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Proteins synthesized by the rat endometrium during early pregnancy*

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Summary. Rat uteri from animals at pro-oestrus and Days 3–6 of pregnancy were incubated *in vitro* in the presence of [^{35}S]methionine. Labelled endometrial proteins from each sample were separated by two-dimensional gel electrophoresis and resolved by fluorography. A group of 16 proteins that are synthesized only on specific days was defined. On Day 5, the day of embryo implantation, only 3 of these 16 proteins were synthesized. Of 8 proteins synthesized on both Days 3 and 6, 5 were also found on Day 4, but only 1 was synthesized on Day 5. These results demonstrate that during the interval in which implantation is initiated, no unique proteins are produced but several protein species are no longer synthesized by the endometrium.

Introduction

In the rat, embryo implantation is initiated only during a few hours on the 5th day of pregnancy (Psychoyos, 1973). Ovarian secretion of oestradiol between the 3rd and 4th days of pregnancy is necessary for implantation to occur (Psychoyos, 1973), but the mechanisms through which oestradiol mediates this process are unknown. Several lines of evidence imply a role for RNA and protein synthesis in the initiation of implantation. The classic mode of action of oestradiol is to effect gene transcription and subsequent protein synthesis (O'Malley & Means, 1974; Clark & Peck, 1979), and these responses to oestradiol have been repeatedly demonstrated in the rat uterus (reviewed by Segal, Scher & Koide, 1977). RNA and protein synthesis increase in the endometrium just before and during blastocyst attachment on the 5th day of pregnancy (O'Grady & Bell, 1977). In addition, RNA extracted from uteri of ovariectomized, oestradiol-treated rats can stimulate uterine oestrogenic responses (Fencl & Villee, 1971), and can induce implantation of blastocysts in rats during delayed implantation when placed in the uterine lumen or in the parametrial uterine fat (Segal *et al.*, 1977; Lejeune, Puissant, Camus & Leroy, 1982b). Administration of actinomycin D to ovariectomized, pregnant rats maintained in delayed implantation by progesterone treatment has also been found to precipitate blastocyst implantation (Leroy, Schetgen & Camus, 1980). It has been suggested that implantation is prevented by inhibitory proteins whose synthesis depends on the increased levels of progesterone secreted after mating (or administered to maintain delay of implantation). Both actinomycin D and oestradiol (administered or naturally secreted) might act by inhibiting the progesterone-dependent transcription of endometrial RNA which is responsible for preventing implantation (Leroy *et al.*, 1980). Biochemical evidence that oestradiol can restrict as well as induce gene expression in the rat uterus has been provided by measurements of available RNA initiation sites on uterine chromatin from hormone-treated animals. Progesterone administration to ovariectomized rats resulted in a 5-fold stimulation in the number of RNA chains

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initiated, but subsequent administration of oestradiol reduced this number to below control levels within 4 h (Glasser & McCormack, 1979). These experiments demonstrate that oestradiol can counteract the effect of progesterone on transcription.

The experiments described here examine endometrial gene expression at the translational level. Proteins synthesized by rat endometria during pro-oestrus and Days 3–6 of pregnancy were compared following two-dimensional gel electrophoresis.

Materials and Methods

Animals. Virgin Sprague–Dawley rats (225–300 g, Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) were maintained at 23°C in a photoperiod of 14 h light (05:00–19:00 h) and 10 h dark with free access to food and water. Vaginal smears were obtained each morning and only animals demonstrating 3 consecutive 4-day oestrous cycles were used. Each female was placed with one male on the afternoon of pro-oestrus for mating. Animals were assumed to be pregnant if spermatozoa were present in the vaginal smear the morning after mating which was designated Day 1 of pregnancy. On Day 6 of pregnancy animals were injected intravenously with Pontamine blue 20 min before killing (Psychoyos, 1960). Rats were killed by decapitation, and uteri were removed immediately, placed on ice, trimmed of adhering fat and weighed.

