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Some features of mitochondrial ribonucleic acid from rye (*Secale cereale*) leaves

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There is now extensive evidence that mitochondria contain RNA and DNA in molecular forms, suitable for the systems involved in the transmission of genetic information and protein biosynthesis. The presence of ribosomes in mitochondria (Rendi 1959; Rifkin et al. 1967) as well as of enzymes such as aminoacyl-RNA synthetases together with corresponding tRNA (Barnett, Brown 1967: Barnett et al. 1967) and of DNA-dependent RNA polymerase (Luck, Reich 1964) was demonstrated. The difference of mitochondrial ribosomes and DNA from cytoplasmic ribosomes (Rifkin et al. 1967; Dure et al. 1967) and nuclear DNA (Luck, Reich 1964; Reich, Luck 1966) indicates that mitochondria contain an autonomic protein synthesizing system, similarly as is the case with chloroplasts (Szarkowski 1965). The problem of synthesis and origin of mitochondrial nucleic acids is still rather controversial.

This paper deals with mitochondrial RNA of primary rye leaves. Both the nucleotide composition and molecular type of this RNA were studied. A chromatographic characteristic of mitochondrial RNA derived from green and etiolated rye leaves was obtained. Ribonucleolytic activity bound with mitochondria has also been studied.

MATERIAL AND METHODS

Plants. Rye (*Secale cereale*) var. ‚Dańkowskie' was cultivated as described previously (Ombach et al. 1966) under different conditions of lighting: in a day - night cycle (green plants) and in darkness (etiolated plants). The age of the culture was 14 days, the size of the primary leaves — ca. 12 cm.

Mitochondria. Leaves (200 g) were cut into about 1 cm. fragments and homogenized with 300 ml. of a solution containing 0.25 M sucrose, 0.01 M $CuSO_4$, 0.01 M magnesium acetate and 0.01 M Tris-HCl buffer, pH 7.3 in a "Turmix" homogenizer at maximum speed for 60 sec. The homogenate was filtered through four layers of cheese-cloth and subjected to differential centrifuging. Nuclei, plastids and cell debris were sedimented at 1000 g for 15 min. The mitochondria were sedimented from the supernatant at 12 000 g for 15 min. The mitochondrial pellet was thoroughly resuspended in 40 ml. of the same solution with a teflon homogenizer

and centrifuged at 12 000 g for 15 min. The washing procedure was repeated twice with a fresh portion of the solution.

Isolation of RNA. RNA was isolated from mitochondria by the phenol method of Click and Hackett (1966) by the use of 0.1 M Tris-HCl buffer, pH 7.3. For determination of nucleotide composition, RNA was isolated according to the procedure of Stanley and Yee (1966). Before isolation of RNA the material was purified by the method of Nieman and Poulsen (1963). The white powder yielded was extracted with 15 ml. of 1 N perchloric acid for 6 hours at 4°C as described previously (Gołaszewski et al. 1967).

Base composition of RNA. RNA was hydrolyzed with 1 N HCl at 100°C for 1 hr. The separation of nucleotides was performed by paper chromatography (ascending) on Whatman No 3 paper with a mixture of methanol, ethanol, hydrochloric acid, water (50:25:6:19) (Kirby 1955) as a solvent system. The separated compounds were eluted from paper with 0.1 N HCl for 18 hrs (Markham, Smith 1950).

Fractionation of RNA on methylated albumin column (MAK). Methylated albumin-covered infusorial earth was prepared according to Mandell and Hershey (1960) and placed in a glass column fitted with sintered glass filter disc (Schott G-3). The column was connected to a water pump and equilibrated with 0,1 M NaCl containing 0.05 M phosphate buffer, pH 6.7 according to Sueoka and Cheng (1962). The size of the column was 6×2 cm. The solutions of RNA were applied on the column in 0.05 M phosphate buffer, pH 6.7. Substances not adsorbable on methylated albumin were washed off with 0.1 M NaCl containing 0.05 M phosphate buffer, pH 6.7. RNA was eluted with increasing concentrations of NaCl (0.2—1.3 M) in solutions containing 0.05 M phosphate buffer, pH 6.7 according to Sueoka and Cheng (1962). The flow rate was regulated by applying a water pump.

