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NUCLEIC ACIDS OF PISUM SATIVUM

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The preparation of native samples of nucleic acids (NA) from plants is difficult since they are poor in nucleic acids and rich in structural formations of nonprotein nature. Seed germs and plantules, which consist predominantly of embryonic tissue, are generally used to obtain NA.

In the cell, the greater part of the nucleic acids is bound in structural protoplasms. In order to extract them completely the protein component, with which they form nucleoproteins, must be destroyed. This is achieved by Kirby's method of treating the tissue with phenol [1]. However, the total extraction of the NA deprives the investigator of the possibility of investigating the heterogeneity of the NA with respect to their physicochemical state in the cell. For this purpose we must use fractional extraction enabling the NA to be differentiated with respect to the nature of their bond with other cell components. The choice of extractants and the procedure for extracting the NA from tissue may differ according to the aim of the investigation. We have used successive extraction of the NA with the following extractants: 0.14 M NaCl, 1.5 M NaCl, cold phenol treatment, and hot phenol treatment after previous suspension in 1.0 M NaCl and 0.1 M NaCl, respectively. Fraction 1 includes the bulk of the RNA of the tissue and a small amount of the DNA. This includes the free t-RNA, the ribonucleoprotein particles, and the DNA bound in structures in labile form and capable of passing into solution of low ionic strength.

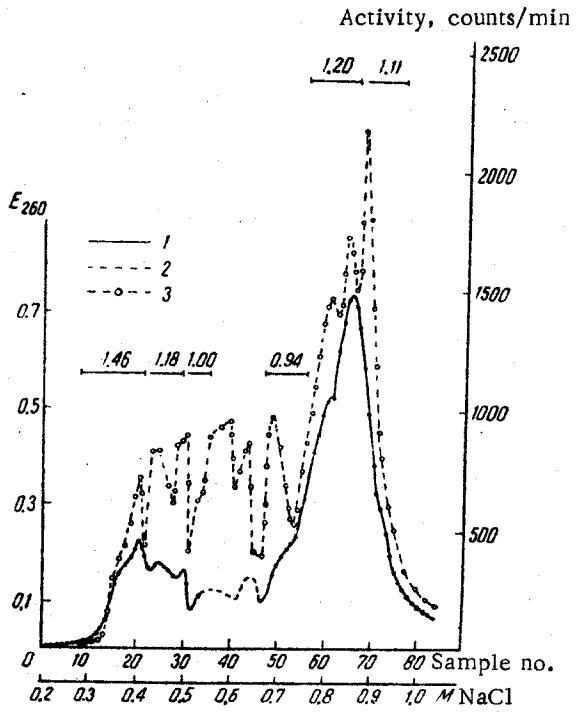

Fig. 1. Chromatographic profile of the RNA of the 0.14 M NaCl extract of 2-day-old pea plantules on MA. The figures above the peaks denote the specificity coefficients. 1) Optical density at $E_{260m\mu}$; 2) DNA zone; 3) ^{32}P-including activity.

Fraction 2 includes the bulk of the DNA of the tissue and comparatively small amounts of RNA. Into it passes the bulk of the deoxyribonucleoproteins of the chromatin of the cell nucleus, which are capable of being dispersed only in solutions of high ionic strength.

Fraction 3 contains mainly the DNA of the deoxyribonucleoproteins firmly bound in the structures of the nucleus. This DNA is liberated only by the destruction of the protein components by treatment with phenol.

Fraction 4 mainly contains RNA. Treatment of the tissue with neutral phenol makes it possible to isolate the structural fibrous ribonucleoprotein. It also contains RNA strongly bound to polysaccharides.

As our investigations have shown, the quantitative ratio of the fractions given depends to a considerable extent on the nature of the tissue and the plant organs. In a study of the component composition of the NA fractions, the best results are obtained by column chromatography with DEAE-cellulose, substituted Sephadexes, histones, or methylated albumin. We have used column chromatography with methylated albumin (MA).

Generally (according to the literature and our results) on elution from MA columns the oligonucleotides appear first, sometimes in a zero peak. The t-RNA are located in the 0.30–0.44 M zone, the DNA in the 0.5–0.1 M zone, and the r-RNA in the 0.74–0.89 M zone. The m-RNA are detected by the activity of a ^{32}P label. More frequently they come after or before the t-RNA. We have isolated them for the first time from the tissues of higher plants.

