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The effect of ultrasonic energy on the separation of cytoplasmic RNA fractions

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Abstract

Plant tissue — 4 days old germs of *Zea mays* and *Pisum sativum* were subjected to ultrasonic energy using generator with a constant frequency of 800 kHz. RNA was isolated by Mieczyński's modification of the method described by Georgiev. Preparations of RNA were separated by electrophoresis on the coloured agar-gel. Depending on the degree of ultrasonication changes in three fractions of RNA: A_1 — 28 S, A_2 — 18 S and A_3 — 4 S were observed.

INTRODUCTION

The problem of ultrasounds and their technical applications has become a developed area of investigations. Examinations conducted on biological material have been many-sided too. Numerous data giving evidence of the destructive effect of ultrasonic energy on bacterial, plant and animal cells as well as on some of their structures have been collected. Changes of growth, developmental and metabolic processes have been examined with changing physical parameters of ultrasonication of organisms. (Ultrazvuk v biologii, 1964). Some our own papers (Adamczyk, 1962; Halicz et al. 1964; Halicz, Gubański 1964; Halicz, Maciejewska-Potapczykowa 1966) contain results of examinations of the effect of ultrasonic energy on content of phosphorus compounds, activity of some enzymes, oxygen uptake intensity and course of initial developmental phases of germinating seeds. The effect of ultrasonic waves on biologically active compounds opened new questions in different scientific disciplines and new possibilities of application of ultrasounds for investigations of structure of polymeric molecules, possible mechanisms of their destruction, joining and other reactions. Nucleic

acids turned out to be a suitable material for examinations from amongst biopolymers (Elpiner 1968). Numerous experiments on the effects of ultrasounds on DNA were conducted, considerably less of time being spent for appropriate RNA investigations.

This fact prompted the authors to undertake experiments on the behaviour of cytoplasmic RNA isolated from germs of selected plants and subjected to ultrasonication, using electrophoresis on the agar-gel.

MATERIALS AND METHODS

Plant tissues — 4 days old germs of *Zea mays* and *Pisum sativum* ultrasonicated in vivo were the starting material for examinations. Generator with constant frequency of about 800 kHz and maximal mean surface intensity approximated to about 4 W/cm² and 8 W/cm² was used for the ultrasonication. The arrangement transferring ultrasonic energy (of Zeiss type) possesed a cylindrical vessel of the volume 125 ml. 5 g of plant tissue was always placed in the vessel which was then filled up to 100 ml with distilled water. Continuous water flow was maintained in the chamber in which the cylindrical vessel had been placed in order to maintain constant temperature during the ultrasonication. The ultrasonication times were 20, 40 and 60 min respectively with the intensities 4 W/cm² and 8 W/cm².

Isolation of cytoplasmic ribonucleic acid

RNA isolation from the plant material was performed according to the method given by Georgiev (1959) and modificated by Mieczyński (1967). The frosen plant tissues in amount of 5 g were homogenized using Potter's homogenizer with 10 ml of 0.1% SDS solution in 0.15 M phosphate buffer of pH 7.0 and 10 ml of freshly distilled 90% phenol pH 6.0. The homogenization was continued for 5 min. The obtained suspension was centrifuged for 10 min at 6000 rpm, at a temperature of 0°—4°C. The cytoplasmic RNA present in the water layer was separated and repeated extraction of the residue was performed. The water layers from both extractions were joined and two volumes of 96% ethyl alcohol was added. The shaggy RNA precipitate was centrifuged after several hours for 10 min at 3000 rpm. The obtained RNA sediment was dissolved in 0.4 ml of phosphate buffer of pH 7.0 or in 0.14 M NaCl solution. Thus prepared RNA solution was subjected to electrophoresis.

Electrophoresis on the coloured agar-gel

The agar-gel for electrophoresis was prepared according to Grabar (1953) trying to obtain homogenous gel, free of inorganic salt contaminations. Pure 2.5% agar-gel may be preserved in a refrigerator under sterile conditions for several weeks.

Preparation of the coloured agar

100 ml of coloured veronal buffer of pH 8.6 were added to 100 ml of 2.5% agar. The coloured veronal buffer was prepared by dissolving of 10 mg of acridine orange and 10 mg of pyronine B in 100 ml of the buffer (Tyrawska-Spychałowa and Duda 1972). The mixture of molten agar with coloured buffer was heated up to boiling and kept boiling in a water bath during several minutes. Final agar concentration was conducted to 1.25%.

