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DELAYED IMPLANTATION IN ROE DEER (*CAPREOLUS CAPREOLUS*)

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Summary. The results presented in this paper indicate that delayed implantation in the roe deer is due to a lack of certain essential factors which are needed to induce and support the process of embryonic growth. These factors are eventually supplied, in the first weeks of January, as a secretion emanating from the endometrial glands. This secretion contains uterine-specific and serum proteins, about twenty free amino acids, protein-bound glucose and galactose and, rather surprisingly, a free ketose which appears to be fructose. Elongation of the roe deer blastocyst is also correlated with a rise in the concentration of plasma oestrogens, an endocrine change that may stimulate the endometrial glands into secretory activity. However, since simultaneous changes were not observed in the ovaries, the elevated oestrogen levels may be a consequence rather than a cause of embryonic growth.

INTRODUCTION

The roe deer (*Capreolus capreolus*) is a unique and important animal: unique, because it is the only artiodactyl known to exhibit the phenomenon of delayed implantation, and important because by studying this process one can gain insight into the way in which the uterus is able to control the growth of the embryo.

The reproductive cycle of the roe deer is summarized in Text-fig. 1. The entire gestation period lasts about 10 months, beginning with the rut in late July or early August. Delayed implantation or embryonic diapause (Short & Hay, 1966) starts when the blastocyst has lost its zona pellucida, a few days after ovulation, and continues for 5 months until the end of December or the beginning of January. During this period the blastocyst only increases in diameter from 1 to 5 mm, but during the first 2 weeks of January a normal rate of embryonic growth is suddenly resumed and the blastocyst rapidly elongates. Embryonic elongation is followed by placental attachment and a further 5 months of normal gestation. Finally, one, two, or occasionally three, young are born in May.

The purpose of this paper is to describe the way in which the uterus at first restrains and then stimulates the growth of the roe deer blastocyst.

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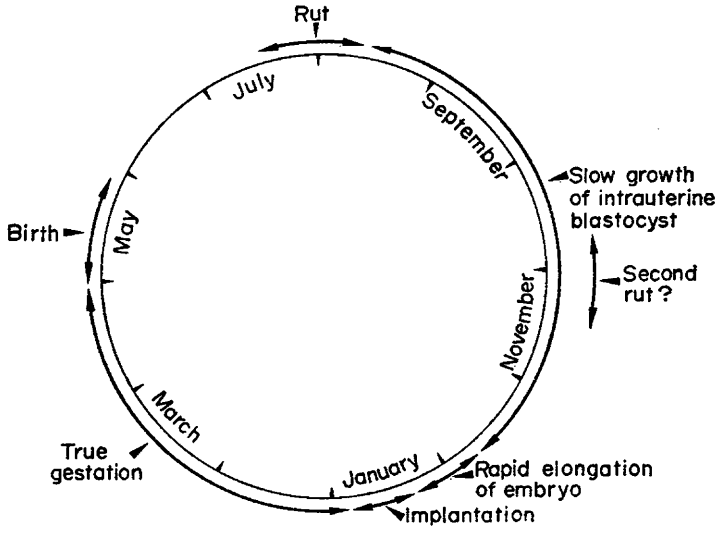
MATERIALS AND METHODS

Animals

This study was based upon the post-mortem examination of roe deer shot during the Forestry Commission cull at Thetford Chase, Norfolk. When an animal had been shot the uterus was removed and put on ice and a blood sample was taken from the heart or jugular vein. The carcase was then transported back to a field laboratory where the remainder of the post-mortem examination was completed within 2 hr of death.

Electron microscopy

The blastocysts and small pieces of uterus were fixed in a cacodylate-buffered mixture of paraformaldehyde and glutaraldehyde (pH 7·0 to 7·2) for 2 hr at 2°C before being transferred to cold cacodylate buffer (pH 7·0 to 7·2) for storage.



TEXT-FIG. 1. The reproductive cycle of the roe deer (*Capreolus capreolus*).

Specimens destined for examination with the transmission electron microscope were post-fixed in cacodylate-buffered osmium tetroxide and dehydrated through a graded series of cold aqueous ethyl alcohols before being embedded in Araldite. Silver-grey sections were cut on a Huxley Cambridge microtome, stained with uranyl acetate and lead citrate, and viewed with a Siemens 1 electron microscope.

