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QUANTITATIVE ISOLATION OF RIBONUCLEIC ACID FROM THE IMMATURE RAT UTERUS—EXAMINATION OF RIBONUCLEIC ACID SYNTHESIS FOLLOWING *IN VIVO* 17β-ESTRADIOL ADMINISTRATION*

CHARLES A. FROLIK† and JACK GORSKI
Departments of Biochemistry and Animal Science, University of Wisconsin, Madison, WI 53706, U.S.A.

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SUMMARY

A CsCl centrifugation procedure was utilized to extract 85%–90% of the total cellular RNA from the immature rat uterus. This total RNA was deaggregated by treatment with sarkosyl and further fractionated by cellulose chromatography and acrylamide gel electrophoresis. Using these techniques, no specific RNA synthesis could be detected following *in vitro* incorporation of [^{3}H]- and [^{14}C]-adenosine into rat uterine RNA 15 min, 30 min and 5 h after *in vivo* 17β-estradiol administration.

INTRODUCTION

Estrogen has long been known to be involved in the growth and differentiation of the female reproductive system. Recent studies [1–2] suggest that the mechanism of action of estrogen involves the synthesis of messenger RNA (mRNA). Indeed, such an induction of a specific mRNA has been observed as an early response to the estrogen stimulation of ovalbumin synthesis in the chick oviduct [3–4]. However, the search for newly formed mRNA during the early time periods following estrogen administration in the immature rat uterus has created controversy [5–8]. A problem involved in these studies is the isolation of a small amount of RNA from a tissue that displays a high RNase activity. The present study was undertaken to devise a method for the quantitative isolation of undegraded RNA from a small amount of tissue. The RNA obtained was further fractionated by cellulose chromatography and acrylamide gel electrophoresis in order to ascertain whether these methods would be sufficient to bring to light an estrogen inducible mRNA species.

MATERIALS AND METHODS

Preparation of uteri. Twenty-one-day-old female rats (Holtzman, Madison, WI) were injected intraperitoneally with 5 μg of 17β-estradiol dissolved in 0.5 ml of 0.9% (w/v) NaCl containing 1.0% (v/v) ethanol. Control animals received an equal vol. of 0.9% NaCl alone. At the designated time intervals after injection, the animals were decapitated and their uteri quickly excised, stripped of all surrounding fatty tissue and immediately transferred to the incubation medium containing radioactive adenosine (exact composition described in the figure legends) prewarmed at 37° and flushed with 95% O_2 and 5% CO_2. Incubations contained 3–5 uteri per stoppered 10 ml Erlenmeyer flask and were maintained at 37° in a shaking water bath for 30 min. After incubation the uteri were blotted on filter paper, rinsed three times in ice cold 0.05 M Tris–HCl, pH 7.6 and were used in the preparation of total RNA as described below.

Extraction of RNA. Total cellular RNA was extracted from the rat uterus using a modified CsCl centrifugation procedure described by Glisin *et al.*[9]. All solutions and equipment were autoclaved prior to use. Five uteri were minced, immediately added to a homogenization buffer containing 0.1 M Tris–HCl, pH 8, 4% (w/v) recrystallized sarkosyl (sodium lauryl sarcosinate, Schwarz–Mann Co.), and 500 μg/ml polyvinyl sulfate and homogenized on ice by means of a Tissuemizer (Model SDT 100 N, Tekmar Co., Cincinnati, OH) set at medium speed using five 10 s bursts of power with a 5 s wait between bursts. The homogenate was mixed with 0.67 g/ml CsCl until all of the CsCl was dissolved. The resulting solution was centrifuged at 4000 *g* for 1–2 min to break up the foam. The solution was then layered over 1.2 ml of a 6.2 M CsCl–0.1 M EDTA (ethylenediamine tetraacetic acid) cushion in a cellulose nitrate centrifuge tube and centrifuged in a Beckman SW 50.1 rotor at 100,000 *g* for 12 h at 2°. After centrifugation, most of the liquid above the RNA pellet was removed with a Pasteur pipet and the tube inverted and allowed to drain. The bottom 1 cm of the tube was then cut off. The RNA pellet was dissolved in the precipitation buffer (0.12 M NaCl, 0.02 M

*From the Departments of Biochemistry and Animal Sciences, University of Wisconsin, Madison, Wisconsin 53706. These studies were supported in part by the College of Agricultural and Life Sciences, University of Wisconsin, Madison and Ford Foundation Grant 630-0505A.

