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The Effects of Oestradiol on the Acid-Soluble Nucleotides of Rat Uterus

By JANET M. OLIVER and A. E. KELLIE
Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London W1P 5PR, U.K.

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1. Techniques have been developed to measure the concentrations of the ribonucleotides of the immature rat uterus *in vivo*. Tissue was frozen rapidly in liquid nitrogen, ground to a fine powder, dispersed in frozen perchloric acid and thawed slowly. Nucleotides were separated from other acid-soluble constituents on short columns of polyethyleneimine-cellulose and the mixture was resolved into individual nucleotides by two-dimensional thin-layer chromatography on polyethyleneimine-cellulose plates. 2. The nucleotides of immature rat uterus consisted of approximately 75% of ATP–ADP, 10–12% each of GTP–GDP and UTP–UDP and less than 2% of CTP. 3. Injection of oestradiol (5μg) promoted a linear decrease in the amounts of purine nucleotides to approximately 60% of control values in 4–5h, followed by a return to greater than control values in 8–10h. Concentrations of the pyrimidine nucleotides remained constant for 4–6h and then increased to 200% of control at 12h after hormone treatment.

It has been established (Hamilton, 1968) that oestradiol causes a rapid increase in the incorporation of radioactive nucleosides into the uterine RNA of ovariectomized and immature rats. However, it has been difficult to determine the effect of hormone treatment on the rate of RNA synthesis because no information has been available on the size of the uterine nucleotide pools through which precursor nucleosides must pass. The present study was undertaken to measure the concentrations of these endogenous pools and to determine whether they are affected by treatment of immature rats with oestradiol.

EXPERIMENTAL

Materials. Oestradiol, DNA, RNA, ribonucleosides and ribonucleotides of purines and pyrimidines were all purchased from the Sigma (London) Chemical Co., London S.W.6, U.K., polyethyleneimine-cellulose for chromatography was obtained from Merck A.-G., Darmstadt, Germany, and polyethyleneimine-cellulose plates for t.l.c. from Baker Chemicals N.V., Deventer, Holland. Ribonuclease was purchased from the Worthington Biochemical Corp., Freehold, N.J., U.S.A., and [^{14}C]glycine from The Radiochemical Centre, Amersham, Bucks., U.K.

Extraction of the acid-soluble fraction of rat uteri. Immature female rats (50–70g) of the Wistar strain were killed by cervical dislocation and the uteri exposed, cleaned of connective tissue *in situ*, and transferred into liquid N_2 within 30s of death. Pooled uteri from approx. 15 rats were ground to a fine powder in a mortar frozen by continuous addition of liquid N_2, ice-cold 4% (v/v) $HClO_4$ (3ml) was added and the frozen mixture of acid and uteri was again pulverized. After thawing slowly to 4°C in an ice-bath, the suspension was centrifuged for 5min at 800*g* at 4°C, the pellet was washed with ice-cold 4% (v/v) $HClO_4$ (2ml) and the pooled supernatants were neutralized with 3M-KOH–0.1M-tris. Precipitated $KClO_4$ was removed by centrifugation and the supernatant layers were stored at −14°C.

Isolation of nucleotides from the acid-soluble fraction. Short columns (8cm × 0.5cm internal diam.) plugged with asbestos wool were packed to approx. 3cm with a slurry of polyethyleneimine-cellulose, and washed with a small volume (1–2ml) of 1.0M-triethylammonium bicarbonate buffer, pH7.4 (Smith & Khorana, 1960).

They were then eluted with at least 50ml of glass-distilled water delivered from an elevated reservoir through lengths of 1mm bore tubing tipped with disposable hypodermic needles that were inserted into the columns through air-tight polythene closures. Samples were applied to the columns by the same method, the reservoir was replaced by the sample solution (approx. 6ml) and the columns were washed with water until the effluent was free of electrolytes. Nucleotides were then eluted with 1.0M-triethylammonium bicarbonate buffer, pH 7.4, (2–5 ml) the highly volatile buffer was removed by freeze-drying and the purified nucleotides were suspended in a minimum volume of water for chromatography.

Thin-layer chromatography of nucleotides. Portions of the aqueous nucleotide solutions, with E_{260} approx. 8, were applied to 20cm × 20cm polyethyleneimine-cellulose plates, the plates were developed by the two-dimensional system of Randerath & Randerath (1964) and u.v.-absorbing areas of the chromatograms were marked. Short columns (4cm × 0.5cm internal diam.) plugged with

asbestos wool were connected to a vacuum pump and u.v.-absorbing spots were scraped from the plates and sucked against the plugs.