Material to be used for scanning electron microscopy was washed in distilled water, frozen with liquid nitrogen and freeze dried. The specimens were then coated with gold-palladium (60/40) alloy in an evaporator and examined with a Cambridge Stereoscan 600 microscope.

Quantitative analysis of uterine flushings

Uterine washings were obtained by flushing each uterine horn from the tubal

end with 20 ml of 0·85% phosphate-buffered saline. The concentration of total hexose in these flushings was determined by the anthrone reaction (Trevelyan & Harrison, 1952) while free glucose was estimated by the glucose oxidase technique (Marks, 1959). The corrected absorption of flushings at 280 nm was used for the assessment of total protein while α-amino nitrogen was measured using dinitrofluorobenzene as described by Goodwin, Schoenfeld & Ryland (1970). Zinc and calcium were determined by atomic absorption spectrophotometry; in the case of calcium, EDTA was used to overcome the suppression of calcium absorption by phosphate ion. The concentration of prostaglandin $F_{2\alpha}$ in the flushings was determined by a gas chromatography–mass spectrometry technique (Kelly, 1973).

Qualitative analysis of uterine flushings

Electrophoresis of the uterine proteins was carried out on columns of polyacrylamide gel at pH 8·9 as described by Davis (1964) and Reisfeld, Lewis & Williams (1962). A 7% solution of acrylamide was used in preparing the gel and polymerization was effected by ammonium persulphate.

Carbohydrates were separated by one-dimensional chromatography on silica gel plates employing the solvent system of 1-butanol (9): acetic acid (6); diethyl ether (3); water (1) (Hay, Lewis & Smith, 1963). The spots were located with thymol (Adachi, 1965) and the presence of ketose confirmed with the following reagents: dimedone (Adachi, 1964), anthrone (Johanson, 1953), β-indolylacetic acid (Heyrovsky, 1956) and phloroglucinol (Menzies & Seakins, 1969). 'Free' and 'bound' carbohydrates were separated by the addition of ethanol to the uterine flushings as described by Mann & Rottenburg (1966) for human semen. In order to hydrolyse the 'bound' fraction, the ethanol-insoluble portion from each flushing was suspended in 0·5 ml distilled water, made acid by the addition of 0·1 ml of 6 N-H_2SO_4, and heated in a boiling water bath for 6 to 8 hr.

Amino acids were isolated by the method of Cook & Luscombe (1960) and separated by two-dimensional chromatography on silica gel plates using the solvent systems of chloroform:methanol:17% ammonia (40:40:20), and phenol:water (75:25). The spots were finally located with a ninhydrin spray. Samples were also analysed with a Locarte automatic amino-acid analyser.

Oestrogen assay

Total unconjugated oestrogens were measured in 1 ml aliquots of plasma by a radioimmunoassay technique as modified by Challis, Heap & Illingworth (1971) from the procedure of Tillson, Thorneycroft, Abraham, Scaramuzzi & Caldwell (1970). The SLC-6X antiserum was used in this assay, diluted 1 : 15,000, together with tritium-labelled oestradiol-17β ([6,7-^{3}H]oestradiol; specific activity > 40,000 mCi/mmol) obtained from the Radiochemical Centre, Amersham. Standard curves were prepared using 8 to 500 pg oestradiol-17β and the samples counted in a Packard Tri-Carb liquid scintillation spectrometer using a scintillation fluid consisting of 0·1 g POPOP (p-bis-2-(5-phenyloxazolyl)-benzene) and 4 g PPO (2,5-diphenyloxazole) per litre AR toluene.

RESULTS

Surface ultrastructure of the blastocyst

Examination of the roe deer blastocyst during delayed implantation by scanning electron microscopy revealed the presence of numerous branched microvilli which formed a delicate network over the surface of the trophoblast. Periodic swellings of up to 2 μm in diameter were often observed along the length of the microvilli and the free tips of the latter also appeared swollen (Pl. 1, Fig. 1). The microvilli were present over most of the blastocyst surface though a few individual cells, possibly undergoing necrosis, possessed a very poor covering.

