METABOLISM OF RABBIT ENDOMETRIUM FOLLOWING THE ADMINISTRATION OF HUMAN CHORIONIC GONADOTROPHIN, AND DURING OESTRUS, PSEUDOPREGNANCY, AND PREGNANCY

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Summary

Changes in endometrial metabolism which occur after the intravenous injection of human chorionic gonadotrophin and during pregnancy (day 9) and pseudopregnancy (day 9) in the rabbit have been measured. During *in vitro* incubation, the endometrium at all stages studied produced more isotopically labelled carbon dioxide from [1-^{14}C]glucose than from [6-^{14}C]glucose.

The endometrium from does injected with human chorionic gonadotrophin either 5 or 10 hr before incubation incorporated less tritiated uridine into ribonucleic acid than did that from rabbits at oestrus. When a larger amount of uridine was added during incubation there was no effect from injection of gonadotrophin. These results were thought to show that the gonadotrophin resulted in the formation of a larger uridine pool in the endometrium. There was more fluid in the uterine horns of the rabbits injected with human chorionic gonadotrophin and the proportion of does showing this fluid was greater at 10 hr than at 5 hr after treatment.

During pregnancy and pseudopregnancy, the endometrium increased its oxygen consumption and carbon dioxide production, but incorporated less glucose into lactate, lipid, ribonucleic acid, and protein.

I. INTRODUCTION

Capacitation, a physiological change that rabbit spermatozoa undergo as a necessary preliminary to penetration into ova, normally occurs in the female genital tract during the transit of the spermatozoa to the site of fertilization (Austin 1967). Soupart (1966, 1967) has shown that the intravenous injection of human chorionic gonadotrophin (HCG) into oestrous does in increasing doses from 25 to 75 i.u. increases the effectiveness of the female genital tract for capacitation. He also observed that the ultrastructure of the endometrium from rabbits of varying capacitating capacity differs widely. In addition, Lutwak-Mann (1962) found fluid free in the uterine horns of rabbits 5–24 hr after the injection of 25 i.u. of HCG or after natural mating.

These responses to HCG suggested that it induces changes in the physiology and biochemistry of the uterine endothelium before the occurrence of ovulation. This response is seen 9·5–14 hr after intravenous injection (Harper 1963; Fox and Krinsky 1968).

Natural mating or the injection of an ovulating dose of HCG have been shown to give an immediate elevation of progestin level in peripheral blood (Forbes 1953)

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and in the ovarian vein (Hilliard, Endroczi, and Sawyer 1961) of the rabbit. Moreover, recent studies of enzymes in the endometrium have shown that the activities of glucose-6-phosphate dehydrogenase and a number of other carbohydrate-metabolizing enzymes are dependent on progesterone (Hafez and White 1967; Murdoch and White 1969) and are altered by pregnancy and pseudopregnancy.

The present investigation is of oxidative and biosynthetic events in the endometrium of the rabbit which may be associated with the above effects of HCG and of progesterone during pseudopregnancy and early pregnancy. Since glucose-6-phosphate dehydrogenase plays an important role in the hexose monophosphate metabolic pathway, in which carbon 1 of glucose is converted to CO_2, specifically labelled glucose was used. These experiments showed that there was no detectable effect of HCG on the endometrial metabolism with glucose as substrate and the work was extended to examine the incorporation of tritiated uridine into RNA and protein.

II. Materials and Methods

(a) *General*

Virgin albino rabbits, aged 6–8 months, were kept in separate cages for at least 4 weeks before use. The animals were killed by cervical dislocation at oestrus (as confirmed by the presence of large follicles in the ovaries), and at 5 and 10 hr and 9 days (pseudopregnant) after the intravenous injection of 50 i.u. of HCG (Pregnyl, Organon). Pregnancy was induced by artificial insemination with 0·2 ml of freshly collected semen at the time of injection of HCG.

(b) *Preparation and Incubation of Endometrium*

After slaughter, the uterus was removed quickly and dissected free of fatty and connective tissues and of the attached oviducts and cervix. The uterine horns from oestrous does and from rabbits treated with HCG 5 and 10 hr earlier were placed on a piece of filter paper, gently squeezed along their length to express their luminal contents, and the presence or absence of fluid in the horn was recorded. In pregnant animals (day 9), only the sections of the uterine horn between implanting blastocysts were used since a preliminary examination revealed that it was difficult to separate effectively the endometrium from the embryonic tissues.

