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EFFECTS OF ESTROGENS ON UTERINE POLY (A)-RICH RNA

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SUMMARY

The working hypothesis that steroid hormones are transferred into the nucleus of the target cells in combination with a protein receptor and there alter the transcription of one or more portions of the genome is supported by experimental data from many laboratories. There are difficulties in identifying and measuring the immediate product of the transcription process, mRNA, and none of the several criteria used to distinguish messenger from ribosomal and transfer RNA has been completely satisfactory. The messenger RNA's of eukaryotic cells appear to contain a covalently linked region of 100 or more nucleotides rich in polyriboadenylic acid linked to the mRNA at the 3'-OH end. The injection of estradiol into an adult castrate rat increases the total amount of uterine poly (A)-rich RNA and the effectiveness of this material in inducing enzyme synthesis when instilled into the uterine lumen. RNA extracted from the uterus of an estrogen treated rat and instilled into the uterine lumen of an immature rat increases protein synthesis and the activities of glucose-6-phosphate dehydrogenase and ornithine decarboxylase. The effective RNA, a very small fraction of the total, is separable from the mass of RNA by chromatography on a sepharose 4B polyuridylic acid column. This suggests that the RNA effective when instilled in utero has a polyadenylic acid portion and may be a messenger RNA. The RNA not bound to poly U sepharose is without effect in stimulating protein synthesis or in increasing the activity of G-6-PDH or ODC when instilled in utero.

INTRODUCTION

The concept that steroid hormones are transferred into the nucleus in combination with a specific protein receptor and within the nucleus function in some way to alter the transcription of one or more portions of the genome has been attractive to many investigators[1]. A great deal of effort has been expended in the past decade in attempts to determine its validity. The difficulty of identifying and measuring the immediate product of the transcription process, messenger RNA, is even greater in mammalian systems than in the simpler prokaryotic systems. Several criteria have been used to distinguish messenger from ribosomal and transfer RNA's. The base composition of messenger RNA is comparable to that of the DNA in the cell; its nucleotide sequence differs from that of ribosomal and transfer RNA; it has a short half-life, a heterogeneous size distribution and, by definition, the capacity to serve as a template for protein synthesis. Of the several methods developed to assay mRNA using these criteria, none has yielded an unambiguous measure of newly synthesized mRNA. The fraction of messenger RNA having the base composition of rRNA is not known and, therefore, analyses of base composition are not satisfactory. In many bacterial species the base composition of ribosomal RNA is quite similar to that of DNA and therefore similar to the base composition of messenger RNA. Analyses of size distribution cannot be quantitated with certainty, for the size distribution of messenger RNA changes with the length of the isotopic labeling period. Measurements of template activity are not suitable for measuring the mRNA content of newly synthesized, labeled RNA because the protein synthesizing machinery cannot distinguish labeled templates from unlabeled templates.

Measurements of the metabolic instability of newly synthesized RNA can be made by adding an inhibitor of RNA synthesis along with, or immediately after, the added radioactive precursor. Interpretation of such experiments is difficult because RNA that is metabolically stable under physiological conditions may be turned over in the presence of a drug such as actinomycin[2]. When inhibitor and isotope are added simultaneously and RNA synthesis continues for a short time, some mRNA undergoes turnover during the incorporation period[3]. Assay conditions that result in instability of ribosomal or transfer RNA

would yield a high value for the content of mRNA, whereas conditions which result in degradation of mRNA during the incorporation period will give a low value. In experiments measuring the fraction of RNA which is unstable the kinetics with which the labeled uridine saturates the intracellular pool must be measured and the amount of uridine incorporated into molecules other than RNA must be known.

Measuring unique nucleotide sequences of RNA by RNA–DNA hybridization should, in theory be the most promising way of quantitating the amount of mRNA in a pulse labeled RNA preparation. Much effort has been expended to make such assays specific and quantitative[4–6]. More recently a covalently linked region of 100–200 nucleotides rich in polyriboadenylic acid has been identified in the rapidly-labeled polyribosome-associated RNA and in the heterogeneous nuclear RNA of a number of eukaryotic cells[7–9], in several specific eukaryotic messenger RNA's[10–12] and in viral messenger RNA's[13]. It has been inferred that most, if not all, messenger RNA's in eukaryotic cells (with the notable exception of the histone messenger RNA's[14]) contain a poly (A) region at the 3'-OH end. The poly (A) region may function in transporting mRNA from nucleus to cytoplasm[15] or it may serve a regulatory role in the translation of messenger RNA or in its stability in the cytoplasm. Poly (A)-rich RNA can be separated from the mass of RNA extracted from a cell by binding it to poly U or poly T bound, in turn, to sepharose or nitrocellulose[16].

