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Ultrastructure of the blastocyst and endometrium of the roe deer (*Capreolus capreolus*) during delayed implantation

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INTRODUCTION

A few days after ovulation in late July or August the roe deer blastocyst loses its zona pellucida and enters a 5 month period of delayed implantation or diapause during which the trophoblast exhibits a minimum of mitotic activity and the inner cell mass remains undifferentiated. The delay phase is terminated in January by the sudden, rapid elongation of the blastocyst and subsequent placental attachment (Bischoff, 1854; Keibel, 1902; Short & Hay, 1966). A preliminary ultrastructural study of the trophoblast during the diapause revealed the presence of numerous electron-dense or granular inclusions, a well-developed covering of branched microvilli and a striking lack of mitochondria, ribosomes, Golgi apparatus and endoplasmic reticulum. During embryonic elongation the inclusions disappeared, the microvilli were reduced in height while the size and number of organelles increased dramatically (Aitken *et al.* 1973; Aitken, 1974*a*).

An ultrastructural examination of the uterus (Aitken *et al.* 1973) suggested that the endometrial glands played a major role in the initial restraint and subsequent stimulation of embryonic growth. This was also indicated by subsequent studies which revealed a highly significant increase in the calcium, protein, carbohydrate and α-amino nitrogen content of the uterine flushings, the dilatation of the endometrial duct openings and the sudden appearance of mucoid material on the uterine surface during embryonic elongation (Aitken, 1974*a*, *b*).

Since the previous ultrastructural studies were limited in scope and based on a very small number of animals, it was decided to increase both the number and scope of the observations in a new investigation of the roe deer uterus and blastocyst.

MATERIALS AND METHODS

Animals

This study was based upon the post-mortem examination of roe deer shot during the annual Forestry Commission cull at Thetford Chase, Norfolk. The uterus was removed from each animal immediately after death and put on ice in a thermos flask for transport back to a field laboratory. Here, the uterine horns were flushed with 20 ml of phosphate buffered saline to recover the blastocysts and elongating embryos.

Table 1. *Details of the animals used in the ultrastructural study of the roe deer blastocyst*

Stage of development	Animal no.	Date shot	Embryos examined
Early diapause	71/7	15 Nov	1 blastocyst, 1 × 1 mm
Early diapause	71/8	17 Nov.	1 blastocyst, 1·5 × 1·5 mm
Mid-diapause	71/14	1 Dec.	1 blastocyst, 2 × 2 mm
Mid-diapause	71/18	10 Dec.	1 blastocyst, 2 × 2 mm
Mid-diapause	71/22	14 Dec.	1 blastocyst, 2 × 2 mm
Mid-diapause	71/23	15 Dec.	1 blastocyst, 2 × 2 mm
Late diapause	71/24	15 Dec.	1 blastocyst, 3 × 3 mm
Late diapause	71/30	20 Dec.	1 blastocyst, 3 × 3 mm
Late diapause	71/32	21 Dec.	1 blastocyst, 3 × 3 mm
Rapid elongation	71/44	4 Jan.	1 elongating conceptus crown-rump length of 10 mm

Electron microscopy

The embryos and small pieces of uterus were fixed in a cacodylate buffered mixture of paraformaldehyde and glutaraldehyde (pH 7·0–7·2) for 2 hours at 2 °C and then transferred to cold cacodylate buffer (pH 7·0–7·2) for storage.

Material to be examined by transmission electron microscopy (TEM) was post-fixed in cacodylate buffered osmium tetroxide (pH 7·0–7·2), dehydrated through a graded series of cold aqueous ethyl alcohols and finally embedded in Araldite. Silver grey sections were cut on a Huxley Cambridge microtome, stained with uranyl acetate and lead citrate, and viewed under a Siemens I electron microscope.

