RIBONUCLEIC ACID SYNTHESIS IN ISOLATED PLANT NUCLEI *IN VITRO*

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ABSTRACT

The evidence for the synthesis of RNA for an extended period of time and the release of synthesized RNA after its post-transcriptional modification into post-nuclear supernatant of the nuclei of *Vigna sinensis* have been presented here. The criteria of purity of the nuclear preparation have been checked by electron microscopy. The kinetics of RNA synthesis showed that the rate of synthesis of RNA using the labelled RNA precursors was biphasic; the initial linear rate was observed for the first 100 min, then a reduced rate for the next 120 min but the release of RNA increases steadily up to 180 min. The gross nature of the released RNA in the post-nuclear supernatant has been characterized. It shows that the released RNA contained an appreciable amount of poly(A) containing mRNA and mRNP-like particle in addition to *r*-RNA, *t*-RNA and poly(A)$^-$ mRNA.

INTRODUCTION

EXTENSIVE investigations have been carried out on the synthesis of RNA and the release of these RNAs into the supernatant after post-transcriptional modification in polysomal fraction from mammalian tissue[1]. Recent reports have shown that nuclei isolated from a variety of cells synthesize RNA for extended periods of time[2-8] and can perform several post-transcriptional events such as polyadenylation, capping and processing of primary transcripts,[2,5-9]. Nucleus is known to contain the machinery for transcription and replication in eukaryotes and can initiate intact cell processes in respect of RNA synthesis[8,9]. It has also been suggested that one of the many possible functions of protein bound forms of eukaryotic mRNAs is their involvement in nucleo-cytoplasmic transport of mRNA[10]. RNA synthesis *in vitro* for extended periods of time in rat liver nuclei has recently been reported[11]. The presence of poly(A)$^+$ mRNAs as well as well-defined mRNPs in the post-nuclear supernatant has also been confirmed from the above studies.

Information on the mechanism of RNA synthesis, gross nature of the synthesized RNA, processing of synthesized RNA and the role of processed RNA in the molecular process in plant system is scanty[12]. Earlier work from this laboratory have shown that nucleus isolated from *Vigna sinensis* (Linn) savi is biologically active with respect to RNA and DNA synthesis[13].

In this communication, we report extended RNA synthesis in isolated nuclei of 48 hr germinating embryos of *V. sinensis* and the subsequent release of *in vitro* synthesized RNA into the supernatant. Efforts have been made to determine the nature of the released RNA.

MATERIALS AND METHODS

Radioactive ribonucleoside triphosphates ([^{3}H]-UTP) was purchased from New England Nuclear Corporation Inc. Unlabelled ribonucleosides were purchased from Calbiochem. Agarose, acrylamide, PPO and POPOP were purchased from Sigma Chemical Co., USA. Oligo(dT) cellulose (type T_3, Collaborative Res. Inc.) was used. All other chemicals used were of analytical grade and were purchased from Merck or BDH, England.

Germination of seeds and isolation of nuclei: Seeds of *V. sinensis* (purchased from local market) were germinated as reported earlier[14]. Nuclei were isolated from the embryos of the germinating seeds according to method reported earlier[13,15].

Fixation and staining for TEM of intact nuclei: Nuclei as isolated by us were fixed and negatively stained with potassium phosphotungstate (pH 7.4) for TEM examination. The isolated nuclei were washed five to six times with 0.1 M saline citrate buffer (pH 7.6) and subsequently centrifuged at 1500 g for 10 min and the pellet was subjected to fixation with 3% glutaraldehyde solution in saline citrate at pH 7 at 4°C for 10 min. The fixation procedure maintains the intactness

of nuclear structures. The nuclei as fixed by glutaraldehyde reagent were spun at 1500 g for 10 min at the same temperature and were suspended in 0.45 M sucrose buffer containing 6 mM Tris-HCl (pH 7.4) having 3 mM $MgCl_2$. This nuclear preparation was centrifuged at 10000 g for 10 min. The pellet thus obtained was resuspended in the same buffer. A definite aliquot Of suspension was mixed with the equal volume of ice-cold 1.5% potassium phosphotungstate (pH 7.4) and the mixture was sprayed on to carbon-coated nitrocellulose grids in the form of droplets using micropipette. After allowing the droplet to settle for a few minutes, the liquid was carefully removed with a filter paper from one end of the grid. The grid was dried and viewed in a transmission electron microscope.

Fixation and staining for SEM: After the isolation of nuclei, these were subjected to fixation and staining for SEM examination. Nuclei were washed in 0.1 M sodium phosphate buffer (pH 7.4) and centrifuged at 1500 g for 10 min. The supernatant was discarded and the process repeated 5 times. The final pellet was suspended in the phosphate buffer and treated with 4% phosphate buffered glutaraldehyde fixative slowly at pH 7.4 at 4°C, kept overnight at the same temperature followed by centrifugation at 1500 g for 15 min to pellet out the fixed nuclei. This was subsequently washed with phosphate buffer, two to three times, to remove the excess fixative. The fixed nuclei were then subjected to post-fixation process in 2% phosphate buffered osmium-tetroxide fixative, pH 7.4 for 30 min. The fixed nuclei were dehydrated with graded concentration of alcohol starting from 35% to 100%. This preparation was ready for SEM analysis. The suspension was taken on a small glass slide (1 × 1 cm) and dried in air. The glass slide was coated with gold (thickness 300 Å) in Edward's Coater (Phillips SEM 500).

Incorporation of [^3H]-UTP by isolated nuclei: The complete assay system and the reaction conditions are given in the legend to the respective figures.

Determination of radioactivity incorporated into nuclear RNA in various experimental conditions: Unless otherwise mentioned, the radioactivity after incubation with labelled UTP, was determined as described by Dube *et al*[16].

