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STUDY ON UTERINE RIBONUCLEIC ACID WITH ESTROGENIC ACTIVITY

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SUMMARY

The influence of intrauterine instillation of uterine RNA on protein, lipid and RNA synthesis in uteri of ovariectomized rats was studied. Extensive purification of the uterine RNA was performed to exclude any residual extradiol-17β in the preparations. RNA was isolated from the uteri of estrogen-stimulated rats by phenol fractionation or diethyloxydiformate treatment and washed twice with 95 per cent ethanol. The RNA preparations (8–10 mg) were dissolved in 20 ml of saline and tested for estrogen-like activity by a single instillation into ligated uterine horns of ovariectomized rats. [^{3}H]uridine (100 μCi) and [^{14}C]glycine (25 μCi) were administered intravenously to ovariectomized rats at various times after the intrauterine instillation of RNA. Rats were killed 30 min after the administration of uridine and 30 or 60 min after glycine. The incorporation of radioactivity into total RNA, lipids and proteins was determined.

Manipulative procedures such as ligation of uterine horns and/or intrauterine instillation of saline stimulated uterine RNA synthesis. When uterine and liver RNA obtained from estrogen-stimulated rats and washed with ethanol but not with ether were administered, however, the increase in RNA synthesis was significantly greater than the procedural control values at 4 and 8 h after the intrauterine instillation. To exclude any residual estrogen the uterine RNA preparation was extracted with ether 6 times. When the sediment from the organic phase was dissolved in oil and assayed, it stimulated uterine RNA synthesis. The uterine RNA obtained from control non-stimulated ovariectomized rats stimulated RNA synthesis. However, the values obtained were equivalent to the ones observed with saline instillation or following ligation of the uterine horns. Uterine lipid synthesis was increased, also, by ligation or intrauterine instillation of saline. The increase in lipid synthesis initiated by uterine RNA was equivalent to the procedural control values. Protein synthesis, however, was increased by uterine RNA obtained after ethanol washing and the sediment of the ether phase.

To analyze for the possible presence of residual estradiol-17β in the uterine RNA preparations, the contents of estradiol-17β in the preparations were determined. [^{3}H] or [^{14}C]estradiol-17β (10 μg) was administered intravenously to ovariectomized rats. The RNA were prepared from the uterus, kidney and liver 4 h after the hormone treatment. The content of estradiol-17β in the RNA preparations as calculated from the radioactivity ranged from 0·05 to 3·5 pg per 10 μg of RNA. It was determined that a single dose of 1 pg of estradiol-17β administered by intrauterine instillation stimulated RNA synthesis at 4 h, increased slightly lipid synthesis at 30 min and accelerated protein synthesis at 4 h. The present results reveal that uterine and liver RNA extracted with phenol and washed with ethanol but not with ether contains small amounts of estrogen which may account for the stimulatory action of such extracts.

After repeated washings of RNA with ether the estrogen content was reduced to less than 10^{-2} pg of estradiol-17β per 10 μg RNA. The ether-extracted uterine or liver RNA did not stimulate RNA synthesis, but did increase protein synthesis at 24 and 48 h after instillation. RNA extracted from estrogen-free proliferative uteri induced by intra-uterine pressure possesses similar biologic activity. These results indicate that the ability to stimulate protein synthesis may be an intrinsic property of RNA from proliferating uteri.

INTRODUCTION

EVIDENCE has been accumulating in recent years indicating that estrogens and other steroid hormones may exert their stimulatory effect on target tissues by activating initially RNA synthesis [1]. This concept is supported by the observation that the stimulation of the uterus with estrogen results in a rapid increase