The columns were then inverted over test tubes and the nucleotides were quantitatively eluted by washing with 1.6M-LiCl (0.5–2ml). Each eluate was drawn into a 1ml disposable syringe, the volume was noted and the sample transferred to a silica cell with a 1cm light-path and internal diameter of 0.1 or 0.2cm. The nucleotide present was identified and the concentration measured from the spectrum recorded with a Unicam SP.800 spectrophotometer. The purity of each nucleotide was confirmed by combining and desalting the same fractions from several chromatograms. No significant contamination of the seven major nucleotides was observed after rechromatography on Whatman no. 1 paper for 16h in isobutyric acid–NH_3–water (66:1:33, by vol.) and in 0.1M-phosphate buffer (pH6.8)–$(NH_4)_2SO_4$–propan-1-ol (50:30:1, by vol.).

Determination of RNA and DNA. The acid-insoluble fractions were washed at 4°C by centrifugation at 800*g* for 5min with successive 5ml portions of 4% (v/v) $HClO_4$, 95% (v/v) ethanol, chloroform–ethanol (2:1, v/v) and ether. After a second ether extraction the lipid-free powders were dried, weighed and divided into replicate 10mg portions. Duplicate portions were assayed for RNA by the spectrophotometric method of Reel & Gorski (1968) after incubation for 2h with ribonuclease (100μg). Further duplicate portions were heated at 70°C for 20min in 10% (v/v) $HClO_4$ (4ml) and 0.4ml portions of the supernatant solutions were used for DNA determination by the modified diphenylamine method of Giles & Meyers (1965).

RESULTS AND DISCUSSION

Assessment of techniques. Bucher & Swaffield (1966) have suggested that ATP/ADP ratios may be used to indicate whether or not the acid-soluble nucleotide composition of an extract accurately reflects that of the original tissue. In the present investigation, this ratio was approx. 0.2 when uteri were extracted by homogenization in perchloric acid at 4°C, 0.5 when uteri were frozen 4min after death and thawed slowly after dispersal in frozen perchloric acid and greater than 2.0 in similar extracts of tissue frozen within 30s of death. The latter value, which is close to the physiological ratio established by Bucher & Swaffield (1966), indicates that the distribution of nucleotides in the present extracts is similar to their distribution *in vivo* in the rat uterus.

Several ion-exchange techniques are available for the separation of tissue extracts into their component nucleotides. Bengtsson (1962) and Bengtsson, Deutsch & Nilsson (1965) used columns of Dowex 1 (formate form) and Ecteola-cellulose to resolve trichloroacetic acid extracts of rabbit vagina into five principal nucleotide fractions and, in this laboratory, perchloric acid extracts of rabbit uterus have been resolved into nine major fractions on DEAE-Sephadex columns by a modification of the method of Caldwell (1969). These methods were impracticable for the current work because a single column elution requires 3 days and because the uteri of approx. 100 immature rats are required for each column analysis.

Preliminary experiments showed that the two-dimensional ion-exchange t.l.c. methods of Randerath & Randerath (1964) separated mixtures of authentic nucleotides in 3h with excellent resolution and greater than twenty-fold increase in sensitivity over column and paper chromatographic methods. However, the inorganic salts in tissue extracts interfered with chromatographic resolution when volumes greater than 0.2ml of an extract of 15 uteri, containing 10 E_{260} units in a total volume of 6ml, were applied directly to polyethyleneimine-cellulose plates. Nucleotides were not separated satisfactorily from these salts by any of the techniques examined. It was not possible to achieve quantitative recovery of nucleotides after adsorption on to charcoal (Hurlbert, 1957); columns of poly-*N*-vinylpyrrolidone (Dougherty & Schepartz, 1969) did not completely separate nucleotides from contaminating potassium perchlorate; and the capillary transfer technique of Foster, Abbot & Terry (1966) was impracticable with large extract volumes. Removal of salts from freeze-dried extracts with acetone–ether (Blumson & Baddiley, 1961) led to some loss of nucleotides in the organic phase. Cross-linked dextrans and polyacrylamide gels are not suitable for the desalting of mononucleotides (Uziel, 1967).

The results in Table 1 show that the ion-exchange desalting technique quantitatively separates nucleotides, that are strongly bound to the polyethyleneimine-cellulose columns, from salts and other acid-soluble compounds such as amino acids, purine and pyrimidine bases and nucleosides. Subsequent chromatography of the nucleotide fraction on polyethyleneimine-cellulose plates develops a pattern of u.v.-absorbing spots identical with that observed with authentic mixtures of nucleotides and with the pattern described by Randerath & Randerath (1964).

