Ribonucleic Acids from Barley Leaves

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1. The total RNA and the RNA present in 27 000g pellet (probably composed of chloroplasts, nuclei and mitochondria) and in 27 000g supernatant (probably composed of microsomes and soluble proteins) fractions (separated by centrifugation at 27 000g of a leaf homogenate prepared in 0.5M-sucrose-0.02M-tris-HCl, pH 7.6) of barley leaves were extracted by phenol-sodium lauryl sulphate and their elution profiles on Sephadex G-200 and on ECTEOLA-cellulose anion-exchanger were examined and their nucleotide compositions and the melting curves were determined. 2. The pellet and the supernatant fractions contained respectively about 55% and 20% of the total RNA, whereas 25% of the total RNA was lost during homogenization of the leaf tissue with sucrose-buffer. 3. The total RNA or the RNA from pellet or supernatant fractions, which by its behaviour on Sephadex G-200 columns was found to be predominantly of high molecular weight (i.e. of ribosomal origin), produced about 13 peaks on ECTEOLA-cellulose columns. The RNA species in the pellet and supernatant fractions probably resembled each other in molecular size or secondary structure or both. However, they were present in relatively different amounts in these fractions. 4. The T_m (i.e. the temperature at which 50% of the maximal increase in extinction had occurred) of total RNA and of RNA from pellet fraction was 64.5° whereas T_m of RNA from the supernatant fraction was 73°. The total RNA and the RNA from pellet fraction also resembled each other in nucleotide composition, and the RNA from the supernatant fraction in accordance with its high T_m had a high GMP + CMP content.

The occurrence of various species of RNA in living cells has been demonstrated by fractionation of RNA by density-gradient centrifugation (Montagnier & Bellamy, 1964; Pollard, 1964; Sporn & Dingman, 1963), gel electrophoresis (Bachvaroff & McMaster, 1964), countercurrent distribution (Goldstein, Bennett & Craig, 1964) and by chromatography on methylated albumin-coated kieselguhr (Mandell & Hershey, 1960; Spiegelman & Hayashi, 1963), protamine-coated kieselguhr (Legault-Démare, Rebeyrotte, Leprieur & Roussaux, 1964), ECTEOLA-cellulose anion-exchanger (Bloemendal, Bosch & Sluyser, 1960; Bosch, Bloemendal & Sluyser, 1960; Goldthwait & Kerr, 1962) or hydroxymethyluracilcellulose (Barber & Jones, 1964) columns. Such studies have, however, been mostly carried out with RNA of bacterial or animal origin and, as far as I am aware, little work on the fractionation of RNA from plants has been done. The present paper therefore deals with the fractionation of total RNA of green barley leaves on ECTEOLA-cellulose anion-exchanger and the study of nucleotide composition, shape of the melting curve and other properties of total RNA. In

addition, RNA present in the 27 000g pellet (probably composed of chloroplasts, nuclei and mitochondria) and in the 27 000g supernatant (probably composed of microsomes and soluble proteins) of the leaf homogenate was studied similarly.

MATERIALS AND METHODS

Barley (*Hordeum vulgare* L. var. Wolfe) plants were grown in soil in pots on greenhouse benches. When the plants were 7-8 days old the first seedling leaves were excised and used for the extraction of RNA.

Extraction of total RNA. Leaves (15g.) were homogenized for 3 min. in a chilled Waring Blendor with 100 ml. of cold 0.02M-tris-HCl buffer containing 0.5% of sodium lauryl sulphate, pH 8.0, and 100 ml. of phenol saturated with water. The homogenate was transferred to a glass-stoppered bottle and shaken for 1 hr. at 23° in a wrist-action shaker. The homogenate was subsequently centrifuged at 16 000g and 2° for 10 min. The upper aqueous layer was carefully pipetted off and cleared of fine fibres by filtering through a peg of glass wool. The RNA in the aqueous layer was precipitated by adding 2 vol. of 95% (v/v) ethanol and 0.1 vol. of 20% (w/v) potassium acetate, pH 5.0, and leaving the contents overnight at -10°. The RNA precipi-

