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Electron Microscopic Autoradiographic Study of RNA Isolated from Apple-Tree Callus Tissue Labelled with 6-Benzylaminopurine-8-C^{14}

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Abstract. In the present paper RNA from apple-tree callus tissue labelled with 6-benzylaminopurine-8-C^{14} (6-B-8-C^{14}) was studied. The RNA isolated from this tissue was prepared as sample for electron microscopic studies and was also used as biochemical control. The electron microscopic autoradiograms obtained showed the labelled structure of RNA, sRNA and rRNA. The incorporation of labelled purine rings was confirmed in all three RNA types by the radioactivity, which was also proved in nucleotides after hydrolysation of RNA fractions. The results were compared with RNA isolated from tissue cultivated on a non-radiactive medium.

The effect of cytokinin on nucleic acid biosynthesis and on the maintenance of polysome structures in separated leaves has been described (SRIVASTAVA and WARE 1965). The incorporation of labelled purine rings deriving from cytokinins into nucleic acids was proved by FOX (1964, 1966) and SRIVASTAVA (1967). ZACHAU *et al.* (1965) isolated s-RNA from brewers yeast proving by chemical method the presence of cytokinins as a minor base in certain sequences in s-RNA. As far as we know there was no mention about the electron microscopic autoradiographic determination of radioactivity from synthetic cytokinin incorporated into RNA.

The autoradiographic method in electron microscopy was worked out by several authors (*e.g.* LIQUIER-MILWARD 1956; CARO, VAN TUBERGEN 1962; CARO 1962, 1964; GRANBOULAN 1963; SALPETER, BACHMAN 1964, 1967; DROZ 1967). On the basis of these findings, the localization of various labelled substances was studied, *e.g.* by NEUTRA and LEBLOND 1966; NORDNYNN and MAN 1966; STEIN and STEIN 1968.

In the present work we studied by electron microscopic autoradiography the incorporation of C^{14}-labelled bases into RNA, isolated from apple-tree callus tissue cultures, cultivated *in vitro* on a substrate to which 6-benzyl-

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aminopurine-8-C^{14} was added. We are of the opinion that it is appropriate to separate RNA, deproteinize it and so obtain a more detailed electron microscopic picture of the localization of radioactivity deriving from labelled cytokinins in these structures.

Material and Methods

RNA was isolated from apple-tree callus tissue cultivated *in vitro* on Murashigo-Skoog medium, to which 6-benzylaminopurine-8-C^{14} was added in a concentration of 1 mg/l. The specific activity of the applied kinin was 18·29 μC/mM.

RNA was isolated by the phenolic method. With regard to the plant material, the RNA extraction was carried out by us in a short heat shock (see FRIEDRICH 1968), or the material was at the beginning of this procedure homogenized in the cold, distilled phenol. After deproteinization the RNA was divided into two parts, one of which was used for electron microscopic and spectrophotometric studies and for the determination of radioactivity. The other part was fractionated on a Sephadex M 200 column. The outflow of the single fractions was checked on a Zeiss UV spectrophotometer and the radioactivity of each fraction was measured.

Electron Microscopy

The resulting RNA suspension in tris buffer was fixed for electron microscopy with 10% neutralized formaldehyde and spread on grids with Formvar films. Since during the development of autoradiograms the Formvar film may not remain tight to the grid and also the copper of the grid could interact with the photographic emulsion, the Silke method was used (1961). In this method the wires of the carrying grid are covered with Formvar ensuring that the space between the wires remains free. The grids were fixed on plexiglass cylinders, covered with gelatine impregnated with chrome alum. A Nucleic Ilford L 4 emulsion was applied to the samples by means of a platinum loop (CARO and VAN TUBERGEN 1962). Then the samples were put into a light tight box containing a drying agent and filled with CO_2, because water and oxygen have an attenuating effect on the latent picture. After a three weeks' exposure the samples were developed in a physical developer and after fixation stained for two hours with a 2% uranylacetate solution. The gelatine layer was removed by the method of REVEL and HAY (1961).

The grids were observed in a Tesla BS 242 and in a JEM 6 A microscope. Simultaneously the RNA suspension was dialyzed against water and used for autoradiography in electron microscopy. The absorptive spectrum of the suspension was determined by SPIRIN's method (1963) and showed the characteristic maximum at 260 mμ.