The column-elution technique described for the measurement of individual nucleotides was tested by chromatography and elution of known amounts of authentic nucleotides. At least 96% of the nucleotide in a u.v.-absorbing spot was measured by this method. Alternative elution techniques described by Randerath & Randerath (1967) were less rapid and gave less reproducible recoveries.

Effect of oestradiol on purine and pyrimidine compounds of the immature rat uterus. Immature female rats were injected intraperitoneally with oestradiol [0.1ml of a 50μg/ml solution in ethanol–0.9% sodium chloride (1:4, v/v)] and were killed

Table 1. *Isolation of nucleotides from an acid-soluble extract of immature rat uterus*

Columns of polyethyleneimine-cellulose were loaded with the mixture indicated and eluted as described in the Experimental section.

E_{260} units added						Recovery in 2ml of water		Recovery in 4ml of triethylammonium bicarbonate buffer, pH7.4		
Uterine extract	Adenine	Adenosine	(ATP+ ADP+ AMP)	NAD	[2-^{14}C]-Glycine added (c.p.m.)	E_{260} units	c.p.m.	E_{260} units	c.p.m.	% of added nucleotides
1.55						0.02		1.51		98
8.32						0.14		8.10		98
1.55	1.23					1.21		1.57		101
1.55		1.04				0.98		1.48		96
1.55			1.32			0.02		2.78		97
1.55				1.24		0.03		2.75		98
1.55					7532	0.02	7462	1.50	4	97

Table 2. *Effect of oestradiol on the purine and pyrimidine compounds of the immature rat uterus*

Immature female rats (50–70g) were injected intraperitoneally with oestradiol (5μg) and killed at 2h intervals for 12h. The extraction and analytical procedures are described in the Experimental section. Results are the average of two separate experiments.

Time after oestradiol (h)	DNA (μg/mg dry wt.)	RNA (μg/mg dry wt.)	Acid-soluble nucleotides: E_{260} units/mg of DNA	Acid-soluble nucleotides: *μg/mg dry wt.	Distribution ratios: RNA/DNA	Distribution ratios: Nucleotides/DNA
0	51	19.7	3.8	7.3	0.39	0.14
2	51	19.8	3.3	6.2	0.39	0.12
4	46	19.8	2.5	4.3	0.43	0.09
6	44	20.0	2.9	4.7	0.46	0.11
8	41	19.8	3.9	5.9	0.48	0.14
10	39	20.0	4.2	6.1	0.51	0.15
12	38	23.8	4.4	6.2	0.63	0.16

* Assuming the nucleotide fraction has ATP/ADP ratio 2, then μg of nucleotides/mg dry wt. = $\frac{E_{260}\text{ units/mg DNA}}{15.4} \times 574 \times$ mg of DNA/mg dry wt. where $15.4 = E_{260}^{mM}$ for adenine nucleotides, 574 = 2/3 (mol.wt. of ATP)+1/3 (mol.wt. of ADP).

in groups of 15 at 2h intervals up to 12h. During this period it was found that the average DNA content per uterus remained constant between 0.20 and 0.23mg, whereas the average wet weight of the uteri increased from approx. 25 to 40mg and the lipid-free dry weight increased by approx. 20% from 4 to 5mg. Table 2 shows an increase in the RNA/DNA ratio after 4h confirming earlier reports of an oestrogen-induced increase in the total amount of uterine RNA (Hamilton, 1968). It also shows an accompanying decrease in the total acid-soluble nucleotides/mg of DNA to 60% of the control value at 4h, followed by a subsequent return to control values in 8h. The uterine distribution ratios for purines and pyrimidines indicate that approx. 10% of these compounds are present in the form of acid-soluble nucleotides.

The present results are in agreement with those reported by Billing, Barbiroli & Smellie (1969), except that these authors did not observe such a significant decrease in the nucleotides per uterus in the first few hours after hormone treatment. However, they homogenized immature rat uteri in cold water before acid extraction and chromatographic separation of nucleosides from nucleotides and reported a high recovery of nucleosides and low values for E_{260} units/uterus; this suggests that extensive degradation of adenosine phosphates may have occurred during nucleotide extraction.

Effect of oestrogen on the nucleotides of the immature rat uterus. Of the 12 nucleotides identified by t.l.c., seven were present in sufficient quantity for accurate spectrophotometric analysis. It is apparent (Table 3) that adenosine phosphates are the

Table 3. *Effect of oestradiol on the major nucleotides of the immature rat uterus*

The acid-soluble nucleotides of the immature rat uterus were analysed by two-dimensional ion-exchange t.l.c. as described in the Experimental section at the indicated times after injection of oestradiol (5 μg). The results are the average of duplicate experiments and are expressed as nmol of nucleotide/mg of DNA.

