{-3}M NaCl at pH 6·3. When however it was dialyzed against 10^{-5}M NaCl for 2 days, kept at room temperature overnight, and brought back to 10^{-3}M NaCl again, the value of E(P) was found to be 7980 at pH 6·3. Thomas[12] has shown that exposure of DNA to low sodium chloride concentration ($< 10^{-3}$M) results in irreversible changes in its structure. Thus the change in E(P) observed indicates that the DNA preparation, as isolated, was not denatured.[13,14]

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Hydrolysis of 325 μg of the DNA preparation with 12 M perchloric acid and separation of the bases by paper chromatography disclosed the presence of guanine, adenine, cytosine, and thymine, but no uracil. Thus there was no apparent contamination of the DNA preparation with RNA. Protein content of the DNA preparation was determined by the Folin-phenol method of Lowry *et al.*[15] Protein contamination was found to be 27 μg/mg DNA.

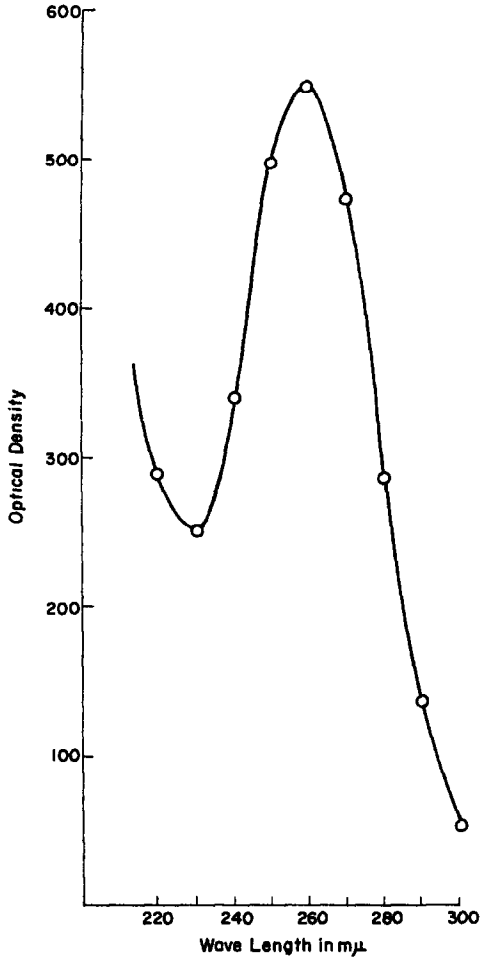

FIG. 2. ACTION SPECTRUM OF THE "NATIVE" DNA EXTRACTED FROM CORN ROOT-TIPS. THE SPECTRUM WAS MADE ON DNA FRACTION IN 10^{-3} M NaCl SOLUTION.
Discussed in text.

A total of 1·3 mg DNA was obtained from 4 g of fresh root tips equivalent to 0·16% of the dry weight. Thus the yield of DNA by this mild extraction procedure was about 20% of that obtainable by strong perchloric acid extraction.[9]

DISCUSSION

In the present report, methods are described for the characterization of RNA and DNA from plant pollen and also for the isolation of an apparently native DNA from root tips of

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Zea mays and Oenothera. The problems encountered are due mainly to the low concentrations of nucleic acids in the plant tissues studied and to the presence of interfering substances. For example, in the case of pollen, the low content of DNA[16] and the presence of large amounts of polysaccharide material make extraction and estimation of base composition difficult. No widely applicable chemical methods of removal of polysaccharides has been reported.[17] Electrophoretic methods, while suitable, result in losses (possibly non-representative) and yield very limited amounts of sample. All of our attempts to precipitate nucleic acids from pollen extracts, and thus separate them from polysaccharides, failed. The methods tried included precipitation with ethanol;[16,18] dilution to low NaCl concentration;[19] acidification of the alkaline digest;[20] use of egg albumin[21] and use of precipitants such as streptomycin[22] or indium chloride.[23]

Although many attempts were made to achieve separation of RNA and DNA by the perchloric acid extraction method,[9] cross contamination between RNA and DNA was always observed. The methods of differential extraction, here described, yield RNA fractions which are free of thymine and DNA fractions free of uracil. Further the RNA fraction was then sufficiently free of impurities so that paper chromatographic methods could be applied to separate the base components. The DNA fraction however still had sufficient carbohydrate impurity so that column chromatographic methods had to be employed.