Radiolabelling and preparation of soluble endometrial proteins. Uterine horns were separated, slit longitudinally, and incubated for 4 h (08:00–12:00 h) at 37°C in an atmosphere of 95% O_2, 5% CO_2 in 35-mm diameter sterile plastic culture dishes. Each dish contained two uterine horns and 3 ml incubation medium (Krebs–Ringer–bicarbonate buffer, pH 7·4 (Krebs, 1950); 8 mM-glucose, 0·1% bovine serum albumin) and 30 μCi [^{35}S]methionine/ml [sp. act. > 1000 Ci/mmol: New England Nuclear (Boston, MA, USA]). Incorporation of [^{35}S]methionine increased linearly over this period. After incubation, uterine horns were rinsed briefly in incubation medium devoid of ^{35}S-labelled methionine, blotted on filter paper, and the endometria removed by scraping with glass slides. Endometria from 2 or more horns were homogenized together in 1·5 ml microfuge tubes containing 50–100 μl isoelectric focussing lysis buffer (O'Farrell, 1975). Ampholines (1·6% pH 5–7, 0·4% pH 3·5–10) were purchased from LKB (Gathersburg, MD, U.S.A.). Homogenates were centrifuged for 1 h at 15 600 ***g***, 4°C to separate soluble proteins. The supernatant volume was determined, aliquants taken to measure radioactivity, and the supernatant frozen in liquid nitrogen and stored at −80°C. To measure acid-insoluble radioactivity, 3 μl and 6 μl volumes were taken from each sample, added to 1 ml of a solution of 2 mg unlabelled methionine/ml, and proteins were precipitated on ice by the addition of 1 ml 25% (w/v) trichloroacetic acid (TCA). Precipitates were collected on glass-fibre filters (GF/A, Millipore, Lexington, MA, U.S.A.), washed with 10% cold TCA, and air-dried in scintillation vials. Scintillation fluid (10 ml Econofluor + 3% Protosol: NEN) was added and samples were kept in the dark overnight before counting. Samples were counted in a Packard Tricarb 460C scintillation counter with 95% efficiency for ^{35}S.

Gel electrophoresis of protein samples. Two-dimensional gel electrophoresis was performed according to the methods described by O'Farrell (1975). Urea crystals were added to all samples to achieve a concentration of 9·5 M and SDS was added to a final concentration of 0·1% to reduce streaking of samples caused by nucleic acids (Van Blerkom, 1978). Isoelectric focussing of samples was conducted on pre-focussed tube gels (3 mm diam., 10–12 cm long). Endometrial protein samples containing 3×10^5 counts were loaded on each gel. One gel in each group focussed contained IEF markers of known isoelectric points (pI) (Pharmacia Chemicals, Piscataway, NJ, U.S.A.). These gels were stained with Coomassie blue and used to calculate the pH gradients for the focussed gels. Focussed gels of protein samples were equilibrated with SDS gel electrophoresis buffer (O'Farrell, 1975) and stored at −80°C. Separation of focussed proteins in the second dimension by SDS gel electrophoresis was conducted on 15-cm long, 1·5-mm thick slab gels

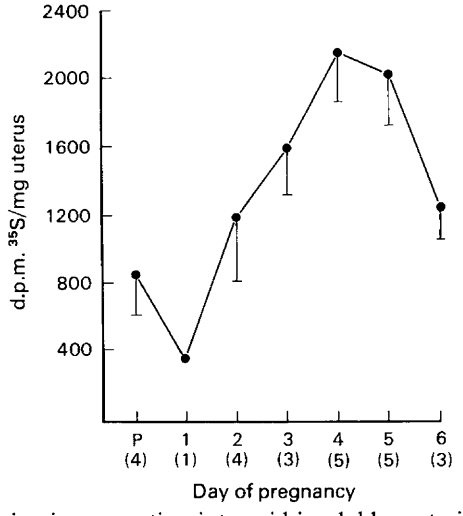
composed of a 7·5–17% acrylamide gradient. ^{14}C-Labelled molecular weight markers (Bethesda Research Laboratories, Bethesda, MD, U.S.A.) were run in a well adjacent to the isoelectric focussing gel. Following electrophoresis in the second dimension, gels were permeated with autoradiography enhancer (EnHance: NEN), dried, and exposed to pre-sensitized, Kodak XAR film for 15 days at −80°C for fluorography (Laskey & Mills, 1975).