in the rate of incorporation of precursors into RNA[2]. The initial increase is in RNA of the nuclear fraction[3]. Numerous investigators suggest that the changes induced in the uterus by estrogen administration are in some manner mediated by the synthesis of this 'new RNA' or possibly messenger-RNA. In support of this hypothesis, they relate the finding that actinomycin D, which is an inhibitor of DNA-dependent RNA synthesis, prevents most of the effects initiated by estrogens[4]. It is conceivable that estradiol-17β may induce new molecules of mRNA which subsequently mediate the effects and that the estradiol may have no further role in causing the transition of target organ from the atrophic to the stimulated state. Evidence to support this hypothesis was obtained by Segal *et al.*[5], who introduced a method for the biological testing of RNA preparations on uterine epithelium of rats *in vivo*. They demonstrated striking differences in response when uterine RNA preparations obtained from estrogen-stimulated and from non-stimulated rats were used. The RNA preparations obtained from estrogen-stimulated uteri instilled into the uterine lumen of castrated rats resulted in hypertrophy of the epithelial cells[5–7, 10], increase in alkaline phosphatase activity[8] and acceleration of protein synthesis[9], whereas RNA extracts from uteri of non-stimulated castrated rats were inactive. The finding that stimulatory activity of uterine RNA was destroyed on treatment with RNase substantiated the hypothesis that the estrogenic property was inherent in the RNA.

The present study was undertaken to evaluate the ability of RNA from estrogen-stimulated uteri to influence intracellular biochemical events. RNA, protein and lipid synthesis in the uterus of ovariectomized rats following intrauterine instillation of the RNA preparations were determined. A rigid procedure to eliminate traces of estrogens in the uterine RNA preparations was carried out.

EXPERIMENTAL

Chemicals

Diethyl pyrocarbonate (diethyl oxydiformate, Eastman Kodak Co., Rochester, New York, U.S.A.) and water-saturated phenol (Mallinckrodt Chemical Works, U.S.A.) were used for preparation of RNA. The following radiochemicals were purchased from New England Nuclear Corporation (Boston, Mass., U.S.A.): [5-^{3}H]uridine (S.A. 26·4 Ci/mmol), [2-^{14}C]glycine (S.A. 36·7 mCi/mmol), [6,7-^{3}H]-estradiol-17β (S.A. 5·4 Ci/mmol) and [4-^{14}C]estradiol-17β (S.A. 52 mCi/mmol).

Donor rats

Adult albino rats of Holzmann strain were ovariectomized at least 3 weeks prior to use. Estradiol-17β (10 mg) was injected intravenously into 35 rats. The rats were killed 4 h later and RNA from uterus, kidney and liver was isolated. The corresponding tissues from 15 non-stimulated animals were prepared and designated as control RNA(O-RNA). 'Non-specific' uterine RNA preparation was obtained from uteri of 8 ovariectomized rats which were stimulated by stretching the uterine horns and by injecting 50 μl of saline into the ligated horn. The RNA was isolated 4 h after the manipulation and designated as 'pressure-induced' RNA (P-U-RNA).

Preparation of RNA

RNA was prepared according to the phenol method of Kirby [5 method A, 13]

or the diethyloxydiformate procedure described by Fedorcsák *et al.*[14]. The latter method is used to isolate messenger RNA. The RNA was precipitated with 95 per cent ethanol containing potassium acetate and washed twice with absolute ethanol. The partially purified RNA was dissolved in saline prior to use in the bioassay for estrogenic property. Since RNA isolated by the two methods showed identical results, the data obtained with both types of RNA are presented together. To eliminate traces of estradiol the RNA preparations were dissolved in saline solution and extracted with equal volumes of ether six times. The ratio of the absorbance of the RNA preparations measured at 280 and 260 nm ranged from 0·48 to 0·50.

Determination of estrogen content of RNA

In the first experiment 3 ovariectomized rats were injected intravenously with 40 μCi (210 μg) of [4-^{14}C]estradiol-17β and killed 4 h later. The RNA of uterus and liver was isolated by the method of Fedorcsák *et al.*[14]. The majority of the radioactivity (65 per cent) was located in the precipitated fraction during the initial treatment with diethyloxydiformate. The initial two ethanol washings of the precipitate removed a considerable amount of radioactive materials (21–27 per cent). The counts in the third ethanol phase and in the ether phase were very low. The amount of estradiol in the ethanol-washed RNA that was calculated from the radioactive counts corresponded to approximately 0·3 pg per 10 μg of RNA. The ether-extracted RNA possessed insignificant amounts of radioactivity, probably due to the low specific activity of [^{14}C]estradiol-17β. In parallel experiments 200 μCi of [6,7-^{3}H]estradiol-17β equivalent to 10 μg was administered intravenously to 16 ovariectomized animqls. The rats were killed 4 h later: the estradiol content calculated from the total radioactivity detected in the RNA isolated by the method of Fedorcsák *et al.*[14] and extracted with ethanol was equivalent to 1·6 pg (uterus), 3·5 pg (liver) and 0·9 pg (kidney) per 10 μg. The RNA isolated by the method of Kirby[13] contained approximately 1·0 pg (uterus), 0·5 pg (liver) and 1·0 pg (kidney) per 10 μg. This experiment was repeated with 15 ovariectomized rats. The results are presented below. The RNA preparation obtained was tested for biological activity by intrauterine instillation. The radioactivity of the samples was determined by combusting the materials as described by Gupta[16] to minimize any quenching effect during counting.