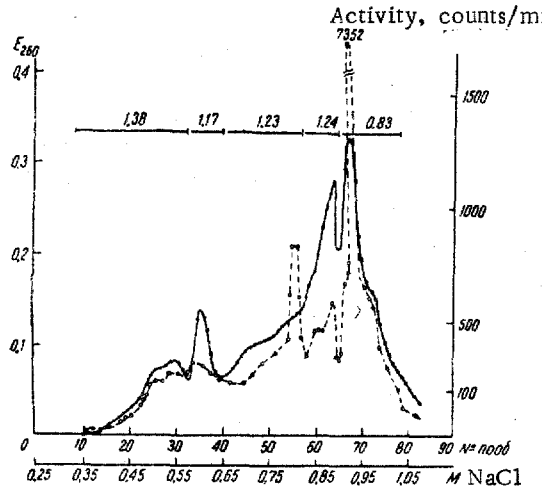

Fig. 2. Chromatographic profile of the RNA of the hot-phenol fraction of 2-day-old pea plantules on MA. 1) Optical density at $E_{260m\mu}$; 2) DNA zone; 3) ^{32}P - including activity.

Figures 1–4 show chromatographic profiles of the NA of two-day-old plantules of peas with an indication of the specificity coefficient and the metabolic activity of the RNA of the individual zones and peaks of these profiles. The molecular weights were determined viscosimetrically [2] and also by means of the electron microscope.

The secondary structure of the NA molecules was evaluated from the size of the hyperchromic effect, the absorption spectrum, and the luminescence spectrum of NA – acridine orange complexes. The native nature of the RNA fractions was checked by Spirin's method [3] from the temperature dependence of the reduced viscosity.

The metabolic activity of the NA was determined from the rate of inclusion and subsequent dilution of a ^{32}P label. The inclusion of the label was measured 24 hr after the addition of the ^{32}P and the replacement of the label 3–4 days after the first measurement.

The main forms of the RNA are described below.

0.14 M NaCl fraction. RNA predominates in the chromatographic profile of the fraction (see Fig. 1). For pea plantules it is mainly t-RNA with a molecular weight of 20 000–30 000 and a specificity coefficient (G + C)/(A + U) = 1.46, and r-RNA with a molecular weight of $1.5–2 \times 10^6$ and a specificity coefficient of 1.2. Between the r-RNA and DNA peaks there is RNA of the AU type with a specificity coefficient of 0.94. Its molecular weight is about 700 000. The right part of its peak possesses a high label-including activity. There is also high label activity in the RNA zone located between the t-RNA and the DNA. Its specificity coefficient is about unity and the molecular weight varies from the left edge of the zone to the right over the range from 120 000 to 180 000. In respect of their nucleotide composition, these fractions belong neither to t-RNA nor to r-RNA. Their heterogeneity with respect to nucleotide composition, molecular weight, and metabolic activity is a weighty argument in favor of the polyfunctionality of tissue RNA. They were found in particularly large amounts in tissues with intensive synthesis. For example, they form a considerable part of the RNA of the weak-salt extract from the seeds in the ripening phase. It is possible that the metabolically active components of this group of RNA bear the function of messenger RNA.

The 1.5 M NaCl fraction contained a small amount of RNA. The chromatographic profile of this RNA is similar to that of the RNA of the weak-salt fraction, but the ^{32}P-label-including activity in the RNA of the 1.5 M NaCl extract is considerably less than that of the weak-salt extract.

The cold-phenol fraction contained a small amount of t-RNA and r-RNA. The first was extremely heterogeneous in the metabolic respect and the second was characterized by a high activity.

The hot-phenol fraction consisted almost completely of RNA, amounting to 4–24% of the whole RNA of the tissue (cf. Fig. 2). It is characteristic that the RNA of this fraction is present in particularly large amount in tissues the cell nuclei of which have large nucleoli (the cells of the cotyledons and the parenchyma of young pea stems). This shows convincingly that it is inherent in the nucleoli and the chromatin structures connected with it. We obtained a direct proof of this from an analysis of a native preparation of the chromatin of the cell nucleus isolated by Bonner's method from 2-day-old pea plantules (Fig. 3). The hot-phenol fraction of the RNA of the chromatin amounted to 70–86% of the total RNA of the chromatin.