Analysis of two-dimensional gels. Three or four gels from each day were compared and only protein spots found on at least 3 duplicate gels were considered to have been synthesized during the 4-h incubation on a given day. No attempt was made to quantitate separated proteins. Approximate molecular weights were determined from a regression of log molecular weight against distance migrated of ^{14}C-labelled standard proteins and were consistent amongst gels. pH gradients were determined by plotting known pI values of the marker proteins against distance migrated during isoelectric focussing. Approximate pI values for proteins were determined from linear regressions of these graphs. Calculated pI values varied from gel to gel and pH focussing ranges have been given for specific proteins in lieu of mean pI values.

Results

Incorporation of [^{35}S]methionine into endometrial protein

The amount of label incorporated into endometrial proteins of uteri from rats at pro-oestrus and Days 3–6 of pregnancy is shown in Text-fig. 1. The amount of labelling increased steadily from pro-oestrus to Day 4 and was greatest in uteri from Days 4 and 5 of pregnancy. Labelling in Day-6 tissue was less than in Day-5 endometria.



Text-fig. 1. [^{35}S]Methionine incorporation into acid-insoluble material in the endometria of uteri incubated for 4 h *in vitro*. Animals were in pro-oestrus (P) or 1–6 days pregnant. Each sample (*n*) contained tissue from 2 animals. Values are mean ± s.e.m. for the no. of samples indicated in parentheses.

Electrophoretic patterns of labelled endometrial proteins

Representative fluorograms of endometrial protein gels from uteri during pro-oestrus or Days 3–6 pregnancy are shown in Plates 1–3. The majority of the protein spots for each day focussed between pH 4 and pH 7 and had molecular weights $< 70\,000$. Most of these proteins were synthesized on every day examined and may represent cell 'maintenance' proteins. However,

Table 1. Mean molecular weights, pH ranges, and days of synthesis for specific rat endometrial proteins

Protein spot	Mean mol. wt	pH range	Days synthesized				
			Pro-oestrus	3	4	5	6
1	35 700	5·95–6·95	+	+	+	+	+
2	34 900	6·15–6·35	+	+	+	+	+
3	31 600	7·00–7·55	+	+	+	−	+
4	46 500	5·30–5·90	−	+	+	+	+
5	32 200	4·85–5·80	+	+	+	−	−
6	31 200	4·70–5·70	+	+	+	−	−
7	35 100	6·70–7·20	+	+	−	−	+
8	49 200	4·00–5·95	+	+	−	−	+
9	50 200	6·05–6·40	+	−	−	+	+
10	26 500	5·55–5·95	−	+	+	−	+
11	31 900	6·65–6·80	−	+	+	−	+
12	60 100	4·85–5·95	−	+	+	−	+
13	45 000	4·45–5·85	−	−	+	+	+
14	85 000	5·30–5·90	−	+	−	−	+
15	44 000	5·75	−	+	−	−	−
16	44 000	5·30	−	+	−	−	−
17	34 800	5·70	−	+	−	−	−
18	27 000	4·80	−	+	−	−	−

several proteins were synthesized only on specific days (Plates 1–3). In Table 1 the mean molecular weight, pH focussing range, and days on which each protein was synthesized are listed.

The principal difference amongst protein patterns from different days was the absence of several protein spots on Day 5 (Spots 3, 5–8, 10–12), or Days 4 and 5 (Spots 7, 8, 14: Pl. 2, Fig. 3; Pl. 3, Fig. 4). All of these spots except Nos 5 and 6 reappeared on Day 6 (Pl. 3, Fig. 5). However, Spot 13 was synthesized only on Days 4, 5 and 6, and Spot 9 appeared only on Days 5, 6 and pro-oestrus. Four proteins were synthesized only by Day-3 endometria (Spots 15–18; Pl. 2, Fig. 2), but proteins unique to other days were not observed. Gels from Days 3 and 6 appeared to have the most proteins in common. Spots 1 and 2 were synthesized by all stages, although in samples from Days 4 and 5 (Pl. 2, Fig. 3; Pl. 3, Fig. 4), Spot 2 appeared to be shifted to the left in relation to neighbouring spots when compared to samples from pro-oestrus and Day 3 (Pl. 1, Fig. 1; Pl. 2, Fig. 2). The intensity of these two spots was also decreased in Day-5 samples (Pl. 3, Fig. 4), both in relation to other Day-5 spots and to Spots 1 and 2 on gels from other days. These changes were consistent amongst all gels examined for each day.