A number of marked changes were observed on the surface of the trophoblast at the beginning of elongation. The intricate arrangement of branched microvilli became severely reduced, with the result that the trophoblast surface was now covered by a system of interlocking ridges (Pl. 1, Fig. 2). Periodic swellings were sometimes observed along the length of these ridges and occasional microvilli with swollen tips were also seen. The sudden increase in cell number at the time of rapid embryonic growth was associated with a decrease in trophoblast cell size. A 2 × 2 mm blastocyst during delayed implantation was made up of polygonal trophoblast cells, the longest diameter of which ranged from 44 to 57 μm, while trophoblast cells of an 11 × 2·5 mm blastocyst, at the beginning of elongation, had a longest diameter of 19 to 27 μm.

EXPLANATION OF PLATES 1 TO 3

PLATE 1

FIG. 1. Surface of a roe deer blastocyst, measuring 2·5 × 2·5 mm, recovered during delayed implantation on 20 December 1972. Note the swollen tips (long arrows) and periodic swellings (short arrow) along the length of the branching microvilli. Bar = 2 μm.

FIG. 2. Surface of a blastocyst at the beginning of elongation measuring 11 × 2·5 mm and recovered on 15 December 1972. The network of branching microvilli has been reduced to a system of interlocking ridges. Note occasional free microvilli with swollen tips (arrowed). Bar = 4 μm.

PLATE 2

FIG. 3. Roe deer endometrial gland during delayed implantation. × 8000. Two blastocysts measuring 1 × 1 mm and 1·2 × 1·2 mm were recovered from this animal on 15 November 1971. Note the accumulation of electron-lucent vesicles above the nucleus (arrowed).

FIG. 4. Roe deer endometrial gland during the phase of rapid embryonic growth. × 7000. Two embryos with crown–rump lengths of 2·3 and 2·1 cm were recovered from this animal on 14 January 1972. Note the marked absence of electron-lucent vesicles and the well developed rough endoplasmic reticulum (arrowed).

PLATE 3

FIG. 5. Microvillar surface of the roe deer endometrium during delayed implantation. This animal was shot on 4 December 1973 in circumstances that prevented the recovery of the blastocysts. Bar = 2 μm.

FIG. 6. Dilated duct opening observed during the phase of rapid embryonic growth. Two elongating embryos, the extra-embryonic membranes of which measured about 10 cm, were recovered from this animal on 16 January 1973. Bar = 4 μm.

FIG. 7. Bulging uterine epithelial cells observed during the phase of rapid embryonic growth in the roe deer. Two embryos with a crown-rump length of about 2 cm were recovered from this animal on 19 January 1973. Note the presence of mucus-like material on the surface of the epithelial cells (arrowed). Bar = 4 μm.

FIG. 8. The protein pattern obtained by polyacrylamide gel electrophoresis from uterine flushings collected at the time of rapid embryonic growth. Note the major uterine-specific protein (arrowed); a = albumin.

