EFFECTS OF HUMAN CHORIONIC GONADOTROPHIN ON THE INCORPORATION OF URIDINE INTO NUCLEOTIDE POOLS AND RIBONUCLEIC ACID IN THE REPRODUCTIVE TRACT OF THE RABBIT DOE

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Abstract

The effects of injection of human chorionic gonadotrophin, and of a previous ovulation, on the *in vivo* incorporation of [5-^{3}H]uridine into ribonucleic acid by the reproductive tract of oestrus does have been observed. At 6 hr after administration of gonadotrophin the ovaries contained slightly less ribonucleic acid than did those from control animals, but with a fivefold increase in specific radioactivity following injection of [5-^{3}H]uridine 1 hr before slaughter. Injection of gonadotrophin also resulted in increased incorporation of uridine into the free nucleotides in the ovaries.

In the tubular organs (fallopian tube, uterus, cervix, vagina) the specific radioactivity of ribonucleic acid and free nucleotides resulting from uptake of tritiated uridine was increased following injection of chorionic gonadotrophin. Although both the ribonucleic acid and nucleotide pools showed small decreases following gonadotrophin treatment, these changes were not statistically significant.

The tubular organs in does ovulated 4 weeks previously showed a greater incorporation of uridine into ribonucleic acid than the similar organs from does that had not previously ovulated. Does that had previously ovulated also contained more ribonucleic acid in the mucosal tissue, but not in the outer muscular coat of these organs, compared with animals that had not previously ovulated.

Generally the mucosal tissues on extraction yielded more ribonucleic acid of a higher specific radioactivity than did the muscular tissues.

These results are interpreted as being caused by the increased 17β-oestradiol released by the ovary in response to luteinizing hormone acting on the rest of the reproductive tract.

I. INTRODUCTION

In a previous study (Murdoch and O'Shea 1970) it was shown that the endometrium from does injected with human chorionic gonadotrophin (HCG) incorporated less tritiated uridine into ribonucleic acid (RNA) during *in vitro* incubation than did that from rabbits at oestrus. In the present work, *in vivo* incorporation of tritiated uridine by the whole reproductive tract was measured to obtain comparisons between different tissues, and between treatment groups under more physiological conditions, as differences between *in vitro* and *in vivo* RNA metabolism have been reported (Gorski and Nicolette 1963). Total free nucleotide was also examined,

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as in the previous experiments indications of changes in the precursor pools for RNA were thought to be present.

In addition, the effect of a previous ovulation was looked at to see if it might be a cause of the quantitative between-experiment variation noticed before (Murdoch and O'Shea 1970).

II. Materials and Methods

(*a*) *Treatment of Rabbits*

The animals used were virgin does, aged 5–6 months, which had been separately caged for 4 weeks. Two does were injected i.v. with 25 i.u. of HCG, and two does were not injected, so that animals were available which had or had not previously ovulated.

Four weeks later rabbits from each group were either injected i.v. with 62·5 i.u. of HCG (HCG treated) or not injected (control) to give a doe in each of the four treatment groups. After 5 hr all were injected i.v. with 0·5 mCi of [5-^{3}H]uridine and killed 1 hr later by cervical dislocation followed by exsanguination. The reproductive tracts were quickly removed, dissected free of fatty and connective tissues, and placed in ice.

(*b*) *Preparation of Samples*

After the state of the ovaries was checked, the chilled reproductive tract was divided into ovaries, fallopian tubes, uterine horns, cervix, and upper vagina. The luminal contents of the uterine horns were gently squeezed into weighed vials. The uterine horns, cervices, and vagina were opened longitudinally and separated into essentially mucosal plus submucosal tissue, and muscle tissues, by scraping off the inner layer (Murdoch and O'Shea 1970). Samples (up to 1 g) were minced with fine scissors where necessary, weighed, and frozen in dry ice.

(*c*) *Separation of Tissue Fractions*

With some modifications, this was by procedures previously described (Hutchinson and Munro 1961; Murdoch and O'Shea 1970). Except where noted, all steps were carried out at 0–5°C. The sample was pulverized at −79°C, homogenized in 10 vol. of ice-cold 0·2N perchloric acid (PCA), and centrifuged. The acid-insoluble pellet was washed twice with 0·2N PCA. The three supernatants were combined as the acid-soluble fraction.