Discussion

The pattern of incorporation of [^{35}S]methionine into endometrial protein during Days 1–6 of pregnancy in uteri incubated for 4 h *in vitro* (Text-fig. 1) is similar to that reported by Reid & Heald (1970) for the incorporation of [^{3}H]leucine in whole uteri *in vivo* measured 30 min after intravenous injection of the labelled precursor. With both methods an increase in protein labelling occurred between Days 2 and 5 of pregnancy and a decline was measured on Day 6. In uteri labelled *in vivo* the decrease in labelling on Day 6 was less in implantation site tissue than in uterine segments between implantation sites (Reid & Heald, 1970). In uteri labelled *in vitro* total endometrium was used on Day 6 and an overall decline in labelling was observed when compared to endometria from Days 4 and 5. Since the incorporation of labelled amino acids into protein on different days of pregnancy is similar in uteri exposed to labelled amino acids *in vivo* and *in vitro*, in-vitro incubation of uteri appears to be a valid method with which to label endometrial proteins being synthesized on particular days of pregnancy. In addition, in-vitro incubation of uteri precludes the inclusion of

PLATE 1

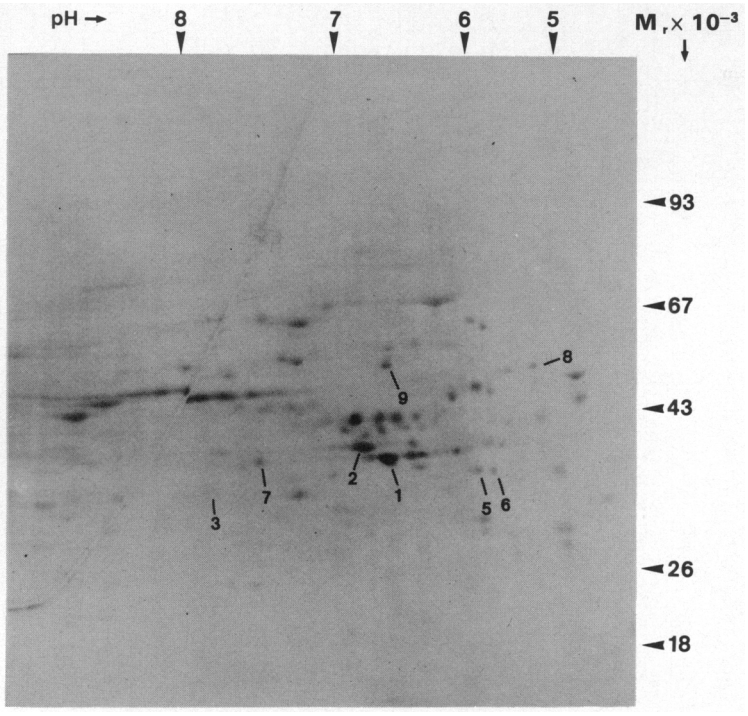


Fig. 1. Two dimensional gel fluorograms of radiolabelled endometrial proteins synthesized by uteri incubated *in vitro*, from pro-oestrous rats.

PLATE 2

Two dimensional gel fluorograms of radiolabelled endometrial proteins synthesized by rat uteri incubated *in vitro*.

Fig. 2. Proteins synthesized by uteri on Day 3 of pregnancy. Spot 9 is no longer present, but many new spots have appeared. Spots 15–18 are found only on Day 3.

Fig. 3. Proteins synthesized by uteri on Day 4 of pregnancy. Spot 13 appears for the first time, but Spots 7, 8 and 14 are no longer present.