Each uterine horn was opened down the mesometrial side and the exposed endometrium scraped off using a scalpel blade and fine forceps. The tissues from two or more animals in each treatment group were pooled and weighed into lots of 500 mg. The endometrium was incubated in Warburg flasks of 16 ml capacity containing 2 ml of calcium-free Krebs–Ringer phosphate solution (Umbreit, Burris, and Stauffer 1959) (pH 7·4) and 2 mg (0·5 μCi/mg) of either D-[1-^{14}C]glucose or D-[6-^{14}C]glucose. In experiments designed to assess the rate of incorporation of labelled uridine into RNA and protein, the flasks contained 500 mg of endometrium, 2 ml of calcium-free Krebs–Ringer phosphate, 2 mg of unlabelled glucose, and either 0·2 mg or 2·0 mg (12·5 μCi/mg) of [5-^{3}H]uridine. The centre well of the flasks contained 0·1 ml of 20% (w/v) potassium hydroxide and glucose oxidation was determined by assay of $^{14}CO_2$ trapped. Oxygen consumption was measured with air as the gas phase over a 2-hr incubation period at 37°C and with a shaking rate of 114 strokes/min.

To provide background values that would correct for any passive uptake of labelled material by the tissue, flasks containing 5 ml of chilled 10% (w/v) trichloroacetic acid (TCA) plus materials identical to those of the experimental flasks were stoppered and immersed in an ice-water mixture during the 2 hr incubation. These aliquots were put through the same extraction procedures as the experimental samples.

(c) *Separation of Tissue Fractions*

Except where noted, centrifuging was carried out at 4°C for 10 min at 1000 *g*, and the separation was based on a procedure described by Hutchinson and Munro (1961).

Following incubation, 5 ml of ice-cold 10% (w/v) TCA were added to the contents of each flask, and, after standing for 10 min at 0°C, the samples were centrifuged. When incubations were carried out in the presence of radioactive glucose, the incorporation of labelled carbon into lactate was measured. The acid-soluble fraction (supernatant) was neutralized with NaOH, the glucose was removed by two $CuSO_4$–$Ca(OH)_2$ precipitations (Barker and Summerson 1941; O'Shea and Wales 1967), and aliquots of the resultant solutions chromatographed on paper using butanol–acetic acid–water (4 : 1 : 5 v/v) solvent (Lugg and Overell 1948). The only radioactive peak, which coincided with authentic lactate, was extracted with water and assayed.

The acid-insoluble fraction was placed in solid carbon dioxide and pulverized in the frozen state in a percussion–mortar device. The fragments were homogenized in 5 ml of ice-cold 10% TCA in an all-glass homogenizer and centrifuged. The supernatant was discarded and the acid-insoluble material washed again in 10% TCA. Lipids were then extracted with one wash (5 ml) of 95% ethanol, two washes of ethanol–chloroform (3 : 1 v/v), one wash of ethanol–ether (3 : 1 v/v), and one wash of ether. The pooled organic solvents were evaporated to dryness, dissolved in 4 ml of chloroform–ethanol (2 : 1 v/v), and washed with an equal volume of water to obtain a constant low level of radioactivity in the background samples. Aliquots were taken for lipid phosphate analysis (0·05 ml) (Bartlett 1959) and for isotope counting (0·4 ml).

The RNA in the acid-insoluble, defatted residue was assayed by hydrolysing in 5 ml of 0·3N NaOH for 1 hr at 37°C (Fleck and Munro 1962). The hydrolysate was acidified with 1N HCl to pH 1 and centrifuged. The supernatant was deproteinized and cleared by shaking and washing at room temperature with chloroform. Washing was continued until no precipitate formed at the interface of the chloroform and aqueous layers, and then traces of chloroform were removed by one wash with ether (Sevag, Lackmann, and Smollens 1938). The RNA in this deproteinized supernatant was determined by measuring the ultraviolet absorption at 260 nm.

The precipitate was dissolved in 1N NaOH and the protein measured by the method of Lowry *et al.* (1951).

(*d*) *Measurement of Radioactivity*

Usually 0·8 ml of solution was counted by liquid-scintillation techniques in 10 ml of a scintillator consisting of toluene–triton X-100 (2 : 1 v/v) (Patterson and Greene 1965) containing 0·4% (w/v) 2,5-diphenyloxazole and 0·01 (w/v) 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene. Counts were corrected for quenching by internal standardization with labelled toluene. 0·4 ml of the 1N NaOH solution of protein was counted in 10 ml of the toluene–triton X-100 scintillator with the addition of 0·4 ml of 1N HCl to obtain a clear mixture.

(*e*) *Statistical Methods*

The significance of the results was assessed by analysis of variance using the within-group variance as error. Where there was heterogeneity of variance, the raw data were converted to logarithms. In order to save space, summaries of the analyses of variance are not tabulated in all cases. Chi-squared was determined as described by Snedecor (1956).