Preparation of the agar-block for electrophoresis

Clean, degreased object glasses were covered with 2 mm thick layer of the 1.25% coloured agar-gel. After cooling down starting channels with dimensions 2×20 mm at a distance of 15—20 mm from the glass end were cut out. Microscopic glasses were covered with very thin layer of 3% agar solution before pouring of 1.25% agar for the sake of obtaining of the agar-block smooth surface.

Performance and results of the electrophoresis

The electrophoresis was carried according to Tsanev (1962, 1964, 1965), making use of the apparatus described by Mieczyński (1967). A tank made of organic glass with dimensions 300×200×60 mm, divided into three chambers by oblong partition walls constituted this apparatus. Two extreme chambers filled with veronal buffer of pH 8.6 served as electrode vessels. The central part filled with ice was the cooling electrophoretic chamber on which microscopic glasses were placed. The glasses with agar were arranged in a row on a chamber with ice thus to their ends be grounded on partition walls. The electrods were of thin platinum wire. Strips of filter paper laying on slantwise placed plates of organic glass in the electrode vessels provided the gel contact with the electrode vessels.

In order to perform the electrophoresis, 0.1—0.2 ml of RNA solution was poured into a starting channel and the process was conducted under current strength of 4 mA per one glass and constant strain of 220 V. All the process proceeded 90 min. Estimation of separation of RNA

fractions was made during the electrophoresis duration. One might observe migration of individual fractions in UV light using 260 nm filter, due to origin of fluorescing complexes between positively charged dye molecules and negatively charged RNA molecules. Electrophoregrams were read with a densitometer (type VEB Medizinische Gerätefabrik, Berlin), after termination of the electrophoresis. Individual fraction per cent contents were counted planimetrically taking the whole graph surface as 100%. Individual fractions were characterized on the basis of data from Tsanev's laboratory and works conducted in the Department of Biophysics of Łódź University (T y r a w s k a - S p y c h a ł o w a, D u d a, 1972).

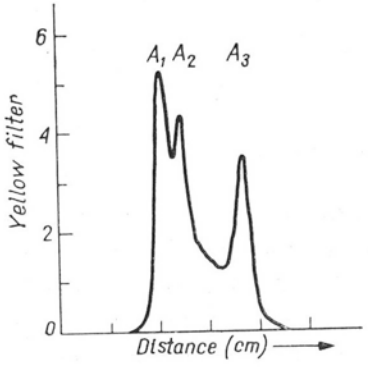

Fig. 1

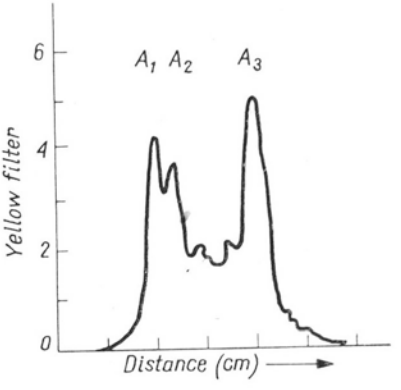

Fig. 2

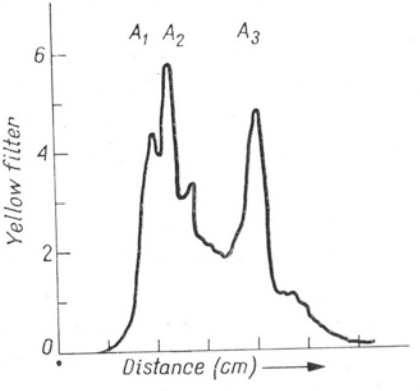

Fig. 3

Fig. 1—3. The densitometric graphs of RNA fractions: 1 — of control maize (*Zea mays*) germs which were not subjected to ultrasonic energy action; 2 — of maize (*Zea mays*) germs subjected to ultrasonic energy action with the intensity of 4 W/cm² for 60 min.; 3 — of maize (*Zea mays*) germs subjected to ultrasonic energy action with the intensity of 8 W/cm² for 40 min.