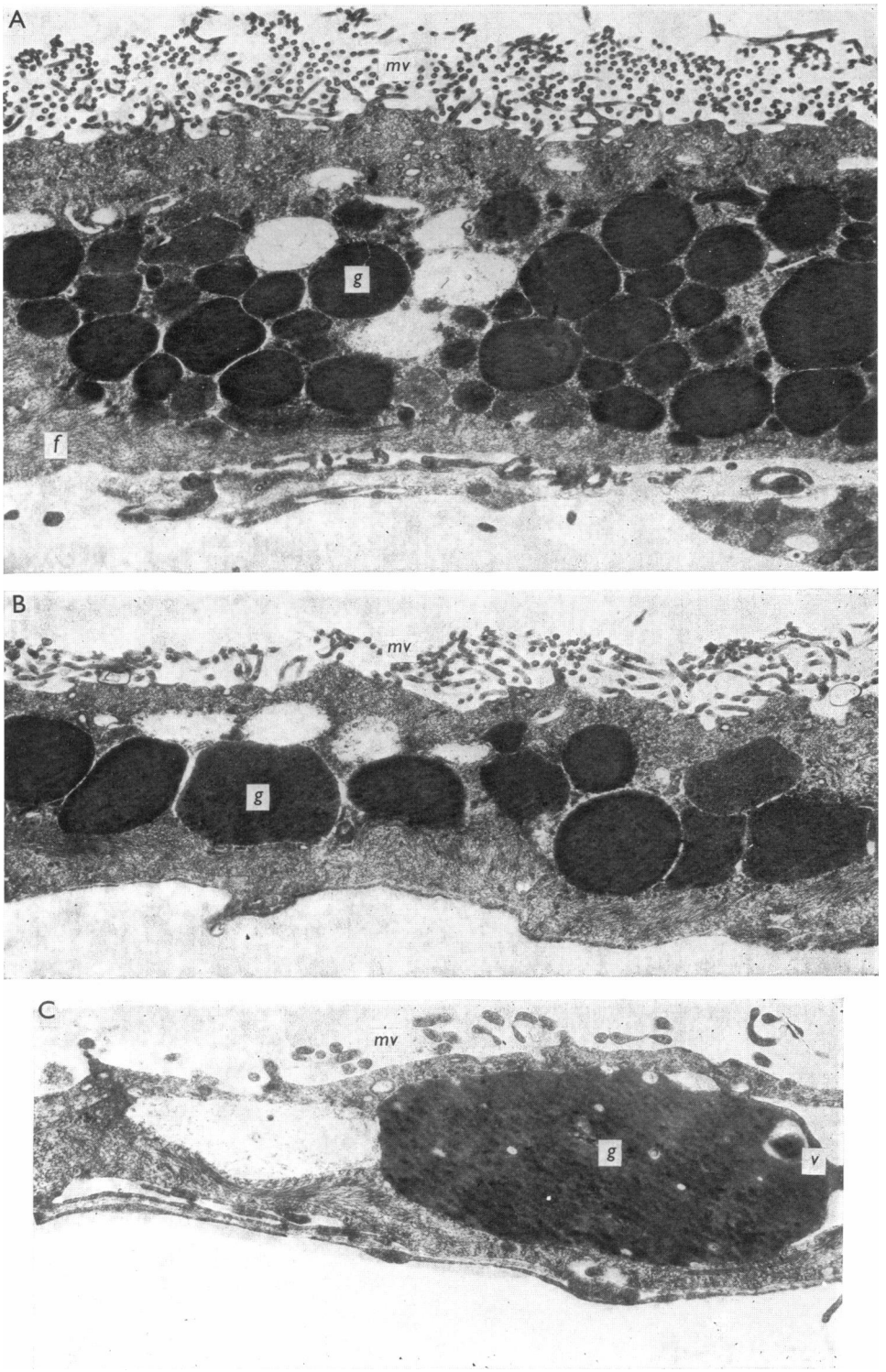
Specimens to be used for scanning electron microscopy (SEM) were washed in distilled water, frozen with liquid nitrogen and freeze dried. The samples were then coated with gold palladium (60/40) alloy in an evaporator and examined with a Cambridge Stereoscan 600 microscope.

RESULTS

Electron microscopy of the embryo

Some detailed information about the animals used in this study is presented in Table 1. All observations were confined to the trophoblast.