III. Results

(*a*) *Effect of HCG on the Metabolism of Glucose by Endometrium*

Table 1 shows that substantial amounts of both carbon 1 and carbon 6 of glucose were converted to CO_2 and incorporated into lactic acid, lipid, RNA, and protein fractions of endometrium when incubated *in vitro* with specifically labelled sugars and indicates that, under these conditions, oxidative, glycolytic, and biosynthetic events in the tissue were proceeding at measureable rates.

The metabolism of endometrial tissue from rabbits during oestrus and 10 hr after the injection of HCG was similar. More $^{14}CO_2$ was formed from [1-^{14}C]glucose than from [6-^{14}C]glucose ($P<0·05$). The oxygen uptake of the endometrium from the does treated with HCG was slightly less than that from oestrous does ($P<0·05$).

The radioactivity in the fractions of lactate, lipid, RNA, and protein following incubation was recorded as specific activity (see Barker and Warren 1966). Variations between treatments for these parameters were not significant.

TABLE 1

METABOLISM OF SPECIFICALLY LABELLED GLUCOSE BY THE ENDOMETRIUM OF RABBITS DURING OESTRUS AND 10 HR AFTER THE INJECTION OF 50 i.u. OF HUMAN CHORIONIC GONADOTROPHIN (HCG)

Values are the means ± standard errors of the means of four replicates

Substrate	Oxygen Uptake (μl/flask)	^{14}C in CO_2 (nCi/flask)	^{14}C in Lactate (nCi/flask)	^{14}C in Lipid (nCi/mg lipid P)	^{14}C in RNA (nCi/mg)	^{14}C in Protein (nCi/mg)
			During oestrus			
[1-^{14}C]Glucose	145± 5·2	5·07±0·51	9·45±0·8	11·06±1·1	0·86±0·15	0·070±0·008
[6-^{14}C]Glucose	155±10·4	3·82±0·35	10·59±1·0	12·14±0·5	0·76±0·09	0·068±0·002
			10 hr after HCG injection			
[1-^{14}C]Glucose	123±11·8	5·27±0·84	8·26±1·5	8·20±1·7	0·64±0·16	0·064±0·012
[6-^{14}C]Glucose	125±14·4	3·23±0·75	8·80±1·8	9·66±3·2	0·53±0·12	0·053±0·011

TABLE 2

METABOLISM OF [5-^{3}H]URIDINE BY THE ENDOMETRIUM OF RABBITS DURING OESTRUS AND AFTER THE INJECTION OF 50 i.u. OF HCG

Values are the means ± the standard errors of the means of four replicates (expts. 1 and 2) or five replicates (expt. 3). The specific activity of the tritium-labelled uridine was 12·5 μCi/mg. 0·2 mg of labelled uridine added in experiments 1 and 2 and 2·0 mg in experiment 3

Treatment	Oxygen Uptake (μl/flask)	^{3}H in Protein (nCi/mg)	^{3}H in RNA (nCi/mg)
		Experiment 1	
Oestrus	137±25	0·24±0·04	3·07±0·47
10 hr after HCG	119±19	0·24±0·04	1·75±0·49*
		Experiment 2	
Oestrus	169±11	0·31±0·01	6·16±1·56
5 hr after HCG	159±12	0·30±0·04	4·33±1·26*
Oestrus	182± 8	0·35±0·01	9·61±1·12
5 hr after HCG	199±12	0·45±0·11	8·19±1·31*
		Experiment 3	
Oestrus	163± 8	4·51±0·76 } **	42·51±4·75 } ***
5 hr after HCG	197±14	5·19±1·02 } **	42·29±5·71 } ***

* Significantly different from oestrus, $P < 0·05$.

** Significantly different from 0·2 mg uridine, $P < 0·01$.

*** Significantly different from 0·2 mg uridine, $P < 0·001$.

(*b*) *Effect of HCG on the Metabolism of Uridine by Endometrium*

The incorporation of tritiated uridine into the RNA-enriched fraction and into protein of endometrial tissue from does in oestrus and after the injection of HCG was measured. In the first two of these experiments (Table 2), where a low level of uridine

(0·2 mg) was added to each flask, the RNA fraction from oestrous rabbits had a higher specific activity than that from rabbits either 10 hr or 5 hr after the injection of HCG ($P<0·05$).