In the extraction with neutral hot phenol, the RNA is isolated in the form of nucleoprotein with a very well-expressed fibrous structure. Judging as a whole, it consists of the structural ribonucleoprotein of the chromatin of the cell nucleus. Acid proteins predominate in it, and it also contains histone-like proteins. After deproteinization, the RNA of the structural nucleoprotein gives a large zone 0.54–0.5 M on the chromatographic profile with a molecular weight of about one million. In this zone there are three peaks of ^{32}P-label-including activity. Part of the RNA issues in the zone of the low-molecular-weight RNA (50 000). It is also heterogeneous metabolically.

The hot-phenol fraction is very rich in polysaccharides, which issue from the column with the zero peak. They are generally accompanied by a fraction of oligonucleotides with an unusually active ^{32}P label. A small part of the RNA is very firmly bound to carbohydrates and is liberated only by repeated phenol treatment. The profile of the total RNA (see Fig. 4) isolated by the phenol method with detergents [1] was used as a control for the chromatographic profiles of the RNA fractions described above.

At the present time it is difficult to state whether all the RNA which is close to DNA in nucleotide composition is messenger RNA. Of the chromatographic components of the RNA that we have considered, great interest is attached to the high-molecular-weight RNA of the hot-phenol fraction forming structural ribonucleoprotein. It must be assumed that some of it participates in the molecular organization of the chromatin of the cell nucleus.

According to established ideas, DNA is homogeneous. Nevertheless, information on its heterogeneity with respect to nucleotide composition, the inclusion of a radioactive label, and its physicochemical state in the nucleus periodically appears in the literature [4–9].

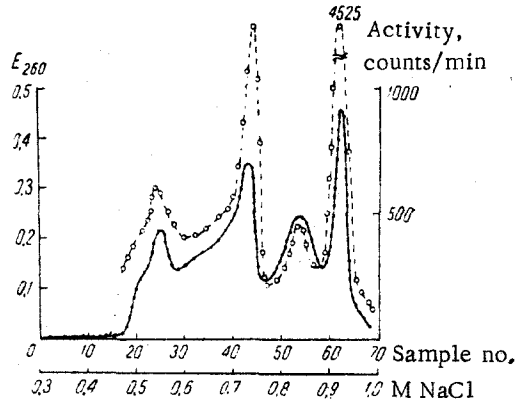

Fig. 3. Chromatographic profile on MA of the hot-phenol fraction of RNA isolated from a native preparation of the chromatin of the cell nucleus (symbols as for Fig. 1). 1) Optical density at $E_{260m\mu}$; 2) DNA zone; 3) ^{32}P-including activity.

We have succeeded in discovering the nature of the heterogeneity of the DNA to some extent by using the above-described approach of fractionation of the NA with their subsequent study on a column of methylated albumin. As has been found, part of the DNA of plants is extracted from the tissue with 0.14 M NaCl, i.e., by a solution of low ionic strength. Consequently, it is weakly bound to the cell structures. This fraction we have called labile DNA. It is possible that in the cell it interacts predominantly with a small amount of protein of the nonhistone type. There is much labile DNA in the intensively functioning differentiated tissues.

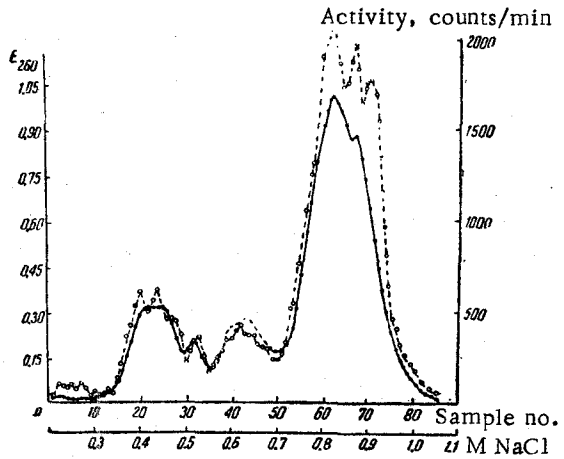

Fig. 4. Chromatographic profile of the total RNA of 2-day-old pea plantules on MA (symbols as for Fig. 1). 1) Optical density at $E_{260m\mu}$; 2) DNA zone; 3) ^{32}P-including activity.