Studies over the past decade have shown that when RNA is extracted from steroid stimulated tissue, purified and reintroduced into a comparable tissue, it can induce changes which mimick the effect of the steroid itself[17, 18]. This was initially shown in our laboratory with the response of the seminal vesicle to RNA from the seminal vesicles of testosterone-stimulated rats[19, 20] and, more recently, in the response of the uterus to the instillation of RNA isolated from an estrogen stimulated uterus[21, 22]. In our initial experiments we measured the increase in protein synthesis by the incorporation of labeled amino acids into proteins. More recently we have found[23] that instilled uterine RNA results in increased activity of specific enzymes such as glucose-6-phosphate dehydrogenase and ornithine decarboxylase that respond to estradiol administered *in vivo*. The experiments described in this paper indicate that this effect is localized in a small fraction of total uterine RNA separable from the mass of RNA by chromatography on sepharose 4B polyuridylic acid columns. This suggests that the effective uterine RNA has a polyadenylic acid portion by which it is bound to the polyuridylic acid sepharose and that it is in fact a messenger RNA.

EXPERIMENTAL

Adult (200 g) ovariectomized CD female rats and 22–25 day old CD female rats were purchased from Charles River Breeding Laboratories, Wilmington, Mass. Estradiol 17β, glucose-6-phosphate, 6-phospho-D-gluconate, $NADP^+$, β mercaptoethanol, dithiothreitol and pyridoxal phosphate were purchased from Sigma Chemicals, St. Louis. RNAse-free sucrose was purchased from Schwartz-Mann, Orangeburg, New York, DNAse from Worthington Biochemicals, Freehold, New Jersey, and DL (carboxy ^{14}C)-ornithine hydrochloride (4·62 mCi mmole^{-1}), uniformly labeled ^{14}C-amino acid mixture (100–400 mCi mmole^{-1}) and hyamine hydroxide from New England Nuclear Corp., Boston, Mass.

Preparation of RNA

Adult rats ovariectomized four or five days previously were injected subcutaneously with 0·1 ml propylene glycol or 0·1 ml propylene glycol containing 10 μg estradiol-17β 4 to 16 hr prior to the extraction of RNA. The animals were killed, their uteri were removed and stripped of fat, then rinsed in 0·9% saline and frozen in a mortar in dry ice to be pulverized. The pulverized tissue was homogenized in buffer A (0·02 M sodium acetate, 0·05 M NaCl, pH 5·1, containing 400 μg/ml each of dextran sulfate and polyvinyl sulfate and 0·5% sodium dodecyl sulfate). An equal volume of buffer-saturated phenol containing 0·1% 8-hydroxyquinoline was added, shaken at 4° for 10 min and centrifuged at 10,000 *g* for 5 min. The aqueous phase was transferred and re-extracted with phenol three more times or until no protein was visible at the interphase. Following the final phenol extraction the solution was made 0·2 M in NaCl and the RNA was precipitated by adding two volumes of ethanol. The solution was stored at −20°C for 16 hr, then centrifuged 15 min in the cold. The RNA precipitate was dissolved in buffer A and treated with 60 μg DNAse/ml for 1 hr at room temperature. The solution was subjected to another phenol extraction and the RNA was precipitated by the addition of ethanol; the mixture was kept at −20°C for 4 hr. The RNA was purified by being redissolved in buffer A and reprecipitated with ethanol at −20°. The precipitate was washed three times with ethanol–ether 3:1; the final traces of ethanol ether were removed with an air stream. The RNA was dissolved in 0·9% sterile saline and an aliquot was measured in a Zeiss spectrophotometer at 230, 260 and 280 nm.