tate was removed by centrifugation (16000g for 5 min. at 2°), dissolved in 0.02M-tris-HCl buffer, pH 7.6, and reprecipitated by adding 2 vol. of cold 95% (v/v) ethanol and letting the contents stand for 3-4 hr. at -10°. The RNA was collected by centrifugation in the cold, dissolved in 15 ml. of buffer (0.02M-tris-HCl, pH 7.6, containing 1mM-MgCl₂) and incubated with 30 µg. of deoxyribonuclease (twice crystallized, purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) at 37° for 20 min. After incubation the mixture was chilled and the RNA precipitated by adding 2 vol. of cold ethanol and letting the contents stand for 4-5 hr. at -10°. The final RNA precipitate was removed by centrifugation in the cold, dissolved in 15-18 ml. of 0.02M-tris-HCl, pH 7.6, put in a seamless cellulose bag (Fisher Scientific Co.) and dialysed for 24 hr. at 2° against two changes of 1 l. of 0.02M-tris-HCl, pH 7.6. The dialysed solution was clarified by centrifugation for 15 min. at 20000g at 0° and the clear solution was made up to 25 ml. with tris buffer. Normally 8 ml. of this solution was used immediately for chromatography on ECTEOLA-cellulose exchanger, and 1 ml. diluted to 15-20 ml. with tris buffer was used for determination of RNA concentration (by measurement of extinction), melting curves and for the study of other properties of RNA. The remaining RNA solution was stored at -20° for future nucleotide analysis and other purposes.

Separation of 27000g pellet and 27000g supernatant fractions and isolation of RNA from these fractions. Leaves (15g.) were homogenized for 2-3 min. in a chilled Waring Blendor with 0.02M-tris-HCl, pH 7.6, containing 0.5M-sucrose and 0.1% (w/v) of bentonite (purified according to the procedure of Fraenkel-Conrat, Singer & Tsugita, 1961). The homogenate was squeezed through six layers of cheese-cloth and the filtrate was centrifuged at 27000g and 0° for 30 min. The 27000g pellet was extracted with 50 ml. of 0.02M-tris, containing 0.5% of sodium lauryl sulphate, pH 8.0, and 50 ml. of phenol saturated with water, by shaking for 1 hr. at 23°. The 27000g supernatant (about 100 ml.) was mixed with 100 ml. of phenol saturated with water containing 0.5% of sodium lauryl sulphate and the mixture was shaken for 1 hr. at 23°. The phenol-buffer mixtures from both pellet and supernatant fractions were centrifuged at 16000g and 2° for 15 min. The aqueous layer was pipetted off and the RNA in the aqueous layer was precipitated by adding 2 vol. of 95% (v/v) ethanol and 0.1 vol. of 20% (w/v) potassium acetate (pH 5.0) and by leaving the contents overnight at -10°. Subsequent steps for the isolation of RNA from pellet and supernatant fractions were the same as described above for the isolation of total RNA.

Chromatography of RNA on ECTEOLA-cellulose anion-exchanger. The ECTEOLA-cellulose exchanger was purchased from Nutritional Biochemicals Corp. and was purified by washing successively with N-NaOH, N-HCl, deionized water, N-NaOH and deionized water. A column of ECTEOLA-cellulose (1.2 cm. × 17 cm.) was packed under gravity and, before use, was washed successively with 500 ml. of N-NaOH, 1 l. of deionized water and 1 l. or more of 0.02M-tris-HCl, pH 7.6, until the effluent was of pH 7.6. The same procedure (i.e. wash with alkali, water and buffer) was used every time the column was regenerated after a run. If the column was prepared as described above, highly reproducible chromatographic patterns were obtained even after repeated use of the column. For