Bioassay procedure

The recipient rats were ovariectomized 3 weeks prior to use. A median longitudinal incision was made on the abdomen of etherized rats. Both uterine horns were ligated with 5-0 silk just above the bifurcation, avoiding injury or tying of the periuterine blood vessels. The instillation of RNA into these singly ligated uterine horns was made under the ligature and injected toward the cephalic end. In the majority of rats a second ligature was tied at the upper end of the uterus. This uterine preparation was then designated as a double-ligated uterine horn. In these rats the instillation was performed by inserting the needle under the upper ligature toward the caudal end. A solution of RNA was instilled containing 8–20 μg in 20 μl. Two control specimens were used: the cervical portion of the uterus which remained below the caudal ligatures and the opposite uterine horn which was instilled with saline. At ½, 4, 8, 24 and 48 h after the intrauterine instilla-

tion, 100 μCi of [^{3}H]uridine and 25 μCi[^{14}C]glycine were administered intravenously. The rats were killed 30 min later. Uteri were excised, frozen immediately and weighed. The preparation of these tissues for the determination of RNA, lipid and protein was performed as described by Schneider[12] with slight modification.

RESULTS

Effect of operative procedures

Any operative procedure or manipulation of the viscera appears to influence the synthesis of uterine RNA, protein and lipid (Table 2). The performance of an incision and closure of the abdomen without manipulating the viscera or uterus increased slightly the biosynthetic processes in the uterus. The greatest change occurred in lipid synthesis. Ligation of the uterus alone induced an increase in the synthesis of uterine RNA and protein. These effects were additive. The instillation of saline into a uterine horn (Table 2) with or without a single ligature effected identical changes in RNA, protein and lipid synthesis. With a double-ligated uterine horn, significant increases in RNA (360 per cent), protein (280 per cent), and lipid (220 per cent) synthesis were observed following saline instillation when compared with non-operated ovariectomized rats.

Effect on incorporation into acid soluble fraction

When uterine (U) or liver (L) RNA was instilled into the uterine lumen, radioactivity in the trichloroacetic acid fraction which contains free uridine nucleotide and its degradation products showed an increase of 100–200 per cent within 30 min (Fig. 1) as compared to untreated control rats. At 4 h after the intrauterine instillations a marked decrease in the radioactivity of the acid soluble fraction was observed which returned to the control level at 24 h. These results indicate that any abdominal operative procedure increases the incorporation of uridine into the acid soluble fraction. The effect is present at least 8 h post-operatively.

The intrauterine instillation of RNA did not influence significantly the incorporation of [^{14}C]glycine into the acid-soluble fraction. The incidence is equivalent to the values observed with untreated control rats.

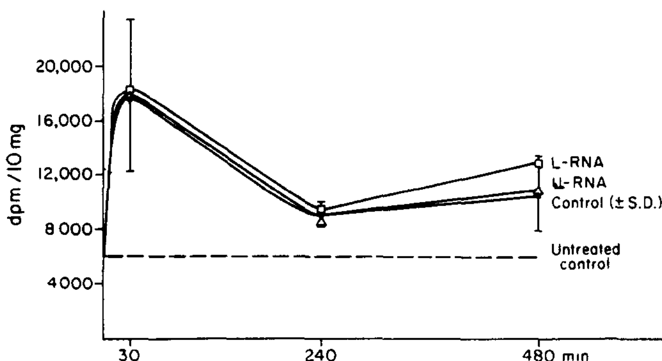

Fig. 1. [^{3}H]-radioactivity in the uterine TCA-soluble fraction following intravenous injection of [^{3}H]-uridine.