Lipids were extracted from the acid-insoluble fraction with one wash of 95% ethanol plus 1% (w/v) sodium acetate, two washes of ethanol–chloroform (3 : 1 v/v), one wash of ethanol–ether (3 : 1 v/v), and one wash of ether. RNA was extracted from the defatted residues by incubation for 1 hr at 37°C in 0·3N KOH. After alkaline hydrolysis the samples were chilled and brought to pH 1 with PCA, stood in ice for 10 min, and centrifuged. The precipitate was washed twice with 0·2N PCA and the supernatants combined as the RNA fraction.

Except for two replicates, the acid-soluble material was separated into nucleosides plus other unabsorbed material and total free nucleotides by absorption onto, and elution from, Dowex 1-X8 (Cl^-) ion-exchange resin (Cohn 1955; Billing *et al.* 1969).

Two ways of purifying the RNA fraction were compared in samples from four rabbits. Method A was by washing with chloroform (Murdoch and O'Shea 1970), and method B was by absorption onto and elution from Dowex-2 resin, essentially as described by De Deken-Grenson and De Deken (1959). In 40 tissue samples method A resulted in solutions in which measurement by u.v. absorption at 260 nm gave a mean value of 202 μg RNA/ml. This was greater ($P < 0{\cdot}001$) than the mean value of 148 μg RNA/ml obtained by pentose measurement (Ceriotti 1955). The solutions also contained protein (Lowry *et al.* 1951). The mean values using method B were 107 and 111 μg RNA/ml by u.v. and pentose measurements respectively. Protein was not detected in any samples after Dowex purification. Absorption spectra of solutions obtained by method B consistently gave higher ratios of absorbance at 260 nm to that at 230 nm (De Deken-Grenson and De Deken 1959) as compared to method A.

Despite the recovery of a lesser amount of radioactivity (method A 9·7 nCi/mg RNA; method B 8·9 nCi/mg RNA; $P < 0{\cdot}001$), as method B gave a much purer extract of nucleotides it was used for the remainder of the experiments, and all results reported pertain to method B.

(*d*) *Assay Techniques*

Radioactivity was measured as previously described (Murdoch and O'Shea 1970). RNA was determined by measuring the u.v. absorption at 260 nm, with yeast RNA (Sigma type X1) as standard. Acid soluble nucleotides were determined by measuring the u.v. absorption at 260 nm and expressed in terms of adenosine monophosphate standards, as most of the free nucleotides in the uterus are adenosine nucleotides (Gorski and Mueller 1963; Oliver and Kellie 1970; Miller and Baggett 1972).

(*e*) *Statistical Methods*

Due to deaths and the occurrence of spontaneous ovulation the treatment groups were of unequal size and the significance of the results was assessed by analysis of variance using the method of unweighted means for disproportionate subclass numbers (Snedecor 1956). However, the mean body weight was similar in all four treatment groups (mean 3·1 kg).

III. Results

(*a*) *Accumulation of Fluid in the Uterus*

If free luminal fluid was present in one uterine horn it was present in the other and the significance of this data was assessed by χ^2 tests on a per doe basis. Uteri from does that had not previously ovulated, at 0 and 6 hr after injection of HCG showed 0 of 6 and 4 of 5 with fluid contents respectively, whereas in does that had previously ovulated the corresponding figures were 2 of 6 and 5 of 8. The overall effect of HCG injection was a significant increase in the proportion of does with free fluid ($P < 0{\cdot}01$) but, as shown by the significant χ^2 test for the interaction of a previous ovulation with HCG treatment ($P < 0{\cdot}05$), this was due mainly to the increased proportion with fluid in does that had not previously ovulated.

The radioactivity in this fluid was similar in all four treatment groups when expressed as nCi/g fluid (mean 74; S.E. 15 nCi/g). There were also no differences in the radioactivity of the blood sera from the four treatment groups (mean 136; S.E. 9 nCi/ml serum).