Fractionation of RNA on Sephadex G-200 column. The molecular type of RNA was estimated by gel filtration on Sephadex G-200 as described by Amano (1967). RNA was dissolved in 0.15 M acetate buffer (550 μg/0.5 ml) containing 0.15 M NaCl, pH 5.0. A known volume of the solution of RNA was layered on the top of the column (1.2×50 cm.). The column was eluted with 0.15 M phosphate buffer, pH 5.0 containing 0.15 M NaCl. The flow rate was 6 ml/hr., 2-ml fractions were collected.

Sucrose density gradient centrifugation of RNA. RNA was centrifuged through a sucrose concentration gradient (5—20 %) (Spencer, Whitfeld 1966). The sucrose was dissolved in 0.025 M Tris-HCl buffer, pH 7.0 containing 0.1 M NaCl. The gradient was prepared in a 5 ml. nitrocellulose tube. A Spinco Model L-2 ultracentrifuge was used with an S. W. 39 rotor. The sample was centrifuged at 37 000 rpm for 5 hours. The fractions (5 drops) (0.07—0.08 ml.) were taken from the bottom of the tube. The RNA content in individual fractions was calculated from the extinction values taken at 260 mμ after adjusting the volume of each fraction to 1 ml with water.

Enzyme activities. The activity of glucose-6-phosphatase (EC 1.9.3.1) was estimated by the method described by Swanson (1955). Ribonucleolytic activity was

studied by the method of Anfinsen et al. (1954), the proportions of the reactants being the same as described previously (Gołaszewski, Szarkowski 1964; Gołaszewski et al. 1967). RNA from yeast (BDH, England) was used as the substrate after the purification by Kunitz's (1940) method in Woodward's modification (1944).

Analytical methods. Protein was determined by the method of Lowry et al. (1951), commercial trypsin (Nutritional Biochemical Corporation, Cleveland, Ohio, USA) being used as the standard after purifying it by dialysis against 0.001 N HCl at 4°C. The insoluble residue was centrifuged off and the supernatant lyophilized and stored at −20°C. Phosphorus was estimated by the method of Fiske and Subbarow (1925). The content of RNA was calculated from the extinction at 260 mμ by the use of the coefficient of 24,0 $mg^{-1} \cdot ml^{-1} \cdot cm^{-1}$ (Nathans, Lipmann 1961). DNA was estimated by the diphenylamine method according to Burton (1956), calf-thymus DNA being used as the standard (Type I, Sigma Chemical Co., USA). For calculating the amounts of nucleotides in acid hydrolysate of RNA that were separated by paper chromatography, the following millimolar extinction coefficients were used: adenine, 13.6 at 260 mμ; guanine, 10.0 at 250 mμ; UMP, 9.89 at 262 mμ; CMP, 13.0 at 280 mμ. The extinctions were measured in a Unicam SP 500 spectrophotometer.

RESULTS

Characteristics of mitochondria. Mitochondria isolated from the primary green and etiolated rye leaves contained but little contamination of microsomes. For estimating the amount of microsomal impurity the activity of glucose-6-phosphatase in the original homogenate was compared with that bound to the mitochondrial fraction. The recovery of glucose-6-phosphatase activity in the mitochondrial fraction was about 2.7 %, thus within the limits of the results obtained with mitochondrial preparations from other tissues (Borst et al. 1967).

Ribonucleolytic activity. Digestion of RNA by mitochondrial preparations from green and etiolated plants was studied at various pH values with the same amount of protein per sample. Within the range of pH 4.0—7.0, 0.1 M citrate-phosphate buffer was used and 0.1 M Tris-HCl buffer for pH 7.0—8.0. The highest ribonucleolytic activity of mitochondrial preparations from green and etiolated leaves was observed at pH 5.8 (Fig. 1). A second, smaller peak was observed at pH 6.8. It has been shown by us in the previous paper (Gołaszewski et al. 1967) that ribonucleolytic activity of cytoplasmic ribosomes from green and etiolated rye leaves exhibits an optimum activity at pH 6.8. It seems therefore that the lower peak of ribonucleolytic activity of mitochondrial preparations might be due to contamination by the microsomal fraction. A small irregularity can be observed on the ascending part of the activity curve between pH 4.0 and pH optimum, in the region of pH 5.2. This might be due to the contamination of mitochondrial preparations with a very active ribonuclease (EC 2.7.7.17) having an optimum activity at pH 5.2, previously found in rye leaves (Gołaszewski et al. 1967).

