The Uptake of [^3H]Uridine into the Nucleic Acids of Rat Uterus During Early Pregnancy

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1. Changes in content and uptake of [^3H]uridine into the nucleic acids of rat uterus during the first 9 days of pregnancy were studied. 2. From day 6 implantation sites were separated from the rest of the uterine tissue for independent analysis. 3. Up to day 5 of pregnancy no changes were found in the total dry matter or in RNA and DNA content/unit dry matter nor in the RNA/DNA ratios. 4. From day 6, when implantation sites are visible, the water content of the implantation sites increased by 2-3%, and the RNA content/unit dry wt. and the RNA/DNA ratios increased. The DNA content/unit dry wt. did not increase in the implantation sites until day 8. 5. Uptake of [^3H]uridine into the acid-soluble fraction of the tissues was markedly higher in implantation sites than in non-implantation sites. 6. Uptake of [^3H]uridine into RNA was significantly increased on day 3 of pregnancy and again on day 5. 7. On days 6 and 7, the incorporation into RNA of implantation sites was significantly higher than in the remainder of the uterine tissue but decreased on days 8 and 9 to the same value as that of the normal tissue. 8. No change occurred in uptake into DNA until day 6, when there was an increase in uptake by the implantation sites. 9. It is suggested that the increase in RNA synthesis on day 3 is a preparation of the uterus for the onset of implantation on day 5, and that increased synthesis in implantation sites on days 6 and 7 is the elaboration of new RNA necessary for this early stage of pregnancy to commence.

After a fertile mating in the rat, the fertilized ova mature and divide in the oviduct until, 3-3½ days after mating, the blastocysts are shed into the uterus and by day 6 become attached to the uterine epithelial tissue. This latter process is known as implantation.

Implantation has been extensively studied endocrinologically (cf. Mayer, 1965) and, since it is accompanied by a rapid growth and development of the uterus at the points of implantation, changes in DNA and RNA have been studied during this period in naturally mated pregnant and pseudopregnant rats (Miller, Owen & Emmens, 1968; Malinowska, Greenstreet & Fotherby, 1969), and in rats in which pseudopregnancy was induced by electrical stimulation of the cervix (Shelesnyak & Tic, 1963). It was shown that the content and concentration of RNA increased from days 5-6 of pregnancy, but that the concentration of DNA remained essentially constant. Moreover, incorporation of [^3H]uridine into the total uterine RNA was increased on days 3-4 of pregnancy, declined (though not significantly) on day 5 and again increased steadily from day 6 onwards (Miller *et al.* 1968).

However, measurements of change in total

uterine content do not permit any analysis of the more detailed events that are occurring. Thus from midnight on day 5 of pregnancy the sites of implantation are clearly visible and different from those of the surrounding uterine tissue (Psychoyos, 1963). Further, such sites possess increased permeability to large molecules, e.g. iodinated albumin (Psychoyos, 1963), and an increased vascular permeability, which enables a ready detection of the site to be made by means of the differential uptake of dyes such as Pontamine Blue or Niagara Blue 2B (Psychoyos, 1963; O'Grady & Heald, 1969). Such differences could lead to equal differences in the ease with which [^3H]uridine passes into the uterine cells, and consequently by affecting the probable size and specific radioactivity of the [^3H]uridine pool presumably could alter the apparent uptake into RNA.

Since the early stages of pregnancy involve rapid growth and development, initially in selected areas of the uterus, the present study has examined the uptake of [^3H]uridine into RNA and DNA in the uteri of rats, and comparison has been made between implantation and non-implantation areas during the first 9 days of pregnancy. In addition attempts have been made to evaluate both the size

of the intracellular [^3H]uridine pools and the contribution made to them by potential changes in vascularity.

MATERIALS AND METHODS

Rats. The rats were a Holtzman-derived Sprague-Dawley strain with a 5-day oestrous cycle. They were maintained in windowless rooms with a lighting pattern from 6.0 a.m. to 8.0 p.m. and a mean temperature of 22°C. Adult virgin females were mated with normal males and the morning that sperm was found in the vaginal smear was designated as day 1 of pregnancy.

Uptake of [^3H]uridine. All injections were performed between 9.30 a.m. and 11.30 a.m. on any one day. Animals were anaesthetized with ether, and 100 μCi of [^3H]uridine (specific radioactivity 26 Ci/mmol) in 0.1 ml of 0.9% NaCl was injected via the femoral vein, followed immediately by 1.0–1.5 ml of a suspension (in 0.9% NaCl) of rat erythrocytes labelled with [^{51}Cr]chromate (see below). Then 50 min later the animals were again lightly anaesthetized and 2.0 ml of a solution of Niagara Blue 2B (2%, w/v, in 0.9% NaCl) was injected, usually via a tail vein. After 10 min the animals were again anaesthetized with ether and a blood sample (2–4 ml) was removed from each by heart puncture. The animals were immediately killed, without recovering consciousness. All animals received Niagara Blue 2B to ensure uniformity of treatment, though it was recognized that implantation sites were not visible until late in the evening of day 5.