Separation of Poly (A)-rich RNA

A sepharose 4B polyuridylic acid column was prepared from 1 g of cyanogen bromide activated sepharose 4B which was swollen and washed for 15 min on a glass filter with 200 ml of 10^{-3} M HCl to

remove the dextran and lactose present as stabilizers. Polyuridylic acid (10·43 mg) was dissolved in 5 ml of 0·1 M $NaHCO_3$ containing 0·5 M NaCl. The sepharose was added and the mixture was shaken gently overnight in the cold. The gel was rinsed with 0·1 M $NaHCO_3$–0·5 M NaCl to remove unbound poly U and then exposed at room temperature to 1 M ethanolamine pH 8 for 2 hr to react any remaining active groups. The gel was finally washed with three cycles of 0·1 M acetate buffer, pH 4, and 0·1 M borate buffer, pH 8, each containing 1 M NaCl, to remove any non-covalently bound proteins. The gel was placed in 0·02 M sodium acetate–0·05 M sodium chloride, pH 5·1, and packed on the column. RNA 3–5 mg dissolved in acetate buffer pH 5·1 was placed on the column for 18 hr in the cold. The unbound RNA was then eluted with pH 5·1 acetate buffer. After some 50 ml of buffer had passed through the column, the eluate was tested and found to have essentially no absorbance at 260 nm. The eluting buffer was replaced with 100 mM Tris buffer, pH 9·0, and 20 additional 1 ml samples were collected. The peak of material absorbing at 260 nm, extending over tubes 5–9, was combined, desalted, and concentrated on biofiber or minicon B15 with an exclusion size of 15,000. The mass of RNA eluted before the pH change was precipitated by the addition of sodium chloride and ethanol and kept at −20° overnight. This fraction, the 'total-poly (A) RNA' was rinsed with ethanol, dried under air, and dissolved in sterile saline.

Instillation of RNA

The RNA to be tested was dissolved in sterile saline and 10 or 15 μl of solution was instilled into the lumen of one uterine horn of an immature rat and an equal volume of sterile saline or of RNA from the uterus of an adult castrate rat dissolved in sterile saline was instilled into the contralateral horn. The animals were sacrificed 4 or 24 hr later and the uteri were removed, rinsed in saline, and weighed. The uteri were incubated in a buffer containing amino acid mixture to measure protein synthesis or the tissues were homogenized to assay for glucose-6-phosphate dehydrogenase or ornithine decarboxylase activity.

Measuring protein synthesis

RNA from the uteri of estrogen treated rats was instilled into one uterine horn of 21-day-old rats and RNA from the uteri of control rats was instilled into the contralateral horn. The rats were killed 24 hr later, the uteri were removed and sliced, then the sliced uterine horns were incubated for an hour in a medium containing a mixture of uniformly labeled amino acids. The uterine proteins were isolated by the method of Fencl and Villee[21], dissolved in NCS solubilizer and the radioactivity was measured in a scintillation spectrometer. The amount of protein was measured by the method of Lowry *et al.*[24].

Glucose-6-phosphate dehydrogenase assay

Uterine horns were homogenized in 0·04 M Tris HCl buffer, pH 7·5, containing 0·1 M KCl, 0·004 M $MgCl_2$ and 5 mM β mercaptoethanol. The homogenates were centrifuged in a Sorvall at 27,000 *g* for 15 min. A portion of the supernatant fluid was removed for protein determination[24] and other portions, 0·2 or 0·4 ml, were assayed for glucose-6-phosphate dehydrogenase activity[25]. The reaction mixture contained 100 μmole Tris–HCl pH 7·5, 20 μmole $MgCl_2$, 1 μmole $NADP^+$, 0·2 μmole phospho-D-gluconate with and without 1 μmole D-glucose-6-phosphate in a final volume of 3·0 ml. The enzyme was added last. The initial velocity of the reaction mixture containing only 6 phospho-D-gluconate was taken as 6 phosphogluconate dehydrogenase activity. Glucose-6-phosphate dehydrogenase activity was taken as the initial velocity of the reaction mixture containing both substrates after the value for 6 phosphogluconate dehydrogenase was subtracted.

Ornithine decarboxylase activity

Ornithine decarboxylase was assayed by the method of Kaye *et al.*[26]. Uteri were homogenized in 5 volumes of 0·25 M sucrose containing 10 mM Tris–HCl buffer, pH 7·5, 5 mM dithiothreitol and 0·1 mM sodium EDTA. The homogenate was centrifuged at 38,000 *g* for 10 min. A portion of the supernatant was removed for protein determination[27] and other portions, 0·1, 0·2 or 0·4 ml, were used for enzyme assays. The reaction mixture consisted of 50 mM Tris–HCl buffer, pH 7·8, 0·05 mM pyridoxal phosphate, 5 mM dithiothreitol, 0·5 mM DL ornithine (1–3 mCi mmole^{-1}) and enzyme preparation in a total volume of 1 ml. The incubations were carried out at 37°C for 1 hr in 25 ml reaction flasks containing polypropylene center wells suspended from rubber stoppers. Hyamine hydroxide (0·2 ml) was added to the centre wells to trap the CO_2. After an hour incubation 1 ml of 1 N H_2SO_4 was added to the reaction mixture to ensure complete release of the CO_2. After a further 30 min incubation the center wells were removed and placed in vials containing scintillation fluid and counted in a scintillation spectrometer.