Effect of intrauterine administration of estradiol-17β on RNA synthesis

The administration of various doses of estradiol from 1 to 100 pg into the uterine lumen effected a slight increase in RNA synthesis in 30 min (Table 1). An increase of 61 per cent is observed at 4 h after intrauterine administration of 1 pg of estradiol (Table 2) and an increase of 276 per cent at 8 h. Doses of 0·1 and 0·01 pg of estradiol did not influence the rate of RNA synthesis. To determine whether or not the routes of administration altered the results, 10 μg of estradiol was injected intravenously. At 4 h after estradiol injection, RNA synthesis of both non-ligated and ligated uteri was increasing significantly over the corresponding control values (Table 2). The increased rate of synthesis induced by the ligation itself was enhanced further but not dramatically by the estradiol treatment. Estradiol in amounts as low as 1 and 10 pg instilled into the horns of a singly ligated or non-ligated uterus increased significantly RNA synthesis when measured 4 h later (Table 2). Elevated levels of RNA synthesis were observed 8 and 24 h after the administration of 1 or 100 pg of estradiol (Table 4). A dose of 0·1 pg of estradiol had no detectable influence on RNA synthesis (Tables 2, 4).

Estradiol effect on lipid synthesis

Estradiol in amounts of 1 pg or greater increased significantly the incorporation of [^{14}C]glycine into lipids 30 min after intraluminal instillation into ligated uteri (Table 1). The level of lipid synthesis remained elevated for 24–48 h after the instillation (Tables 2–4). Although estradiol in amount of 0·1 pg stimulated

Table 1. Incorporation of [^{3}H]uridine into RNA and [^{14}C]glycine into lipids and proteins 30 min after intrauterine instillation of RNA, estradiol-17β and other extracts

	No.	Specific activity of RNA (d.p.m./mg)	Lipid-^{14}C (d.p.m./10 mg of tissue)	Specific activity of protein (d.p.m./mg)
Control (uterine cervical segment)	3	[48753 ± 11478(6635)]	[54·2 ± 1·7(1·0)]	[1868 ± 203(117)]
U-RNA, 14μg†‡	3	70772 ± 28902(16707)	49·1 ± 2·5(1·5)	1689 ± 190(110)
L-RNA, 13 μg†‡	3	49820 ± 14556(8414)	46·6 ± 11·1(6·5)	1450 ± 259(150)
Control (uterine cervical segment)	10	[18286 ± 8943(2828)]	[81·6 ± 21·3(6·7)]	[1812 ± 545(172)]
E_2, 100 pg‡	3	** 44752 ± 21202(12241)	72·9 ± 20·2(10·1)	1454 ± 533(267)
E_2, 1 pg‡	2	31941 ± 1661(1175)	** 139·9 ± 20·8(14·7)	2307 ± 497(352)
Ether-extract of U-RNA‡	3	*** 57442 ± 7761(4481)	96·4 ± 25·0(14·5)	2180 ± 340(196)
U-RNA, ether washed (40 μg)‡	3	21727 ± 11295(6521)	90·4 ± 30·8(17·8)	2094 ± 340(196)

Abbreviations: U-RNA, RNA obtained from uteri of estrogen-treated rats; L-RNA, RNA obtained from livers of estrogen-treated rats; K-RNA, RNA obtained from kidneys of estrogen-treated rats; Y-RNA, yeast RNA; O-U-RNA, RNA obtained from uteri of ovariectomized non-stimulated rats; P-U-RNA, RNA obtained from uteri stimulated by intraluminal pressure; ether-RNA, RNA washed six times with ether after two ethanol washings; E_2, estradiol-17β; i.v., intravenous injection.

*$P < 0·05$; **, $P < 0·01$; ***, $P < 0·001$ as compared to corresponding control radioactivities (▭).

†50 μl of RNA-saline solution.

‡Uterine horns were tied with two ligatures.

Values are mean ± S.D. (S.E.M.).