The uteri were rapidly removed, placed on a filter pad moistened with 0.9% NaCl and trimmed free from fat and connective tissue. Up to day 5 the uteri were cut into small segments with scissors, and representative segments from each horn were taken for the determination of dry weight and of DNA and RNA as described by Heald, O'Grady & Howie, 1970. Samples (equivalent to 15 mg wet wt. of tissue) for the determination of DNA and RNA were precipitated with HClO_4 during this procedure, and the radioactivity due to [^3H]uridine remaining in the supernatant (acid-soluble fraction) was determined.

Samples of the solutions and residues containing the separated nucleic acids (from 15 mg of tissue) (Heald, O'Grady & Howie, 1970) were dried *in vacuo* and dissolved by overnight (18 h) incubation at 37°C in 0.4 ml of Hyamine hydroxide (hydroxide of Hyamine 10X; Packard Instrument Co. Inc., La Grange, Ill., U.S.A.). The solutions containing DNA, RNA and acid-soluble radioactivity were washed into counting vials with 14.6 ml of butyl-PBD [5-(4-biphenyl)-2-(4-*tert*-butylphenyl)-1-oxa-3,4-diazole (British Drug Houses Ltd., Poole, Dorset, U.K.)] scintillator solution.

Additional samples of uterus (approx. 50 mg) were taken for the determination of radioactivity due to ^{51}Cr , to assess the degree of contamination by blood.

Determination of blood in uterine extracts. Blood was determined by using erythrocytes labelled with [^{51}Cr]chromate. On the morning of any experiment a rat was lightly anaesthetized with ether, and blood (2–3 ml), obtained by heart puncture, was mixed with 5 ml of a citrate–dextrose solution [0.3% D-glucose and 1.6% (w/v) trisodium citrate]. After centrifuging, the erythrocytes were washed once with the citrate–dextrose solution and were then suspended in 2–3 ml of 0.9% NaCl containing

50–60 μCi of $\text{Na}_2^{51}\text{CrO}_4$ (specific radioactivity 150 mCi/mg). After standing for 20 min at room temperature, the cells were centrifuged and washed four or five times with fresh 0.9% NaCl (5 ml) to remove excess of radioactive chromate. Completeness of removal was checked by measuring the radioactivity of the final washings. The cells were suspended in 0.9% NaCl and a known volume (usually 1.0–1.5 ml) was injected into the experimental animals immediately after the injection of [^3H]uridine. Then 1 h later a known volume of blood was taken with a syringe, moistened with aqueous heparin, by cardiac puncture under anaesthesia and immediately before death of the animal. The erythrocytes were centrifuged from the plasma and the ^{51}Cr in the erythrocytes and plasma together with ^3H in the plasma sample were determined.

Measurement of radioactivity. ^{51}Cr in homogenates of uteri and in the erythrocyte suspension was determined by counting in a well-type scintillation counter (Ecko Electronics Ltd. type N550A scintillation counter). ^3H was determined by liquid-scintillation counting in a Packard series 4000 liquid-scintillation spectrometer. ^3H and ^{51}Cr radioactivities in the same samples were calculated by the channels-ratio method, and quench corrections were made by use of the automatic external standard on the instrument.

Not less than 10000 counts above background were collected for any one determination, and each sample was counted three times, the results being averaged.

Statistical methods. Statistical procedures for comparison between groups and between pairs were those described by Snedecor & Cochran (1967).

RESULTS

Effect of Niagara Blue 2B on the uptake of [^3H]uridine into RNA. Preliminary experiments were conducted to determine whether the injection of Niagara Blue 2B was likely to produce a major alteration in the uptake of uridine into uterine RNA. Three groups of rats were compared, one receiving uridine only, one receiving 0.9% sodium chloride and one receiving the dye in 0.9% sodium chloride. The results (Table 1) showed that the injection of saline or dye had no significant effect on the uptake of uridine into RNA.