RESULTS

Glucose-6-phosphate dehydrogenase

Experiments in several laboratories have provided evidence of the increased activity of specific enzymes in target tissues in response to androgen[27] or estrogen

Table 1. Glucose-6-phosphate dehydrogenase activity of immature rat uteri: effect of 17β estradiol and uterine RNA

Experiment	Treatment	G6PDH activity (O.D. units/min/mg protein)
1	Control uterus[6]	0·083 ± 0·009
	Uterus from rats injected with 17β-estradiol[6]	0·120 ± 0·013
2	RNA instilled	
	Total RNA from control uteri[24]	0·043 ± 0·003
	Total RNA from uteri of E_2-treated rats[24]	0·059 ± 0·004
3 and 4	RNA instilled	
	28s RNA, control uteri	0·033 0·036
	28s RNA, E_2-treated uteri	0·043 0·047
	18s RNA, control uteri	0·040 0·039
	18s RNA, E_2-treated uteri	0·050 0·048
	5–6s RNA, control uteri	0·042 0·041
	5–6s RNA, E_2-treated uteri	0·044 0·042

Female rats 21 days old were anesthetized. RNA, ~74 μg, from uteri of adult castrate rats was instilled into one uterine horn and RNA from uteri of adult castrate rats injected with 10 μg 17β estradiol 16 h previously was instilled into the contralateral horn. Twenty-four hours later the rats were killed, the uterine horns were removed and glucose-6-phosphate dehydrogenase activity was measured by the method of Moulton and Barker (1971).

[28]. The activity of hexokinase in the rat prostate is increased following the administration of testosterone [29]. The activity of glucose-6-phosphate dehydrogenase in the uterus is markedly increased following the administration of estradiol[25, 30]. In comparable experiments we confirmed this finding, obtaining a 45% increase in uterine glucose-6-phosphate dehydrogenase activity 16 hr after the injection of estradiol (Table 1). The instillation of RNA from the uteri of estradiol-treated adult castrate rats in one horn and RNA from the uteri of control adult castrate rats in the other horn led to a 37% increase in glucose-6-phosphate dehydrogenase activity in the horn receiving uterine RNA from estradiol-treated rats compared to the control (Table 1). In subsequent experiments RNA from the uteri of estrogen-treated adult castrate rats and RNA from the uteri of control adult castrate rats were extracted and separated by sucrose density gradient centrifugation into three peaks of roughly 28, 18 and 5s RNA. The corresponding peaks from each of 6 gradients were pooled, precipitated, repurified, then instilled into the uteri of 21-day-old rats. One uterine horn received a specific RNA peak from the uteri of control rats and the contralateral horn received the comparable RNA peak from the uteri of estrogen-treated rats. Twenty-four hours later the glucose-6-phosphate dehydrogenase activity of the uterus was not altered by the instillation of 5s RNA from estrogen-treated as compared to control rats. However, the instillation of either 18s or 28s RNA from the uteri of estrogen treated rats led to a greater glucose-6-phosphate dehydrogenase activity in the uteri of the recipient rats than in the uterine horn receiving the comparable RNA from control uteri. The average increase obtained with 18s RNA was 45% and the average increase using 28s RNA was 35% (Table 1). In another series of experiments the control horn was instilled with saline rather than with RNA from the uteri of control castrate rats. In this series the instillation of 28s or 18s RNA led to increased glucose-6-phosphate dehydrogenase activity in the uterus whereas the instillation of 5s RNA did not.