PLATE 3

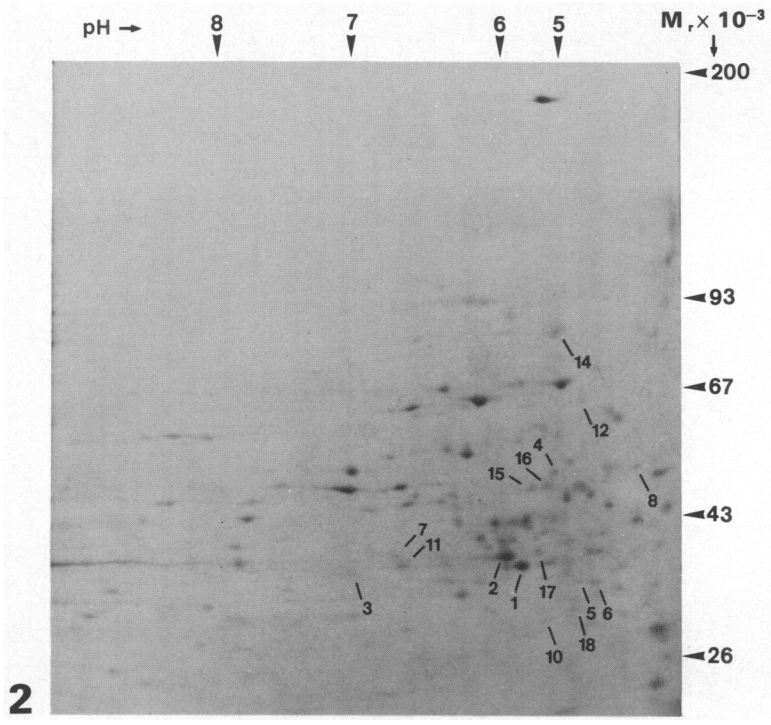
Two dimensional gel fluorograms of radiolabelled endometrial proteins synthesized by rat uteri incubated *in vitro*.

Fig. 4. Proteins synthesized by uteri incubated on Day 5 of pregnancy. Spot 9 reappears and Spot 13 is still present, but Spots 3, 5, 6, 7, 8, 10, 11, 12 and 14 are not present.

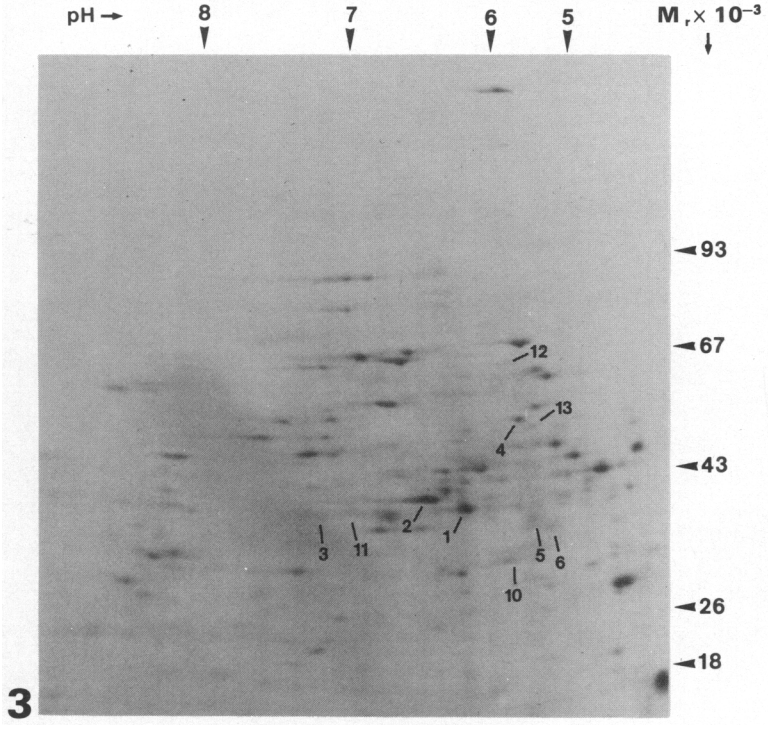
Fig. 5. Proteins synthesized by uteri incubated on Day 6 of pregnancy. Spots 3, 7, 8, 10, 11, 12 and 14 reappear and Spots 9 and 13 are still present.