To see if this effect was due to a larger pool of endogenous precursors in the endometrial tissue of the treated animals, in experiment 3 a comparison was made of the addition of either 0·2 mg or 2·0 mg of uridine of the same specific activity. The increase in the amount of labelled uridine markedly raised the specific activity of the protein ($P<0·01$) and RNA ($P<0·001$) fractions (Table 2). In the aliquots incubated with 0·2 mg of uridine, the endometrium from the rabbits treated with HCG incorporated less tritium into RNA ($P<0·05$). However, the specific activity of the RNA in the tissue from oestrus and HCG injected animals was the same when the flasks contained 2·0 mg of uridine.

In these experiments some protein was still present in the RNA fraction but to an extent that would account for only a small fraction of the radioactivity.

(*c*) *Effect of HCG on the Accumulation of Fluid in the Uterine Horns*

The presence or absence of free luminal fluid in each uterine horn of the rabbits used in the above experiments was noted. Horns from rabbits at oestrus and at 5 and 10 hr after the injection of HCG showed 21 of 139, 54 of 68, and 64 of 64 with fluid contents respectively. These values were all significally different ($P<0·001$) by the χ^2 test.

Although no measurements were made of the fluid volumes the amounts expressed from the uteri of the treated does were obviously greater than the small volumes obtained from those horns of the oestrous rabbits containing fluid.

(*d*) *Metabolism of Glucose by Endometrial Tissue from Does during Oestrus, Pseudopregnancy, and Pregnancy*

The results of an experiment designed to examine the oxidative metabolism of specifically labelled glucose by endometrial tissue from does during oestrus, pseudopregnancy (day 9), and pregnancy (day 9) and the incorporation of the labelled carbon into the various tissue fractions at these stages are given in Table 3. In all treatment groups more $^{14}CO_2$ was produced from and more protein labelled by [1-^{14}C]glucose than [6-^{14}C]glucose.

Pseudopregnancy resulted in increased production of $^{14}CO_2$ and utilization of oxygen, but decreased incorporation of the labelled carbons into lactate, lipid, RNA, and protein. Similar effects were seen in the pregnant animals with oxygen uptake, and incorporation of the labelled carbons into lactate, RNA, and protein. The pool sizes of the parameters did not vary significantly within the endometrial tissue between the various reproductive stages studied.

IV. Discussion

The present paper confirms the report of Lutwak-Mann (1962) that injection of HCG causes increased uterine fluid within 5 hr and a further increase after 10 hr. Sugawara and Takeuchi (1964) reported an increased uterine flow of fluid in rabbits at oestrus and showed an even greater flow from the oviducts. They demonstrated

increased oviductal fluid following injection of HCG (Sugawara and Takeuchi 1967), suggesting that some of the uterine fluid increase may be derived from the oviducts. This secretory response to HCG may, in part, explain the claim by Soupart (1966, 1967) that the injection of ovulating doses of HCG in the rabbit stimulates the effectiveness of the female genital tract for capacitation.

TABLE 3

METABOLISM OF SPECIFICALLY LABELLED GLUCOSE BY THE ENDOMETRIUM OF RABBITS DURING OESTRUS, PSEUDOPREGNANCY (DAY 9), AND PREGNANCY (DAY 9)

Values are means for four replicates

Substrate	Oxygen Uptake (μl/flask)	^{14}C in CO_2 (nCi/flask)	^{14}C in Lactate (nCi/flask)	^{14}C in Lipid (nCi/mg lipid P)	^{14}C in RNA (nCi/mg)	^{14}C in Protein (nCi/mg)
			Oestrus			
[1-^{14}C]Glucose	111	3·43	18·9	7·66	0·428	0·037
[6-^{14}C]Glucose	109	2·07	18·1	6·87	0·317	0·023
			Pseudopregnancy			
[1-^{14}C]Glucose	202	7·71	9·8	4·71	0·237	0·020
[6-^{14}C]Glucose	183	2·86	13·4	4·84	0·212	0·018
			Pregnancy			
[1-^{14}C]Glucose	188	5·99	12·9	6·85	0·294	0·025
[6-^{14}C]Glucose	184	2·04	9·7	5·68	0·236	0·019

Summary of Analysis of Variance

Source of Variation	D.F.	Variance Ratios					
		Oxygen Uptake	CO_2	Lactate	Lipid	RNA	Protein
Treatment (A)	(2)						
Oestrus *v.* pseudopregnancy	1	20·5**	12·1**	4·6*	4·8*	7·7*	10·4**
Oestrus *v.* pregnancy	1	17·4**	2·1	5·2*	0·8	4·1	5·1*
Substrate label (B)	1	0·3	29·8**	0·0	0·4	2·2	7·0*
Interaction $A \times B$	2	0·1	2·9	0·3	0·2	0·3	1·6
Within-group error	18	*998*†	*1·59*†	*206*	*5·13*	*0·011*	*0·00005*

* $P < 0·05$. ** $P < 0·01$. † 12 degrees of freedom due to missing values.