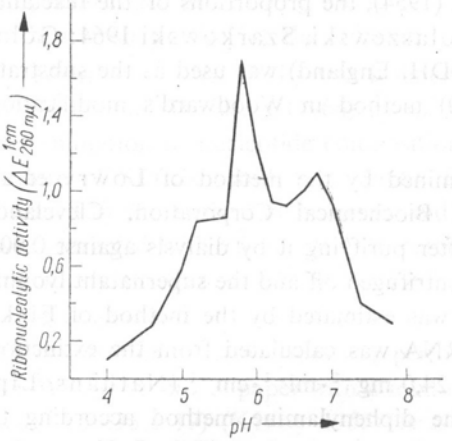

Fig. 1. Ribonucleolytic activity of mitochondria isolated from green rye leaves, versus pH.

Enzymic activity was determined as described under Material and Methods. The following buffers were used: 0.1 M citrate-phosphate buffer and 0.1 M Tris-HCl buffer in pH range 2.0—7.0 and 7.0—8.0 respectively.

Nucleotide composition of mitochondrial RNA. Purification of the mitochondrial pellet from chlorophyll impurities and free nucleotides and defatting by the method of Nieman and Poulsen (1963) yielded a white powder, from which RNA was extracted twice with 1 N perchloric acid. The isolated RNA was then hydrolyzed with 1 N HCl at 100°C during 1 hr. in sealed glass tubes. The hydrolysate was chromatographed on paper in the solvent system of Kirby (1955). The method enabled the separation of the four main components of the hydrolysate, i.e., adenine, guanine, CMP and UMP. The individual spots were eluted with 0.1 N HCl during 18 hours at room temperature (Markham, Smith 1950) and the amount of UV-absorbing material was estimated quantitatively. The results are presented in Table 1.

Table 1

Nucleotide composition of mitochondrial RNA of green rye leaves (in moles per 100 moles of nucleotide in RNA)

	Mitochondria	Ribosomes*
Guanine	34,0 (0,9)	32,6
Adenine	25.0 (0.7)	23.5
Cytidylic	20.0 (1.0)	21.2
Uridylic	21.0 (0.6)	22.6
$\frac{G+C}{A+U}$	1.17	1.17
n	5	

* Hydrolysis of RNA was made with 1 N HCl at 100°C for 1 hr. The hydrolysis products were separated by paper chromatography in methanol:ethanol:hydrochloric acid:water (50:25:6:19, by vol.) solvent. For other details see Material and Methods. Values in parentheses represent standard deviation; n represents the number of preparations. * Data from Gołaszewski et al. (1967).

The data obtained indicate that RNA isolated from mitochondria of green leaves is rich in guanine, and cytidylic acid is less abundant in the material. For comparison the nucleotide composition of RNA isolated from cytoplasmic ribosomes of the primary leaves of rye seedlings (Gołaszewski et al. 1967) is also included in Table 1. On comparing the data a similarity can be seen between the nucleotide composition of both acids. Ribonucleic acid of cytoplasmic ribosomes is also rich in guanine and poor in cytidylic acid.

The G+C/A+U ratio is the same in both acids and equals 1.17.

Characteristics of mitochondrial RNA. RNA isolated from mitochondria of green and etiolated leaves by the phenol method of Click and Hackett (1966) exhibited an absorption maximum at 260 mμ and a minimum at 230 mμ at pH 7,0. The E_{280}/E_{260} ratio was 0.50.