chromatography of total RNA or the RNA from 27000g pellet or 27000g supernatant fractions the ECTEOLA-cellulose column, equilibrated with 0.02M-tris, pH 7.6, was charged with 8-16 ml. of RNA solution ($E_{260}^{1\%}$ 35-106) in tris buffer and subsequently washed with 200 ml. of the same buffer. The stepwise elution of the column was done with 0.02M-tris, pH 7.6, containing increasing concentrations of NaCl as indicated in Figs. 2 and 3. Subsequently the column was eluted with 0.1N-NaOH and finally with N-NaOH. The chromatography was carried out in the cold room at 2° and 5 ml. fractions were collected. The column was allowed to flow under gravity at a rate of 0.6 ml./min. The elution pattern of RNA was followed by reading the extinction of the 5 ml. fractions at 260 mµ in a Beckman model DU spectrophotometer. The recovery based on extinctions at 260 mµ was almost 100% of the RNA applied to the column.

Chromatography of total RNA and of RNA from 27000g pellet and 27000g supernatant fractions on Sephadex G-200. Sephadex G-200 was purchased from Pharmacia Fine Chemicals Inc. and was allowed to swell for at least 24 hr. in 0.02M-tris-HCl, pH 7.6, containing 0.1M-NaCl, before packing in a column (18 cm. × 1.7 cm.). During swelling the supernatant buffer was replaced several times to remove the fine particles. Before use the column was washed overnight with the tris-NaCl in a cold room at 2°. For chromatography of RNA solution on Sephadex G-200, 2-8 ml. of RNA solution in 0.02M-tris, pH 7.6, was applied to the column and the column was subsequently eluted with 0.02M-tris, pH 7.6, containing 0.1M-NaCl. Fractions (5 ml.) were collected and their extinctions read at 260 mµ. The Sephadex G-200 column used above was calibrated by chromatographing the soluble RNA from *Torula* (mol.wt. 20000-30000, purchased from California Biochemicals Corp.).

Determination of the melting curves of RNA. The melting curves of RNA were determined by heating RNA solution in stoppered quartz cuvettes in a Beckman model DU spectrophotometer equipped with a dual thermospacer. The extinction at 260 mµ was measured at intervals during heating. The temperature was raised at about 1°/min. by using a water-circulating bath.

Determination of the nucleotide composition of RNA. The RNA was hydrolysed with 0.3N-NaOH for 16 hr. at 37° (Smillie & Krotkov, 1960). The hydrolysate was cooled to room temperature and made free from Na⁺ ions by treatment with Dowex 50 (H⁺ form; 200-400 mesh) until the pH was between 5.5 and 6.5 (Cantoni, Gelboin, Luborsky, Richards & Singer, 1962). The resin was removed by filtration and the filtrate was concentrated to about 1 ml. at 37° in a rotary vacuum evaporator. The four RNA nucleotides were separated by two-dimensional paper chromatography with the solvents of Pabst Laboratories (1956) no. I (10 ml. of concentrated ammonia, 329 ml. of water, 661 ml. of isobutyric acid; first solvent) and no. III (600 g. of ammonium sulphate, 1 l. of 0.1M-sodium phosphate buffer, pH 6.8, 20 ml. of propan-1-ol; second solvent). The nucleotide spots were located on the paper with ultra-violet light and eluted with 0.1N-HCl overnight at 23°. The extinction of the 0.1N-HCl solution of the nucleotides was read against appropriate blanks at λ_{max} of the nucleotides. The concentration of the nucleotides was calculated from the ϵ_{max} values of nucleotides published by California Biochemicals Corp.