Changes in uterine dry weight and DNA and RNA contents. Changes in uterine dry weight during the first 9 days of pregnancy are shown in Fig. 1. Up to the early evening of day 5 implantation does not occur, although the difference between implantation sites and non-implantation sites is clearly visible on day 6. There was little or no change in the dry-matter content up to day 5 in the whole uterus, and up to day 9 in the non-implantation sites. On the other hand, the dry-matter content of the implantation sites is apparently lower on day 5 (though not significantly; $P=0.4$) and was significantly lower ($P<0.02$) on days 7–9 than that of the non-implantation sites. Also, during days 6–9 the dry-matter content remained constant in the implantation sites, although the sites themselves grow rapidly during

Table 1. Uptake of [³H]uridine into uterine RNA of pregnant rats in the presence and absence of Niagara Blue 2B

Rats were mated and treated on day 7 of pregnancy as described in the Materials and Methods section. Each group consisted of three rats. The control group received uridine only, the saline-treated group received 2.0 ml of 0.9% NaCl and the dye-treated group received 2.0 ml of 2.0% (w/v) Niagara Blue 2B in 0.9% NaCl, in each case 10 min before the animals were killed. Values are given $\pm$ s.d.

Experimental group	Implantation sites			Non-implantation sites		
	RNA (μ g/mg of tissue)	Radioactivity		RNA (μ g/mg of tissue)	Radioactivity	
		(c.p.m./ μ g of RNA)	(corrected c.p.m./ μ g of RNA*)		(c.p.m./ μ g of RNA)	(corrected c.p.m./ μ g of RNA*)
Control	6.02 $\pm$ 2.14	7.7 $\pm$ 1.3	24.7 $\pm$ 10.1	4.05 $\pm$ 1.24	5.1 $\pm$ 1.7	15.4 $\pm$ 7.3
Saline-injected	6.02 $\pm$ 1.0	7.9 $\pm$ 1.7	27.4 $\pm$ 1.7	3.73 $\pm$ 0.70	5.4 $\pm$ 0.85	15.6 $\pm$ 12.9
Dye-injected	5.36 $\pm$ 1.18	8.0 $\pm$ 2.0	24.0 $\pm$ 8.3	4.37 $\pm$ 2.16	3.2 $\pm$ 2.0	13.7 $\pm$ 9.7

* Corrected to a standard count of 1000 c.p.m. in the acid-soluble fraction from 1.0 mg of tissue.

Fig. 1. Change in dry-matter content of uterine tissue during early pregnancy in the rat. From day 6 onwards the implantation areas were dissected and weighed separately. ●, Whole uterus to day 5 and non-implantation areas thereafter; ○, implantation sites.

Fig. 2. Change in DNA/unit of dry matter in pregnant-rat uterus. ●, Whole uterus to day 5 and non-implantation areas thereafter; ○, implantation areas.

this period. The absence of a significant difference between the implantation sites and non-implantation sites on day 6 is attributed to the very considerable variation occurring in samples taken on this day of pregnancy.

Changes in DNA content are shown in Fig. 2. Here the content/unit dry wt. showed no significant change until day 8, when a difference was apparent between the implantation sites and non-implantation sites. Thus at this stage the total DNA concentration in the implantation sites was significantly above that of the non-implantation sites ($0.05 > P > 0.02$).

Changes in RNA content/unit of dry matter are shown in Fig. 3. The total RNA content did not

change significantly between days 1 and 5. On day 6 no differences were measurable between implantation sites and non-implantation sites ($P=0.2$), but on days 7–9 highly significant differences ($P<0.01$) were found between the RNA contents of the two sites. Thus the RNA content of the implantation site rose whereas that of the non-implantation site decreased.

When calculated in terms of RNA/unit of DNA (Fig. 4) it became apparent that changes in the RNA content/cell were already measurable on day 6 of pregnancy. Thus on day 6 and subsequently, the concentration of RNA/unit of DNA in the implantation sites rose markedly and was significantly increased ($P<0.001$) over that of the non-implantation sites, in which the RNA/DNA ratio decreased on days 8 and 9.

In terms of total content, it was clear that major and significant changes took place in the RNA/DNA ratios near the earliest time detectable in the commencement of implantation.

Fig. 3. Change in RNA/unit of dry matter in pregnant-rat uterus. ●, Whole uterus to day 5 and non-implantation areas thereafter; ○, implantation areas.

Fig. 4. Changes in RNA/unit of DNA in pregnant-rat uterus. ●, Whole uterus to day 5 and non-implantation areas thereafter; ○, implantation areas.

[³H]Uridine content of the acid-soluble fractions of the tissues. The acid-soluble fraction of the tissues contained appreciable amounts of tritiated material presumed to be free uridine. Although there was no detectable variation in the content between days 1 and 5, highly significant differences in [³H]uridine content were found when implantation sites were compared with non-implantation sites in the same uterus. Table 2 shows the combined mean values for radioactivity.