Determination of protein and DNA: Protein was determined by the method of Lowry *et al*[17] and DNA determined by the method of Burton[18].

Extraction of RNA from nuclear pellet and released RNA from the post-nuclear supernatant for electrophoresis: The nuclear pellet and the post-nuclear supernatant fractions were separated by centrifugation (10 min, 0–4°C) from the pooled incubation mixtures after 120 min of incubation with [^3H]-UTP under standard conditions. RNA was isolated from each fraction by hot SDS phenol method[20] as described by Brawerman[19], and kept suspended in the minimum volume of 10 mM Tris-HCl buffer at neutral pH and stored at −20°C for electrophoresis.

Preparation of sample for polyacrylamide and agarose gel electrophoresis: The RNA sample extracted from nuclear pellet and post-nuclear supernatant fractions taken in neutral Tris-HCl buffer were analyzed on agarose-polyacrylamide gels according to Loening[21] and modified by Roy *et al*[11]. A gel electrophoresis system was used containing 2% polyacrylamide (20:1 acrylamide/methylene bis acrylamide) and 0.5% agarose (9 × 0.6 cm) in 0.4 M Tris-HCl buffer (pH 7.8) with 0.02 M sodium acetate, 2 mM EDTA, and 0.1% SDS in 350 µl of 10% ammonium persulfate (w/v), and 35 µl TEMED (2.5%). Electrophoresis was then carried out for 3 hr at 5mA/gel and 4.5 volts/cm at room temperature. Bromophenol blue (0.01%) was included in the sample as a visual marker. After electrophoresis, the gels were cut into 2 mm slices. The slices were solubilized with 200 µl of 30% H_2O_2 overnight at room temperature and counted in aquasol scintillation fluid in a scintillation counter[22].

Determination of poly(A)$^+$ mRNA and poly(A)$^+$ mRNP in the post-nuclear supernatant by oligo(dT)-cellulose column chromatography: Cell-free RNA synthesis was carried out at 30°C for 120 min in a complete system as described in the legend to figure 2. After incubation the reaction mixtures were chilled in ice and pooled. The resulting mixture was then centrifuged at 1500 g for 10 min at 0°C to separate the nuclear pellet from the supernatant fraction. The nuclear pellet was further washed two to three times with homogenizing buffer A (0.25 M sucrose, 6 mM Tris-HCl pH 7.4, 3 mM $MgCl_2$) and the first wash was added to the supernatant fraction. One portion of this pooled supernatant is used for isolating poly(A) containing RNA by passing through oligo-dT column. Another portion is first subjected to deproteinization[19] and the same fraction was used for isolating poly(A) containing RNA. The samples containing RNA were subjected to oligo-dT cellulose chromatography as described by Roy *et al*.[11].

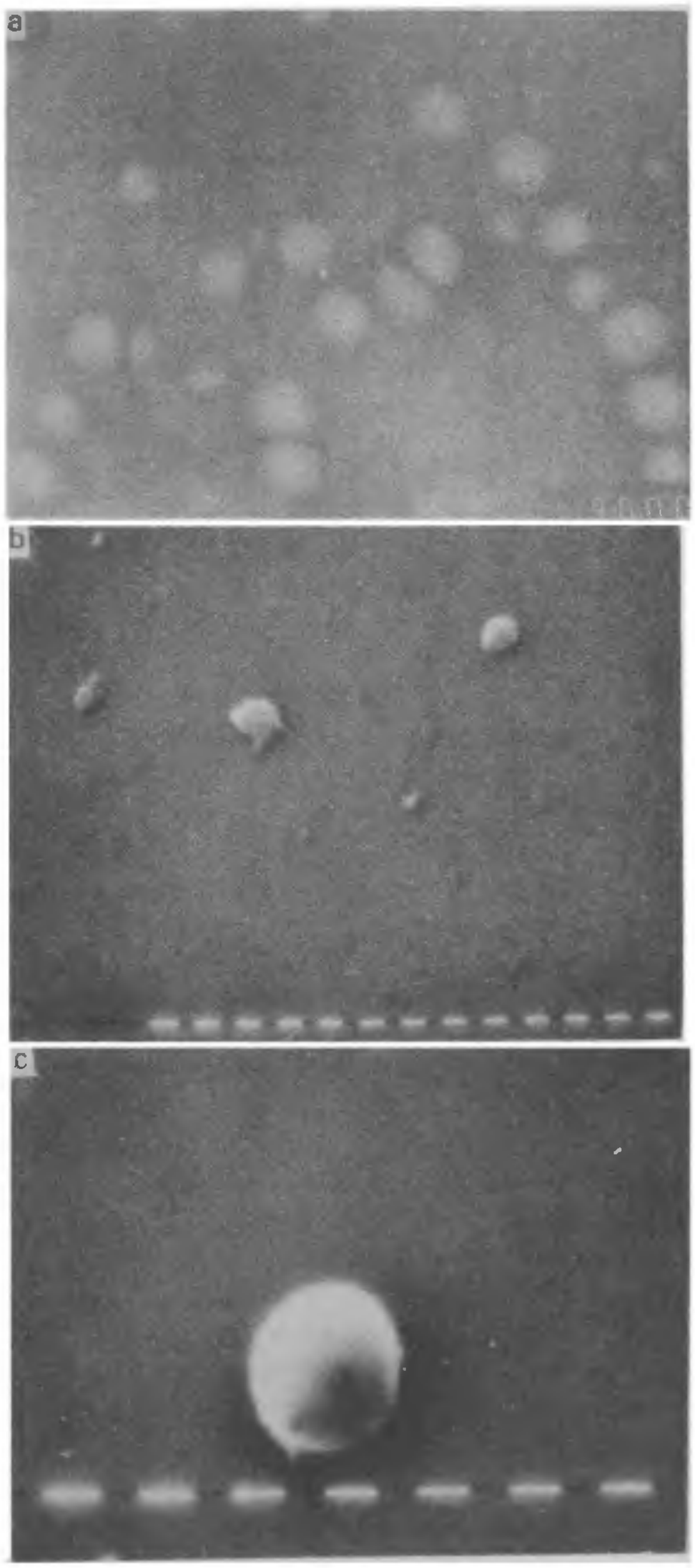
These post-nuclear supernatant fractions were dialyzed at 4°C overnight against the binding buffer