We have recently shown that the chromatin isolated from tissues rich in labile DNA relative to the total DNA is poor in histones and contains much histone-like proteins with a large amount of dicarboxylic amino acids and hydroxy amino acids [19].

The labile DNA is eluted from a column of methylated albumin in a single peak in the 0.52–0.65 M NaCl zone with the maximum activity (in the case of 2-day-old pea plantules) in the first half of the peak (Fig. 5). In comparison with the DNA of the 1.5 M extract [(A + T)/(G + C) = = 1.47], it [(A + T)/(G + C) = 1.17] is somewhat richer in GC pairs and has a higher melting point of the helices, but it has almost the same molecular weight. Its distinguishing feature is a very high activity in the inclusion and replacement of a radioactive label (^{32}P), which shows its high metabolic activity. For the labile DNA from differentiated tissues, for example, from the bases of the stems, on separation on MA the activity profile coincides with the profile in UV light.

The second DNA fraction (stable DNA), extractable with 1.5 M NaCl, is insoluble in solutions of low ionic strength before deproteinization. In the cell nucleus it forms the bulk of the nuclear, histone-containing deoxyribonucleoprotein [10]. Apparently the DNA of the salt extract is the main component of the chromatin. Decomposition of the

latter into nucleoprotein particles takes place under the influence of the salt solution of high ionic strength. In view of this fact and also the low metabolic activity, we propose to call this fraction stable DNA.

The stable fraction of DNA generally issues from a MA column in two peaks with maxima at 0.58 and 0.67 M NaCl. In the first peak (in the course of a concentration gradient of NaCl), the DNA is characterized by a high content of GC pairs [(A + T)/(G + C) = 1.24)], a higher melting point of the helices, and a comparatively active inclusion of a label. The predominating part of the DNA of the 1.5 M NaCl extract is represented by the second peak with a high content of AT pairs [(A + T)/(G + C) = 1.50] and a low activity in the inclusion and replacement of a label.

The third DNA fraction is extracted in the phenol treatment of the cell structures previously suspended in 1.5 M NaCl. Consequently, to extract this DNA not only solvation but also the destruction of the protein complex firmly keeping the nucleic acid in the chromatin of the cell nucleus is necessary. The phenol fraction of DNA may be called firmly-bound DNA.

The DNA of the cold-phenol extract, like the DNA of the 1.5 M NaCl extract, issues mainly in two peaks, the DNA of the second peak predominating in embryonic tissues. In respect to nucleotide composition, the DNA of the cold-phenol extract is similar on the whole to that of the salt fraction, but in respect to metabolic activity and nucleotide composition the DNA of the first peak of the cold-phenol fraction is similar to the DNA of the weak-salt extract.

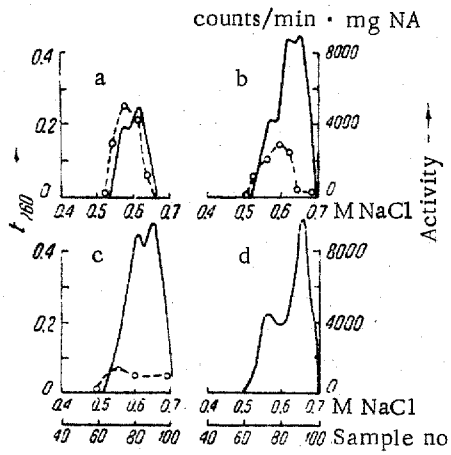

Fig. 5. Chromatographic profiles of DNA fractions from 2-day-old pea plantules on MA after treatment with ribonuclease. a) Labile; b) stable; c) strong; d) DNA of the chromatins. The broken line indicates ^{32}P-including activity.

Experimental

Extraction of the NA. A. 0.14 M NaCl. The material after treatment for 3 min in 0.5% Khloreks (antiseptic) solution and careful washing with distilled water was rapidly homogenized in the cold in a mixture of 0.14 M NaCl, 0.01 M $MgCl_2$, 0.015 M sodium citrate, and a suspension of bentonite (1–3 mg/ml). The extract was separated by centrifuging at 0 to +2° C. The extraction was repeated several times. The NA were deproteinated with water-saturated phenol at pH 7.8 with the use of chloroform, dodecyl sulfate, and PAS [11, 12].

B. 1.5 M NaCl. The residue after the removal of fraction 1 was suspended in two volumes of a mixture of 1.5 M NaCl, 0.015 M sodium citrate, and bentonite.