† National Institutes of Health Fellow No. 1-F22-AM-03129-01. Present address: National Cancer Institute, Bldg. 37, Room 3C02, Bethesda, MD 20014.

Tris–HCl, pH 7.6, 0.5% SDS, 1.5% sarkosyl), heated at 65° for 2 min to break up aggregated RNA and then precipitated with 2 vol. of ethanol. The final pellet was dissolved in 0.3 ml H_2O.

Cellulose chromatography. Separation of poly(A) containing RNA from non-poly(A) rich RNA was performed on Sigma cell Type 38 cellulose (Lot number 126B-1831-9) using a modified procedure from Schutz *et al.*[10] and Sullivan and Roberts[11]. One half g cellulose was slurried in a high ionic strength buffer (10 μM Tris–HCl, pH 7.6, 500 mM KCl, 0.2 mM $MgCl_2 \cdot 6H_2O$) that had been preheated to 45°. The slurry was packed into a column (4 cm high × 0.7 cm inside diameter) and held at a temperature of 45°. The column was then washed with 20 ml of high ionic strength buffer. The total RNA sample (0.25–0.3 ml) to be applied was made 1.2–1.5% in sarkosyl and heated at 65° for 2 min to avoid nonspecific absorption of non-poly(A) rich newly synthesized RNA to the cellulose. After heating, 3 ml of high ionic strength buffer was added to the sample and the temperature adjusted to 45° before application to the column. After the sample had been applied, the non-poly(A) rich RNA was eluted from the column with high ionic strength buffer, and 14 fractions (containing 25 drops per fraction) were collected. At fraction 15, the elution buffer was changed to a low salt buffer (0.01 M Tris–HCl, pH 7.6) to elute the poly(A) containing RNA. After collecting 5 fractions also at 25 drops/fraction, the column was stripped with a formamide wash (11% 1 mM Tris–HCl pH 7.6, 89% formamide). Tubes containing the nonabsorbed RNA were combined and the RNA was precipitated with 2 vol. of ethanol. The fraction containing the low salt wash poly(A) rich RNA was made 0.1 M in NaCl. Then 0.58 OD_{260} units of pituitary tumor RNA (obtained using the CsCl centrifugation procedure described earlier) was added as carrier and the RNA precipitated with 2 vol. of ethanol. The resulting RNA fractions were used for polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. 2% Acrylamide–0.5% agarose gels were prepared by combining methods used by Bishop *et al.*[12] and by Peacock and Dingman[13]. Gels were prepared by combining 3.33 ml of an aqueous stock solution of 15% acrylamide, 0.75% bisacrylamide with 8.33 ml 3E buffer (0.12 M Tris, 0.06 M sodium acetate, 0.003 M sodium EDTA adjusted to pH 7.2 with glacial acetic acid). The solution was warmed and then 0.02 ml of N,N,N′,n′tetramethylethylenediamine (TEMED) and an agarose solution (prepared by dissolving 0.125 g agarose in 13.12 ml boiling H_2O) was added. Immediately 0.2 ml of fresh aqueous 6.25% ammonium persulfate solution was added, the resulting mixture swirled and immediately transferred to Plexiglass tubes (0.6 cm internal diameter) to a height of 11 cm. After the gels were allowed to polymerize for 1 h, they were partially extruded from the tube and the top 1 cm was cut off to form a flat surface. The gels were electrophoresed in E buffer (1/3 concentration of 3E) containing 0.25% recrystallized sarkosyl at constant current (5 mA/gel) for 30 min prior to application of the sample. The RNA fraction to be electrophoresed was made 4 mM in Tris–HCl, pH 7.6, 14 mM in sodium EDTA, and 1.8% in sarkosyl. The resulting solution was heated at 65°C for 2 min, quickly cooled on ice and then made 8.6% in sucrose and colored with bromophenol blue. The sample was layered on top of the gel and electrophoresed until the dye front had traveled 8 cm (approximately 2 1/2 h). After electrophoresis, the gels were scanned in the Gilford Model 2410 Linear Transport (Gilford, Oberlin, OH) at 260 μ to locate the 28*s* and 18*s* ribosomal peaks. The gels were then frozen on dry ice and sectioned into 1.8 mm discs with a multiple razor blade slicer. Each slice was incubated at 45° for 2–4 h in a vial containing 0.5 ml of 90% NCS–10% H_2O. Five ml of toluene-based scintillation fluid (15 g 2,5-diphenyloxazole, 0.9 g 1,4 *bis*[2(4 methyl-5-phenyl-oxazolyl)] benzene in 3 l. toluene) was added and the sample counted in an Isocap/300 liquid scintillation spectrophotometer (Nuclear Chicago, Des Plaines, IL). Four percent acrylamide gels [14] were prepared from 6.66 ml stock acrylamide solution, 8.33 ml 3 E buffer, 9.79 ml water, 0.02 ml TEMED and 0.2 ml of 10% ammonium persulfate. After polymerization for 1 h the gels and samples were treated as described above for the 2% acrylamide–0.5% agarose gels. The samples were allowed to electrophorese until the dye band had just eluted from the gel (approximately 3 1/2 h). The gels were then scanned and sliced as described above.