Fig. 1. Transmission electron microscopy of the trophoblast during delayed implantation. A, The trophoblast during early diapause (animal 71/7). Note the well-developed covering of branched microvilli (*mv*), the abundance of granular inclusions (*g*), the presence of fibrillar material (*f*) and the marked absence of cytoplasmic organelles such as mitochondria, endoplasmic reticulum, Golgi apparatus and ribosomes. × 10750. B, The trophoblast during mid-diapause (animal 71/14). The development of the microvilli (*mv*), the number of granular inclusions (*g*) and the height of the trophoblast cells are all reduced compared with the early diapause specimen. × 12435. C, The trophoblast during late diapause (animal 71/30). There has been a further reduction in the development of the microvilli (*mv*), the number of granular inclusions (*g*) and the height of the trophoblast cells compared with the early diapause specimen. The few granular inclusions remaining are perforated by electron-lucent vesicles (*v*) × 16900.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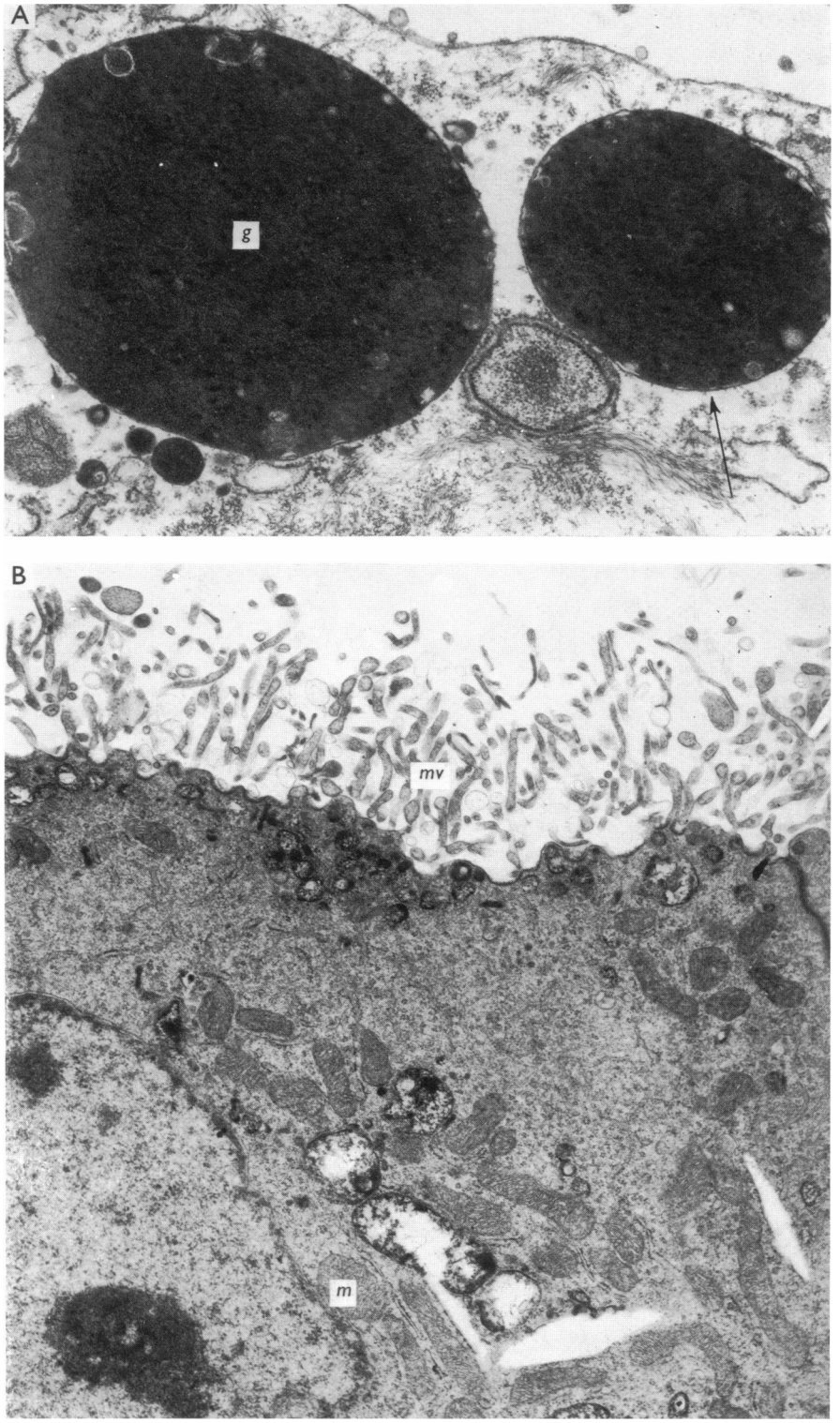


Fig. 2. Transmission electron microscopy of the trophoblast during late diapause and embryonic elongation. A, the trophoblast during late diapause (animal 71/32). Note the large granular inclusions (*g*) which are clearly membrane-bound (arrowed) and perforated by clear vacuoles. × 26195. B, the trophoblast at an advanced stage of elongation (animal 71/44); Note the well-developed covering of microvilli (*mv*) and the presence of numerous apical mitochondria (*m*). × 14700.

Table 2. *Details of the animals used in the ultrastructural study of the roe deer uterus during delayed implantation*

Stage of development	Animal no.	Date shot	No. of embryos	Embryo size
Diapause	71/7	15 Nov.	2 blastocysts	1 × 1 mm diameter 1·2 × 1·2 mm diameter
Diapause	71/9	24 Nov.	2 blastocysts	2 × 1·2 mm diameter 1 × 1·2 mm diameter
Diapause	71/12	26 Nov.	2 blastocysts	2 × 2 mm diameter 2 × 2 mm diameter
Diapause	71/17	8 Dec.	2 blastocysts	2 × 2 mm diameter 2 × 1·8 mm diameter
Diapause	71/24	15 Dec.	2 blastocysts	3 × 3 mm diameter 3 × 3 mm diameter
Diapause	71/30	20 Dec.	1 blastocyst	3 × 3 mm diameter
Diapause	71/32	21 Dec.	2 blastocysts	3 × 3 mm diameter 3 × 3 mm diameter