The similar levels of radioactivity from [1-^{14}C]glucose and [6-^{14}C]glucose in the endometrial lipid fraction suggest that both carbon 1 and carbon 6 of the glucose molecule may be metabolized and incorporated into lipid via the same pathways. However, the higher level of radioactivity from [1-^{14}C]glucose than from [6-^{14}C]glucose in RNA and protein is opposite to that which would be expected in association with the greater formation of $^{14}CO_2$ from [1-^{14}C]glucose and suggests that some incorporation of label into these fractions occurs by way of carbon dioxide fixation. A similar dichotomy between carbon dioxide production and incorporation of label has been reported for the rat uterus by Barker and Warren (1966).

Significant changes in endometrial metabolism have been shown to occur in the present investigation after HCG injection. Endometrial tissue from does injected with HCG 5 and 10 hr before incubation incorporated less tritiated uridine into RNA than endometrium from oestrous does. However, when a larger amount of uridine was added during incubation there was no effect of HCG, suggesting that the gonadotrophin may increase the size of the endometrial uridine pool. Mueller, Herranen, and Jervell (1958) reported a striking increase in the size of the uridine–monophosphate pool in the uterus of the rat following oestrogen treatment.

The effects of HCG on secretion and endometrial metabolism may result from the action of the progestin, 20α-hydroxypregn-4-en-3-one (20α-OHP), which is secreted by the ovarian interstitium in response to the luteinizing hormone (LH) properties of the gonadotrophin (Simmer, Hilliard, and Archibald 1963; Hilliard, Penardi, and Sawyer 1967; Hilliard, Spies, and Sawyer 1968). The concentration of 20α-OHP is increased in the ovarian venous effluent within minutes after coitus or LH injection and high levels continue throughout most of the preovulatory period. Although Hilliard, Penardi, and Sawyer (1967) have indicated that the progestin appears to play a positive role in prolonging LH discharge from the pituitary gland in the mated rabbit, the extent to which it participates in regulating endometrial metabolism is essentially unknown and further studies are clearly warranted.

Implantation of the conceptus in the rabbit occurs between 7 and 9 days after mating (see Böving 1963) and depends upon progesterone secretion from the corpora lutea. The secretion of progesterone increases steadily in the first week of pregnancy and becomes maximal during the third week (Mikhail, Noall, and Allen 1961; Hilliard, Spies, and Sawyer 1968). The increased oxygen consumption and decreased lactate production of endometrial tissue from pregnant and pseudopregnant rabbits in the present investigation may, therefore, reflect changes in the activities of progesterone-dependent enzymes such as succinic dehydrogenase and lactic dehydrogenase (Hafez and White 1967; Murdoch and White 1969). Mounib and Chang (1965) also reported that the oxygen uptake of rabbit endometrium incubated with glucose increased until day 11 of pseudopregnancy but did not find a significant decrease in lactate production. These workers, however, expressed their data on a dry matter basis and measured total lactate formation rather than lactate accumulating from added glucose. In view of the complex series of interactions occurring between the maternal tissues and blastocyst during implantation, the slight differences between pseudopregnant and pregnant animals are not surprising and may reflect differences in hormonal status.

Glucose-6-phosphate dehydrogenase is a key enzyme in the hexose monophosphate shunt and a parallelism between the rate of activity of this pathway and the rate of protein, pentose sugar, and nucleic acid production has been demonstrated in both mature and actively dividing cells (Beaconsfield and Reading 1964; Beaconsfield and Ginsburg 1964; Beaconsfield, Ginsburg, and Jeacock 1964). The increased glucose-6-phosphate dehydrogenase activity of endometrium from pregnant and pseudopregnant rabbits reported by Hafez and White (1967) and Murdoch and White (1969) does not appear to be paralleled by an increase in hexose monophosphate shunt activity, as measured by incorporation of radioactive glucose into RNA and protein in the present investigation. However, since the transport of sugars and the concentrations of glycogen, glucose, and oligosaccharides in the uterus

are influenced by ovarian hormones (Brody and Westman 1958; Vaes and Van Ypersele 1960; Gregoire and Gibbon 1965; Bitman *et al.* 1967; Roskoski and Steiner 1967), the decreased incorporation of labelled glucose into RNA and protein at these reproductive stages may be due to an increase in the pool size of endometrial carbohydrates which compete with the labelled sugar as RNA and protein precursors.

Further work is required to reveal the possible implications which these metabolic changes in the endometrium may have in association with secretory activity, capacitation, and nutrition of zygotes.

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