Miscellaneous procedures. The amount of poly(A) present in the various RNA fractions was determined by the method of Adesnik and Darnell[15]. Protein was determined by the procedure of Lowry *et al.*[16], DNA by the technique of Schneider[17], and RNA by absorbance at 260 μ (assuming 40 μg RNA per absorbance unit) or by the orcinol reaction as described by I.-San Lin and Schjeide[18]. The two methods of RNA determination produced similar results. [2, 8-^{3}H]-adenosine (40.8 Ci/mmol) was obtained from New England Nuclear, uniformly labeled [^{14}C]-adenosine (542 mCi/mmol) was from Amersham–Searle, and [5, 6-^{3}H]-uridine (45 Ci/mmol) was also from New England Nuclear.

RESULTS

In order to accurately examine changes in uterine RNA synthesis, a RNA extraction procedure was needed that would give a quantitative and reproducible yield of uterine RNA that was free from protein and DNA contamination. Of the various extraction methods tested (chloroform–phenol extraction [19], diethyl pyrocarbonate precipitation [20] and CsCl centrifugation [9]), the CsCl centrifugation technique fulfilled the necessary qualifications. In 32 separate extractions, this procedure yielded an average ± stan-

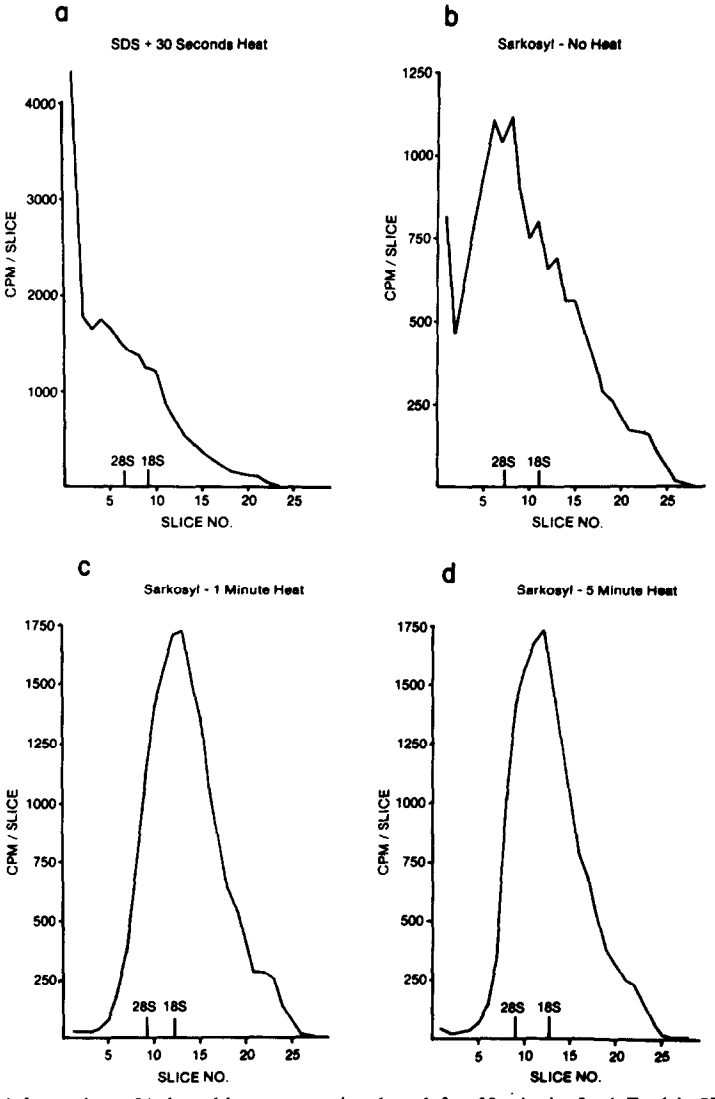

Fig. 1a. Uteri from three 21-day-old rats were incubated for 30 min in 2 ml Eagle's HeLa medium containing 50 μCi [5, 6-^{3}H]-uridine (similar results were obtained using [2, 8-^{3}H]-adenosine). An aliquot of the total RNA, extracted as described in Methods, was made 4 mM in Tris–HCl, pH 7.6, 14 mM in sodium EDTA and 3.3% in sodium dodecyl sulfate and heated at 65° for 30 s and quickly cooled on ice before application to a 2% acrylamide–0.5% agarose gel.