Table 3. *Details of the animals used in the ultrastructural study of the roe deer uterus during embryonic elongation and placental attachment*

Stage of development	Animal no.	Date shot	No. of embryos	Embryo size
Embryonic elongation	71/39	30 Dec.	2 elongating blastocysts	Approx. 150 mm long Approx. 150 mm long
Embryonic elongation	71/42	4 Jan.	2 elongating blastocysts	Approx. 150 mm long Approx. 150 mm long
Initiation of placental attachment	71/38	30 Dec.	2 elongated embryos	5·5 mm C-R length 5·5 mm C-R length
Placental attachment	71/61	14 Jan.	2 elongated embryos	22 mm C-R length 22·5 mm C-R length
Placental attachment	71/65	14 Jan.	2 elongated embryos	23 mm C-R length 21 mm C-R length

C-R = Crown-rump

Early diapause (animals 71/7 and 71/8). During the early stages of delayed implantation (Fig. 1*a*) the blastocysts were covered with a well developed layer of long, slender, branched microvilli, amongst which occasional membrane-bound vesicles could be seen. Large numbers of caveolae and clear, micropinocytotic vesicles were associated with the outer membrane of the trophoblast cells while the cytoplasm contained numerous electron-dense droplets, occasional granular inclusions and a few large, clear vesicles. All these structures appeared to be at least partly membrane-bound (Fig. 2*a*). The cells were largely devoid of organelles although mitochondria and a poorly developed granular endoplasmic reticulum were sometimes observed at the periphery of large groups of electron-dense droplets. A great deal of fibrillar material was observed near the basal membrane and also making contact with the desmosomes of the lateral cell borders.

Table 4. *Details of the animals used in studying the surface ultrastructure of the roe deer uterus*

Stage of development	Animal no.	Date shot	No. of embryos	Embryo size
Diapause	72/7	4 Dec.	1 blastocyst	Not measured
Embryonic elongation	72/33	16 Jan.	2 elongating blastocysts	Approx. 100 mm long Approx. 100 mm long
Initiation of placental attachment	72/35	16 Jan.	2 elongated embryos	6·5 mm C-R length 6·5 mm C-R length
Initiation of placental attachment	72/39	19 Jan.	2 elongated embryos	20 mm C-R length 20 mm C-R length
Placental attachment	72/41	21 Jan.	1 elongated embryo	68·5 mm C-R length

C-R = crown-rump

Mid-diapause (animals 71/14, 71/18, 71/22 and 71/23). By mid-diapause (Fig. 1*b*) the height of the trophoblast cells and the number of microvilli, caveolae and micropinocytotic vesicles associated with the outer membrane were reduced. A similar reduction in the number of electron-dense droplets was associated with a marked increase in the number of granular inclusions present in the cytoplasm. Considerable fusion of these granular inclusions had also taken place, so that extensive areas of the cytoplasm were now occupied by large continuous, composite vesicles. Since the electron-dense droplets and granular inclusions were both membrane-bound, and were of similar size and position, it was concluded that these structures were possibly not, as previously suggested (Aitken *et al.* 1973) separate entities. The formation of the granular inclusions seemed to involve the dispersion of the electron-dense material within the droplets. Progressive stages of dispersal were represented by variations in electron density both within and between the inclusions.

Late diapause (animals 71/24, 71/30 and 71/32). This stage was marked by a further reduction in the height of the trophoblast cells and in the number and height of the apical microvilli (Fig. 1*c*). The number of caveolae, micropinocytotic vesicles and granular inclusions was also reduced, although a few extremely electron-dense inclusions remained. The latter were often very large and were perforated by numerous clear vacuoles, some of which were membrane-bound (Fig. 2*a*).

Advanced stage of embryonic elongation (animal 71/44). In contrast to the attenuated state of the trophoblast during late diapause, the cells were now columnar with centrally placed nuclei. Numerous tall, slender, branched microvilli were again present on the outer surface (Fig. 2*b*) amongst which many membrane-bound vesicles could be seen. The number of caveolae and micropinocytotic vesicles had also increased, but differed from those present in diapausing blastocysts in containing much electron-dense, granular material. A dramatic increase in the number of cell organelles had also occurred and lipid deposits were now evident, particularly in the basal region of the trophoblast cells. The number of granular inclusions and the amount of fibrillar material were seriously reduced at this stage.