PLATE I

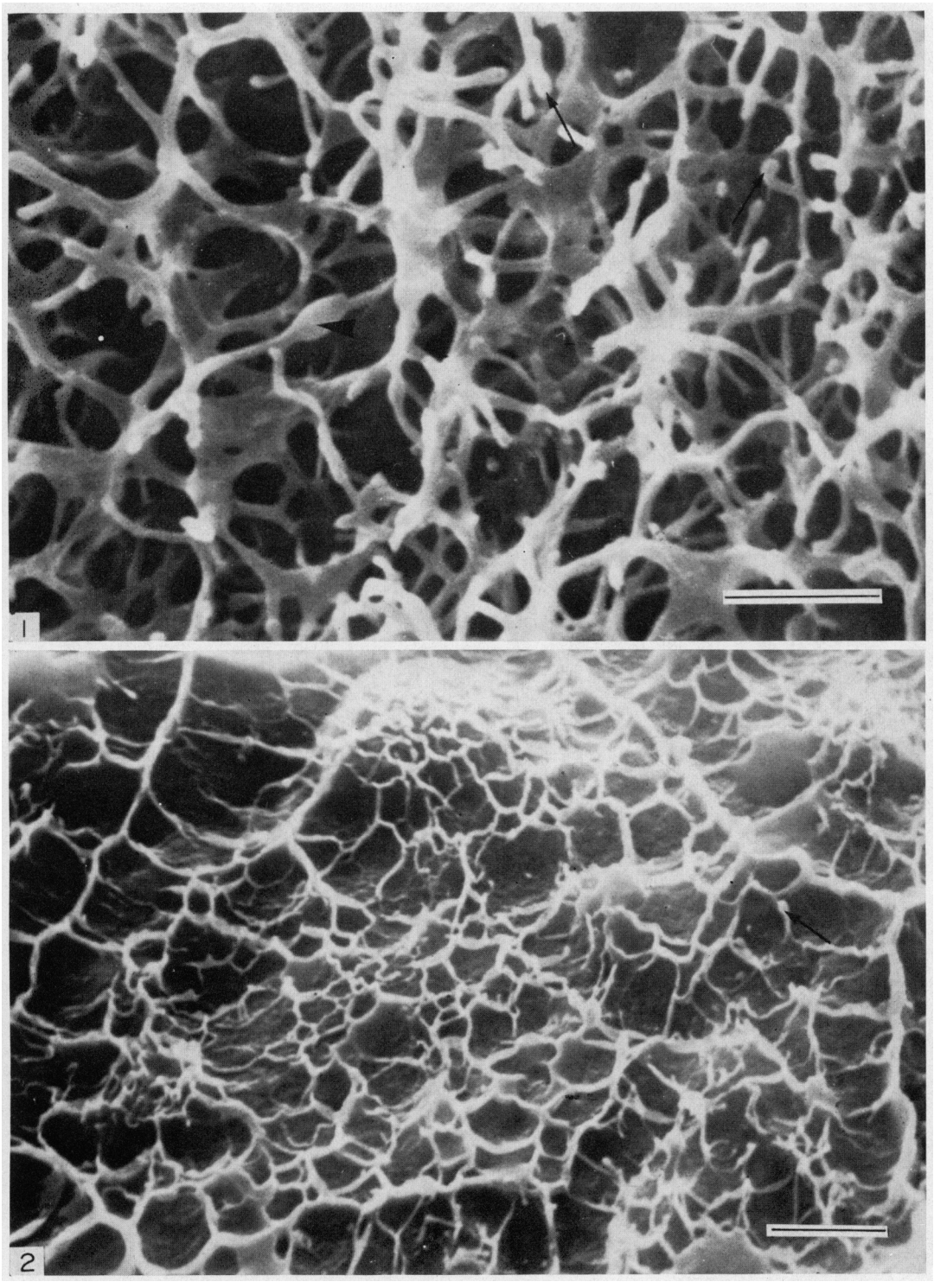


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PLATE 2