Figs. 1b and 1c. Uteri from five 21-day-old rats were incubated as in (a) except the medium contained 100 μCi [2, 8-^{3}H]-adenosine. An aliquot of the total RNA was made 4 mM in Tris–HCl, pH 7.6, 14 mM in sodium EDTA and 1.2% in sarkosyl. The sample was either not heated (1b) or heated at 65° for 1 min (c) or 5 min (d) and quickly cooled on ice prior to application to 2% acrylamide–0.5% agarose gels.

dard error of the mean of 92 $\pm$ 2 μg RNA per uterus. Assuming that in an immature 21–22-day-old rat uterus there is 105 μg of RNA present [6, unpublished observations]. This amounted to 88 $\pm$ 2% yield. The direct colorimetric test for protein indicated that less than 0.1% of the total protein found in an immature rat uterus was present in the final RNA sample. Similarly, of the total DNA present, less than 1% remained in the final RNA solution. Experiments have also indicated that this technique will give an RNA preparation from the rat pituitary that displays prolactin mRNA activity in a cell free protein synthesizing system [21] and, from the rat uterus, a preparation that shows general mRNA activity (Stone, unpublished data). These results, along with the appearance of ribosomal 28*s* and 18*s* RNA in an approximate ratio of 2:1, argue against possible RNA breakdown due to RNase activity.

Isolation of uterine RNA using this method followed by separation of the RNA on acrylamide–agarose gels revealed that only what appeared to be very high mol wt RNA was being labeled with [^{3}H]-uridine or [^{3}H]-adenosine *in vitro* during the early time periods following estrogen stimulation (Fig. 1a). These results appeared to agree with those obtained earlier by other laboratories [22, 23] who found only very high mol wt RNA labeled with [^{3}H]-uridine and [^{3}H]-guanosine up to 1 h post estrogen. Treatment