(*Facing p.* 398)

PLATE 2

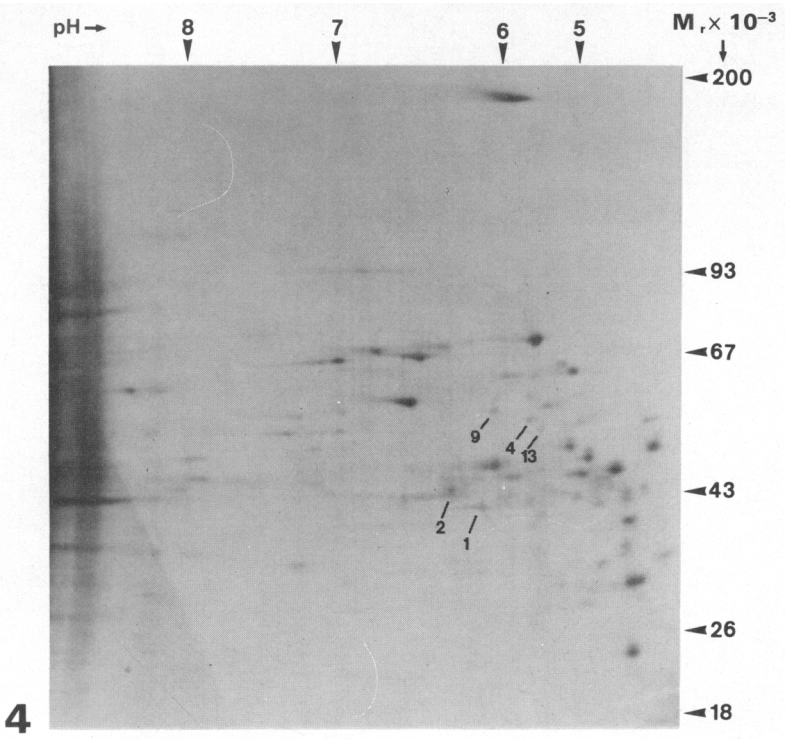


2

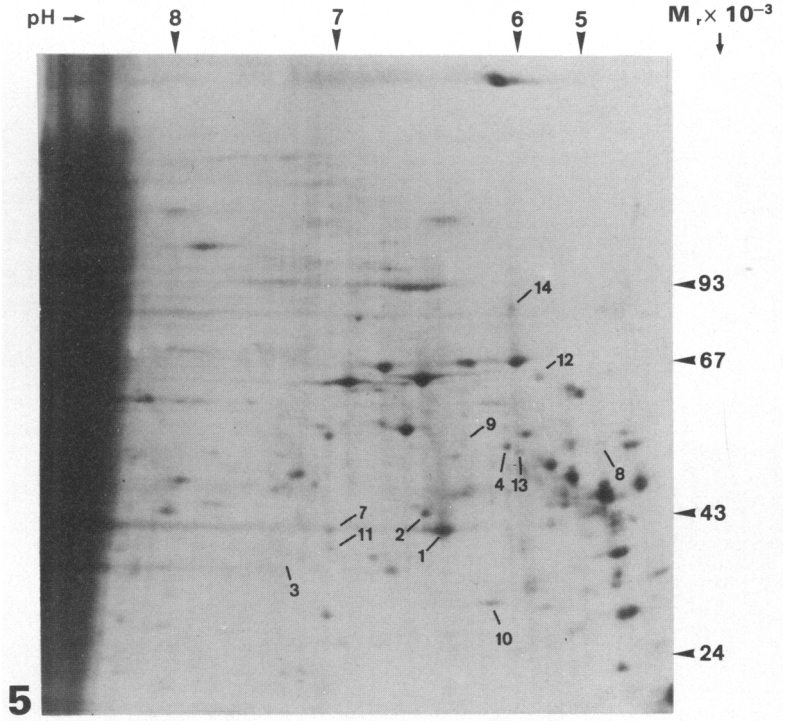


3

PLATE 3



4



5

labelled serum proteins in the endometrial protein samples as noted by Lejeune, Lecocq, Lamy & Leroy (1982a), and also exposes all uteri to an identical, known concentration of labelled amino acids.