TABLE 1

EFFECT OF PREVIOUS OVULATION AND INJECTION OF HCG ON THE INCORPORATION OF [5-^{3}H]URIDINE BY THE OVARY OF THE RABBIT DOE

Values are means for 3–8 rabbits

Treatment	RNA		Free nucleotide		Free nucleoside fraction (nCi/g tissue)
	mg/g tissue	nCi/mg RNA	mg AMP equiv/g tissue	nCi/mg AMP equiv.	
Control					
Not previously ovulated	5·23	6·06	1·40	142	89
Previously ovulated	4·82	6·30	1·44	140	71
6 hr after HCG					
Not previously ovulated	3·80	34·25	1·16	961	147
Previously ovulated	4·39	30·68	1·36	748	150

(*b*) *Ovary*

There were no effects of previous ovulation on the parameters measured (Table 1). As compared to control animals the ovaries of does at 6 hr after injection

of HCG had significantly less RNA ($P < 0{\cdot}01$), but of much higher specific radioactivity ($P < 0{\cdot}001$). The free nucleotide was not significantly altered in amount but also had a fivefold increase in specific radioactivity ($P < 0{\cdot}001$).

Radioactivity in the free nucleoside fraction increased ($P < 0{\cdot}01$), but to a lesser extent.

(c) Effect of HCG and Previous Ovulation on RNA in the Tubular Organs

The data for changes in specific radioactivity of RNA and the summary of the analysis of variance are given in Table 2. Considering the tract as a whole, both

TABLE 2

EFFECT OF INJECTION OF THE RABBIT DOE WITH HCG ON THE INCORPORATION OF [5-^{3}H]URIDINE INTO THE RNA OF THE REPRODUCTIVE TRACT

Values are means expressed as nCi/mg RNA

Tissue	Doe not previously ovulated		Doe previously ovulated	
	Control ($n = 6$)	HCG ($n = 5$)	Control ($n = 6$)	HCG ($n = 8$)
Fallopian tube	6·24	7·95	7·39	11·81
Endometrium	7·29	18·55	11·23	12·35
Myometrium	6·57	11·05	8·25 ($n = 5$)	9·86
Cervical mucosa	8·28	10·44	10·17	13·32
Cervical muscle	5·30 ($n = 4$)	8·65 ($n = 3$)	6·70	8·82
Vaginal mucosa	8·82	10·62	12·48	15·92
Vaginal muscle	2·47 ($n = 4$)	3·36 ($n = 3$)	4·39	4·07

Summary of analysis of variance

Source of variation	D.F.	Variance ratio
Injection of HCG	1	20·04***
Between tissues		
Endometrium *v.* myometrium	1	7·74**
Cervical mucosa *v.* muscle	1	6·70*
Vaginal mucosa *v.* muscle	1	46·54***
Uterus *v.* fallopian tube	1	4·65*
Uterus *v.* cervix	1	3·74
Uterus *v.* vagina	1	10·94***
Previous ovulation	1	5·28*
Interactions	19	1·20
Within-group error variance	137	*3·0241*

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

injection of HCG (10·48 *v.* 7·54 nCi/mg RNA) and previous ovulation (9·77 *v.* 8·26 nCi/mg RNA) resulted in a greater specific radioactivity in the RNA. In all parts examined, i.e. uterus, cervix, and vagina, the mucosal tissue had higher values than the muscle tissue. The uterus as a whole had a higher specific radioactivity than either fallopian tube or vagina.

With regard to the amount of RNA (Table 3) the results are not as clear due to significant interactions. Overall HCG apparently caused a decrease in RNA but this was mainly due to the cervical tissues which were the only ones to show a consistent

TABLE 3

EFFECT OF INJECTION OF THE RABBIT DOE WITH HCG ON THE LEVEL OF RNA IN THE REPRODUCTIVE TRACT

Values are means expressed as mg RNA/g tissue

Tissue	Doe not previously ovulated		Doe previously ovulated	
	Control ($n = 6$)	HCG ($n = 5$)	Control ($n = 6$)	HCG ($n = 8$)
Fallopian tube	3·46 ($n = 5$)	3·75	4·73	4·33
Endometrium	5·24	5·01	5·74 ($n = 5$)	6·54
Myometrium	4·41	4·42	4·39 ($n = 5$)	4·25
Cervical mucosa	4·07	2·81	5·35	4·96
Cervical muscle	3·05 ($n = 4$)	2·49 ($n = 3$)	2·84	2·35
Vaginal mucosa	3·00	2·55	4·34	3·51
Vaginal muscle	2·49 ($n = 4$)	2·62 ($n = 3$)	3·02	2·70