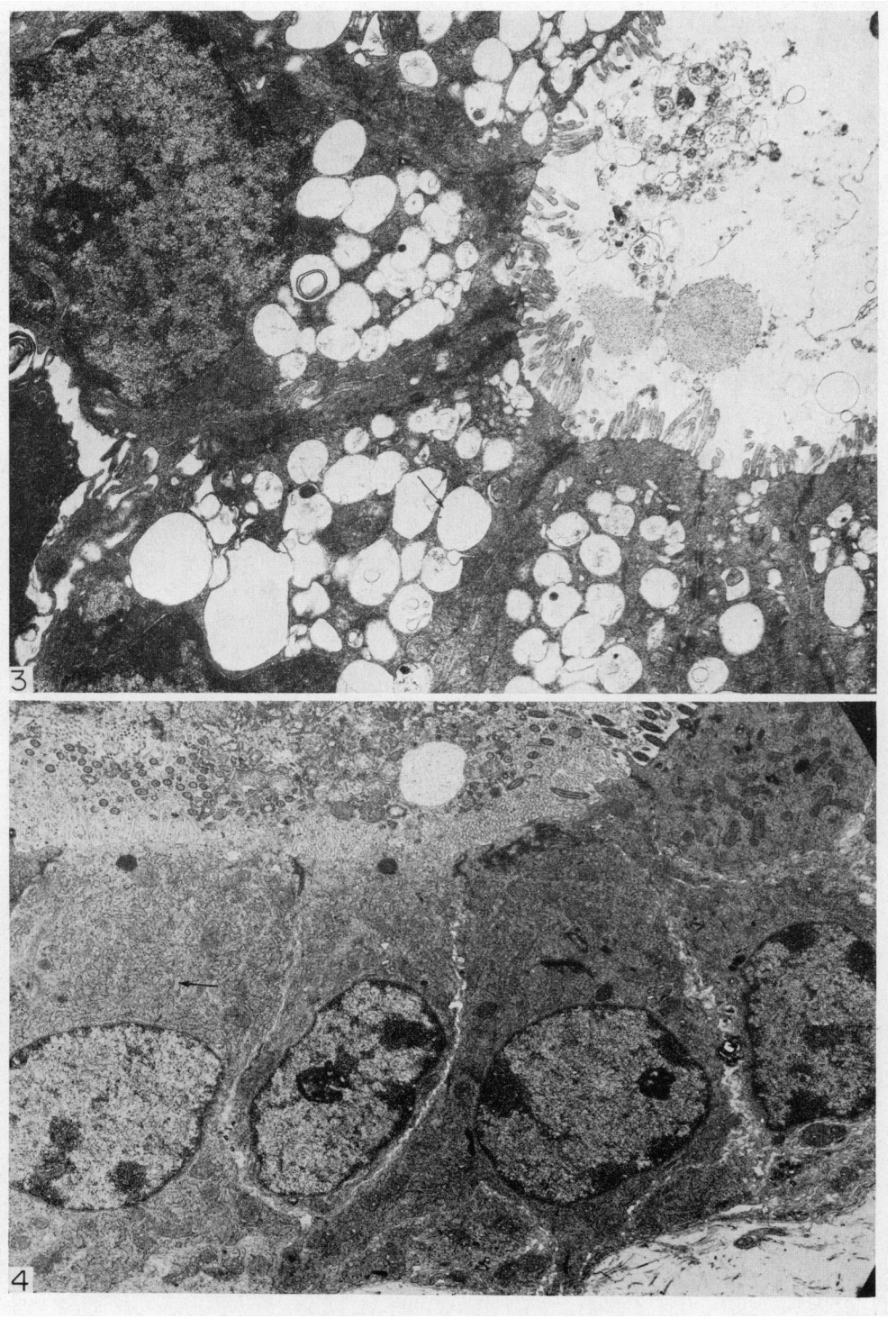
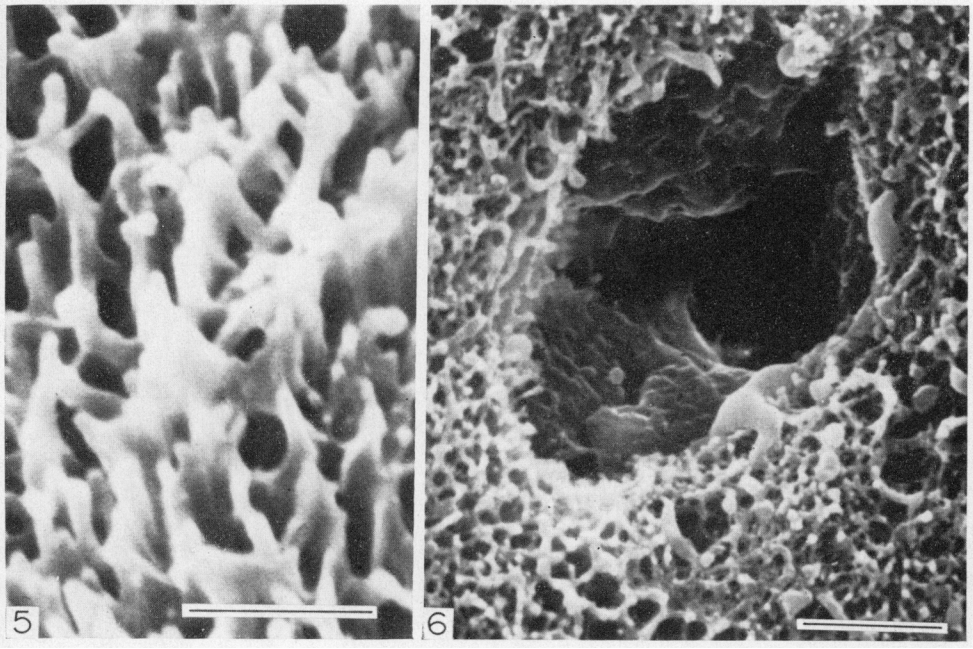