Ornithine decarboxylase

The activity of uterine ornithine decarboxylase increases dramatically in response to injected estrogen [31]. We confirmed this phenomenon and obtained a two-fold increase in uterine ornithine decarboxylase activity after the injection of 0·5 μg of estradiol into immature rats (Table 2). There was a marked effect of the estrogen treatment on uterine ornithine decarboxylase activity whether the rat was aged 23, 24 or 30 days. The uterus of untreated rats 23 days old had no detectable ornithine decarboxylase activity. The RNA extracted from the uterus of an estrogen-treated rat and instilled into a control uterine horn increased uterine ornithine decarboxylase if the RNA was instilled for 4 h (Table 2). However, if the RNA was instilled for a longer time, for 24 hr, there was no difference in enzyme activity between control and RNA treated uteri, although there was an increased protein synthesis. It is known from the experiments of Russell and Snyder[32] that ornithine decarboxylase has a remarkably short half-life, about 10–20 min. The

Table 2. Ornithine decarboxylase activity of immature rat uteri: effects of 17β estradiol and uterine RNA

Experiment	Treatment	Rat age (days) 23	24	30
1	Control uterus	0	1·6	1·3
	Uterus from rat injected with estradiol	4·7	4·3	3·7
2	RNA instilled 4 h			
	Total RNA from control uteri		1·3	
	Total RNA from uteri of rats E_2-treated 4 h		3·7	
3	RNA instilled 24 h			
	Total RNA from control uteri		1·4 ± 0·12	
	Total RNA from uteri of E_2-treated rats		1·2 ± 0·11	
4	RNA instilled 4 h			
	Total RNA from control uteri		1·6 ± 0·10	
	Total RNA from uteri of rats E_2-treated 2 h		1·6 ± 0·13	
	Total RNA from uteri of rats E_2-treated 4 h		2·7 ± 0·09	

Mean ± standard error of ornithine decarboxylase activity, expressed as nmol CO_2/h/mg protein.

Table 3. Immature rat uteri: Stimulation of protein synthesis, glucose-6-phosphate dehydrogenase and ornithine decarboxylase activities by poly (A)-rich RNA

Poly (A) RNA instilled	Control horn	Experimental horn
Protein synthesis, ^{14}C-amino acid incorporated, dpm/μg protein[12]	84 ± 7	130 ± 9
G6PDH Activity, ΔOD/min^{-1}/mg protein[12]	0·030 ± 0·004	0·072 ± 0·007
ODC Activity, nmol CO_2/h^{-1}/mg protein[4]	2·5 ± 0·2	4·1 ± 0·3
Total-poly (A) RNA instilled		
Protein synthesis, ^{14}C-amino acid incorporated, dpm/μg protein[8]	102 ± 11	100 ± 10
G6PDH Activity, ΔOD/min/mg protein[12]	0·037 ± 0·005	0·036 ± 0·005

Mean ± standard error. Number of experiments in parenthesis. Poly (A)-rich RNA eluted from poly U-Sepharose column. Control horn instilled with RNA from uterus of castrate adult rat; experimental horn instilled with RNA from uterus of castrate adult rat injected with estradiol 4 or 24 h previously.

half-life of the messenger RNA for this enzyme may also be very short and this may explain the lack of effect at 24 h.

Effects of poly (A)-rich RNA

The 'Poly (A)-rich RNA', after desalting and concentration was instilled into one uterine horn of an immature rat in amounts of 0·1–0·2 μg RNA per horn. Seventy-five microgram aliquots of the 'total-poly (A) RNA' was instilled into one uterine horn of other rats and saline was instilled as control into their contralateral uterine horn. Twenty-four hours after the RNA was instilled, the rats were killed and some uterine horns were incubated in a ^{14}C-amino acid mixture to measure protein synthesis. Other horns were taken for an assay of glucose 6-phosphate dehydrogenase activity. The results, summarized in Table 3, show that the total-poly (A) RNA is essentially without activity when instilled, whereas the poly (A)-rich RNA causes marked stimulation of protein synthesis and of glucose-6-phosphate dehydrogenase activity when instilled in amounts that are 500–1000 times smaller than the amount of unfractionated RNA required to elicit the response.

Segments of immature uteri maintained in organ culture showed greater protein synthesis, as measured by the increased incorporation of labeled amino acids, when 0·1 μg poly (A)-rich RNA from estrogen-treated

Table 4. Immature rat uteri: stimulation of protein synthesis by poly (A)-rich RNA instilled or added in organ culture

	Poly (A) RNA from castrate uteri	Poly (A) RNA from estrogen injected castrate rats
Poly (A) RNA instilled into immature uteri	197 ± 21	255 ± 23
Poly (A) RNA added to culture medium containing explants of immature uteri	48 ± 3	60 ± 5

Poly (A)-rich RNA eluted from poly-U-Sepharose column. Poly (A)-rich RNA, 0·1 μg, from uterus of castrate adult rat or from uterus of castrate adult rat injected with 17β estradiol 16 h previously instilled into immature uterus or added to organ culture medium 1066 containing explants of immature uteri. Values are expressed as dpm/μg protein.

uteri was added to the incubation medium than when an equivalent amount of poly (A)-rich RNA from the uteri of castrate rats was added (Table 4).