	Time after oestradiol administration (h)						
Nucleotide	0	2	4	6	8	10	12
ATP	103.9	79.8	59.0	74.0	106.5	116.4	126.6
ADP	52.0	37.3	32.8	27.0	31.1	37.5	46.8
AMP	4.4	3.2	1.9	1.9	2.9	4.2	3.1
GTP	16.5	9.5	8.1	9.9	15.3	17.8	21.8
GDP	6.7	4.8	4.4	4.9	8.1	9.0	8.9
UTP	13.0	13.3	13.6	13.9	17.1	22.4	27.3
UDP	6.0	6.6	7.0	6.4	8.6	10.4	10.2

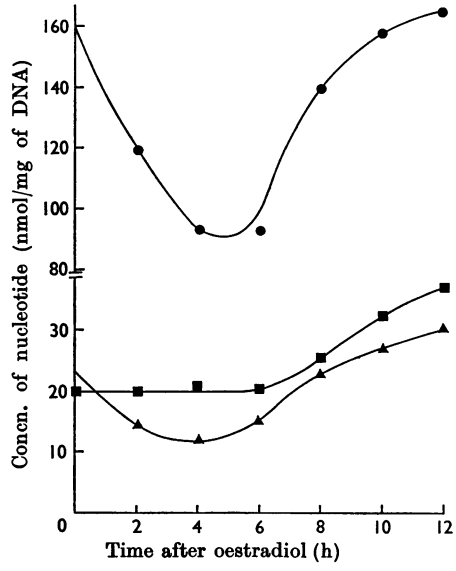

Fig. 1. Effect of oestradiol on the amounts of ATP+ADP+AMP (●), GTP+GDP (▲) and UTP+UDP (■) in the immature rat uterus. The analytical procedure was as described in Table 3.

predominant nucleotides of the rat uterus, with ATP alone accounting for approx. 50% of the total nucleotide. Guanosine and uridine phosphates each account for about 12% of the total nucleotide, whereas CTP, the only cytosine phosphate identified, formed approx. 1.5% of the total nucleoside triphosphate and 1% of the total nucleotide. Very small amounts of GMP and UMP were observed and NAD and two uridine diphosphate sugars were present at similar concentrations to AMP. The identification of significant amounts of guanine nucleotides and small amounts of cytosine nucleotides is of interest since these have not been measured previously in uterine extracts (Bengtsson, 1962; Gorski & Mueller, 1963; Bengtsson *et al.* 1965).

Fig. 1 illustrates the effect of oestradiol on the concentrations of the nucleotides of adenine, guanine and uridine. The hormone caused a rapid and linear decrease in the concentrations of the purine nucleotides to less than 60% of the control values in 4h, followed by an increase to control concentrations in 8–10h. The concentrations of uridine nucleotides remained constant for several hours and then increased linearly to about 200% of control at 12h after hormone treatment. The concentration of CTP appeared to increase in a similar manner.

These results confirm the report by Aaronson, Natori & Tarver (1965) who showed, by the luciferin–luciferase method that the ATP concentration in the uteri of immature rats decreases in a linear manner to approx. 50% of control values in 4h. These findings also agree with the results of Bengtsson (1962) on the changes in several nucleotides in the ovariectomized rabbit vagina at 4–8 and 14–18h after oestradiol injection. Extracts were prepared from tissue frozen within 30s of death and column analysis showed ATP/ADP ratios of approx. 2. There was a steady increase in the concentration of uridine phosphates after 4h and an initial decrease followed by a rapid increase in that of adenosine phosphates. Gorski & Mueller (1963) observed an increase in the content of uridine nucleotides in the uteri of ovariectomized rats 4h after the injection of oestradiol, but found little change in the concentration of ATP at this time. Their data are difficult to assess since extracts were prepared by homogenization in perchloric acid at 4°C and the concentrations of ADP, AMP and adenosine were not reported.

The present results indicate that radioactive

nucleosides used to measure rates of uterine RNA synthesis may be diluted in the endogenous nucleotide pools to very different extents at different time-intervals after hormone treatment. Hence, data expressed as c.p.m. of nucleoside incorporated/mg of RNA may be misleading. The methods developed in this study could be used to analyse the specific radioactivity of the nucleoside triphosphate being incorporated into RNA and the rates of RNA synthesis could be expressed more accurately as μmol of nucleoside triphosphate incorporated/mg of RNA.

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