When the differences between individual pairs (i.e. non-implantation sites versus implantation sites in the same uterus) were analysed statistically (Snedecor & Cochran, 1967) the radioactivity of the acid-soluble fraction was significantly higher ($P < 0.01$) in the implantation sites than in the non-implantation sites. In terms of percentage, the implantation sites accumulated 20–40% more radioactive uridine in the acid-soluble fraction than did the non-implantation areas. These differences are not considered to be due to an increase in blood volume in the implantation sites. Indeed radioactivity due to ⁵¹Cr in the tissue homogenates was uniformly difficult to detect. Thus in only one instance was it possible to detect radioactivity due to ⁵¹Cr rising significantly above the background count of the well-type scintillation counter when using up to 50 mg of tissue. Calculated on the basis of the counts obtained, the blood volume in this instance was 2% (v/v) of the tissue volume, and contributed [³H]uridine to the extent of 9.0% of the total tissue radioactivity. Thus even a doubling of blood volume would not have increased any contaminating uridine radioactivity by more than 9%, whereas the increased radioactivity in the acid-soluble fraction was in the range 20–40%.

Uptake of [³H]uridine into RNA and DNA. The incorporation of uridine into RNA of the uterine

Table 2. Radioactivity in the acid-soluble fraction of uterine tissues at different stages of pregnancy after a dose of [³H]uridine

Pregnant rats were injected with 100 µCi of [³H]uridine and the tissues treated as described in the Materials and Methods section. Values are given as d.p.m./10 mg wet wt. of tissue $\pm$ s.d.

Day	No. of rats	(Radioactivity (d.p.m./10 mg of tissue))		
		Whole tissue	Implantation sites	Non-implantation sites
1	3	2323 $\pm$ 222	—	—
2	3	1987 $\pm$ 263	—	—
3	3	2029 $\pm$ 600	—	—
4	3	2312 $\pm$ 179	—	—
5	4	2545 $\pm$ 192	—	—
6	6	—	2597 $\pm$ 547	2150 $\pm$ 218
7	3	—	3410 $\pm$ 130	2387 $\pm$ 79
8	3	—	2826 $\pm$ 150	1860 $\pm$ 340
9	3	—	2412 $\pm$ 211	1684 $\pm$ 60

samples is shown in Fig. 5. In this and in subsequent figures the results are calculated on the basis of a standard count in the acid-soluble fraction in order to eliminate error arising through the implantation sites possessing a higher intracellular ³H]uridine radioactivity than the non-implantation site.

There are two major features. First, although the incorporation of ³H]uridine into RNA on day 2 was not significantly different from incorporation on day 1 ($P>0.05$), there was a highly significant increase between days 2 and 3 ($P<0.01$) and an equally significant decrease between days 3 and 4 ($P<0.01$). Thereafter the incorporation into RNA increased significantly between days 4 and 5 ($P<0.01$) (Table 3).

On day 6 the uptake into RNA in the implantation sites was very significantly greater ($P<0.001$) than into RNA of the non-implantation areas. This

was true also on day 7, yet by days 8 and 9 no differences were detectable between the uptake into RNA in the two types of tissue ($P=0.20$).

In contrast with RNA, there was no enhanced uptake into DNA on day 3 of pregnancy (Fig. 6), or indeed until day 6, when there was a marked increase ($P<0.001$) in the uptake of radioactivity into DNA in the implantation sites. Thereafter, although the total uptake in the implantation sites was lower than on day 6, the differences between the implantation sites and non-implantation sites were always significant ($P<0.02$). It is recognized that ³H]uridine is not incorporated directly into DNA and the radioactivity has been presumed to be that of cytidine formed from uridine in the uterus or elsewhere in the rat tissues.

Fig. 5. Uptake of ³H]uridine into RNA in pregnant-rat uterus. ●, Whole uterus to day 5 and non-implantation areas thereafter; ○, implantation areas.

Fig. 6. Uptake of radioactivity into DNA of pregnant rat uterus after the injection of ³H]uridine. ●, Whole uterus to day 5 and non-implantation areas thereafter; ○, implantation areas.

Table 3. Changes in the incorporation of ³H]uridine into uterine RNA during the first 5 days of pregnancy in the rat

Rats were mated, and uterine tissue was removed and analysed as described in the Materials and Methods section. Values are c.p.m./μg of RNA corrected to a standard tissue count of 1000 c.p.m. Results are quoted ± s.d. Statistical procedures followed those described by Snedecor & Cochran (1967).