RNA subjected to column chromatography on methylated albumin with discontinuous NaCl gradient concentration was separated into 10 fractions (Fig. 2). The

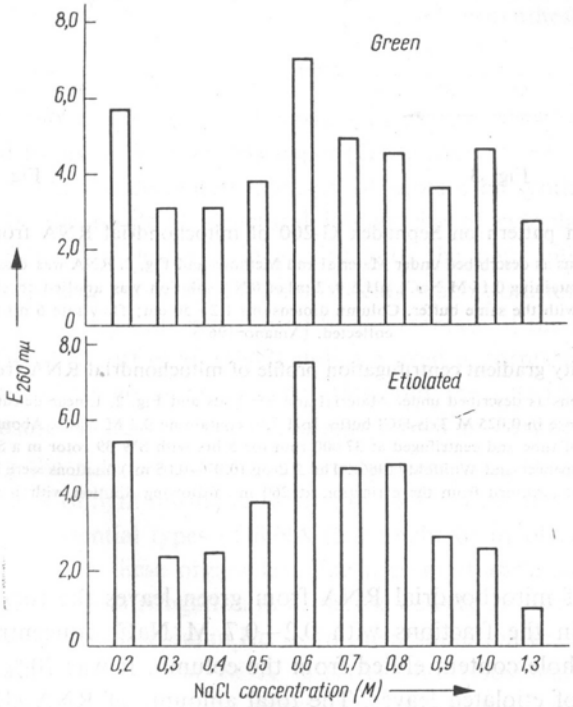

Fig. 2. Elution pattern of mitochondrial RNA of green and etiolated rye leaves on MAK-column

RNA was isolated by the phenol method of Click and Hackett (1966) using 0,1 M Tris-HCl buffer, pH 7.3. About 2 mg of RNA in 0.05 M phosphate buffer, pH 6.7, was applied into the column. The stepwise elution was carried out with increasing concentrations of NaCl (from 0.2 to 1.3 M) in 0.05 M phosphate buffer, pH 6.7, according to Sueoka and Cheng (1962). The volume of eluent was 40 ml per step. The flow rate was regulated by applying water pump. Column dimensions: 6×2 cm; temperature 20°C

separation pattern of mitochondrial RNA was almost the same in the case of green and etiolated leaves. Only slight quantitative differences in the amounts of individual fractions eluted with various concentrations of NaCl were observed. According

to Sueoka and Cheng (1962) on the basis of the NaCl concentrations used, it can be affirmed that RNA isolated from mitochondria comprises two main types of molecules. The first one, comprising the fractions eluted by 0.2—0.7 M NaCl, would correspond to soluble RNA, while that eluted by higher concentrations of NaCl would represent the ribosomal type of RNA.

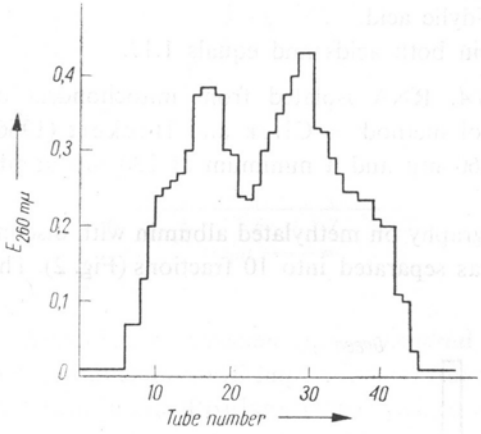

Fig. 3

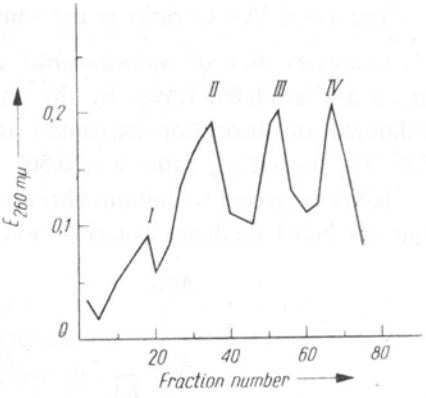

Fig. 4

Fig. 3. Gel filtration pattern on Sephadex G-200 of mitochondrial RNA from green rye leaves

RNA extraction conditions as described under Material and Methods and Fig. 2. RNA was dissolved in 0.15 M acetate buffer (550 μg/0.5 ml) containing 0.15 M NaCl, pH 5,0. 2 ml of RNA solution was applied to the top of the column. The column was eluted with the same buffer. Column dimensions 1.2×50 cm; flow rate 6 ml/hr; 2 ml fractions were collected. (Amano 1967)