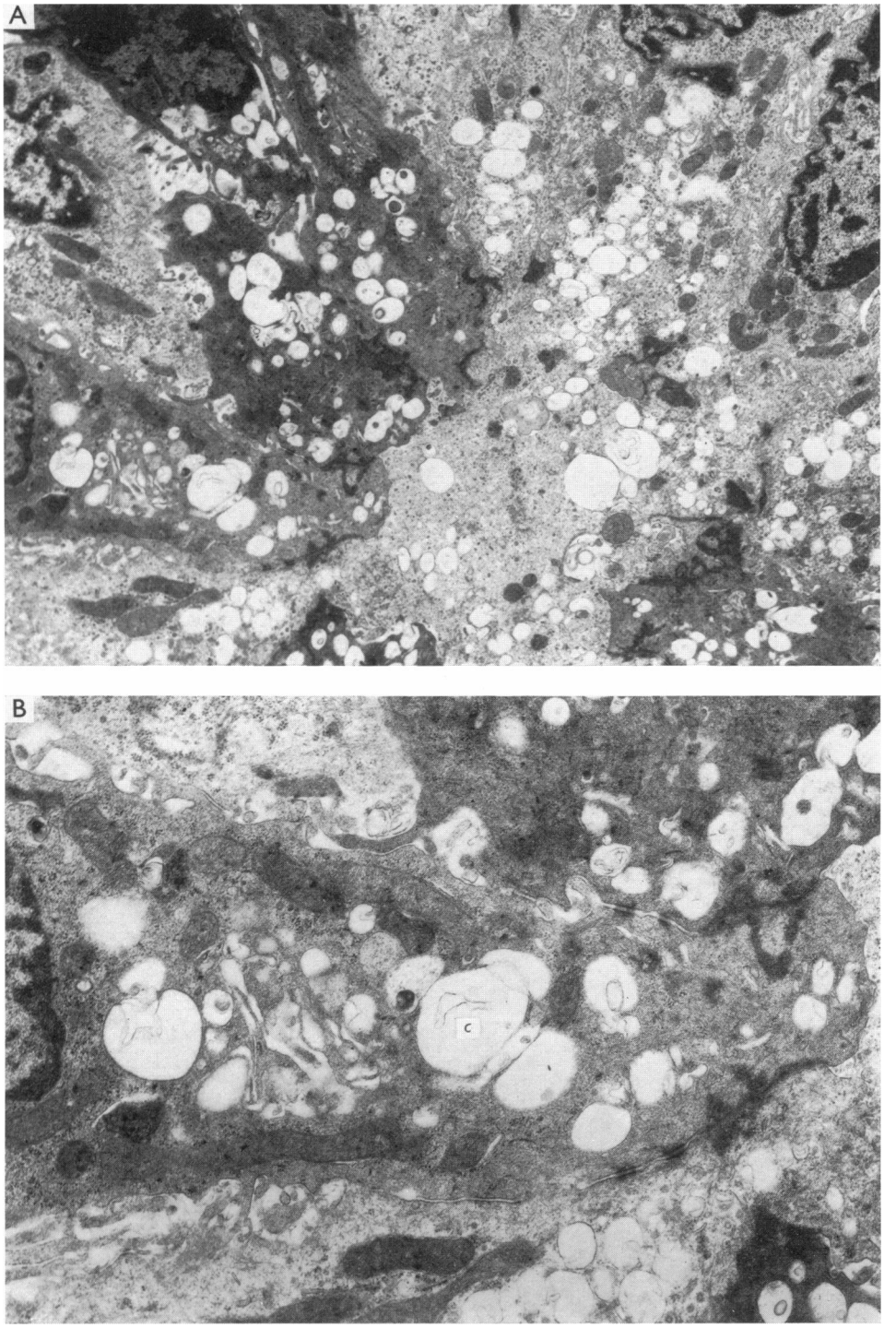


Fig. 3. Transmission electron microscopy of the endometrial glands during diapause. A, Endometrial gland cells during the late stages of delayed implantation (animal 71/32). Note the accumulation of clear vesicles in the supranuclear region of the cells. × 5340. B, Supranuclear region of an endometrial gland cell during late diapause showing vesicles (*c*) (animal 71/32). × 14415.

Electron microscopy of the endometrium

Some details about the animals used in this study are presented in Tables 2–4.

The endometrium during diapause (animals 71/7, 71/9, 71/12, 71/17, 71/24, 71/30, 71/32 and 72/7)

During diapause the cells of the tightly coiled glandular fundi were dominated by the accumulation of abundant, clear, supranuclear vesicles evidently derived from the Golgi apparatus. The cells also contained numerous apical mitochondria, abundant free ribosomes and occasional basal lipid deposits. However, the granular endoplasmic reticulum was poorly developed and the apical cell membranes were largely devoid of microvilli (Fig. 3). The non-ciliated cells of the ductal epithelium showed a similar but less marked accumulation of clear supranuclear vesicles during diapause, but differed from the gland cells in possessing numerous subnuclear mitochondria and more prominent basal lipid deposits (Fig. 4). In the few ciliated cells examined many small electron-dense mitochondria and a few dense lysosome-like granules were observed. However the granular endoplasmic reticulum and Golgi apparatus were poorly developed and clear supranuclear vesicles were absent.