Table 2. Incorporation of [^{3}H]uridine into RNA and [^{14}C]glycine into lipids and proteins 240 min (4 h) after intrauterine instillation of RNA, estradiol-17β and other extracts

	No.	Specific activity of RNA (d.p.m./mg)	Lipid-^{14}C (d.p.m./10 mg of tissue)	Specific activity of protein (d.p.m./mg)
No operation*	18	3307 ± 1689(398)	30·7 ± 11·2(3·0)	279 ± 82(22)
Abdominal incision no ligation*	3	4947 ± 1374(793)	68·6 ± 11·9(6·9)	333 ± 112(65)
Saline no ligation*	4	7097 ± 1786(893)	86·3 ± 17·4(8·7)	803 ± 169(85)
Saline one ligature†	10	8548 ± 1611(509)	84·4 ± 16·5(5·2)	826 ± 106(57)
Two ligatures‡	3	13833 ± 7906(4565)	83·0 ± 13·6(7·9)	666 ± 189(109)
Saline two ligatures‡	12	15326 ± 5436(1569)	99·7 ± 34·1(9·8)	956 ± 448(129)
E_2, 10 μg i.v. (no operation)*	16	18042 ± 7162(1798) ***	92·8 ± 23·1(10·9) ***	737 ± 221 (55) ***
E_2, 10 μg i.v. (two ligatures)‡	14	37804 ± 19289(5155) ***	100·8 ± 30·0(8·0)	1238 ± 529(142)
E_2, 10 pg†	5	11631 ± 2782(1244) *	94·9 ± 23·5(10·5)	836 ± 207(93)
E_2, 1 pg‡	13	24696 ± 6109(1694) ***	132·2 ± 35·9(9·9) *	1353 ± 427(118) *
E_2, 0·1 pg‡	3	15877 ± 6025(3479)	165·9 ± 6·8(3·9) **	1796 ± 426(246) *
E_2, 0·01 pg‡	3	13501 ± 4267(2464)	126·4 ± 22·4(12·9)	911 ± 107(62)
U-RNA, 8–20 μg‡	13	32074 ± 15870(4402) **	107·0 ± 43·6(12·1)	956 ± 250(69)
L-RNA, 8–20 μg‡	8	25339 ± 7411(2620) **	90·0 ± 33·3(11·8)	1027 ± 168(60)
U-RNA, 8·8 μg†	4	12357 ± 968(484) ***	91·4 ± 13·7(6·8)	948 ± 106(53)
Ether-U-RNA 9·6 μg†§	5	8788 ± 2511(1123)	141·7 ± 21·5(9·6)	764 ± 47(21)
O-U-RNA, 8·8 μg†	4	7804 ± 2422(1211)	95·4 ± 27·2(13·6)	770 ± 145(72)
Ether-L-RNA, 9·6 μg†§	4	8529 ± 3592(1796)	149·4 ± 30·2(15·1)	768 ± 61(31)
P-U-RNA, 8·6 μg†§	5	8243 ± 2620(1172)	148·2 ± 22·8(10·2)	779 ± 81(36)

Abbreviations are as given in Table 1.

*No ligature.

†One ligature as described under method.

‡Two ligatures as described under method.

§Rats were killed 60 min after the i.v. injection of [^{14}C]glycine and 30 min after the injection of [^{3}H]uridine.

lipid synthesis at the 4th hr (Table 2), the rate returned to control values by the 24th h (Table 4). No effect on lipid synthesis was observed with a dose of 0·01 pg. Estradiol (10 μg) injected intravenously induced a 3-fold increase in lipid synthesis 4 h later (Table 2). No increase was observed, however, when the same amount of estradiol was injected intravenously into rats whose uterine horns were ligated.

Estradiol effect on protein synthesis

Estradiol in amounts of 1 or 100 pg increased significantly glycine incorporation into the protein fraction when measured 8, 24 and 48 h later (Table 3–4). Effects on protein synthesis were not observed at 30 min at any dose of estradiol (Table 1). At the 4th h 1 and 0·1 pg of estradiol effected a slight increase in total protein synthesis (Table 2).