Parallel RNA samples were treated with basic hydrolization and the resulting mononucleotides were divided electrophoretically on Whatman 4 paper for 6 hours at 500 V. The single mononucleotides were identified in UV light and their radioactivity measured on a Frieseke-Hoepfner counter.

As control, RNA was isolated from apple-tree callus tissue, cultivated on the same substrate, but the added 6-benzylaminopurine was not labelled.

This RNA suspension was treated in the same way as other samples in all repeated experiments and was always chemically analyzed.

Results

Direct positive staining of the RNA suspension without using the technique of ultrathin cuts of tissue or sediment enabled us to obtain very detailed electron micrographs of RNA. It is known that RNA macromolecules change their shape. According to the properties and temperature of the solution, they change from ballike shapes over sticks up to more or less developed, non-interrupted polynucleotide chains (LITTAUER *et al.* 1960; DANON *et al.* 1961; SPIRIN 1963). On the base of electron microscopic studies we can confirm these findings. Because of the high resolving power of the electron microscope it is possible to use in autoradiography finer silver grains, thus obtaining a localization of radioactivity in terms of 0·1—0·2 μm. The great depth of the focus makes it also possible to observe the structure of RNA and the silver grains in the same level. Figure 1 shows a characteristic structure of deproteinized non-divided RNA with contrasting traces of radioactivity. The developed grains were in all preparations bound only to the RNA structure and appeared generally at its middle. The electronmicrographs were deliberately overexposed to obtain a less contrasting RNA. The minor magnification gives a general view of the localization of traces of activity on the RNA structure. In a direct magnification 48,000 (Fig. 2, 3) deproteinized RNA fibres, about 30 Å thick, are distinguishable, which corresponds to published data (HART 1958; HALL 1959; SPIRIN 1963). Figure 4 shows the detailed structure of deproteinized RNA, where part of the filament, 19 Å is spiralized.

For the study of 6-benzylaminopurine-C^{14} incorporation, RNA was further divided into r-RNA and s-RNA (see Methods). Autoradiograms of both r-RNA and s-RNA show traces of radioactivity. Fig. 5 was made with a JEM 6A electron microscope. Also in this picture contrasting traces of activity are visible. The average thickness of the fibre is again around 30 Å. Fig. 6 is an autoradiogram of s-RNA with contrasting spots of activity, which again on all preparations was bound to the RNA structure.

Fig. 7 was made as control with non-radioactive RNA. The preparation was prepared for autoradiography in the same way, as the others (no traces of radioactivity were found).

On the basis of electron microscopic autoradiography, the radioactivity was determined in non-divided RNA as well as in r-RNA and s-RNA. The determination of these nucleic acids proves the incorporation of radioactivity. Adenylic acid and guanylic acid (for details viz FRIEDRICH 1968) were always found to be radioactive. The incorporation was also verified by measuring the radioactivity of RNA before and after dialysis against distilled water with $MgCl_2$.

Discussion

Descriptions have been published of different techniques of preparation of autoradiograms, differing in the emulsion and the developer applied and

consequently in resolution power. In our experiment we used cytokinin labelled with C^{14}, emanating beta particles of 0—155 KeV energy. The diameter of the RNA fibres is around 30—40 Å. The autoradiograms were therefore developed with physical developers, because this technique enables one to determine the position of the latent image with an error of about 200 Å, *i.e.* with an error, which is five times smaller than that obtained when using a fine grain Microdol X developer. Under these circumstances it is not possible to localize precisely the incorporation of radioactivity on the RNA fibres, because the size of the traces of radioactivity are in terms of 200—600 Å. It is, however, striking that the traces of radioactivity are always located in the center of the RNA structure and never on the edge. Since the Figures 2, 3, 4, 5, 6 and 7 were equally magnified, it is conspicuous that the structure of both r-RNA and s-RNA are fractions of non-divided RNA (Fig. 2). It is interesting to notice that the maximum size of the single traces of radioactivity is different for non-divided RNA and s-RNA.

Finally it can be assumed that the labelled base applied in the form of 6-benzylaminopurine-8-C^{14} to the cells of apple-tree callus tissue is incorporated into RNA with a certain special regularity.