The lumina of the glandular ducts and fundi were generally clear but occasionally contained small amounts of an electron-dense, granular material and cell debris.

The highly convoluted endometrial surface was uniformly covered by numerous simple microvilli while the few duct openings observed rarely exceeded 2 μm in diameter.

The endometrium during embryonic elongation (animals 71/39, 71/42 and 72/33)

The onset of embryonic elongation was associated with a decline in the height of the glandular epithelium evidently as a result of the sudden release of the clear apical vesicles into the glandular and thence into the uterine lumen.The cells appeared to be less active at this stage; the Golgi apparatus and granular endoplasmic reticulum were poorly developed and there were no lipid deposits.

Large numbers of clear supranuclear vesicles were also discharged from the non-ciliated cells of the ductal epithelium during embryonic elongation. However, these cells showed a much less marked decline in activity (Fig. 5). Free ribosomes, mitochondria and some basal lipid deposits were still present and several of the non-ciliated cells now contained electron-dense, lysosome-like granules. Continuity between the perinuclear space and the tubules of the granular endoplasmic reticulum could also be demonstrated at this stage and the latter was frequently dilated in the vicinity of the enlarged mitochondria. Large apical protrusions projecting from the surface of several duct and luminal epithelial cells also suggested apocrine secretion at this time. (Figs. 6 and 7).

This stage was characterised by the presence of clear vesicles, much cellular debris and an electron-dense granular material in the lumina of the glands and ducts.

The endometrium during the early stages of placental attachment (animals 71/38, 71/61, 71/65, 72/35, 72/39 and 72/41)

The most striking feature of the endometrium during this phase was the development of the granular endoplasmic reticulum in the non-ciliated duct cells (Figs. 6 and 7).