[10 mM Tris-HCl (pH 7.6)/0.5 M KCl/1 mM EDTA] containing heparin at 100 μg/ml and 0.5 mM phenylmethyl-sulfonyl fluoride. It was then applied to an oligo(dT) cellulose column (6 × 0.5 cm) previously equilibrated with the binding buffer at 4°C. After the supernatant was charged into the column, the column was extensively washed with binding buffer at 4°C until the UV absorbance at 260 nm of the fractions (1 ml) was reduced to the background level *i.e.* zero absorbance. The bound materials were eluted stepwise with a low salt buffer [10 mM Tris-HCl (pH 7.6)/10 mM KCl/1 mM EDTA] first at 4°C and subsequently the 2nd wash using the same buffer carried out at 45°C. The labelled RNA of each fraction was precipitated by cold CCl_3COOH at 4°C using 150 μg of yeast RNA as carrier[16].

RESULTS

Preparation of biologically active nuclei for its subsequent use in the study of RNA synthesis: The plant nuclei in its highest degree of purity has been isolated and purified (figures 1A, 1B and 1C). These purified plant nuclei showed complete absence of lysed nuclei, whole cell contaminants and impurities as revealed by TEM and SEM analysis. The nuclear preparation is fully active as far as *in vitro* ribonucleic acid synthesis is concerned[13]. Using this purified nuclear preparation the mode of synthesis of RNA and the release of processed RNA in the supernatant fraction have been studied.

Incorporation of [^3H]-UTP into nuclear RNA and the release of this labelled nuclear RNA into post-nuclear supernatant: Figure 2 represents the incorporation of [^{3}H]-UTP into nuclear RNA and also the release of this labelled RNA in the post-nuclear supernatant as measured by acid precipitable material trapped into glass fibre filter paper disc. In this experiment the incorporation of [^{3}H]-UTP into nuclear RNA is biphasic as the rate of synthesis of RNA linearly increases up to 100 min whereas a reduced rate is observed beyond 100 min reaching a steady level at about 120 min thereafter it reduces (figure 2). On the other-hand, the release of RNA from nucleus to post-nuclear supernatant reaches a steady level at about 180 min. To establish the fact that RNA which is released from the nuclei does not leak out of the nuclei after lysis, the intactness of the nuclei has been examined after 60 min of routine incorporation under phase contrast microscope.



Figures 1a–c. a. Transmission electron microscopic view of isolated plant nuclei. The plate represents the electron micrograph of PTA stained plant nuclei (3000 ×) **b.** Scanning electron microscopy of isolated purifield plant nuclei (Scale length – 1 μ). **c.** Scanning electron microscopy of isolated purified plant nuclei (Scale length – 1 μ).