RESULTS AND DISCUSSION

The total RNA as well as the RNA from 27000g pellet or 27000g supernatant fraction had an absorption minimum at 230m μ , a maximum at 260m μ , extinction ratio of 260m μ to 280m μ equal to 1.9 and extinction ratio of 260m μ to 230m μ equal to 2.2. The RNA showed hyperchromicity of 33% on alkaline hydrolysis, hyperchromicity of 22% on heating from 25° to 98° and showed a gradual increase of hyperchromicity with a 5m μ shift of maximum to the higher wavelength on reaction with formaldehyde at 25°. Similar properties of native RNA from different sources have been reported (Fraenkel-Conrat, 1954; Hall & Doty, 1959; Kodama & Bancroft, 1964; Montagnier & Bellamy, 1964; Zachau, Lawson & Schweiger, 1961).

The amounts of total RNA and of RNA present in 27000g pellet and 27000g supernatant fractions (expressed as $E_{260(1ml)}/g$ fresh wt. of barley leaves) are as follows: total, 21.3; pellet, 11.8; supernatant, 4.3. The data presented above show that, in spite of the use of bentonite, about one-quarter of the leaf RNA was lost during homogenization of the leaf tissue with the sucrose-buffer. The barley leaves are very high in ribonuclease activity (Srivastava & Ware, 1965) and loss of RNA by the action of ribonuclease during homogenization of the plant tissues with buffers has been reported (Giles & Meyers, 1964). Alternatively, the loss of RNA may be the result of incomplete extraction of the fibrous tissue, which is usually hard to macerate thoroughly. Although microsomal RNA is supposed to represent the major fraction of the total cell RNA, in the present work, the 27000g pellet, which probably consisted of chloroplasts, nuclei and mitochondria, was found to be richer in RNA compared with the 27000g supernatant, which probably consisted of microsomes and soluble proteins. Greater amounts of RNA in chloroplast-nuclear plus mitochondrial fraction compared with microsomal plus soluble fraction has also been reported for green tobacco leaves (Sugiura, Umemura & Oota, 1962).

The elution profiles of total RNA and those of RNA from 27000g pellet and 27000g supernatant fractions on Sephadex G-200 are presented in Fig. 1. These profiles show that RNA from all the three sources was predominantly of high molecular weight (i.e. of ribosomal origin). The presence of high-molecular-weight RNA in the pellet fraction is in agreement with the reported occurrence of ribosomes in chloroplasts (Braverman, 1963) and nuclei (Revel, Delemen & Mendel, 1963; Sporn & Dingman, 1963).

The elution profile of total RNA on the ECTEOLA-cellulose exchanger is presented in Fig. 2 and that of RNA from pellet and supernatant

fractions in Fig. 3. The total RNA produced about 13 peaks (designated a-m in Fig. 2) on the ECTEOLA-cellulose column. The RNA from both pellet and supernatant fractions produced about the same number of major peaks as the total RNA. The three elution profiles (Figs. 2 and 3), however, differed from each other in the relative amount of different RNA peaks. Since fractionation on ECTEOLA-cellulose is supposed to depend on the secondary structure (Goldthwait & Kerr, 1962) and the size of the nucleic acid molecule (Rosenkranz & Bendich, 1959), the RNA species present in pellet and supernatant fractions probably resembled each other in this respect. The relative amounts of different RNA species in the pellet and supernatant fractions were, however, different.

Fig. 1. Elution profiles of (a) total RNA and of RNA from (b) 27000g pellet and (c) 27000g supernatant fractions from barley leaves on Sephadex G-200. The elution profile of *Torula* soluble RNA (mol.wt. 20000-30000) is presented in (a) for comparison. Column size 1.7cm. $\times$ 18cm.; 5ml. fractions.

Fig. 2. ECTEOLA-cellulose elution profile of total RNA from 4.8g. fresh wt. of barley leaves. Column size 1.2cm. $\times$ 17cm.; 5ml. fractions.

Fig. 3. ECTEOLA-cellulose elution profile of RNA from 27000g pellet (A) and of RNA from 27000g supernatant (B), each from 9g. fresh wt. of barley leaves. Column size 1.2cm. $\times$ 17cm.; 5ml. fractions.