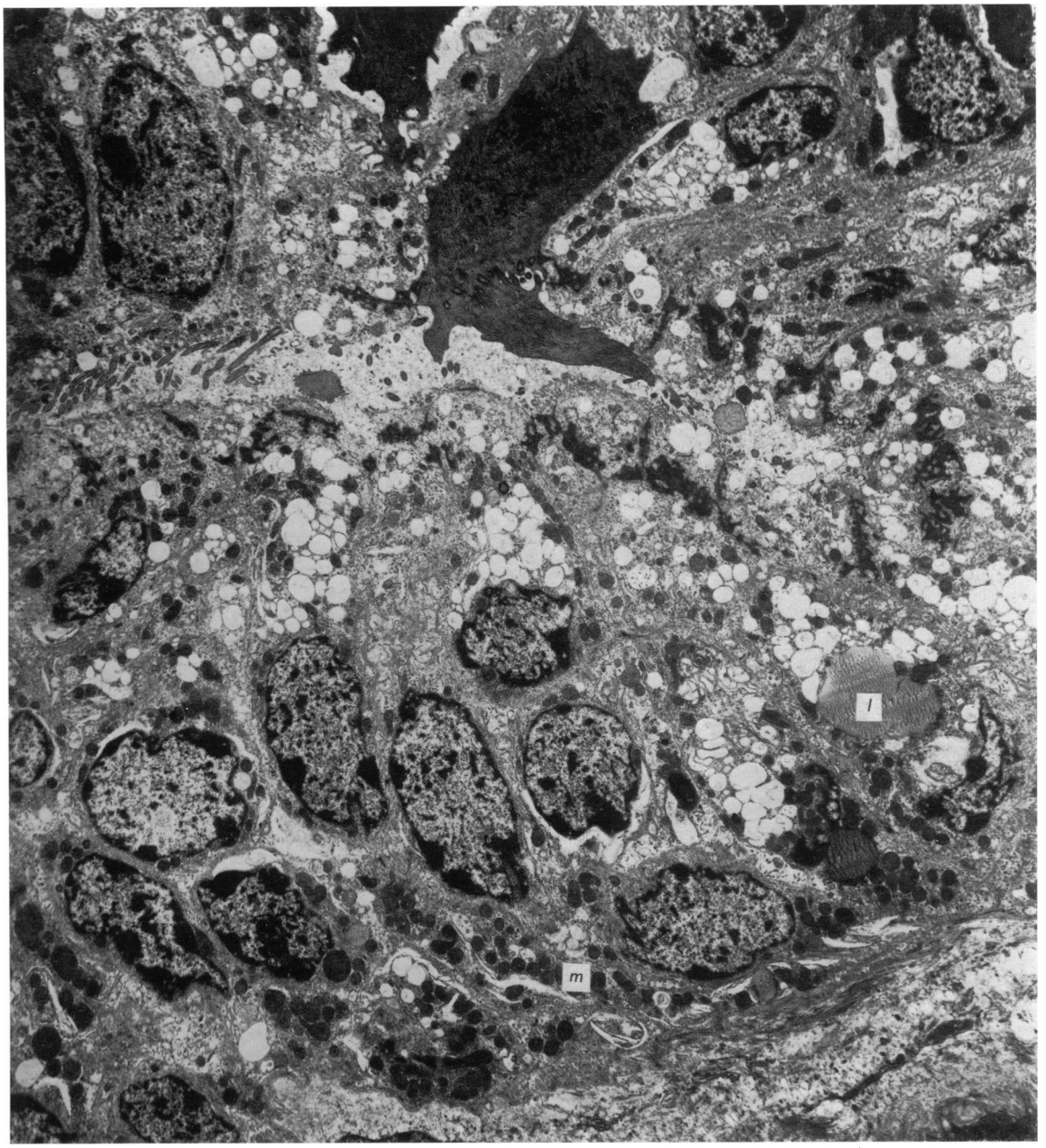


Fig. 4. Transmission electron microscopy of the endometrial ducts during diapause (animal 71/7). Note the accumulation of clear vesicles in the non-ciliated duct cells and the presence of basal lipid deposits (*l*) and numerous mitochondria (*m*). ×4780.