Table 3. Incorporation of [^{3}H]uridine into RNA and [^{14}C]glycine into lipids and proteins 480 min (8 h) after intrauterine instillation of RNA, estradiol-17β and other extracts

	No.	Specific activity of RNA (d.p.m./mg)	Lipid-^{14}C (d.p.m./ 10 mg of tissue)	Specific activity of protein (d.p.m./mg)
U-RNA, 8–20 μg‡	10	32433 ± 11985(3790) **	99·8 ± 35·3(11·2)	2528 ± 1495(473) *
Ether-extract of U-RNA‡	3	47668 ± 18120(10462) **	221·3 ± 79·1(56·0)	3342 ± 190(135) ***
Ether-U-RNA, 12 μg‡	5	10313 ± 3249(1453)	123·1 ± 22·2(9·9)	1086 ± 220(99)
O-U-RNA, 8–20 μg‡	4	11092 ± 3883(1942)	98·6 ± 14·8(7·4)	1443 ± 212(106) *
P-U-RNA, 8·6 μg†	4	10862 ± 2241(1120)	132·0 ± 27·3(13·6)	948 ± 191(96)
L-RNA, 8–20 μg‡	6	36619 ± 9173(3745) ***	105·4 ± 37·9(15·5)	1138 ± 228(93)
E_2, 100 pg‡	3	56258 ± 10530(6080) ***	235·8 ± 37·8(21·8) **	5095 ± 1565(904) ***
E_2, 1 pg‡	3	38401 ± 14945(8629) **	170·2 ± 82·1(47·4)	3670 ± 2190(1265) *
Control (uterine cervical segment)†	26	19417 ± 9690(1900)	99·1 ± 35·9(7·0)	1994 ± 1205(236)
Saline‡	5	10216 ± 2912(1302)	121·6 ± 22·8(10·2)	973 ± 180(80)
U-RNA RNase treated‡	4	18532 ± 15210(7605)	97·7 ± 12·9(6·4)	2483 ± 923(461) **
U-RNA Pronase treated‡	4	18780 ± 4063(2032) **	94·8 ± 24·6(12·3)	3527 ± 1557(779) **

Abbreviations and legend are given in Tables 1 and 2.

Table 4. Incorporation of [^{3}H]uridine into RNA and [^{14}C]glycine into lipids and proteins 24 h after intrauterine instillation of RNA, estradiol-17β and saline

	No.	S.A. of RNA (d.p.m./mg)	Lipid-^{14}C (d.p.m./ 10 mg of tissue)	S.A. of protein (d.p.m./mg)
Ether-U-RNA, 9·6 μg†§	5	5583 ± 1620(724)	76·7 ± 12·2(5·5)	888 ± 117(53)*
P-U-RNA, 8·6 μg†§	5	5061 ± 1956(875)	77·0 ± 15·9(7·1)	852 ± 29(13)*
Ether-K-RNA, 9·7 μg†§	5	6587 ± 1512(676)	76·8 ± 7·7(3·4)	655 ± 108(48)
Ether-L-RNA, 9·6 μg†§	5	4067 ± 1017(455)	80·1 ± 19·8(8·9)	1067 ± 472(211)
E_2, 1 pg‡§	5	6585 ± 1243(556)	73·3 ± 9·1(4·1)	888 ± 111(50)*
E_2, 1 pg†§	5	10540 ± 1766(790)**	97·1 ± 17·6(7·9)	939 ± 80(36)**
E_2, 0·1 pg†§	5	4126 ± 1635(731)	84·8 ± 22·7(10·2)	844 ± 188(84)
Saline†§	5	5442 ± 2002(895)	78·9 ± 9·7(4·3)	717 ± 88(39)

Abbreviations and legends are as given in Tables 1 and 2.

Estrogen content of RNA after ethanol extraction: studies with labeled estradiol

The radioactivity in the RNA fraction was assumed to be associated with estradiol and its metabolites. The estrogen content of U-RNA prepared 4 h after the injection of labeled estradiol and washed twice with ethanol, as calculated from the radioactivity, varied from 0·24 to 1·6 pg per 10 μg RNA. The content in liver (L) and kidney (K) RNA ranged from 0·07 to 3·5 pg and from 0·05 to 0·9 pg per 10 μg of RNA, respectively. It should be pointed out that the lowest values

(0·05–0·3 pg per 10 μg RNA) were obtained when RNA dissolved in saline was extracted with ethanol. When the washing was carried out on solid RNA, the extraction was exceedingly poor and considerable amounts of estrogen remained in the RNA preparation (0·5–3·5 pg per 10 μg RNA).