Summary of analysis of variance

Source of variation	D.F.	Variance ratio
A. Injection of HCG	1	4·64*
B. Between tissues		
1. Endometrium *v.* myometrium	1	28·41***
2. Cervical mucosa *v.* muscle	1	46·16***
3. Vaginal mucosa *v.* muscle	1	7·38**
4. Uterus *v.* fallopian tube	1	20·57***
5. Uterus *v.* cervix	1	81·01***
6. Uterus *v.* vagina	1	138·22***
C. Previous ovulation	1	29·63***
Interactions:		
A × B		
A × B5	1	5·49*
Others	5	0·88
A × C	1	0·02
B × C		
B1 × C	1	5·47*
B2 × C	1	16·00***
B3 × C	1	3·15
Others	3	0·89
A × B × C	6	0·98
Within-group error variance	136	*0·1127*

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

drop 6 hr after HCG administration. Does that had ovulated 4 weeks before had more RNA in their mucosal tissues than does that had not previously ovulated, whereas the muscular tissues were unaffected. Also, the levels in mucosal tissues were higher than those of the muscle with more than the above tissue × ovulation inter-

action being involved. The uterus contained a higher level of RNA than the fallopian tube, cervix, or vagina.

(*d*) *Effect of HCG and Previous Ovulation on the Free Nucleotides in the Tubular Organs*

In the table showing the changes in specific radioactivity (Table 4) first-order interactions that were non-significant, or that were rendered non-significant by the

TABLE 4

EFFECT OF INJECTION OF THE RABBIT DOE WITH HCG ON THE INCORPORATION OF [5-^{3}H]URIDINE INTO THE ACID-SOLUBLE NUCLEOTIDES OF THE REPRODUCTIVE TRACT

Values are means expressed as nCi/mg AMP equivalent

Tissue	Doe not previously ovulated		Doe previously ovulated	
	Control ($n = 4$)	HCG ($n = 3$)	Control ($n = 4$)	HCG ($n = 6$)
Fallopian tube	155	180	155	232
Endometrium	206	415	212 ($n = 3$)	293
Myometrium	154	320	122 ($n = 3$)	214
Cervical mucosa	188	258	187	261
Cervical muscle	125	190	217	152
Vaginal mucosa	297	207	298	499
Vaginal muscle	72	88	102	107

Summary of analysis of variance

Source of variation	D.F.	Variance ratio
A. Injection of HCG	1	13·69***
B. Between tissues		
1. Endometrium *v.* myometrium	1	5·58**
2. Cervical mucosa *v.* muscle	1	2·46
3. Vaginal mucosa *v.* muscle	1	48·55***
4. Uterus *v.* fallopian tube	1	4·51*
5. Uterus *v.* cervix	1	3·58
6. Uterus *v.* vagina	1	1·98
C. Previous ovulation	1	0·61
Interactions:		
A × B		
A × B5	1	4·56*
Others	5	1·66
A × C plus B × C	7	1·93
A × B × C		
A × B3 × C	1	4·90*
A × B6 × C	1	6·49*
Others	4	0·77
Within-group error variance	89	*2236*

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

second-order interactions, were combined for ease of presentation. Injection of HCG gave an overall increase in specific radioactivity of the free nucleotides but had no

effect on vaginal muscle, the reverse effect on vaginal mucosa of does that had not previously ovulated, and a smaller effect on the cervix than the uterus. Again the specific radioactivity was lower in muscle than in mucosa, with this difference being significant in the uterus and vagina.