Fig. 4. Sucrose density gradient centrifugation profile of mitochondrial RNA from green rye leaves

RNA extraction conditions as described under Material and Methods and Fig. 2. Linear density gradient was formed between 5 and 20% sucrose in 0,025 M Tris-HCl buffer (pH 7,0) containing 0,1 M NaCl. About 500 μg RNA solution was layered on the top of tube and centrifuged at 37 000 rpm for 5 hrs with SW 39 rotor in a Spinco model L-2 ultracentrifuge according to Spencer and Whitfeld (1966). The 5 drop (0,07—0,08 ml) fractions were collected and the RNA content was determined from the extinction at 260 mμ following dilution with 1 ml of water

In the case of mitochondrial RNA from green leaves the total amount of the material eluted in the fractions with 0.2—0.7 M NaCl concentration amounted to 64 % of the whole content eluted from the column. It was 70 % in case of mitochondrial RNA of etiolated leaves. The total amounts of RNA eluted in fractions with 0.8—1.3 M NaCl concentrations were 36 and 30 %, respectively.

Fractions eluted from the column with 0.2 and 0.7 M NaCl contained small amounts of DNA which was absent from other fractions.

On Fig. 3 the elution pattern of mitochondrial RNA from a Sephadex G-200 column is shown. Two separate peaks can be seen, however not clearly resolved from one another. This might be due to the presence of partially degraded RNA in the preparation studied. Nonetheless, similarly as in the case of RNA separation on methylated albumin, the elution profile when using Sephadex G-200 shows

two types of RNA — one of highly polymerized molecules that would correspond to ribosomal RNA (rRNA) and the other with a lower degree of polymerization that would correspond to soluble RNA (sRNA).

RNA from mitochondria of green leaves was separated into four fractions on centrifuging in the sucrose density gradient (Fig. 4). The fractions obtained comprise two types of RNA. On comparing the data obtained with the results of other authors (Spencer, Whitfeld 1966) it can be assumed that peaks I and II might represent RNA with a higher degree of polymerization corresponding to ribosomal RNA and peak IV would correspond to sRNA. Peak III contained RNA fraction that, most probably, seems to be a degradation product of ribosomal RNA.

DISCUSSION

It is commonly known that the lack of light limits the growth and development of plant. This is related among others things to a limited biosynthesis of proteins — particularly the plastidial ones (De Deken-Grenson 1954). The similarity between the chemical structure of cytoplasmic ribosomes of the primary leaves of seedlings grown in dark and under conditions of normal light with a day - night cycle has been demonstrated by us in the previous paper (Gołaszewski et al. 1967). It was, therefore, assumed that the decreased intensity of protein biosynthesis in etiolated plants could not be the result of structural immaturity of cytoplasmic ribosomes and their RNA. This conclusion is in accord with the data inferred by Mans (1966), that an increased intensity of amino acids incorporation by ribosomes isolated from illuminated plants was caused by the increase of the messenger RNA level. Recent studies of Stout et al. (1967) indicate even a correlation between the increase of activity of amino acids incorporation and the activity of RNA polymerase after exposure to light of corn seedlings grown in darkness.

On considering the role of mitochondria in protein biosynthesis the question arose whether tha lack of light during growth of the plants does not cause differences in the pattern of the essential types of RNA that might be involved in the process of protein biosynthesis in these organelles. The present studies, such as gel filtration on Sephadex G-200, centrifugation in a sucrose density gradient and chromatographic analysis on the MAK column led us to the assumption that two main components corresponding to soluble RNA (sRNA) and ribosomal RNA (rRNA) are present in the mitochondria of rye leaves. This is in accord with the results of other authors (Barnett, Brown 1967; Borkowski et al. 1967; Dure et al. 1967; Rifkin et al. 1967).