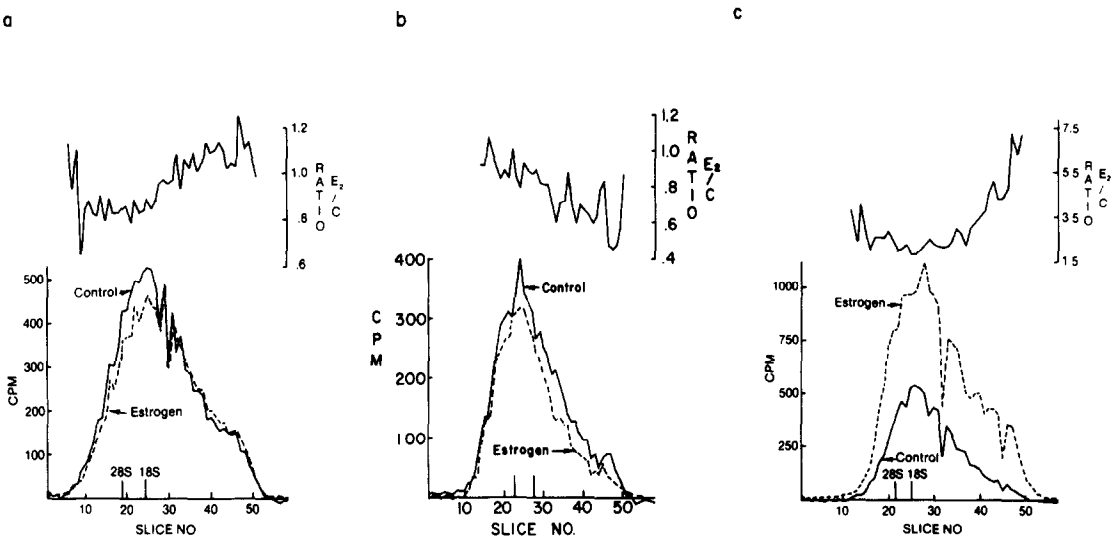

Fig. 2. Separation of uterine RNA by acrylamide–agarose gel electrophoresis. Uteri were obtained from 21-day-old rats that had previously been intraperitoneally injected with either 5 μg 17β-estradiol or vehicle only and sacrificed at the times indicated. Five control uteri from each time point were placed into 2.5 ml Eagle's HeLa medium containing 250 μl (250 μCi) [2, 8-^{3}H]-adenosine and 140 μl of a solution of unlabeled adenosine to give a final adenosine concentration of 7.8×10^{-5} M. Five estrogen treated uteri were added to 2.5 ml Eagle's HeLa medium containing 0.39 ml (125 μCi) [U-^{14}C]-adenosine (7.9×10^{-5} M). After incubation at 37° for 30 min, 2.5 control and 2.5 experimental uteri from the same time point were combined, extracted using the CsCl centrifugation method and electrophoresed on 2% acrylamide–0.5% agarose gels as described in Methods. (a) 15 min estrogen treatment (b) 30 min estrogen treatment (c) 5 h estrogen treatment. (——)-^{3}H c.p.m. (control); (– – –)-^{14}C c.p.m. (estrogen treated).

of the RNA with either dimethyl sulfoxide [24], formamide [25], formaldehyde [26], or sodium dodecyl sulfate [9] caused no change in the radioactively labeled RNA profile after acrylamide–agarose gel electrophoresis (Fig. 1a). However, addition of sarkosyl to the RNA sample plus heating the sample for 1–5 min at 65° and cooling quickly on ice prior to application to the gel, caused a marked, reproducible shift of the radioactively labeled material to lower mol wt values (Fig. 1, b–d). The radioactive profile appeared the same after both 1 min and 5 min of heating, indicating that RNase activity in the preparations was minimal and that the shift in mol wt was not due to decomposition of the RNA during heating. Therefore, in all experiments, RNA samples were heated in the presence of sarkosyl prior to acrylamide gel electrophoresis.

To determine whether or not the above procedures were sensitive enough to detect an estrogen inducible RNA species that may be synthesized during the early time periods following estrogen administration, experiments were performed in which control uteri were incubated in medium containing [^{3}H]-adenosine and estrogen treated uteri in medium containing [^{14}C]-adenosine. No appreciable difference in the RNA profiles on 2% acrylamide–0.5% agarose gels after 15 min, 30 min or 5 h of *in vivo* estrogen treatment (Fig. 2) was observed. Further separation of the total RNA into poly(A)rich and non poly(A)rich RNA by cellulose chromatography followed by 2% acrylamide–0.5% agarose gel electrophoresis produced a similar lack of increase in any RNA species (data not shown). That the cellulose chromatography was indeed isolating the poly(A)rich RNA was shown by the fact that 80–90% of the poly(A) in the total RNA sample was accounted for in the low salt wash [poly(A)] fraction (Table 1).