TABLE 5

EFFECT OF INJECTION OF THE RABBIT DOE WITH HCG ON THE LEVEL OF ACID-SOLUBLE NUCLEOTIDES IN THE REPRODUCTIVE TRACT

Values are means expressed as mg AMP equivalent/g tissue

Tissue	Doe not previously ovulated		Doe previously ovulated	
	Control ($n = 4$)	HCG ($n = 3$)	Control ($n = 4$)	HCG ($n = 6$)
Fallopian tube	0·94	1·11	1·15	1·04
Endometrium	0·83	0·66	0·89 ($n = 3$)	0·94
Myometrium	0·56	0·65	0·79 ($n = 3$)	0·48
Cervical mucosa	1·02 ($n = 3$)	0·83	1·14	1·16
Cervical muscle	0·54	0·46	0·71	0·69
Vaginal mucosa	0·91	0·98	0·90	1·00
Vaginal muscle	0·99	0·70	0·89	0·77

Summary of analysis of variance

Source of variation	D.F.	Variance ratio
A. Injection of HCG	1	8·61**
B. Between tissues		
1. Endometrium *v.* myometrium	1	33·98***
2. Cervical mucosa *v.* muscle	1	146·95***
3. Vaginal mucosa *v.* muscle	1	9·32**
4. Uterus *v.* fallopian tube	1	114·24***
5. Uterus *v.* cervix	1	13·42***
6. Uterus *v.* vagina	1	42·32***
C. Previous ovulation	1	25·62***
Interactions:		
A × B		
A × B3	1	15·94***
Others	5	0·86
A × C	1	0·01
B × C	6	2·86*
A × B × C	6	6·29***
Within-group error variance	87	*0·00262*

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

Although HCG resulted overall in an apparent decrease in the free nucleotide pool (Table 5) the interactions with site and previous ovulation removed the statistical significance from this effect. There was a tendency for the mucosal tissues to contain more nucleotide than the corresponding muscle samples but, after considering the interactions, this was only significant for the cervix. Similarly the uterus contained less nucleotide than the fallopian tube or the vagina. Due to the various interactions, previous ovulation had no effect on concentration of nucleotide.

(*e*) *Effect of HCG and Previous Ovulation on the Free Nucleosides in the Tubular Organs*

As the nucleoside fraction from the acid-soluble extract was a mixture of several types of compounds, only the radioactivity per gram of tissue was considered. No significant effects were seen. The radioactivity in the total acid-soluble fraction (i.e. before separation into free nucleosides and nucleotides), expressed as nCi/g tissue, gave a pattern of results fairly similar to those for free nucleotide alone.

IV. Discussion

Following coitus or injection of luteinizing hormone in the rabbit there is a very rapid increase in 17β-oestradiol and 20-α-hydroxypregn-4-en-3-one (20α-OH) in rabbit ovarian venous plasma (Hilliard *et al.* 1964, 1968; Hilliard and Eaton 1971; Mills *et al.* 1972). These hormones increase to reach a peak at 1·5–4 hr while progesterone stays at a constant level, and then all three steroids decline to reach minimal values at ovulation (Hilliard and Eaton 1971; Mills *et al.* 1972). The 17β-oestradiol is produced by the ovarian follicles (Eaton and Hilliard 1971; Mills *et al.* 1971; Younglai 1972) and appears to be the active ovarian oestrogen in the rabbit (Gabb and Stone 1972). The interstitial tissue is the major source of 20α-OH in the rabbit (Eaton and Hilliard 1971; Oxender *et al.* 1971*a*, 1971*b*).

In the present experiment, does killed at 6 hr after injection of HCG would have been exposed to these peak levels of 17β-oestradiol and 20α-OH (Hilliard and Eaton 1971; Mills *et al.* 1972). The increase in oestradiol is likely to have caused accumulation of fluid in some of the tissues (Mueller *et al.* 1958). As the levels of RNA and nucleotides in the present experiments were expressed per gram of wet tissue this may have obscured changes in these parameters.