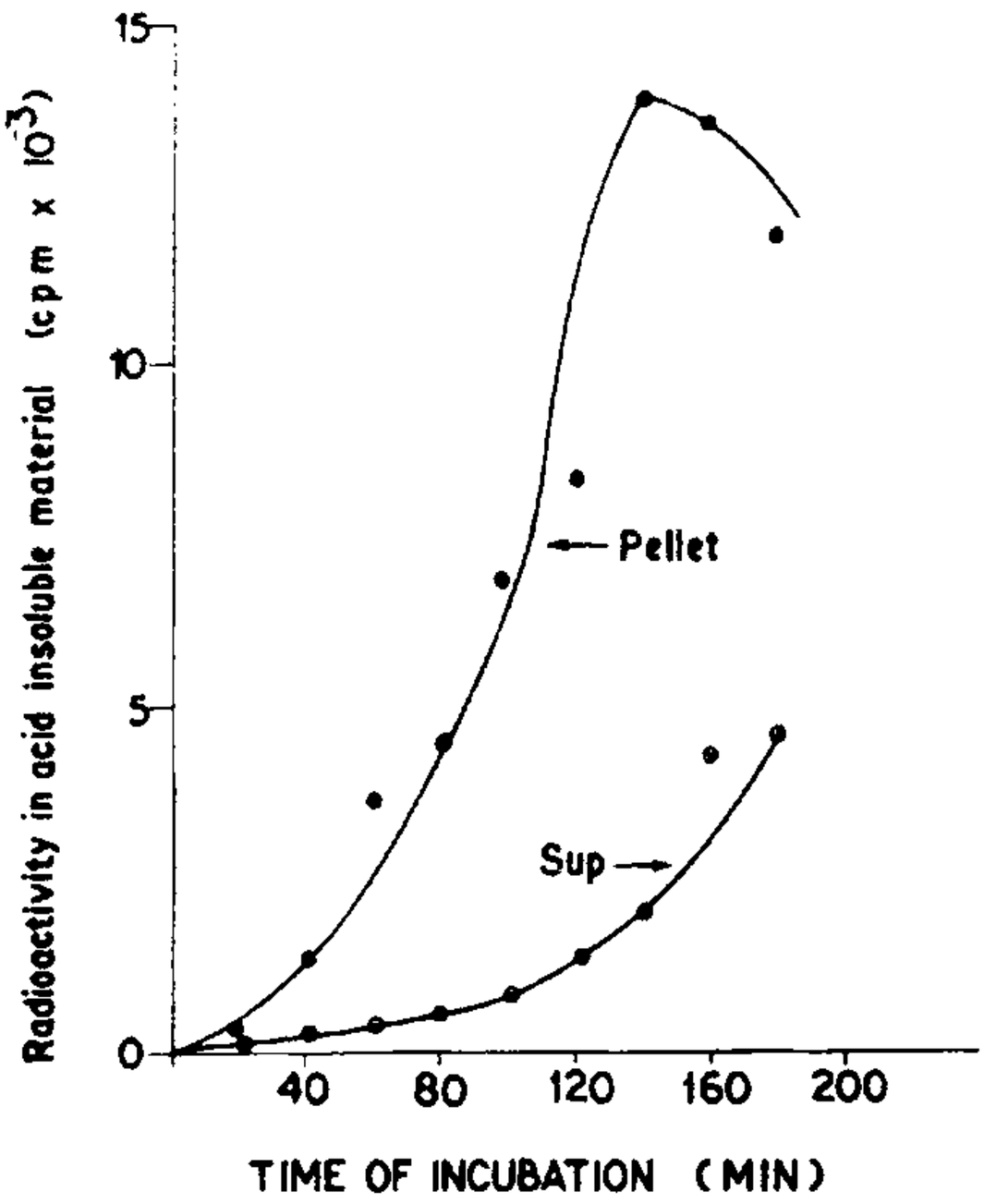


Figure 2. Incorporation of [^{3}H]-UTP into RNA by isolated plant nuclei and rate of release of [^{3}H]-UTP labelled nuclear RNA into supernatant. Cell free RNA synthesis was carried out in a total volume of 0.3 ml containing 250 mM sucrose, 16.7 μM of [^{3}H]-UTP (specific activity 65 cpm/pmole), 0.5 mM DDT, 50 mM Tris-HCl (pH 8.0), 16.7 μM each of ATP, CTP and GTP, 0.05 mM PEP, 10 mM $MgCl_2$ and 140 μg nuclear protein ($\approx$ 53 μg DHA). All the incubation were carried out in duplicate at 30°C for different time points. The reactions were stopped by additions of 0.8 ml of ice cold isotonic sucrose buffer A containing 0.001 ml of 0.5 mM cold UTP and the system were spun at 1500 g for 10 min to separate the nuclear pellet from the supernatant fraction. The supernatant thus obtained after centrifugations containing released labelled RNA after post transcriptional modifications. The pellet was then suspended in 0.8 ml of isotonic sucrose buffer. The reactions in nuclear pellet and supernatant were stopped with equal volume of ice cold 10% TCA (w/v) and then precipitated materials were processed as already mentioned in Materials and Methods Section. ●——● Nuclear pellet o——o Supernatant.

RNase digestion and alakline hydrolysis of the acid insoluble product from the supernatant: Radioactivity present in the post-nuclear supernatant is due to the release of *in vitro* synthesized nuclear RNA as alkaline hydrolysis of the supernatant resulting after incubation of [^{3}H]-UTP with that nuclei does not retain any radioactivity in the fibre disc after its filtration. On the other hand, RNase digestion of the parallel samples retains significant radioactivity, thus indicating the presence of RNase-resistant particle in the total population of RNA.

Electrophoretic analysis of extracted RNA from nuclear pellet and supernatant: Finally attempts have been