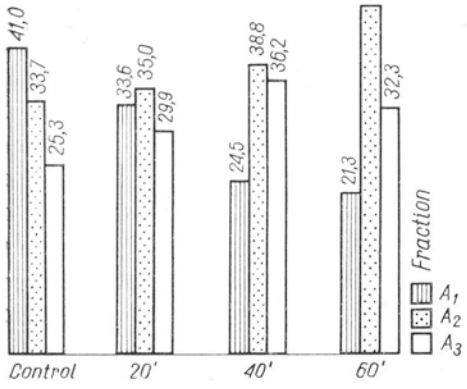

Fig. 4

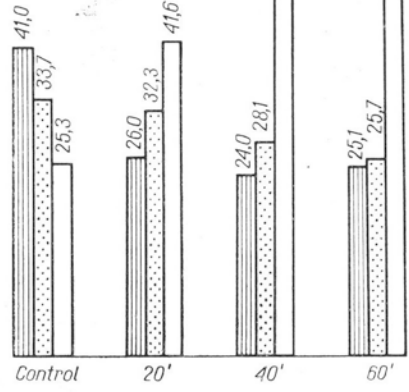

Fig. 5

Fig. 4. The per cent contribution of individual RNA fractions of maize (*Zea mays*) control germs and ones subjected to ultrasonic energy action with the intensity of 4 W/cm² for 20, 40, 60 min.

Fig. 5. The per cent contribution of individual RNA fractions of maize (*Zea mays*) control germs and germs subjected to ultrasonic energy action with the intensity of 8 W/cm² for 20, 40 and 60 min.

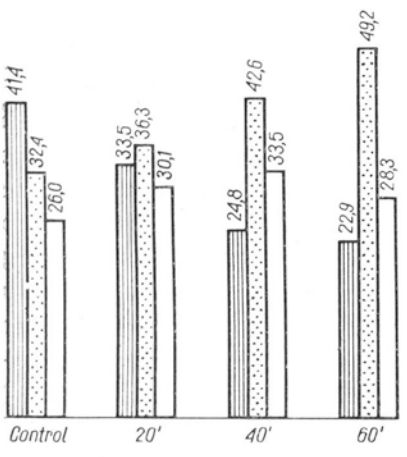

Fig. 6

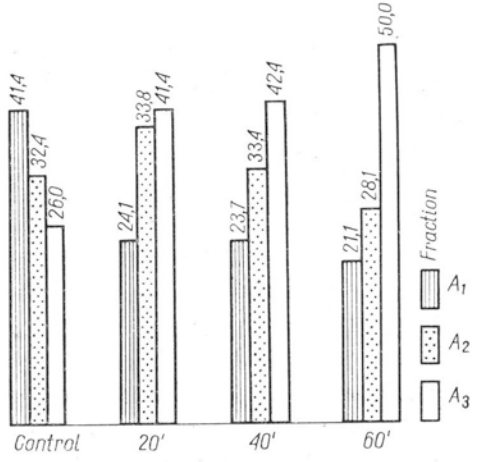

Fig. 7

Fig. 6. The per cent contribution of individual RNA fractions from pea (*Pisum sativum*) control germs and ones subjected to ultrasonic energy action with the intensity of 4 W/cm² for 20, 40 and 60 min.

Fig. 7. The per cent contribution of individual RNA fractions from pea (*Pisum sativum*) control germs and germs subjected to ultrasonic energy action with the intensity of 8 W/cm² for 20, 40 and 60 min.

1. Fraction A_1 — the least mobile, fluorescing in a bricky colour corresponded to highly polymerized RNA molecules with sedimentation coefficient of about 26 S.

2. Fraction A_2 — fluorescing in orange-yellow colour corresponded to RNA with sedimentation coefficient of about 16 S.

3. Fraction A_3 — fluorescing in sea-green colour corresponded to RNA with a sedimentation coefficient of about 4 S.

If the preparation was contaminated with DNA, darkgreen, almost immobile fraction appeared near the starting channel. Estimation of individual fractions was made using the formula established by Tsanev. The presented results (figures 1—7) are from 6 RNA preparations. Their mean per cent values were obtained from 20 fractionations of each preparation.

All the calculations were conducted in relation to control material — 4 days old germs of *Zea mays* and *Pisum sativum*. All the control plants were reared and examined simultaneously with the experimental ones.

The obtained results of examinations of RNA prepared from control and experimental plants are shown in the figures 1—7, in which densitometric graphs of RNA fractions with changeable time and ultrasonic energy parameters in the experimental material and in the control one are presented.