In the preparation of polymerized DNA from root tips, considerations of yield were not as important. Thus, unlike the methods described above for quantitative extraction and estimation of the base components, it was possible to selectively precipitate the DNA with streptomycin sulfate.[22] Following removal of the streptomycin by dialysis, DNA of sufficiently high purity for further physiological experimentation is obtained. In the present procedure an initial addition of 18 μmoles streptomycin to 15 ml DNA preparation precipitated over 95% of the DNA, while the second addition showed only opalescence, and the third and fourth produced no turbidity. Most of the RNA had been removed from the tissue homogenate by the prior extraction with 0·14 M NaCl solution.

EXPERIMENTAL

Isolation and Hydrolysis of Nucleic Acids from Pollen

The procedure used in determining the base composition of RNA and DNA of pollen may be divided into the following six steps.

1. *Preparation of fat-free powder.* Four grams of frozen pollen were boiled in 95% ethanol for 1·5 min, collected by centrifugation at 13,000 *g* for 20 min and then washed repeatedly with 95% ethanol until the supernatant fluid following centrifugation, was colorless. The resultant residue was suspended in 0·05 M sodium citrate and homogenized for 5 min in a conical glass homogenizer. The suspension, thus obtained, was centrifuged and the pellet dispersed in cold 70% ethanol and then reground for 5 min with a hand mortar. The homogenate was next centrifuged and the resultant pellet washed with 50% ethanol containing acetate at pH 4·5, then with 0·2 M $HClO_4$, and finally with an alcohol series (70, 80, 95,

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and 100%). The material then was suspended in a mixture of ethanol:ethyl ether (2:1, by volume), and allowed to stand for 30 min at 50°. The suspension was centrifuged and the residue, after two more washings with ether, dried in vacuo.

2. *Preparation of sodium nucleate.* The defatted sample was dispersed in a Pyrex ignition tube together with a minimal volume of 10%NaCl containing 0·02 M tris buffer at pH 7·5. The tightly stoppered tube was placed in a boiling water bath and after 30 min cooled, centrifuged and the supernatant fluid retained. This procedure was repeated three times, the extracts pooled, dialyzed against water and then lyophilized to obtain the dry sodium nucleate.[24]

3. *Separation of* RNA *and* DNA. Sodium nucleate was hydrolyzed with 0·5 M KOH at 37° for 18 hr[25] and insoluble substances removed by centrifugation. The insoluble material was extracted three times with alkali. The combined supernatant solutions were then dialyzed against a weak alkaline solution (10^{-3} M) until the dialysate had a low and constant optical density.[26] The sample retained in the bag was saved for the determination of DNA composition and the dialysate used for RNA characterization.

4. *Resolution of* RNA *components.* The dialyzed ribonucleotide sample was lyophilized, and hydrolyzed with 72% perchloric acid for 160 min in a water bath at 100°. After cooling, the mixture was diluted with 5 vol. cold distilled water and centrifuged. The resulting clear supernatant solution was decanted, neutralized with K_2CO_3, and then filtered. The residue was dissolved in a small flask containing a volume of about 3 ml 0·1 M HCl, and filtered after agitation for 60 min at room temperature. The two successive filtrates were combined and dried in a flash evaporator. The dried base mixture was dissolved in a small volume of 0·1 M HCl and 20-μl aliquots of the sample solution applied to Whatman No. 1 paper and developed with a solvent containing isopropanol:HCl:H_2O (170:41:39).[27] The separated bases were eluted with 0·1 M HCl and the quantity of each base determined spectrophotometrically.[28]