Because of a recent report from Wira and Baulieu[27] suggesting a possible increase in the relative incorporation of radiolabeled uridine into a specific RNA fraction that migrated in the 16*s* region after acrylamide gel electrophoresis, this size of RNA was more closely examined. However, no estrogen inducible RNA was detected after electrophoresis of either the total RNA or the poly(A)rich RNA on 4% acrylamide gels. The gels were run for maximal separation of RNA between 6*s* and 18*s* in size with the transfer RNA being electrophoresed from the gel and the 18*s* ribosomal RNA just entering the gel (Fig. 3). The ratio change that Wira and Baulieu detected was located in a region of the gel with low c.p.m. and no peak of radioactive material corresponded to the observed increase in ratio. Artifactual changes in the ratio of two isotopes are not unusual under such conditions. Reversal of the isotopes in the labeling of the RNA did not change the results shown here.

The estrogen to control ratio is much higher at 5 h than at 15 or 30 min (Figs. 2 and 3). This increased incorporation of labeled adenosine into RNA in the experimental uteri appeared to be general in nature and distributed throughout the range of RNA sizes. Only in those areas of the gel where the radioactivity

Table 1. Cellulose chromatography of total RNA

Sample	Fraction	% of applied d.p.m.	% of applied OD_{260}	pmol [^{3}H]-adenosine incorporated into poly(A)	% of total poly(A)
I	Applied	—	—	1.08	100
	Nonabsorbed	101	88	0.17	15
	Low salt wash	5.4	1.0	0.97	89
	Formamide wash	1.3	—	0.15	14
II	Applied	—	—	1.27	100
	Nonabsorbed	94	89	0.22	17
	Low salt wash	4.8	0.9	1.04	82
	Formamide wash	1.4	—	0.17	14

Total RNA, extracted as described in Methods from 5 21-day-old rat uteri that had been incubated for 30 min in 2 ml Eagle's HeLa medium containing 150 μCi [2, 8-^{3}H]-adenosine, was separated by cellulose chromatography. Aliquots of each fraction were analyzed for radioactivity, OD_{260} absorbing material and for the presence of poly(A).

fell below 50 c.p.m. was a rise in the ratio observed. Because of the low counts involved and their irreproducibility, these areas were regarded as artifacts and ignored.

CONCLUSION

The CsCl centrifugation method for isolating RNA appears to be a useful procedure for the quantitative isolation of apparently undegraded RNA from the rat