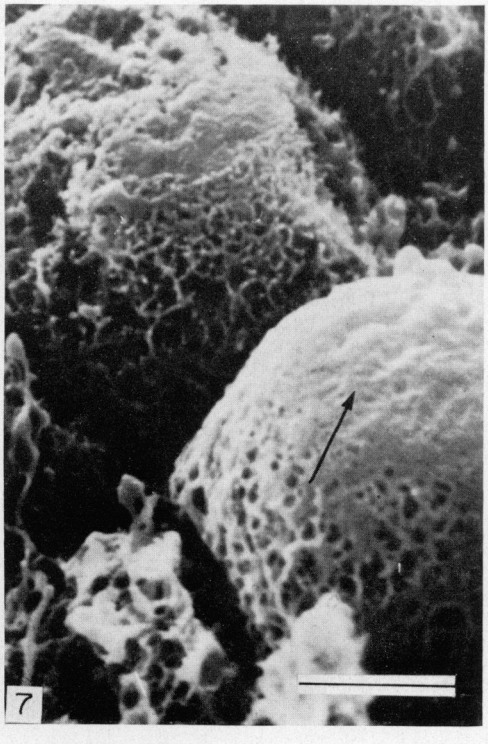
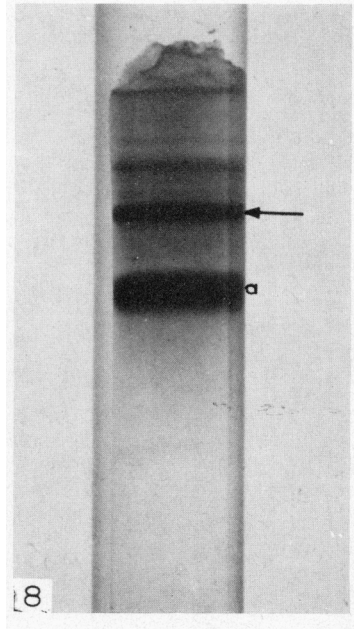
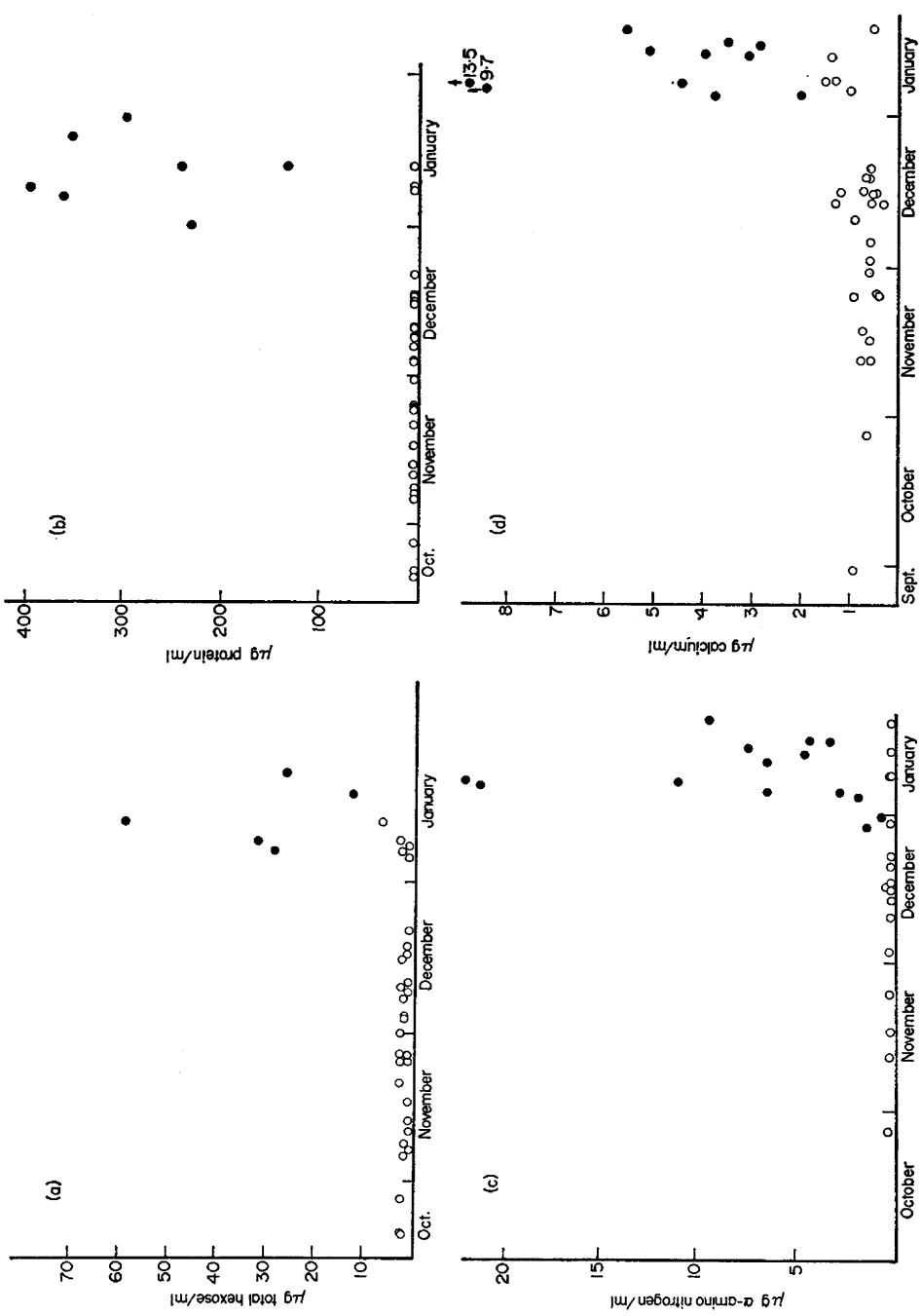