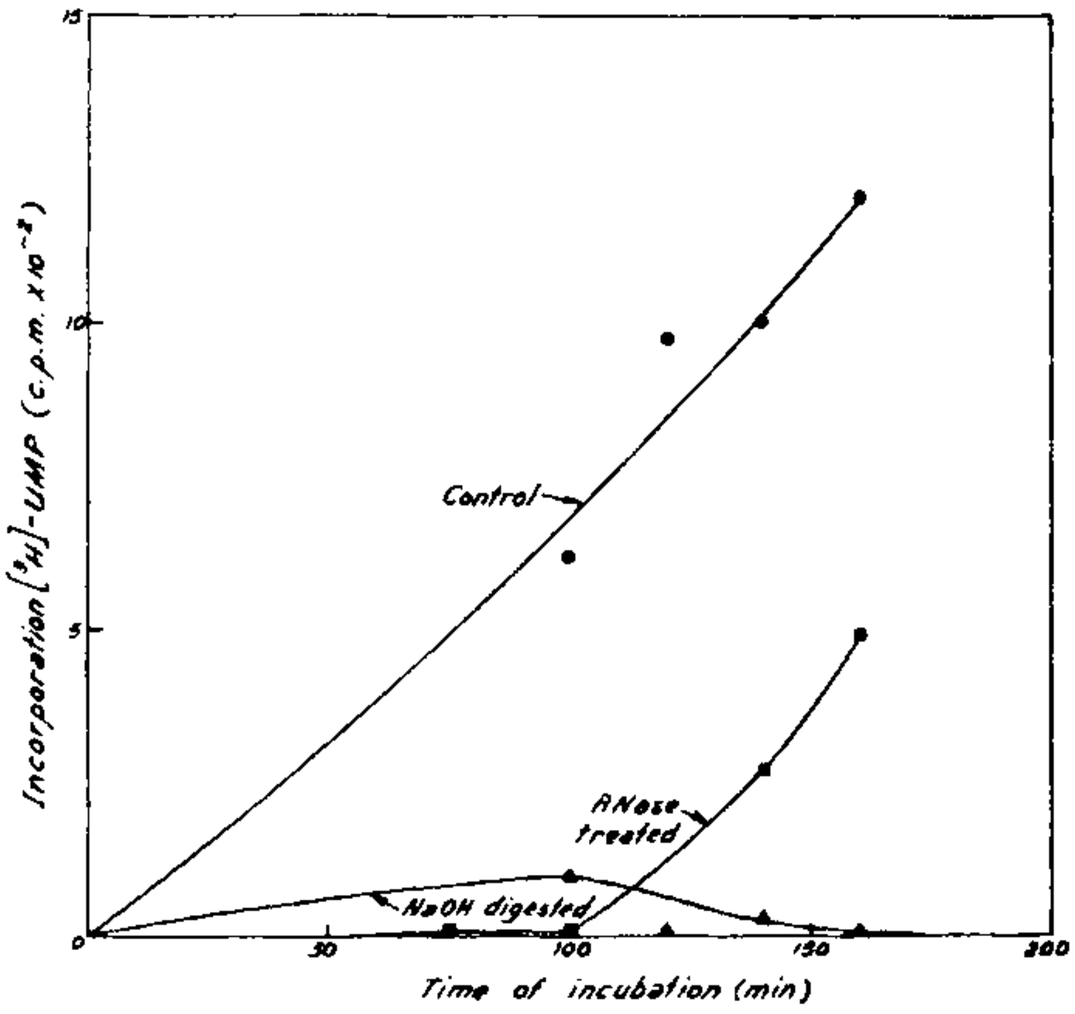


Figure 3. RNase digestion and alkaline hydrolysis of the acid insoluble product from supernatant. The incubation system and the reaction conditions were same as given in the legend to figure 2 excepting the content of nucleoprotein was 110 μg ($\approx$ 38 μg DNA). The incubations at different time points were stopped as per standard method as mentioned in the legend to figure 2. Radioactivity in the supernatant were determined as mentioned earlier. Equivalent amount of aliquot from the supernatant were subjected to: (a) Alkaline hydrolysis was carried out with 0.5 ml of 0.2 N NaOH. The mixture was heated in boiling water bath for 30 min and processed as mentioned in Materials and Methods Section. (b) Digestion with 1 μg/ml pancreatic RNase and 1 μg/ml Rnase T_2 at 30°C for 20 min. The reaction was stopped as mentioned in the legend to figure 2 after addition of 0.1 ml of BSA (1 mg/ml). Radioactivity was determined as given in Materials and Methods section. ●——● post nuclear supernatant ▲——▲ NaOH digested supernatant ■—■ RNase digested supernatant.

made to determine the gross nature of RNA species and electrophoretic studies carried out with the sample of RNA extracted from the nuclear pellet and post-nuclear supernatant fraction by hot SDS phenol method[20] after incorporation of [^{3}H]-UTP according to the standard procedure[13] (figure 4). After electrophoresis the gels were cut into 2 mm pieces. On solubilization of the gel pieces the counts were taken as described by Bouman[22]. In this connection, it could be mentioned that RNA samples extracted with hot SDS phenol substantially retain poly(A)$^+$mRNA in the aqueous phase together with other species of RNA excluding contaminating DNA fractions.

The plot of radioactivity against fraction numbers represents a graph having several peaks. Of these, four may be considered to be major peaks. As far as the nature of the peaks are concerned there is not much difference between the RNA from either pellet or supernatant. But the recovery of radioactivity appears to be slightly higher in the case of RNA sample extracted from nuclear pellet as compared with nuclear supernatant. Although this experiment cannot provide any difinite clue on the exact nature of different RNA species synthesized in the plant nuclei and its release in the post-nuclear supernatant, the results definitely show a hetrogeneous distribution of different RNA species with different molecular weights.

Oligo(dT)-cellulose column chromatography of released RNA in the supernatant: Oligo(dT)-cellulose column chromatography was employed to find out the nature of macromolecule, more specifically various species of RNA present in the post-nuclear supernatant. It is possible to separate poly(A)$^+$mRNP from poly(A)$^+$mRNA by oligo(dT)-cellulose by using a low salt elution buffer at temperature 4°C and 45°C[24,25]. Figure 5A shows that the major portion of the radioactivity in the post-nuclear supernatant was