Although Goldthwait & Kerr (1962), using chromatographic conditions different from those used in the present work, obtained some RNA peaks on ECTEOLA-cellulose which they considered as artifacts produced by reaction of RNA with ECTEOLA-cellulose, no decomposition of RNA or DNA on ECTEOLA-cellulose column was reported by several workers (Bloemendal *et al.* 1960; Bosch *et al.* 1960; Kusakari *et al.* 1963; Rosenkranz & Bendich, 1959; Skidmore, Main & Cole, 1963). Bosch *et al.* (1960) found that an RNA component obtained from the ECTEOLA-cellulose column behaved identically on rechromatography. Similarly, in the present work the peak d (Figs. 2 and 3) on rechromatography (after dialysis for 24 hr. at 2° against 2l. of 0.02 M-tris-hydrochloric acid, pH 7.6) on the ECTEOLA-cellulose column was eluted at its original position. Bloemendal *et al.* (1960) have

reported that polyadenylic acid preparations of molecular size 270000 and 400000 respectively were eluted without any degradation from the ECTEOLA-cellulose column with sodium hydroxide. The peak k in Figs. 2 and 3, which was eluted with 0.1 N-sodium hydroxide, was not retarded on chromatography on Sephadex G-200, thus indicating that it had molecular weight greater than 200000.

The melting curves of total RNA and of RNA from pellet and supernatant fractions are presented in Fig. 4. The ultraviolet-absorption of RNA solution changed gradually with the rising temperature as described by other workers for total ribosomal RNA from different sources (Montagnier & Bellamy, 1964; Munro, 1964; Sporn & Dingman, 1963). On cooling the RNA solution the original extinction at 25° was restored, indicating that the bonds concerned were intramolecular rather than between the two chains. If we define the melting temperature, T_m , as that temperature at which 50% of the maximal increase in extinction had occurred, then the T_m of the total RNA was 64.5°, the T_m of RNA from pellet fraction was 64.5° and the T_m of RNA from supernatant fraction was 73°. The similarity in the T_m of the total RNA and of RNA from pellet fraction is also indicated by the similarity of their nucleotide composition (Table 1), and the high T_m of RNA from supernatant fraction is in accordance with its high GMP + CMP content (Table 1). Differences in the nucleotide composition of RNA from chloroplast and microsomal fractions (Braverman, 1963) and even between 17s and 28s particles of microsomes (Pollard, 1964) have been reported. The data presented in Table 1, however, indicate that the RNA which was lost during homogenization of the leaf tissue with sucrose-buffer was probably richer in UMP and poorer in GMP and CMP.

The data presented in this paper show that the total RNA or the RNA from 27000g pellet or 27000g supernatant fractions of the barley leaf was predominantly of high molecular weight (i.e. of ribosomal origin) and was composed of many

Fig. 4. Melting curves of total RNA (●) and of RNA from 27000g pellet (○—○) and from 27000g supernatant (○---○) in 0.02 M-tris-HCl buffer, pH 7.6.

Table 1. Nucleotide composition of total ribonucleic acid and of ribonucleic acid from 27000g pellet and 27000g supernatant fractions from barley leaves

Values are expressed as moles/100 moles.

RNA	AMP	CMP	GMP	UMP	$\frac{\text{AMP}+\text{GMP}}{\text{CMP}+\text{UMP}}$	$\frac{\text{AMP}+\text{CMP}}{\text{GMP}+\text{UMP}}$
Total	24.5±0.2*	25.0±0.3	30.3±0.7	20.0±0.9	1.21	0.98
27000g pellet	23.9±0.6	25.5±0.4	31.2±1.0	19.6±0.9	1.22	0.98
27000g supernatant	24.1±0.5	29.5±0.6	31.9±0.9	14.6±0.9	1.27	1.15

* S.D. (n = 6).

species of RNA. The RNA species in the pellet and supernatant fractions probably resembled each other in molecular size or secondary structure or both but the relative amounts of various RNA species in the two fractions were different.

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