The synthesis of new protein species on Day 3 of pregnancy may result from the increase in circulating progesterone concentration which begins on Day 2 and continues through Day 5 (Watson, Anderson, Alam, O'Grady & Heald, 1975). Blastocysts transferred to Day-3 uteri fail to implant before Day 5 (Psychoyos, 1973), and some of these proteins may be involved in preventing premature implantation.

The absence of unique proteins and the loss of protein species on Days 4 and 5 is surprising as overall RNA and protein synthesis are elevated on these days (Text-fig. 1; review by O'Grady & Bell, 1977). The secretion of oestradiol between Days 3 and 4 which is responsible for implantation may block the synthesis of specific proteins on Days 4 and 5. The proteins not synthesized on these days may have been involved in preventing the attachment or adhesion of embryos to the endometrial epithelium. There appear to be no special proteins synthesized on the morning of implantation which might be involved in mediating these processes, but the apparent change in pI of Spot 2 implies that post-translational modification (e.g. phosphorylation) of this protein occurs on Days 4 and 5. Variations in turnover rates for specific proteins can also affect electrophoretic patterns, but as turnover rates for the proteins described in this study have not been determined, this effect cannot yet be assessed.

Synthesis of the induced protein (IP) in the pregnant rat endometrium is also low on Day 5, but is elevated on Days 4 and 6 (Bell, Hamer & Heald, 1980). Bell *et al.* (1980) have presented evidence that synthesis of IP is more closely correlated with uterine stromal cell proliferation, which is diminished on Day 5, than with circulating oestradiol or nuclear oestradiol receptor concentrations. Although this correlation could not explain the absence of particular proteins on Day 4 of pregnancy, further investigation of the proteins not synthesized on Day 5 (Table 1) might reveal a similar relationship between the synthesis of certain of these proteins and uterine cell proliferation. The cellular origin of these proteins also remains to be disclosed.

Since the concentration of protein in uterine luminal flushings is elevated on Day 5 (Surani, 1977), it is possible that implantation-specific proteins are synthesized on Day 5, but are secreted into the lumen (or culture medium) and were not present in the protein sample. In the experiments described here, the number of acid-insoluble counts in the medium after 4 h of uterine incubation on Day 5 was very low and insufficient for the resolution of proteins by two-dimensional gel electrophoresis. Two-dimensional gels of proteins found in the medium after a longer incubation time could disclose whether the proteins not found in Day-4 and -5 endometria are secreted.

The similarity of protein patterns in endometria from Days 3 and 6 of pregnancy is in part due to the reappearance of some of the proteins not synthesized on Days 4 or 5. These proteins may also be involved in defining the implantation period. Although stromal cell decidualization should be initiated in Day-6 implantation sites, no new proteins appeared in gels from Day-6 samples. Lejeune *et al.* (1982a) have described 4 radiolabelled proteins in two-dimensional protein gels from decidualizing rat endometria. These proteins were synthesized 24–72 h after scratching the endometrium to induce decidualization in hormonally primed animals. In the work described here, animals were killed at 08:00 h on Day 6 and total endometrium, rather than implantation tissue alone, was utilized for protein samples. Although intravenous pontamine blue injection revealed implantation sites in these uteri, new proteins were not seen in Day-6 gels. Synthesis of decidual proteins may not yet have begun, or the amount of decidualizing tissue may have been too small for these proteins to be detected. The basic pattern of labelled endometrial proteins shown by Lejeune *et al.* (1982a) is, however, similar to that seen in Plates 1–3.

The differential synthesis of the proteins described in this paper implies their involvement in the regulation of implantation period although the precise functions of these proteins are not yet known. Some of these proteins may be secreted and interact directly with the blastocysts, or they may be cellular proteins involved in regulating endometrial receptivity to blastocyst attachment.

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Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research council (DHEW publication no. (NIH) 78-23), revised 1978.

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