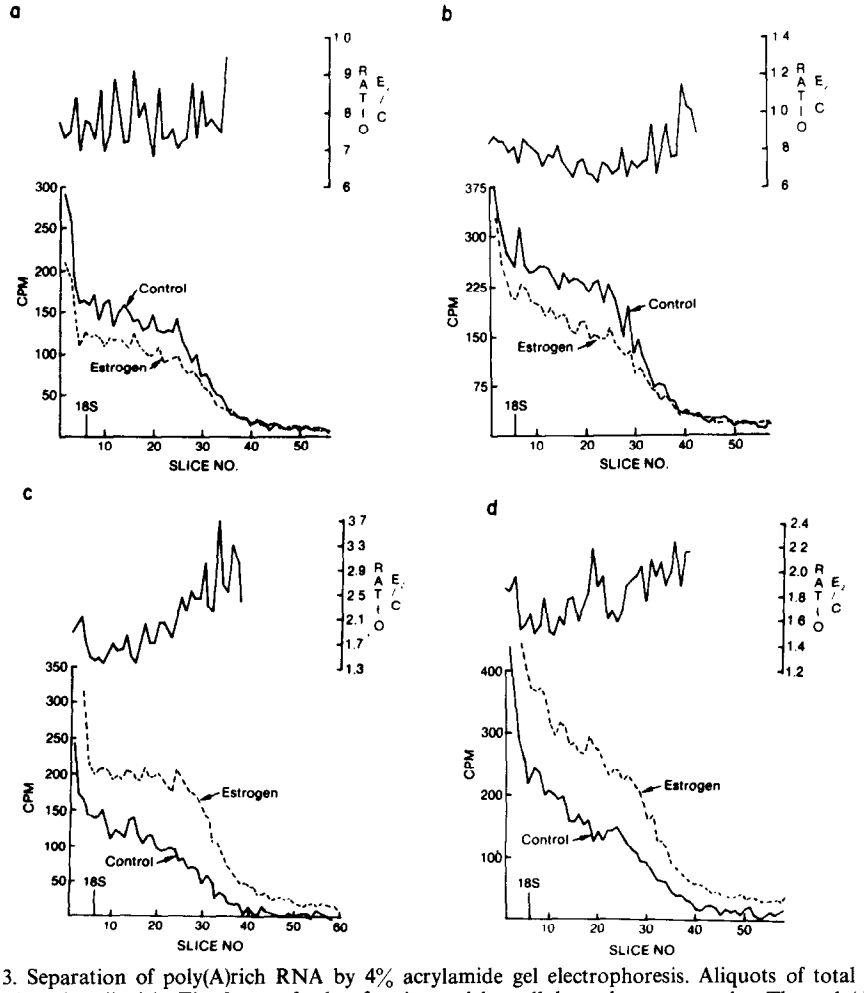

Fig. 3. Separation of poly(A)rich RNA by 4% acrylamide gel electrophoresis. Aliquots of total RNA labeled as described in Fig. 2 were further fractionated by cellulose chromatography. The poly(A)rich (low salt wash) fractions were electrophoresed on 4% acrylamide gels as described in Methods. (a) and (b) Poly(A)rich RNA from uteri treated with estrogen for 15 min. (c) and (d) Poly(A)rich RNA from uteri treated with estrogen for 5 h. (——)-^{3}H c.p.m. (control); (---)-^{14}C c.p.m. (estrogen treated).

uterus. This technique can be of great use in future studies concerning a possible involvement of estrogen at the transcriptional level in the cell nucleus. It is of special merit because of its capability of isolating RNA from small quantities of tissue.

The results in this paper also indicate another method for the deaggregation of RNA. With the current interest in high molecular weight RNA as a possible precursor to mRNA [28], it is important to exhaust all known ways of deaggregating the RNA before making any firm conclusions.

In spite of the utilization of the CsCl centrifugation procedure with its high recoveries, no dramatic change in the synthesis of a particular class of rat uterine RNA was detected during the early time period following *in vivo* estrogen treatment under the experimental conditions employed in this study. When total uterine RNA was examined by acrylamide gel electrophoresis at either 15 or 30 min after estrogen treatment no specific species of RNA appeared to be induced. Separation of the total RNA into poly(A)rich and nonpoly(A)rich RNA followed by gel electrophoresis similarly did not indicate any reproducible increase in a single species of RNA. Also, in experiments not reported here, there was no apparent change in RNA synthesis during a 30 min *in vitro* incubation with 17β-estradiol and [^{3}H]-adenosine under conditions identical to those used for *in vitro* induction of IP synthesis [29].

Nevertheless, there is much indirect evidence that estrogen does induce the synthesis of specific RNAs. In addition to the reports indicating an inhibition of the synthesis of a specific uterine protein and various other estrogen induced responses by several antagonists of RNA synthesis [30–32], (Katzenellenbogen and Ellis, unpublished observations), recent results have also suggested that RNA polymerase II, believed to be responsible for DNA-like RNA synthesis (presumably mRNA) increases to 3- to 4-fold in activity 30 min after estrogen treatment [33–34]. Supposedly this increase in polymerase II activity should result in mRNA synthesis.

It appears from the results presented in this paper that even utilizing sensitive double isotope experiments along with techniques to separate poly(A)rich from nonpoly(A)rich RNA and acrylamide gel electrophoresis direct observation of an estrogen inducible rat uterine RNA species is not possible. Future experiments in this area could perhaps concentrate on the CsCl centrifugation procedure to quantitatively isolate total RNA from the uterus. This could be followed by isolation of the total poly(A) containing RNA and the use of this RNA to make total tritiated complimentary DNA transcripts of the entire mRNA population. This probe could then be used in back hybridization studies with RNA extracted from cells before and after estrogen, to detect the appearance of new sequences—a technique already successfully employed by McKnight *et al.*[35] in the measurement of ovalbumin mRNA sequences in the chick oviduct.

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