On analysing the RNA profile obtained on centrifuging mitochondrial RNA in the sucrose density gradient and comparing it with the profiles obtained by other authors (Spencer, Whitfeld 1966) in similar experimental conditions it can be assumed that peaks I and II correspond to ribosomal RNA 25s and 16s, respectively. Peak IV might represent soluble RNA 4s. Peak III, on the other hand, represents RNA molecules of intermediate size between rRNA and sRNA. As no direct

evidence is available supporting the synthesis of such RNA, it should be assumed that, most probably, this fraction arises as a result of degradation of highly polymerized RNA. This suggestion has also been put forward by Berger (1967) in studies on RNA isolated from chloroplasts of *Acetabularia*. The failure in obtaining in our studies a better separation of the studied RNA on a Sephadex G-200 column was probably due to the presence of this fraction.

On separating, by the use of a MAK-column, RNA of mitochondria derived both from green (grown under normal light regime) and etiolated rye leaves, one can obtain almost the same elution patterns in qualitative and, to some extent, quantitative terms. Thus, the same number of fractions elutable at corresponding NaCl concentrations was found in both cases. Similarly, the approximate percentual composition of various MAK-fractions in all the mitochondrial RNA preparations can also point to general similarities of these RNA's. It may, therefore, be concluded that lack of light does not cause the appearance of differences in mitochondrial RNA. It should also be added that mitochondria from green and etiolated leaves exhibit ribonucleolytic actitivity, the pH optimum of both being at pH 5.8.

It has previously been shown by Suyama (1967) that in the mitochondria of *Tetrahymena pyriformis*, the content of highly polymerized RNA was four times larger than that of sRNA. It is clear from our studies that the unfractionated RNA of mitochondria of green rye leaves has a similar nucleotide composition with a typical abundance of guanylic acid and the same G+C/A+U ratio (1.17) as that of highly polymerized RNA from cytoplasmic ribosomes (Table 1). This similarity allows to assume, that the RNA of mitochondria of the rye leaves studied is in its prevailing part of ribosomal type. The fact that only about 30 % of RNA was eluted from the MAK-column in the range from 0.8 to 1.3 of NaCl molarities, as commonly raported for ribosomal RNA (Sueoka, Cheng 1962), could be explained by the degradation of mitochondrial RNA during its preparation and analysis. More details concerning the nature of mitochondrial RNA will be provided in the course of our studies on its localization within the mitochondrion.

Acknowledgment.

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SUMMARY

It has been found that the nucleotide composition of RNA isolated from mitochondria of primary rye leaves is very similar to that of cytoplasmic ribosomes (Table 1). Mitochondrial RNA can be separated into several fractions both by means of MAK column chromatography (Fig. 2) and sucrose density gradient centrifugation (Fig. 4). At least one third of the total amount of RNA extractable from the organelles by the phenol method appeared to be in highly polymerized, ribosomal-like form. Partial degradation of the remaining part of RNA preparations isolated from mitochondria should be taken into consideration, however, in view of the fact that relatively high

ribonucleolytic activity has been revealed within the organelles (Fig. 1). The light regime of plant cultivation does not seem to have any major effect on the status of RNA bound with the mitochondrial fraction.

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Pewne cechy mitochondrialnego kwasu rybonukleinowego liści żyta (Secale cereale)

Streszczenie

Stwierdzono, że skład nukleotydowy RNA wyizolowanego z mitochondriów pierwszych liści kiełków żyta jest bardzo zbliżony do składu nukleotydowego RNA rybosomów cytoplazmatycznych (Table 1). Mitochondrialny RNA rozdziela się na kilka frakcji zarówno podczas chromatografii kolumnowej przy użyciu metylowanej albuminy (Fig. 2), jak też podczas wirowania w gradiencie gęstości sacharozy (Fig. 4). Przynajmniej 1/3 całkowitej ilości RNA, dającego się wyekstrahować z mitochondriów metodą fenolową, występuje w formie wysoko spolimeryzowanej, podobnej do RNA rybosomów cytoplazmatycznych. Ze względu na wysoką aktywność enzymów rybonukleolitycznych stwierdzanych wewnątrz mitochondriów (Fig. 1), należy jednak wziąć pod uwagę możliwość częściowego rozpadu pozostałej porcji RNA izolowanego z mitochondriów.

Warunki świetlne hodowli roślin nie wpływają znacznie na stan spolimeryzowania RNA tej organelli.