DISCUSSION

The nature and sequence of the molecular events involved in the effects of steroid hormones on their target tissues are becoming clearer. The binding of the steroid to soluble protein receptors present in the nonparticulate fraction of the cell, the temperature-dependent transformation of the steroid receptor complex and its transfer into the nucleus, have been studied in detail for estrogen[33], progestins[34], androgens[35] and corticoids[36]. Although there are some differences from one steroid to the next, the general pattern is similar. The progesterone receptor of the chick oviducts consists of two different subunits, one of which binds to DNA and the other to a nuclear protein[34].

The mechanism by which the steroid receptor complex (or the free steroid) increases the transcription of specific parts of the genome is still wreathed in mystery. However, the product, or one of the products, of the transcriptional process, as the present experiments show, is a poly (A)-rich RNA. The amount of this in the uterine cell is increased following estrogen administration and the poly (A)-rich RNA, when separated from the mass of uterine RNA by sepharose poly U chromatography, increased the synthesis of protein and the activity of glucose-6-phosphate dehydrogenase when instilled into the uterine lumen. Instilling actinomycin D (0·75 μg per horn) along with the RNA did not inhibit the effect, but had a slight stimulatory action on protein synthesis. Perhaps the actinomycin D inhibits the synthesis of endogenous messenger, thus facilitating the action of exogenous RNA. In earlier experiments[37] to test the hypothesis that the RNA instilled may be acting as a template, about 100 μg of polyuridylic, polycytidylic, polyadenylic or polyguanylic acid was instilled into one uterine horn and saline into the contralateral horn. Twenty-four hours later the animals were killed and the uteri removed and incubated 2 hr in a medium containing one microcurie of an amino acid. A horn into which polyuridylic acid had been instilled, when subsequently incubated in the presence of phenylalanine, showed a statistically significant increase in the incorporation of phenylalanine into protein when compared to the contralateral horn that had received saline. Other sections of the uteri incubated in the presence of an amino acid not coded for by the polyuridylic acid, such as glycine, showed no difference in the rates of incorporation of glycine into the two horns. Uterine horns receiving polycytidylic acid showed an increased incorporation of proline, but not of lysine, compared to control uterine horns when incubated *in vitro*. Uterine horns instilled with polyadenylic acid showed an increased incorporation of lysine, but not of glycine, and uterine horns receiving polyguanylic acid showed an increased incorporation of glycine but not of proline when subsequently incubated with the amino acid *in vitro*. The amount of the increased incorporation, 20–25%, produced by the instillation of polynucleotide is similar in magnitude to the increased incorporation of a mixture of labelled amino acids produced by the instillation of RNA isolated from the uteri of estrogen treated rats. These results are consistent with the inference from earlier experiments that the RNA instilled may be acting as some sort of informational RNA. The results are consistent with the hypothesis that this kind of RNA is an intermediate in the effects of estrogens on the metabolism of its target tissue such as the uterus.

Experiments to determine whether cyclic AMP might be involved in the response of the uterus to estrogen yielded negative results. Uteri excised from 22 day old rats were cut into slices and incubated for an hour with cytidine 5^3H- and a ^{14}C: labelled amino acid mixture. The uterine horn had previously been instilled either with theophyllin, with dibutyryl cyclic AMP, with a combination of the two, or with neither.

None of these treatments had any effect on the synthesis of either RNA or protein when the uterine horn was subsequently incubated with labeled substrates.

Experiments by one of my colleagues, Dr. Ira Rappaport, show that only one of the three isoenzymes of uterine glucose-6-phosphate dehydrogenase, the one with medium mobility on agarose acrylamide gel electrophoresis, is increased by the injection of estradiol or by the instillation of RNA from the uterus of an estrogen-treated rat.

The present experiments demonstrate that the RNA effective in stimulating protein synthesis and increasing enzyme activity when instilled in the uterus is a very small portion of the total RNA extracted from tissues with cold phenol. The effective RNA is bound to polyuridylic acid sepharose columns and is eluted with an alkaline buffer; presumably this RNA has a polyadenylic acid portion by which it is bound to the polyuridylic acid. The amount of this material and its radioactivity when the uterus is incubated in the presence of ^{3}H-cytidine are markedly increased in response to the administration of estradiol *in vivo*.