After 15 hr, the extracts were separated by centrifuging and the Na were deproteinated with phenol at pH 7.8–8.

C. Cold phenol treatment. The residue after the separation of the preceding fractions was suspended in 3.5 volumes of 1.5 M NaCl in the cold and cooled phenol at pH 7.8–8 was added; after stirring for 30 min the mixture was separated into layers by centrifuging. The upper (aqueous salt) layer was deproteinated again as for the preceding fractions.

D. Hot phenol treatment. The residue under the layer of phenol after the cold phenol treatment was suspended in 0.1 M NaCl. The suspension was neutralized with acetic acid, treated with an equal volume of phenol at pH 7 and heated to +63° C with stirring. After 20 min, the suspension was cooled and separated into layers by centrifuging in the cold at 2500 g for 30 min. If the hot phenol extraction was carried out at pH 6, RNA free from protein passed directly into the aqueous phase.

The nucleic acids of each extract were precipitated with 2.5 volumes of cooled ethanol. The ethanolic precipitates were washed from traces of phenol with cooled 70% ethanol. The precipitates were dissolved in 0.1 M NaCl and dialyzed in the cold against the solvent. The contents of RNA and DNA were determined spectrophotometrically [17] and also by the pentose reaction [18].

Fractionation of the NA on a column of MA. Serum albumin (fraction V for microbiological purposes) was methylated. The columns were based on kieselguhr according to Mandell and Hershey [13], with columns of adsorbent 2 × 6 cm, 2 × 12 cm, and, for preparative purposes, 3 × 15 cm. The amount of NA deposited was 1, 3, and 10 mg per column in 0.1 M NaCl containing 0.05 M phosphate buffer, pH 6.8. Separation was carried out in a linear concentration gradient of NaCl in the range from 0.2 to 1.2 M. The positions of the peaks in the chromatographic profile were denoted by the molar concentration of the eluate. The latter was determined refractometrically.

Determination of the nucleotide composition. A weighed sample of RNA (10–50 mg) was covered with a 0.75 N

solution of potassium hydroxide in an amount of 1 ml of alkali solution to 10 ml of dry material and was hydrolyzed at 37° C for 18 hr. The further treatment of the solution of ribonucleotides was as described by Vanyushin [14]. The separation of the ribonucleotides was effected electrophoretically in 0.04 M citrate buffer with pH 3.5 at a potential gradient of 30 V/cm for 6 hr. The following millimolar extinction coefficients were used to calculate the content of each nucleotide, reduced to 5 ml of eluate [15]: for adenylic acid 2.84, for guanylic acid 2.36, for cytidylic acid 2.60, and for uridylic acid 1.98. The nucleotide composition of the DNA was determined by paper chromatography after hydrolysis of the samples in 72% perchloric acid [14].

The bentonite and kieselguhr were prepared in the following way. Technical bentonite was carefully ground and suspended in 20 volumes of distilled water. The suspension was centrifuged at 2500 g for 30 min. The precipitate was removed and the bentonite from the supernatant liquid was precipitated by centrifuging at 18 000–20 000 g for 30 min. The precipitate of bentonite was treated with 0.1 M Versene solution (5–8 hr). The Versene was removed by dialysis against distilled water.

Ground kieselguhr was treated by being heated with concentrated hydrochloric acid at the rate of 250 ml per 100 g of kieselguhr for 1 hr. The hydrochloric acid with the metal salts dissolved in it was removed by repeated washing of the kieselguhr with water. The treatment of the kieselguhr with hydrochloric acid was repeated a further three or four times. The purified kieselguhr was calcined in a muffle furnace. To obtain the working fraction (0.25–0.05 mm), the purified kieselguhr was suspended in water and the fraction sedimenting at the rate of 3 cm/min was taken [16]. The working fraction was dried in a thermostated oven at 105°–110° C.

Summary

1. t-RNA, r-RNA, and RNA of the AU type have been isolated from pea plants by fractional extraction and chromatography on methylated albumin and have been studied. The last-mentioned type is heterogeneous with respect to molecular weight, nucleotide composition, metabolic activity, and physicochemical state.

2. The DNA of plants is heterogeneous with respect to its physicochemical state, metabolic activity, and nucleotide composition. With respect to its physicochemical state in the cell, DNA can be divided into labile, stable, and firmly-bound fractions.

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