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V této práci byla metodou elektronmikroskopické autoradiografie studována inkorporace C^{14} značených basí do RNK, isolované z kulusů jabloně, pěstovaných in vitro v mediu se značeným 6-benzylaminopurinem-8-C^{14}. Radioaktivita byla stanovena jak u autoradiogramů sumární RNK, tak r-RNK a s-RNK. Důkazem, že se jedná o skutečnou inkorporaci, je stanovení radioaktivity mononukleotidů, získaných zásaditou hydrolysou těchto nukleových kyselin. Výsledky byly srovnány s RNK isolovanou z tkání, pěstovaných na neznačené živné půdě.

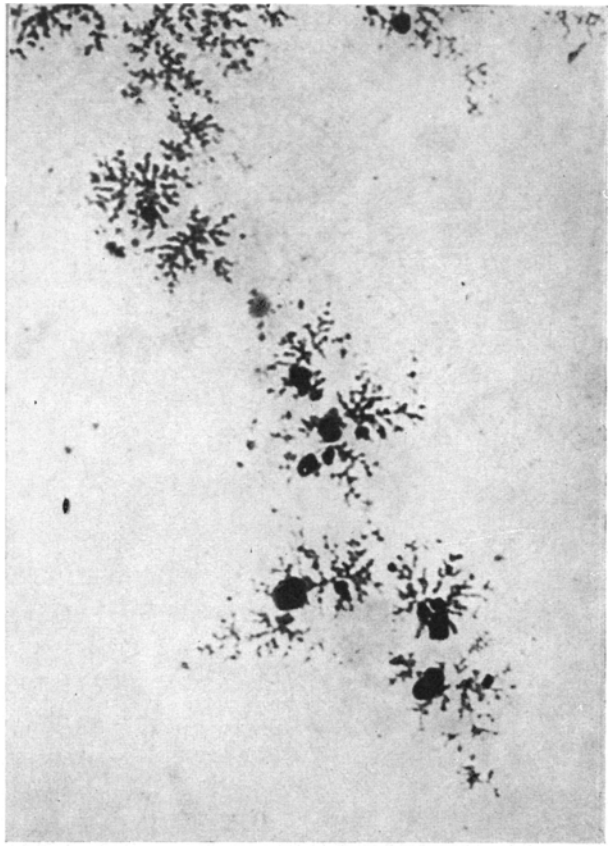

Fig. 1. General view of the non-divided RNA with traces of incorporated radioactivity. Suspension of deproteinized RNA stained with 2% uranylacetate. Magnification 18000×.

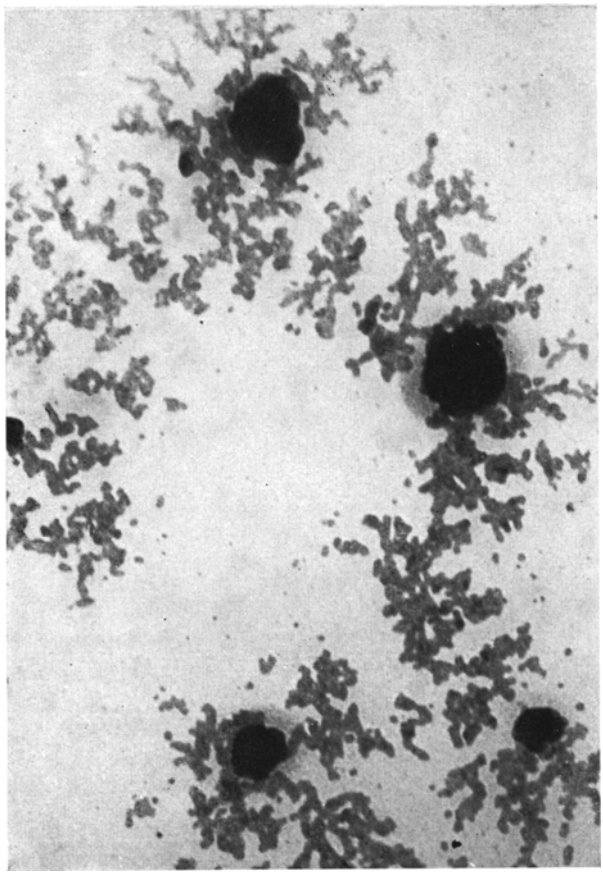

Fig. 2. Major magnification showing details of RNA structure with incorporated radioactivity. Magnification 155000×.

Fig. 3.