REFERENCES

1. Karlson P.: *Perspect. Biol. Med.* **6** (1963) 203–214.
2. Kennell D.: *J. molec. Biol.* **9** (1964) 789–800.
3. Pato M. and von Meyenburg K.: *Cold Spring Harb. Symp. quant. Biol.* **35** (1970) 197–504.
4. Bishop J. O. and Irving M. I.: *Biochem. J.* **121** (1971) 105–108.
5. Kennell D.: *J. molec. Biol.* **34** (1968) 85–103.
6. Gillespie S. and Gillespie D.: *Biochem. J.* **125** (1971) 481–487.
7. Lee S. Y., Mandecki J. and Brawerman G. *Proc. natn. Acad. Sci. U.S.A.* **68** (1971) 1331–1335.
8. Darnell J. E., Wall R. and Tushinski R. J. *Proc. natn. Acad. Sci. U.S.A.* **68** (1971) 1321–1325.
9. Edmonds M., Vaughn M. H. Jr. and Nakazato H.: *Proc. natn. Acad. Sci. U.S.A.* **68** (1971) 1336–1340.
10. Lim L. and Canellakis E. S. *Nature Lond.* **227** (1970) 710–712.
11. Aviv H. and Leder P. *Proc. natn. Acad. Sci. U.S.A.* **69** (1972) 1408–1412.
12. Comstock J. P., Rosenfeld G. C., O'Malley B. W. and Means A. R. *Proc. natn. Acad. Sci. U.S.A.* **69** (1972) 2377–2380.
13. Gillespie D., Marshall S. and Gallo R. E. *Nature New Biol.* **236** (1972) 227–231.
14. Adesnik M. and Darnell J. E. *J. molec. Biol.* **67** (1972) 397–406.
15. Darnell J. E., Philipson L., Wall R. and Adesnik M.: *Science* **174** (1971) 507–510.
16. Brawerman G., Mendecki J. and Lee S. Y.: *Biochemistry* **11** (1972) 637–641.
17. Mansour A. M. and Niu M. C.: *Proc. natn. Acad. Sci. U.S.A.* **53** (1965) 764–770.
18. Segal S. J., Davidson B. W. and Wada C.: *Proc. natn. Acad. Sci. U.S.A.* **54** (1965) 782–787.
19. Fujii T. and Villee C. A.: *Proc. natn. Acad. Sci. U.S.A.* **57** (1967) 1468–1473.
20. Fujii T. and Villee C. A. *Proc. natn. Acad. Sci. U.S.A.* **62** (1969) 836–843.
21. Fencl M. and Villee C. A.: *Endocrinology* **88** (1971) 279–285.
22. Tuohimaa P. J., Segal S. J. and Koide S. S.: *J. steroid Biochem.* **3** (1972) 503–513.
23. Villee C. A. and Loring J. M. Abstract presented at the 53rd Ann. meet. Endocrine Soc., San Francisco, California 1971.
24. Lowry O. H., Rosebrough M. J., Farr A. L. and Randall R. J. *J. biol. Chem.* **193** (1951) 265–275.
25. Moulton B. C. and Barker K. L. *Endocrinology* **89** (1971) 1131–1136.
26. Kaye A. M., Icekson I. and Lindner H. R.: *Biochim. biophys. Acta* **252** (1971) 150–159.
27. Singhal R. L. and Ling G. M. *Can. J. Physiol. Pharmac.* **47** (1959) 233–239.
28. Noack V. I. and Schmidt H. *Endokrinologie* **53** (1958) 291–321.
29. Santti R. S. and Villee C. A. *Endocrinology* **89** (1971) 1162–1170.
30. Moulton B. C. and Barker K. L.: *Endocrinology* **91** (1972) 491–498.
31. Cohen S., O'Malley B. W. and Stasny M.: *Science* **170** (1970) 336–338.
32. Russell D. A. and Snyder S. H.: *Molec. Pharmac.* **5** (1969) 253–262.
33. Jensen E. V., Mohla S., Gorell T., Tanaka S. and DeSombre E. R.: *J. Steroid Biochem.* **3** (1972) 445–448.
34. O'Malley B. W. and Schrader W. T. *J. steroid Biochem.* **3** (1972) 617–629.
35. Liao S., Liang T. and Tymoczko J. L. *J. steroid Biochem.* **3** (1972) 401–408.
36. Munck A., Wira C., Young D. A., Mosher K. M., Hallahan C. and Bell P. A.: *J. steroid Biochem.* **3** (1972) 567–576.
37. Villee C. A.: In *The Uterus* (Edited by H. J. Norris, A. T. Hertig and M. R. Abell). Willams & Wilkins, Baltimore (1973) pp. 80–89.