Similar effects to those obtained with HCG, i.e. increased rate of labelling of RNA and free nucleotides, are seen in ovariectomized animals treated with oestrogen (Gorski and Mueller 1963; Gorski and Nicolette 1963; Miller and Baggett 1972). The greater clarity in the RNA results as compared to those for the free nucleotides may be due to the higher purity of the RNA, more does per group for RNA, and/or the fact that the free ribonucleotide pool was not separated into its individual nucleotides (Mueller *et al.* 1958; Miller and Baggett 1972). It is reasonable to assume that the effects of HCG are mediated through the 17β-oestradiol that is released by the ovarian follicles due to the luteinizing hormone properties of the gonadotrophin. What modifications are caused by the 20α-OH are unknown. This steroid may have a greater role following coitus than after injection of HCG in that it appears to prolong release of luteinizing hormone following natural mating (Hilliard *et al.* 1967). A very low level of progestin may synergize with oestrogens in enhancing capacitation; a high dosage of 20α-OH certainly depresses the capacitation process (Soupart 1970; Plotka and Williams 1971).

Induction of ovulation some 4 weeks before injection of tritiated uridine altered the effect of HCG on fluid production by the uterus, increased the specific radioactivity of RNA, and increased the RNA in mucosal tissues. One possible way in which ovulation may subsequently affect the metabolism of the reproductive tract is through speeding up maturation, as maturation changes have been reported in the RNA of rabbit oviduct by Gupta *et al.* (1970). However, they examined rabbits

which were younger than those of the present work, and also of less than half the weight. It is unfortunate that in the present work no attempt was made to quantify the follicles present, as Eaton and Hilliard (1971) observed that 17β-oestradiol secretion appears to be directly related to the number and condition of the follicles present. Differing status of the ovary could be the cause of the effects of previous ovulation.

The mucosal tissues of the uterus, cervix, and vagina incorporated more uridine into RNA and free nucleotides than did the corresponding muscular coats, and had an increased level of RNA subsequent to ovulation (4 weeks before killing) which had no effect on the muscle. The greater response of the mucosae might be expected from the selective accumulation of oestrogens by endometrium; and more specifically, the high amounts associated with the tubular glands of the endometrium (Ullberg and Bengtsson 1963) correlates well with the rapid increase in fluid secretion by the tract following HCG treatment (Lutwak-Mann 1962; Sugawara and Takeuchi 1967; Murdoch and O'Shea 1970). Differences in extraction efficiency between muscle and mucosa would not cause the observed differences in specific radioactivity between these tissues.

The differences in the metabolism of RNA between the uterus and the other organs suggest a more rapid incorporation of uridine into RNA in this organ. However, in the analysis of the results the mucosal and muscle values were added on a 1 : 1 basis to give organ values, whereas in all organs the muscle formed a bigger proportion than the mucosa. For this reason the organ comparisons, especially of uterus with fallopian tubes, should be treated with caution. Despite this, the higher fertility from uterine horn than from oviducal inseminations (Adams and Chang 1962; Wettemann *et al.* 1971) and the differing capacitation potential between these organs (Bedford 1969) imply such comparisons are worthwhile. Differences between uterus and oviduct in their rate of increase in RNA synthesis due to injection of oestradiol have been reported for the mouse (Bronson and Hamilton 1972).

The increase in uridine incorporation into RNA following injection of HCG (Table 2) is the opposite effect to that seen with *in vitro* incubation of endometrium (Murdoch and O'Shea 1970). Uteri excised 2 hr after oestrogen injection into rats show an enhanced ability to incorporate labelled precursors into RNA *in vivo*, but this stimulatory effect is not seen on *in vitro* incubation of uterine segments (Gorski and Nicolette 1963). The results obtained on *in vitro* incubation vary according to the time of examination following hormone treatment. Munns and Katzman (1971) demonstrated that uteri removed from immature rats, which had been treated with oestradiol 30, 120, and 360 min beforehand, on *in vitro* incubation with tritiated uridine incorporated 163, 101, and 84% respectively as much label as did control uteri. Rabbits killed 5 hr after HCG injection (Murdoch and O'Shea 1970) would have had raised oestradiol levels for about 200 min, and decreased incorporation of uridine with *in vitro* incubation of endometrium parallels the results seen by Munns and Katzmann (1971). The postulation of an increase in RNA precursors following HCG treatment to account for the decrease in *in vitro* incorporation of uridine (Murdoch and O'Shea 1970) is not upheld by the present results, although the more likely possibility of variations in individual nucleotide pools is not precluded (Gorski and Mueller 1963; Miller and Baggett 1972).

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