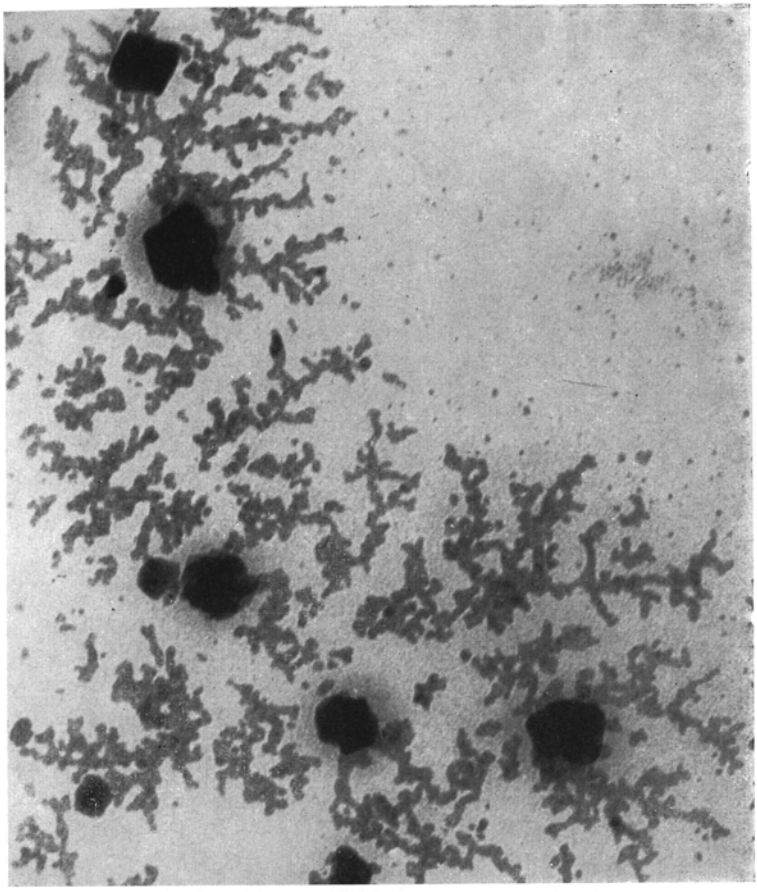

Fig. 4. Magnification 200000×.

Fig. 3, 4. General view with major magnification of the labelled structure of RNA and of the detailed structure of spiralized RNA filament (19 Å). Fig. 3. Magnification 155000×.

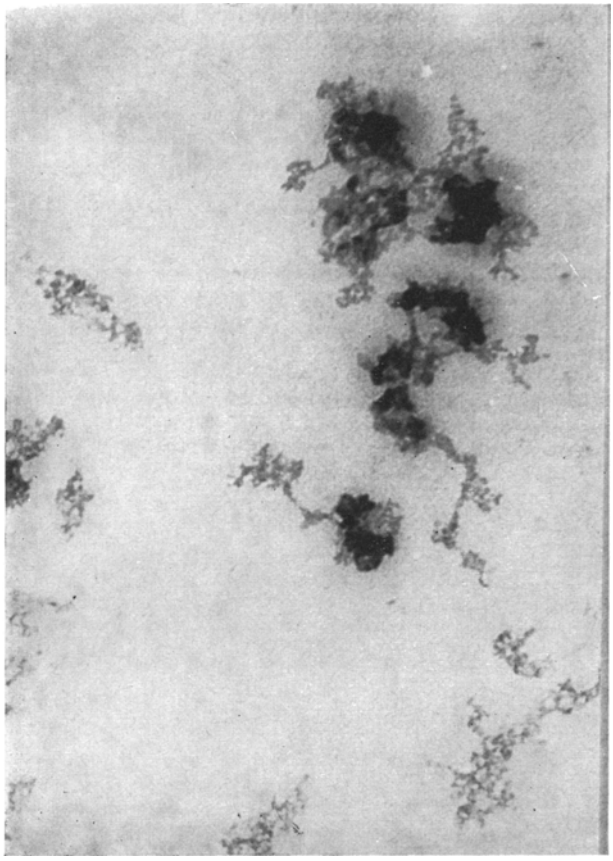

Fig. 5. Fraction of labelled ribosomal RNA. Magnification 155000×.