Days after mating	No. of rats in group	Radioactivity of RNA (corrected c.p.m./μg)	Significance of differences between groups
1	3	3.0 ± 1.5	P > 0.05
2	5	6.5 ± 2.2	
3	5	21.5 ± 7.9	
4	5	8.8 ± 1.8	P < 0.01
5	4	19.3 ± 5.7	P < 0.01

DISCUSSION

Although the differences in dry-matter content (or water content) between implantation sites and non-implantation sites do not appear to have been measured hitherto, such differences were to be expected from the studies of Psychoyos (1963), who showed that the permeability of the implantation sites to dyes and to ^{131}I -labelled albumin was greatly increased over that of the surrounding tissue and of the normal uterus. The present results show that this change is accompanied by an increase in water content of 2–3%. Since no detectable change in blood volume was found, it is concluded that the change in water content is due to an increase in intracellular water rather than to a substantial change in vascularity.

Accompanying the change in water content was an increased uptake of ^3H uridine, the quantities found in the acid-soluble extracts of the tissues being 20–40% greater in the implantation sites than in the non-implantation areas. This finding re-emphasizes that, in order to compare uptake into RNA in uterine tissue at different stages of pregnancy, the total intracellular radioactivity must be taken into account.

Major findings relate to changes in RNA and DNA. The increase in incorporation of uridine into uterine RNA on day 3 of pregnancy is not understood at present. Previous work (Miller, Owen & Emmens, 1967, 1968) has shown that the uptake of uridine into the total uterine RNA of the pregnant rat and mouse increased from day 2 to day 4, although no significant change occurred between days 3 and 5. However, Miller *et al.* (1967, 1968) did not correct their values for the radioactivity in the tissue uridine pool, nor did they calculate their values in terms of DNA or dry matter. In the present work such corrections were applied, and it is considered that the results show a definite increase in nucleic acid synthesis on day 3 of pregnancy. Even so there was no change in dry weight nor in the RNA/unit of dry matter or RNA/unit of DNA ratios. Further, there was no change in DNA/unit of weight, nor in the uptake of radioactivity into DNA.

The significance of the increase in RNA synthesis and its cause require further evaluation. In the rat the fertilized ova are expelled from the oviduct into the uterus by noon on day 3 and it has been suggested (Miller *et al.* 1968) that there is a release of oestrogen from the ovaries during the period days 2–3 of pregnancy. At this time Christie (1966), using histological methods, showed that of a wide variety of phosphatases only adenosine triphosphatase activity was increased by day 3. Yochim & Mitchell (1968) showed that the intrauterine oxygen partial pressure in the rat increased on day 3 and decreased

slightly but not significantly on day 4. Malinowska *et al.* (1969) observed a decrease in the activity of malate dehydrogenase. These changes might be associated with oestrogen production, but it is notable that the response of the uterus to oestrogen includes increases in water content (Astwood, 1938) and in the total RNA/unit of dry matter (cf. Mueller, 1965), neither of which were detected in the present study. Apart from the suggestion by Miller *et al.* (1968), there is in fact no evidence for a release of oestrogen from the ovaries at this time.

In the rat implantation commences on day 5 of pregnancy and the blastocyst makes first contact with the uterine epithelium about noon of that day (Krehbiel, 1937). Results in Fig. 5 show that uridine uptake into RNA also significantly increased in the morning of day 5. Taken together, it is postulated that the increased synthesis of RNA on day 3 is the first phase in the development of the uterine epithelium to a point where it is capable of interacting with the blastocyst, and that the subsequent increase on day 5 is associated with the beginning of the changes in the differentiation of the epithelial cells that occurs during implantation.

The changes occurring in both RNA and DNA at day 6, when implantation sites are visible, are more readily interpreted. It is well established (cf. DeFeo, 1967) that implantation is accompanied by an extensive growth of epithelial tissue in the first instance, and it appears likely that the increase in both amount and rate of metabolism of RNA and DNA reflects this growth. However, it should be noted that with RNA this process, once commenced, does not increase in rate, the RNA content remaining steady while the uridine uptake decreases even though the implantation site is growing rapidly. Such an event suggests that the initial change could be rapid elaboration of a new RNA species necessary for the earliest stages of pregnancy to commence and that, once this has been elaborated, no additional burst of activity is required. Such an explanation would also accord with the finding that the uptake of radioactivity into DNA was markedly and significantly increased at day 6 without any change in the total quantity of DNA/unit of dry matter. Whether the increased RNA system is directly associated with the rapidly growing epithelium or whether it results from another as yet undefined stimulus occurring at the time of implantation is not known.

The results described here clearly require extension in many directions; for example the analyses relate to whole uterine tissue, and no attempts have been made to separate endometrial and myometrial tissue at this time.

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