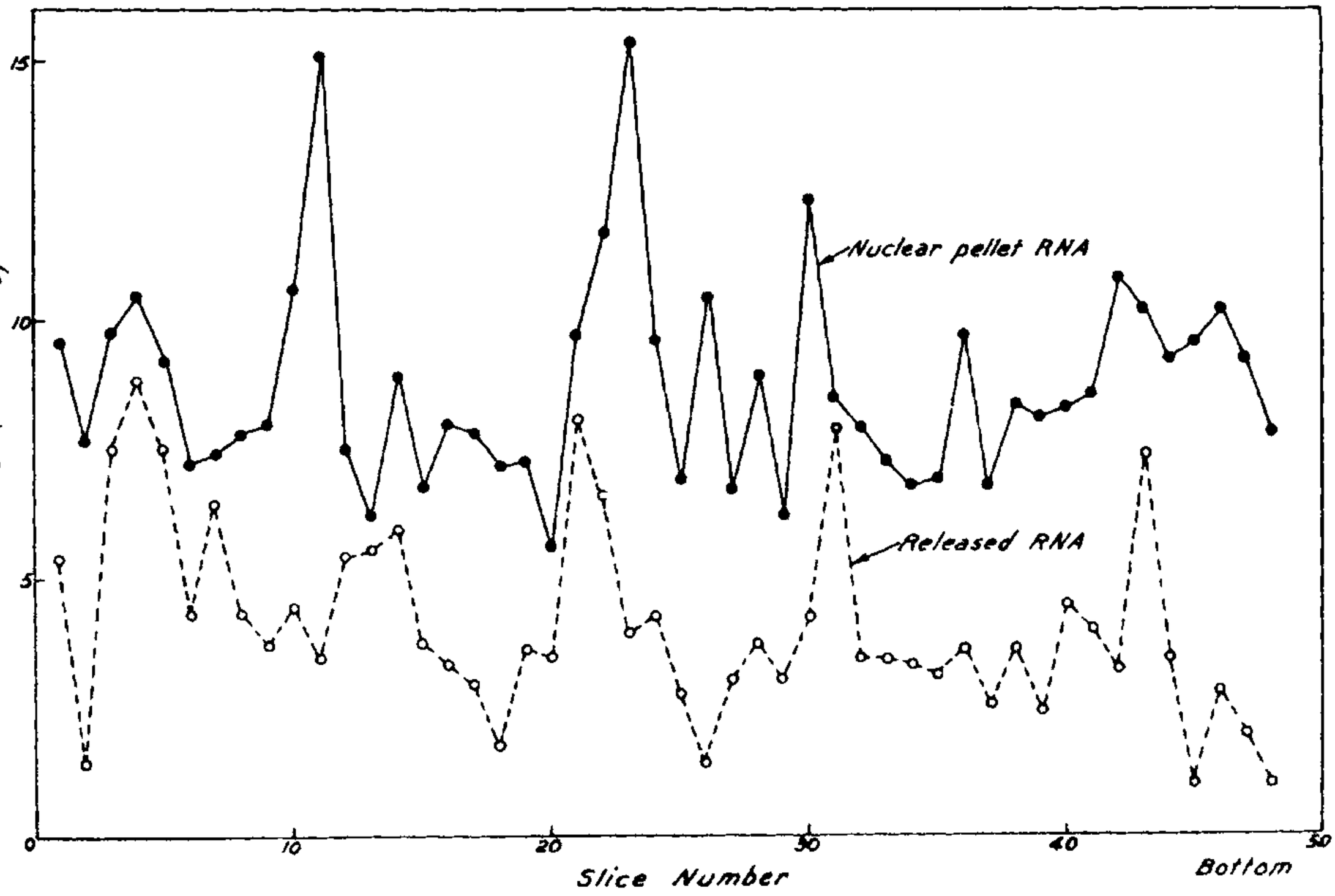


Figure 4. Polyacrylamide gel electrophoresis of RNA synthesized *in vitro*. Experimental details were given in Materials and Methods and in the legend to figure 2. RNA was isolated from nuclear pellet and the post nuclear supernatant. Electrophoresis was carried out for 3 hr in 2% gels supplemented with 0.5% agarose. The gels were sliced (2 mm) and radioactivities were measured as given in Materials and Methods section. ●-- -- ● RNA from nuclear pellet; containing 41600 cpm o----o RNA from post nuclear supernatant; containing 13365 ppm.

recovered in the unbound fraction. This fraction presumably contains *r-RNA*, *t-RNA* and poly(A)$^-$ mRNA. Elution with low salt buffer at 4°C released free poly(A)$^+$ mRNA from the column. Elution of the column with low salt buffer at 45°C releases the bound poly(A)$^+$ mRNP. Total radioactivity recovered in the fractions eluted at 4°C is about 1–2% of the total radioactivity applied to the oligo(dT)-cellulose column. From the chromatographic profile at differential temperature, it is suggested that poly(A)$^+$ mRNP particle is released in the post-nuclear supernatant along with the poly(A) containing mRNA. Figure 5A also shows that approximately 3–5% of the total radioactivity recovered in the bound fraction *i.e.* counts recovered in fractions contain poly(A)$^+$ mRNA and poly(A)$^+$ mRNP. This agrees with the earlier report by Brawerman[23] who showed that the content of poly(A)$^+$ mRNA in the intact cell is not more than 3–5%. This finding in isolated plant nuclei also supports the earlier reports[26] regarding the extended RNA synthesis and the processing of synthesized RNA in the isolated nuclei of animal cell. This may be considered a unique finding in isolated plant nuclei in *in vitro* RNA synthesis and its release after post-transcriptional modification.