5. *Resolution of* DNA *components.* The method is essentially that of Crampton *et al.*[29] with necessary modifications. Separation of the purines and pyrimidines of DNA was attained with a column of Dowex 50-X2, Na^+ form, 200–400 mesh by gradient elution with 0·2–1·0 M citrate buffer at pH 4·0. Additional conditions were: column, 0·9 × 60 cm; temperature, 25–32°; flow rate, 5–9 ml/hr; volume of collected fractions, 2 ml; mixing bottle, 125 ml; buffer reservoir, 1 l.

The sample saved for the analysis of DNA in step 3 was hydrolyzed as described for RNA. The mixture of four bases was dissolved in about 2 ml 0·1 M HCl, adjusted to pH 4 with 0·1 M NH_4OH, diluted to 4-ml sample size with 0·2 M citrate buffer, pH 4·0, and loaded on the column. The absorbancy of each fraction collected was determined at 260 mμ against a water blank. The fractions comprising each peak were pooled and the quantity of base determined spectrophotometrically.

6. *Base identification by paper chromatography.* The pooled samples from step 5 were desalted by loading onto an anion exchanger (10 × 0·8 cm, Dowex 1-2X, COO^- form, 200–400 mesh). The bases were eluted with 1 M HCl. The base-containing fractions were pooled and adjusted to pH 8, with 1 M NH_4OH. The samples were passed through a column of cation exchanger (10 × 0·8 cm, Dowex 50-X2, H^+ form, 200–400 mesh). The desalted

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samples were then subjected to paper chromatography. Each component of the DNA was identified by its R_f value and u.v. absorption as compared with standard samples.

Isolation of "Native" DNA from Roots

All processes were performed in the cold room at 4°. Fresh root-tips (4 g) were collected in 70% ethanol and stored at −20°. Corn root-tips were collected from 4-day-old seedlings while Oenothera root-tips were from young plants grown in a nutrient fog box (Oster Portable Humidifier obtained from John Oster Manufacturing Co., Milwaukee, Wisc.)

The material was homogenized in 70% ethanol with a mortar and pestle for 25 min. Microscopic examination of the homogenate revealed few unbroken cells. The mixture was centrifuged at 13,000 *g* for 20 min and the residue washed successively with 70, 95 and 100% ethanol. The material then was suspended in light petroleum ether (b.p. 60°) for 3 hr and again centrifuged. The sediment was stored at 1° overnight.

The defatted powder was then washed five times with a solution containing 0·14 M NaCl, 0·01 M sodium citrate, and 0·02 M Tris-Cl at pH 7. The sample was then extracted with a minimum quantity of 10% NaCl in 0·02 M tris-Cl at pH 8·5 with stirring, for 35 hr. The resultant suspension was centrifuged at 25,000 *g* for 1 hr and the supernatant liquid dialyzed against 2 l. of solution containing 10^{-3} M NaCl[12, 30] and 0·01 M sodium citrate. This solution was changed 3 times at intervals of 6 hr.

DNA in the clear fluid recovered from the dialysis bag was precipitated by the addition of streptomycin sulfate[21] using 0·3 ml 0·1 M streptomycin to 20 ml of DNA extract. The mixture was kept in the cold room overnight. The fine precipitate was removed by centrifugation at 144,000 *g* for 1 hr. The sediment, upon solution in 0·2 M NaCl, was adjusted to 1·7 M NaCl by the addition of additional salt and to pH 7·5 with 0·1 M sodium bicarbonate. The mixture was agitated by use of a magnetic stirrer overnight. The resulting almost clear solution was centrifuged at 13,000 *g* for 30 min. The supernatant fluid was then subjected to the Sevag procedure[31] until no gelatinous interface could be observed (seven extractions). The water phase, containing the DNA, was dialyzed against an appropriate buffer.

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