DISCUSSION

Rousseau:

Dr. Villee, one interpretation of the lack of effect of RNA devoid of poly A would be that this type of RNA does not penetrate the cells and that poly-A-containing RNA for some reason does. Do you have any experiments about this point using radioactive RNA for instance?

Villee:

We did some experiments with radioactive RNA some time ago when we were investigating the seminal vesicle system. We did find that label got into the cell. We measured that by autoradiography, but it is impossible to know whether that label is still RNA. That's a real problem.

Rousseau:

Did you inject plain poly-A in the uterus to rule out a non-specific effect of the poly-A sequence.

Villee:

We have done that before. We instilled poly-A into the uterus. We then took the uterus out after 24 h and incubated some portions of it in a solution containing labelled phenylalanine and other portions in a solution containing another labelled amino acid. In fact, we tried all of the synthetic polynucleotides-poly-U, poly-A, poly-C and poly-G and each one stimulated the incorporation of the specific amino acid for which it codes but did not stimulate the incorporation of other amino acids.

Lindner:

We also reported some time ago on the estrogen effect on the ornithine decarboxylase in the uterus *in vivo* (*Biochemistry* **12** (1973) 3072), but we have been unable to stimulate ornithine decarboxylase by estradiol in the surviving uterus *in vitro*. Did you get this effect with your poly-A RNA preparation *in vitro* as well?

Villee:

No, we have never tried giving estradiol *in vitro*, but poly-A RNA added *in vitro* does stimulate enzyme activity.

Lindner:

Poly-A-rich RNA works on the surviving uterus also with respect to ornithine decarboxylase? Have you any suggestion then why it does not work with estradiol?

Villee:

I don't know. As I said, we haven't tried adding estradiol *in vitro* and looking for increased ornithine decarboxylase.

Crabbé:

I would like to ask you whether this so-called messenger RNA material which you could extract and properties of which you could demonstrate in uterine horn would also stimulate protein synthesis in other tissues and conversely whether messenger RNA material extracted from other cells such as reticulocytes, for instance, would be able to trigger the synthesis of haemoglobin, in the uterine horn.

Villee:

Well, we did an experiment something like that. It turned out negatively so we haven't tried to publish it. We got some chicks and injected them with diethylstilbestrol daily for more than a week and then with progesterone and prepared RNA. When we instilled this RNA back into the chick oviduct it made avidin. However, when we instilled that RNA into the rat uterus, it did not make avidin. I don't really know how to interpret this. If you inject RNA into an amphibian oocyt, as Gurdon has shown, or add it to a cell free protein synthesizing system, it is translated. There may be something at the cell membrane that excludes non homologous RNA.

Hansson:

Dr. Unhjem in our laboratory in Oslo, showed many years ago that RNA isolated from the uterus of estrogen primed rats stimulated uterine cells after topical application. After fractionation of the RNA by gel filtration, one fraction stimulated epithelial cell height in the uterus whereas the other fraction was inactive. One argument against his study was the difficulty in ruling out the possibility of contamination by trace amounts of estradiol in his RNA preparation. What are your comments to that?

Villee:

Dr. Tuohimaa's work in New York with the Population Council and ours agreed that it is not due to estradiol. The total amount of estradiol that could be contaminating the RNA is something like 3 or 4 orders of magnitude less than the amount of estradiol that's required to give an effect when applied directly into the uterus. Galand and Dupont working in Belgium have also carried out experiments showing that possible estradiol contamination cannot account for the effect. This is, of course, an obvious question and we, as several other laboratories, injected very highly labelled estradiol into rats and prepared RNA from the uteri. The amount of radioactivity in the RNA was background. All we can say is that the amount of estradiol in the RNA instilled was less than about 10^{-12} mg. It takes something like 10^{-6} μg of estradiol applied directly into the uterus lumen to have any effect, so it's quite a few orders of magnitude apart. I think that another important argument is that when you separate RNA into different fractions, some are effective and others are not and presumably if estradiol were a contaminant, it would be smeared over all of these fractions equally.