Effect on RNA synthesis. U-RNA and L-RNA increased slightly uterine RNA synthesis 30 min after intraluminal instillation as compared to that of the control uterine cervical segment of the same rats (Table 1). The sediment from the ether phase of U-RNA induced significant RNA synthesis. Extraction of U-RNA with ether eliminated its capacity to stimulate uterine RNA synthesis. The ethanol-washed U-RNA and L-RNA significantly increased RNA synthesis 4 h after instillation in uteri with one or two ligatures (Table 2). The stimulation of RNA synthesis was observed 8 h after instillation (Table 3). Treatment of U-RNA with RNase or only pronase partially destroyed its capacity to induce RNA synthesis.

Effect on lipid synthesis. None of the RNA fractions had any effect on lipid synthesis. Only the sediment of the ether phase of U-RNA increased slightly glycine incorporation into the lipid fraction of the uterus (Tables 1,3).

Effect on protein synthesis. None of the RNA preparations induced protein synthesis 30 min or 4 h after intrauterine instillation (Tables 1 and 2). On the other hand, uterine protein synthesis was clearly increased 8 h after the instillation of U-RNA or the sediment from the ether phase of U-RNA.

Studies with RNA with low estrogen content: effect of ether extraction on estrogen content of RNA

After two ethanol washings significant amounts of radioactivity were detected in the RNA. Three ether washings removed the radioactivity almost completely. The amount of radioactivity in the final RNA was below the level of sensitivity for liquid scintillation counting. Hence, the radioactivity was measured in gas phase by a proportional counter with low background and high efficiency [17]. If we assume that the residual radioactive materials in the RNA are estradiol and its metabolites, the amounts correspond to $0{\cdot}6 \times 10^{-3}$ pg (uterine RNA), $1{\cdot}6 \times 10^{-3}$ pg (liver RNA) and $3{\cdot}2 \times 10^{-3}$ pg (kidney RNA) per 10 μg of RNA.

Effect on uterine biosynthetic processes. Intrauterine instillation of ether-extracted U-RNA and control of RNA from uteri stimulated by intraluminal pressure had no effect on RNA synthesis up to 24 h (Tables 1, 2, 4). Similarly, ether-extracted RNA preparations did not influence lipid synthesis (Tables 1, 2, 4). Protein synthesis was significantly elevated 24 and 48 h after intrauterine instillation of ether-extracted U-RNA. No rise above control values was observed at 30 min or 4 h after the instillation. Pressure-induced uterine RNA free of exogenous estrogen increased synthesis 24 h after intraluminal instillation.

DISCUSSION AND CONCLUSIONS

Estrogen-like activity of RNA preparations from organs of rats previously treated with estradiol-17β can be due to an intrinsic biologic activity of the RNA itself, to the presence of residual estradiol or its active metabolites, or to other unidentified compounds having estrogen activity. The latter possibility seems unlikely, particularly since previous reports have revealed that the biologic

activity of RNA extracts can be reduced considerably by pre-treatment with RNAase, but not with other hydrolytic enzymes [5, 6, 9].

The present study reveals that RNA preparations from organs of estradiol-treated rats may contain enough estradiol to account for a stimulatory effect on RNA, lipid and protein synthesis in the uterine tissue of rats receiving such extracts by intraluminal instillations.

Extraction with ethanol does not remove all the estradiol from the RNA. The steroids are tightly bound to RNA, though the type of linkage involved is not understood [11]. Ether washing is, however, quite effective in extracting estradiol from RNA in solution. RNA solutions that have been washed with ether continue to stimulate protein synthesis, although they do not elevate the level of RNA or lipid synthesis. With such extracts, biologic activity may be due to an intrinsic property of RNA or to a minute amount of estrogen, too low to be measured by scintillation counting, but which can be detected by sensitive low-background proportional detector.

Previous studies have attempted to establish the minimum dose of estradiol administered by intrauterine instillation required to exert an uterotrophic effect. Galand *et al.*[7] showed that 10 pg of estradiol is the lowest dose which will increase the height of endometrium when instilled into the uterine lumen. The results in the present study show that 10 pg of estradiol is well above the minimal dose required to induce uterine RNA and protein synthesis. A dose of 1 pg estradiol causes a significant increase in uterine RNA and protein synthesis. The minimal effective dose (m.e.d.) of estradiol with respect to these parameters appears to be about 1–0·1 pg. This estimation is probably high for the m.e.d. since loss of steroids by adhesion to glassware may have occurred during the dilution procedure.