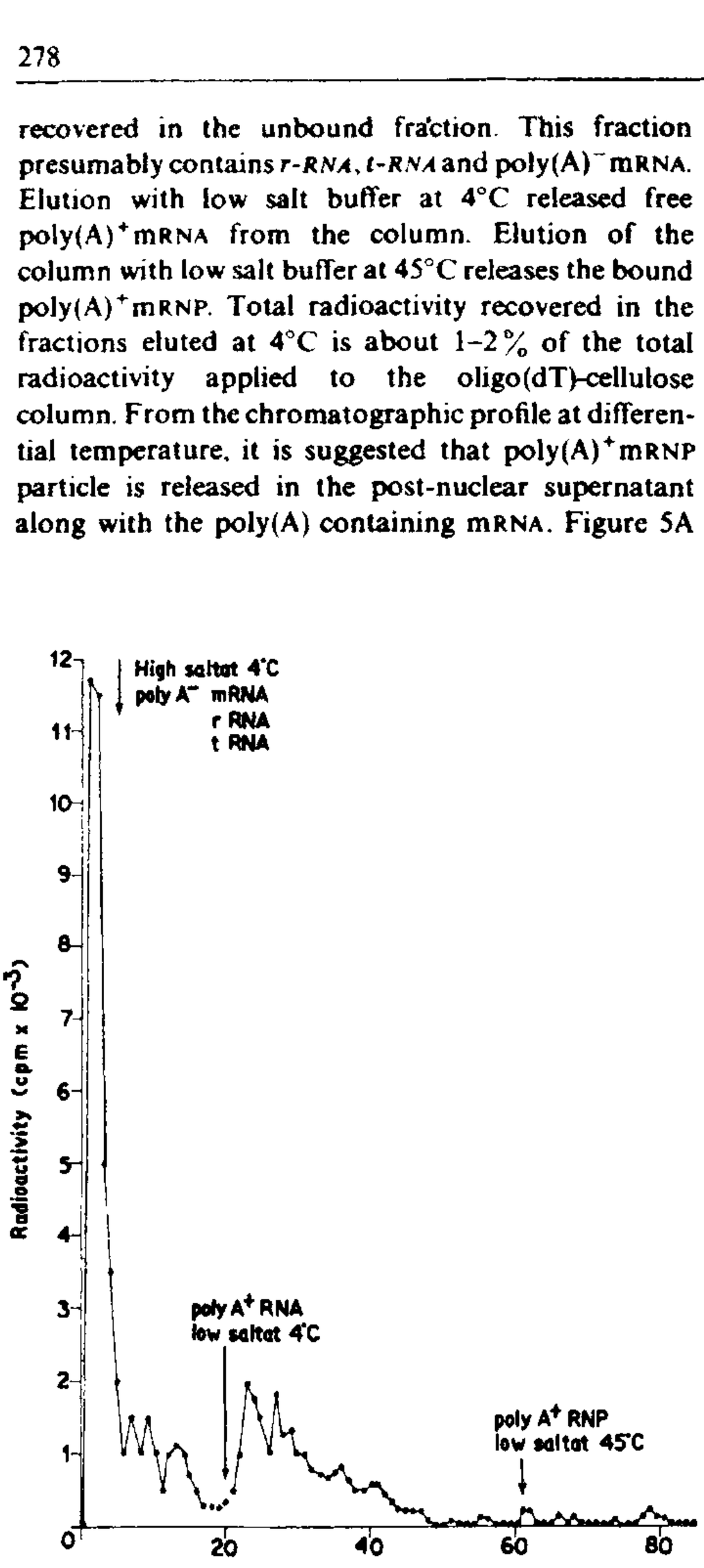


Figure 5. (A) Oligo(dT)-cellulose chromatography of labelled RNA products released into the supernatant fraction from the nuclei. The experimental details were given in Materials and Methods section. Fractions collected were 1 ml. The pooled supernatant fractions were chromatographed. The arrows indicate the positions where the columns were washed or eluted with various buffer. ●——● Elution profile of the supernatant fraction charged into column containing 30310 cpm radioactivity.

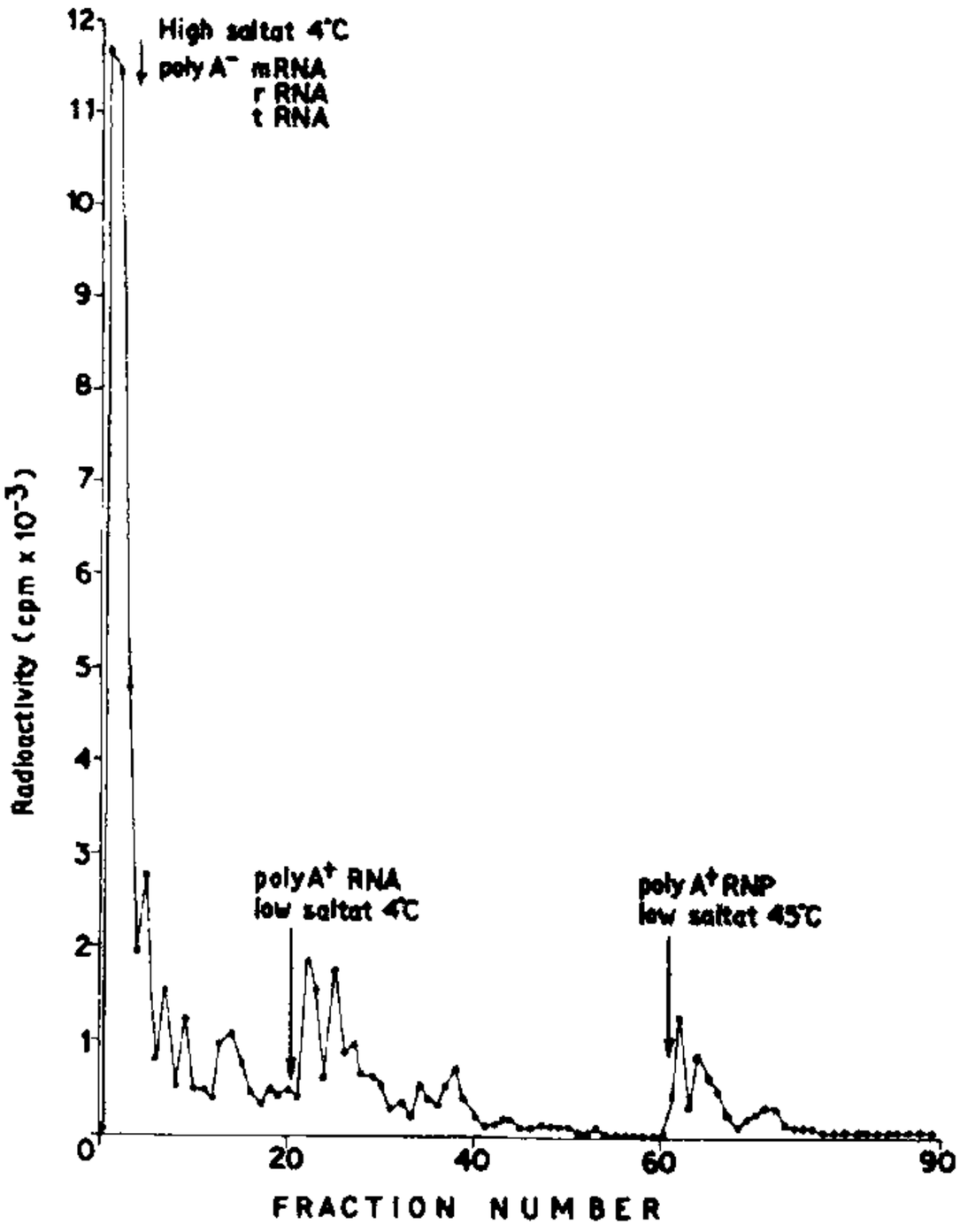


Figure 5. (B) Oligo(dT)-cellulose chromatography of deproteinized labelled RNA products released into the supernatant fraction from the nuclei. The experimental details were given in Materials and Methods section. Fractions collected were 1 ml. The pooled supernatant fractions after deproteinization were chromatographed. The arrows indicate the positions where the columns were washed or eluted with various buffers. ●——● Elution profile of the supernatant fraction charged into column containing 27500 cpm radioactivity.