PLATE 3









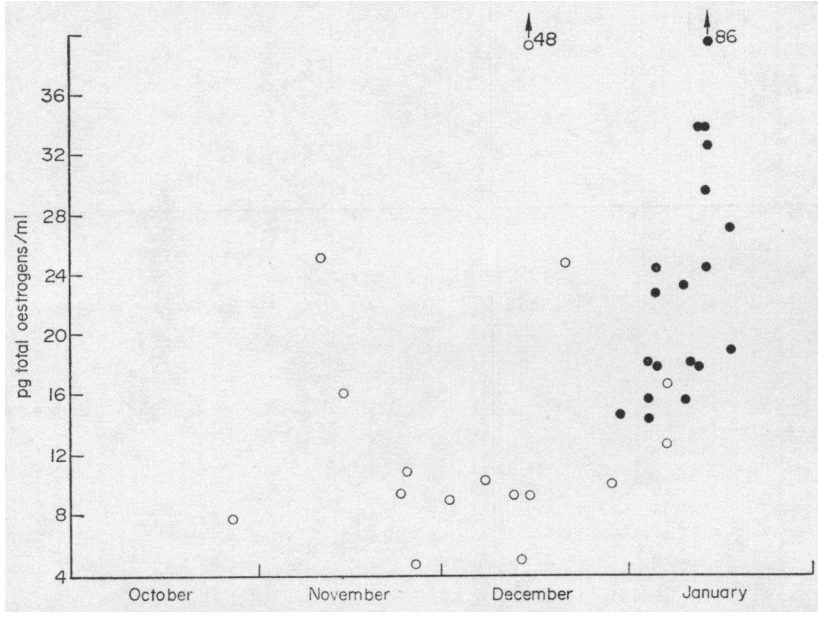
TEXT-FIG. 2. Concentration of various substances in the uterine flushings of roe deer during delayed implantation. ○, Blastocyst in uterus; ●, embryo in uterus. (a) Hexose; (b) protein; (c) α-amino nitrogen; (d) calcium.

Ultrastructure of the endometrial glands

The ultrastructural examination of the roe deer endometrium has revealed a number of important changes occurring during delayed implantation, particularly in the endometrial glands (Aitken, Burton, Hawkins, Kerr-Wilson, Short & Steven, 1973). During delay, clear vesicles, apparently derived from the Golgi apparatus, were seen accumulating above the nucleus of each endometrial gland cell (Pl. 2, Fig. 3). Elongation of the blastocyst was associated with the sudden and dramatic disappearance of these vesicles as they were released into the lumina of the glands (Pl. 2, Fig. 4).

Surface ultrastructure of the endometrium

The surface of the endometrium during delayed implantation was uniformly covered by numerous slender microvilli (Pl. 3, Fig. 5) occasionally



TEXT-FIG. 3. Concentration of total oestrogens in roe deer blood plasma during delayed implantation. ○, Blastocyst in uterus; ●, embryo in uterus.

interrupted by small duct openings which rarely exceeded 2 μm in diameter. The onset of embryonic elongation was associated with a marked increase in the number and diameter of these duct openings together with the appearance of copious amounts of mucoid material, both in the ducts (Pl. 3, Fig. 6) and on large areas of the endometrial surface. The surface of the endometrium also had a very irregular appearance at this time due to the bulging of individual epithelial cells into the uterine lumen (Pl. 3, Fig. 7).