DISCUSSION

Methods of electrophoretic separation of biopolymers on different gels were introduced relatively recently. The electrophoretic profile of nucleic acids on agar-gel has been established by Tsanev in 1965. In this work we applied the method described by that author with the modification consisting in adjusting of electrophoretic reading with a densitometer to the visible light range. The question we attempted to answer was the influence of ultrasounds on cytoplasmic RNA in young plant tissues. For this purpose 4 days old maize (*Zea mays*) and pea (*Pisum sativum*) germs were subjected to the action of increasing doses of ultrasonic energy. Separations of ultrasonicated and control preparations were done simultaneously (see fig. 1).

RNA migration velocity depends on the charge and configuration of molecules (Ostrowski 1965). The agar-gel plays the part of a selective molecular sieve in. Almost linear dependence of the migration of molecules on the agar-gel and on their sedimentation coefficients was established by many authors. The heaviest components, with sedimentation coefficients 45 S and 35 S migrated with the lowest velocity and were found near the starting point, 28 S and 18 S components migrated more quickly and molecules with sedimentation coefficients 5 S and 4 S exhibited the greatest electrophoretic mobility. Summarizing one may say electro-

phoresis on the agar-gel is one of the most convenient analytical and preparative methods which may be applied for investigations of fractions of nucleic acids obtained from animal and plant tissues. (See figs 1, 2, 3).

Nucleic acids appeared to be sensitive to ultrasound action (Dvorkin 1960). There were subjected to depolymerization like other macromolecules. Dvorkin (1960) and Elpiner (1968) found that denaturation or only degradation of macromolecule proceeded depending on the kind of gas by which it was surrounded by. Molecular structure destruction presumably took in place of hydrogen and covalent bonds. It was suggested that destructive ultrasound action was of mechanical nature and that free radical groups created in this process played a considerable role here. This opinion unabled us an explanation of the observed RNA changes.

Analyzing results of per cent contribution of mean values of RNA fractions obtained during the electrophoresis we found that high molecular RNA fractions (A_1) were the most sensitive to ultrasound action. Per cent content of fraction A_1 decreased by about 41% to 21% with a simultaneous almost twofold increase of low molecular fraction (A_3) from 26% up to about 50% with applying maximal field intensity and exposure time. Mean molecular fraction (A_2) increased its per cent contribution with exposure time increase in the case of applying of ultrasonic field of intensity 4 W/cm^2. This phenomenon which is rather difficult to explain may be interpreted as a result of secondary cross-linking of RNA molecules with participation of water dipoles. We may not speak thus of a return to original structure in the case of secondary cross-linking which is the result of disordered joining of molecules. According to Dvorkin and Elpiner nucleic acids are subjected to degradation due to the cleavage of diester phosphate bonds. It is possible that under the conditions of an ultrasonic field of intensity 4 W/cm^2 the supplied energy is sufficient to compensate repulsion of phosphate groups of the same negative charge which with simultaneous revelation of charge of water dipoles, would make the secondary cross-linking phenomenon possible of course through the medium of structurally relaxed environmental water. For explanation of the increase of the fraction A_2 at the expense of fraction A_1 the fact observed by Burnotte and Frederico (1965) is not without importance. They found that RNA degradation due to ultrasound action proceeded mainly in the secondary structure, in the unhelical areas. We suppose a similiar phenomenon took place in the material observed by us (Figs 4, 5, 6, and 7), especially in the case of ultrasonic field of intensity 8 W/cm^2.

The above mentioned tentative explanations of the observed changes of RNA fractions are to a high degree based on theoretical premises and are of hypothetic character.

Further investigations are needed to confirm this hypothesis.

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Wpływ energii ultraakustycznej na rozdział frakcji cytoplazmatycznego RNA

Streszczenie

Tkanki roślinne (4-dniowe kiełki *Zea mays* i *Pisum sativum*) poddano działaniu energii ultraakustycznej przy użyciu generatora o stałej częstotliwości ok. 800 kHz. Izolowanie RNA z materiału roślinnego przeprowadzono metodą Gieorgiewa w modyfikacji Mieczyńskiego. Badania wpływu ultradźwięków na RNA prowadzone były in vivo.

W toku badań zaobserwowano, że w zależności od stopnia nadźwiękowania występowały zmiany w trzech frakcjach RNA: A_1 — 28 S, A_2 — 18 S i A_3 — 4 S.

Analizując wyniki średnich wartości udziału procentowego frakcji RNA podczas elektroforezy stwierdzono, że najbardziej wrażliwe na działanie ultradźwięków są frakcje wysokocząsteczkowego RNA.