Oligo(dT)-cellulose column chromatography of deproteinized released RNA in supernatant: It is noted from figure 5B that when the pooled post-nuclear supernatant fractions after deproteinization by hot SDS phenol method were passed through oligo(dT)-cellulose column, a major portion of the radioactivity of the post-nuclear supernatant was recovered in the unbound fraction. Elution with low salt buffer at 4°C gives a peak of similar nature as in the case of non-deproteinized samples but elution of the column with the low salt buffer at 45°C shows no such peak. Therefore, deproteinization of this post-nuclear supernatant showed absence of poly(A)$^+$ mRNP-like particle.

DISCUSSION

The present results indicate that isolated plant nuclei can synthesize RNA for extended periods of time and allow the release of processed RNA in the supernatant fraction. Preliminary observation[13] from our laboratory showed that the isolated plant nuclear system is fully active as far as *in vitro* RNA synthesis is concerned. Also, [^{3}H]-uridine serves as an efficient precursor for RNA synthesis *in vitro* by this nuclear system. It has clearly been demonstrated that nuclear preparation contains adequate quantities of other NTPs required for RNA synthesis as addition of NTP mixture cannot further stimulate the RNA synthesis[13].

In this investigation the purity of the nuclear preparation has been examined by TEM and SEM. This result is quite satisfactory as far as the intactness and purity of the nuclear preparation are concerned. [^{3}H]-UTP have been used as a labelled precursor for the synthesis of RNA in plant nuclei. It is evident that the rate of synthesis of RNA using labelled RNA precursors reaches maxima at 90–100 min but the release of RNA increases steadily up to 180 min (figure 2).

For characterizing [^{3}H]-UTP incorporated product in post-nuclear supernatant, alkaline hydrolysis was carried out with the cold TCA precipitable material from post-nuclear supernatant. After hydrolysis no radioactivity was found in the TCA-precipitated material. On the other hand, RNase digestion of the parallel sample retains a portion of radioactivity in the precipitate. It seems that a small portion of RNA in the post-nuclear supernatant is bound with protein (figure 3).

Electrophoretic studies, carried out with RNA samples, extracted from the nuclear pellet as well as post nuclear supernatant by hot SDS phenol method[20] as described by Brawerman[19] have clearly indicated the presence of heterogeneous distribution of various labelled RNAs having different molecular weights. The electrophoretic profiles, presented in figure 4, show that the gross nature of the RNA species extracted from either nuclear pellet or post-nuclear supernatant is similar. But the recovery of the radioactivity in the gel slices is higher in nuclear pellet as compared with that of post-nuclear supernatant. Based on these observations, further experiments have been carried out to understand the specific nature of RNA synthesized *in vitro* by the purified isolated nuclei of germinating seeds of *Vigna sinensis*. At the same time, determination of the presence of various RNA species in post-nuclear supernatants might provide new information in plant systems since it has been established that RNAs are released into post-nuclear supernatants after processing of synthesized RNA particularly in the mammalian system[27].

Elution profile obtained after passing the post-nuclear fraction through oligo(dT)-cellulose column suggests that the post-nuclear supernatant contained poly(A) containing mRNA as well as poly(A) containing mRNP-like particle along with poly(A)$^-$ mRNA, t-RNA and r-RNA. The presence of poly(A) containing mRNP particle in the post-nuclear supernatant confirms the event of post-transcriptional modification of *in vitro* synthesized RNA in isolated plant nuclei of *V. sinensis*.

The most striking finding as noted here is that the isolated plant nuclei from *V sinensis* can synthesise RNA for extended periods of time. The *in vitro* synthesized RNA after its post-transcriptional modification gets released into post-nuclear supernatant. The presence of poly(A)$^+$ mRNA and poly(A)$^+$ mRNP-like particle in the post-nuclear supernatant have also been substantiated from the present investigation. Further work may reveal the exact nature of poly(A)$^+$ mRNA and mRNP synthesized in plant system and also the exact role of this RNP particle in the regulation of various molecular processes in the plant system.

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ANNOUNCEMENT

INTERNATIONAL SYMPOSIUM ON PALAEOCLIMATIC AND PALAEOENVIRONMENTAL CHANGES IN ASIA DURING LAST FOUR MILLION YEARS

The above symposium will be held in September/October 1986 at Srinagar/Ahmedabad, with the following general objectives: (a) To intercompare regional and pan-Asian palaeoclimatic patterns during the last four million years vis-a-vis the global trends; (b) Development of quantitative criteria for intercomparison of palaeoenvironmental data; (c) Effects of anthropogenic factors on environmental processes in long and short time perspectives.

The technical sessions will deal with: (i) Palaeoclimatic and Palaeoenvironmental Data, (ii) Palaeoenvironmental Changes and Hominid Evolution, (iii) Chronometric Studies, (iv) Quantitative Techniques, (v) Relevance of palaeoenvironmental Studies, (vi) Correlations and Emerging Patterns.

The title and abstract of the paper should be communicated to the Convener by the *30th April, 1986*.

For details please contact the Convener (D. P. Agrawal), Physical Research Laboratory, Navrangpura, Ahmedabad 380 009.