Quantitative analysis of the uterine flushings

This analysis revealed that the sudden elongation of the roe deer blastocyst was also correlated with a distinct rise in the concentration of total hexose

(Text-fig. 2a), total protein (Text-fig. 2b), α-amino nitrogen (Text-fig. 2c) and calcium (Text-fig. 2d) in the uterine flushings, though the levels of zinc, glucose and prostaglandin $F_{2\alpha}$ remained unchanged.

Qualitative analysis of uterine flushings

The electrophoresis of concentrated uterine flushings on polyacrylamide gel revealed the presence of both uterine-specific and serum proteins in the uterine lumen at the time of embryonic elongation. A major component was a uterine-specific protein present in large amounts and migrating between transferrin and albumin (Pl. 3, Fig. 8). Electrophoresis of concentrated uterine flushings obtained from deer in delayed implantation revealed the presence of a faint albumin band only, occasionally coupled with a trace of the uterine-specific protein mentioned above.

Elongation of the blastocyst was also associated with the appearance of a free hexose in the uterine flushings. The level of free glucose in these flushings was negligible and the sugar was eventually identified as a ketose which migrated parallel with fructose on thin-layer chromatography. Fructose was also identified in the fetal fluids of elongating embryos and measurable amounts were detected in the endometrium throughout delay (38 to 220 μg fructose/g endometrium). Thin layer chromatography of the hydrolysed ethanol-insoluble fraction revealed the presence of two major components which migrated parallel with glucose and galactose.

Analysis of the amino acid fraction of the uterine flushings collected at the time of rapid embryonic growth revealed the presence of twenty amino acids of which glutamine, lysine and alanine were present in the largest amounts. Use of an automatic amino acid analyser demonstrated the presence of eight amino acids in a uterine flushing taken during delayed implantation. In this case, lysine, serine and threonine were quantitatively dominant.

Plasma oestrogen

The results of this assay indicated that the eventual elongation of the roe deer blastocyst was associated with a significant ($P<0{\cdot}05$) increase in the concentration of total oestrogens in blood plasma (Text-fig. 3).

DISCUSSION

The presence of branched microvilli on the surface of the blastocyst during delayed implantation has been confirmed by transmission electron microscopy (Aitken *et al.*, 1973). In contrast to these observations on the roe deer, the surface of the mouse blastocyst during delayed implantation is covered by short, fat, simple microvilli (Bergström, 1972). Furthermore, the various types of imprint left on the surface of the mouse blastocyst as a result of the intimate contact between the uterine epithelium and the trophoblast during delayed implantation (Bergström, 1972) were not observed in the present study. This suggests that the roe deer blastocyst lacks any form of attachment to the uterine epithelium during delayed implantation and lies completely free within the uterine lumen. This conclusion is supported by the ease with which roe deer blastocysts

can be flushed from the uterine lumen and by the high incidence of blastocyst migration between the uterine horns in this species.

The growth of the blastocyst appears to be controlled by the endometrial glands. The results of the ultrastructural examination of the uterus indicate that a secretion gradually accumulates in the apical region of the gland cells during delayed implantation which, when released in January, induces and supports the process of embryonic elongation. The release of a secretion at the time of rapid embryonic growth is also indicated by the changes observed in the surface ultrastructure of the endometrium, i.e. the dilatation of the duct openings and the sudden appearance of quantities of mucus. Many surface features of the roe deer endometrium in January correlate well with the picture of the human endometrium during the secretory phase (Ferenczy, Richart, Agate, Purkerson & Dempsey, 1972; Johannison & Nilsson, 1972). The marked increase in the concentration of total hexose, total protein, α-amino nitrogen and calcium in the uterine flushings at the time of embryonic elongation provides further evidence for the arrival of an endometrial secretion in the uterine lumen at this time. Furthermore, the qualitative analysis of the flushings has yielded important information on the biochemical nature of this secretion.

The endometrial glands may, in turn, be under the control of oestrogen. Thus the elevated levels of oestrogen recorded at the time of rapid embryonic growth may stimulate the release of the secretion from the glands. However, since no obvious changes were observed in the roe deer ovaries during delayed implantation (Aitken *et al.*, 1973) it is also possible that this oestrogen is embryonic in origin.

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