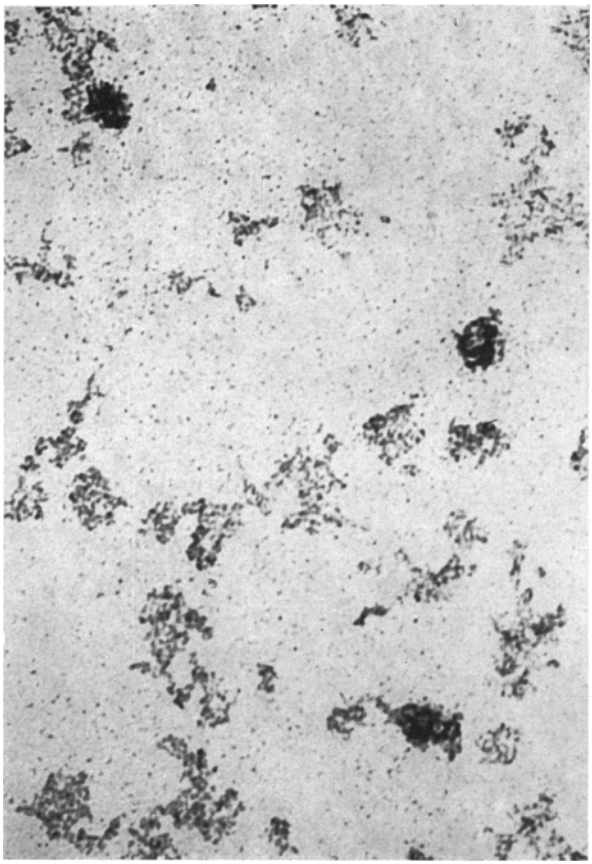

Fig. 6. Fraction of labelled soluble RNA. Magnification 155000×.

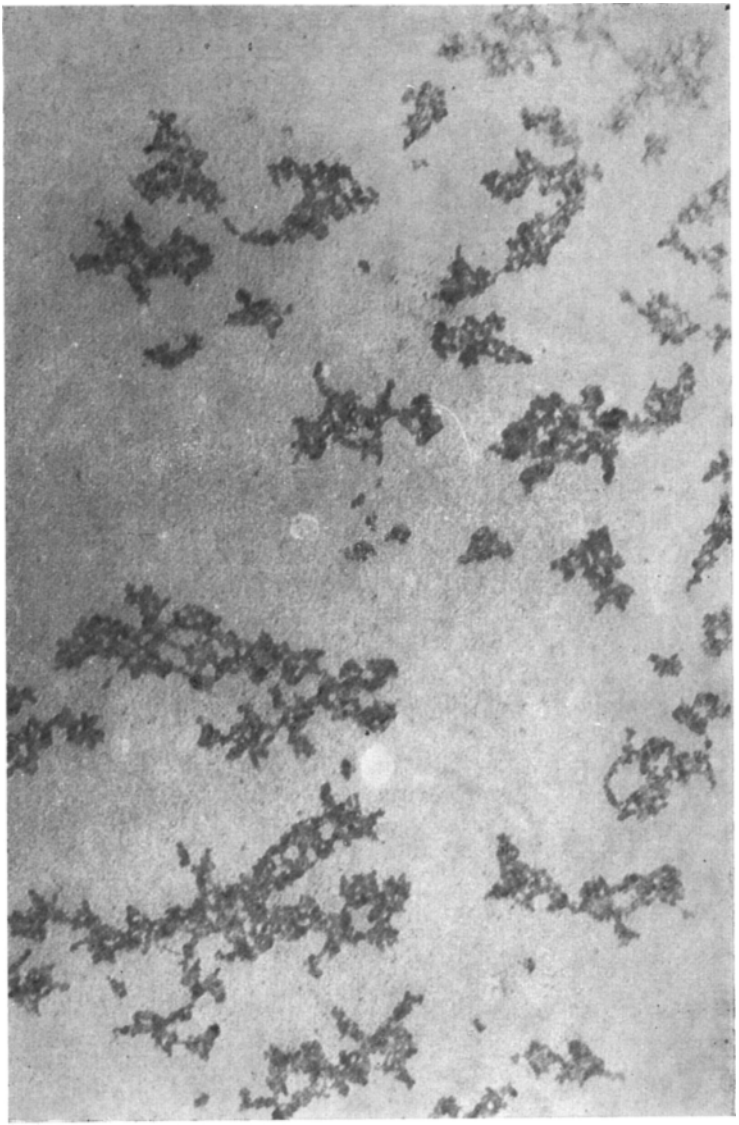

Fig. 7. Control electron autoradiogram of RNA without incorporated radioactivity. Magnification 155000 ×.