After two ethanol washings RNA extracts may contain up to 3·2 pg of estradiol equivalents per 10 μg of RNA. It is recommended, therefore, that ether extraction should be carried out to assure a more complete elimination of contaminating estradiol from tissue RNA extracts. It cannot, of course, be certain that the ether treatment does not denature the RNA or alter the structure so as to reduce inherent biologic activity. However, when the ether extraction is made in liquid/liquid phase there is no alteration of the spectrophotometric characteristics of the RNA solution. (If RNA powder or residue is extracted with ether it turns into an insoluble denaturated material.)

This study shows that uterine protein synthesis is increased by the instillation of RNA obtained from uteri or liver of estrogen-stimulated rats or from RNA obtained from proliferating uteri stimulated by intrauterine pressure. The latter preparation is, by design, free of exogenous estradiol. Villee and Loring[17] showed that total protein synthesis is stimulated after intrauterine instillation of either estrogen-stimulated U-RNA or L-RNA. Neither these findings nor those obtained in the present study mean that the same uterine protein is formed on stimulation with RNA from different sources. RNA from different sources may stimulate different proteins depending on the base ratio and sequence. For example, polyuridylic acid when instilled into the uterine lumen increases the incorporation of phenylalanine into protein [17], but not the incorporation of other amino acids. Further studies are required to determine whether estrogen-stimulated RNA can induce synthesis of specific proteins.

From the results of the present study it can be concluded that:

(1) The intraluminal instillation of RNA preparations from uteri or liver of estrogen-stimulated rats can induce an increase in RNA and protein synthesis in the uterus of ovariectomized rats.

(2) Ethanol washing of the RNA preparations is inadequate to extract completely contaminating estradiol or its active metabolites.

(3) Ether extraction of RNA removes a considerable portion of the contamination. The ether-washed RNA is unable to cause significant changes in the rate of uterine RNA synthesis, but retains the capacity to increase protein synthesis of the uterus.

(4) The stimulation of protein synthesis by ether-extracted RNA may be due to a residual content of estrogens, or the stimulatory activity may be an inherent biological property of the RNA itself. The activity of RNA extracted from proliferating uteri stimulated by intraluminal pressure (not by exogenous estrogen) supports the latter alternative. This interpretation is supported also by the finding that the radioactivity in RNA is equivalent to an amount of estradiol far below the minimum effective dose for the measured response.

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DISCUSSION

Siiteri: Perhaps I missed the point, but did you actually test the ether extract for stimulation?

Tuohimaa: Yes, I did test it.

Siiteri: The combined ether extract was equivalent to the RNA that you had in preparation before?

Tuohimaa: The stimulatory effect of the ether extract was equivalent to that of RNA, before the extraction.
Liao: Do you know whether in Niu's experiment (*Proc. Natn. Acad. Sci. U.S.* **53** (1965) 764) he used ether-washed RNA?
Tuohimaa: He used ether-washed RNA. He has not presented the details of the washing procedure.
Liao: How about the experiment by the Harvard group (*Proc. Natn. Acad. Sci.* **62** (1969) 837) on androgens?
Tuohimaa: Villee and his co-workers wash RNA with ethanol–ether. (*Proc. Natn. Acad. Sci. (U.S.A.)* **57** (1967) 1468 and *Endocrinology* **88** (1971) 279).
Liao: Did they still see the same thing they reported after washing in ether?
Tuohimaa: They did.
O'Malley: Did Villee's group look at RNA synthesis or just protein synthesis?
Tuohimaa: They have studied only protein synthesis. Unhjem, Attramadal and Sölna (*Acta Endocr. (Kbh)* **58** (1968) 227) have extracted uterine RNA with ether and it lost its capacity to induce cellular hypertrophy. They did not determine protein synthesis.
O'Malley: Did you look at cell hypertrophy with ether-extracted RNA?
Tuohimaa: I have not studied that.
Munck: Just to make sure I understand it correctly: what you're saying is that the effect of injected RNA on RNA synthesis is probably an artifact due to the contaminating estrogen, but that the effect on protein synthesis is not. Is that right?
Tuohimaa: That is correct.
Munck: So the effect on protein synthesis is a real one due to the RNA, not to the contaminating estradiol?
Tuohimaa: Yes. There is still some contamination, but it's so low that it seems to be below the minimum dose of estrogen required to induce uterine protein synthesis.