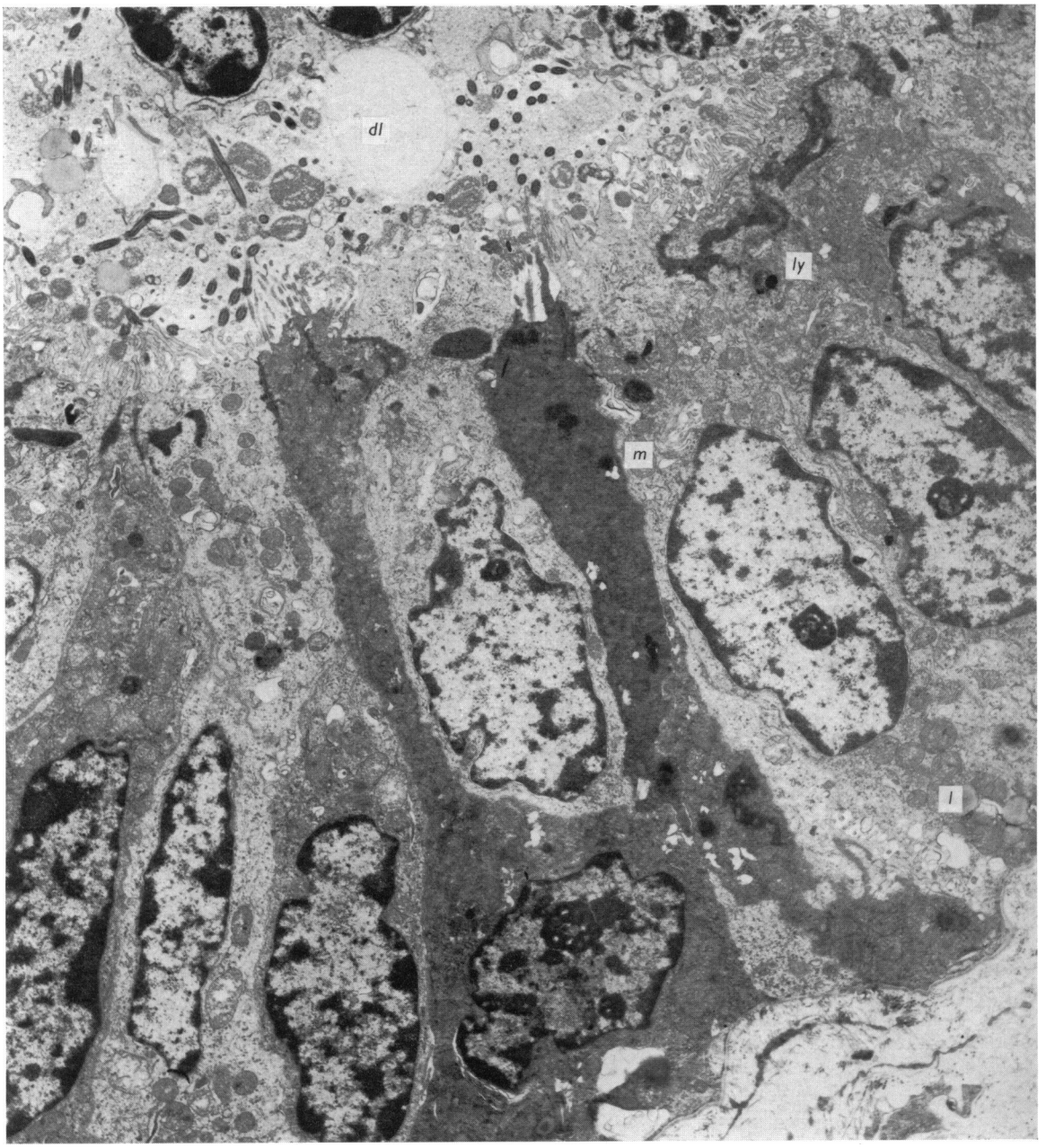


Fig. 5. Transmission electron microscopy of the endometrial ducts during embryonic elongation (animal 71/39). Note the presence of cell debris and granular material in the ductal lumen (*dl*). Clear vesicles are completely absent from the duct cells which now contain abundant mitochondria (*m*), ribosomes, some basal lipid deposits (*l*) and occasional electron-dense, lysosome-like granules (*ly*). × 6130.

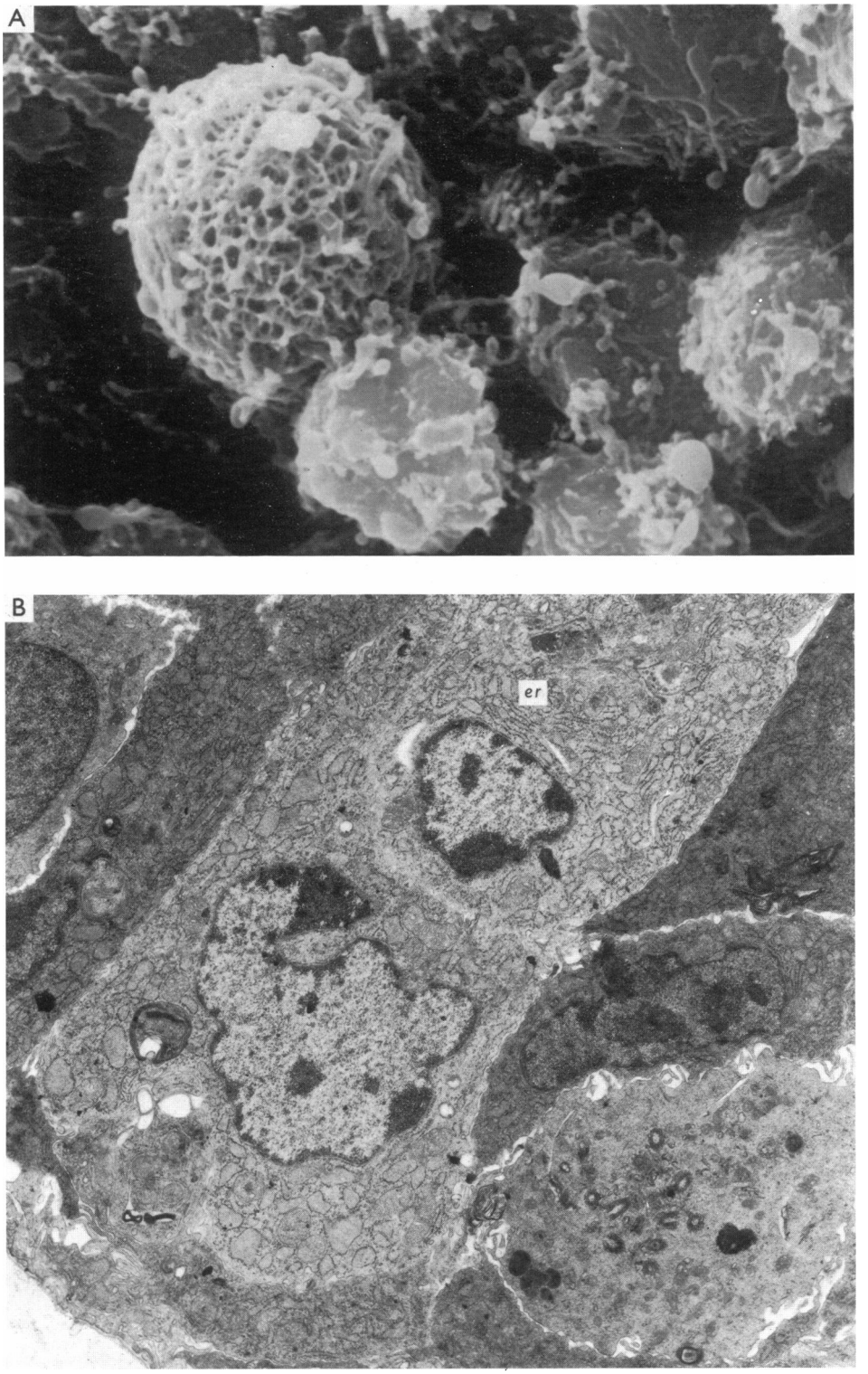
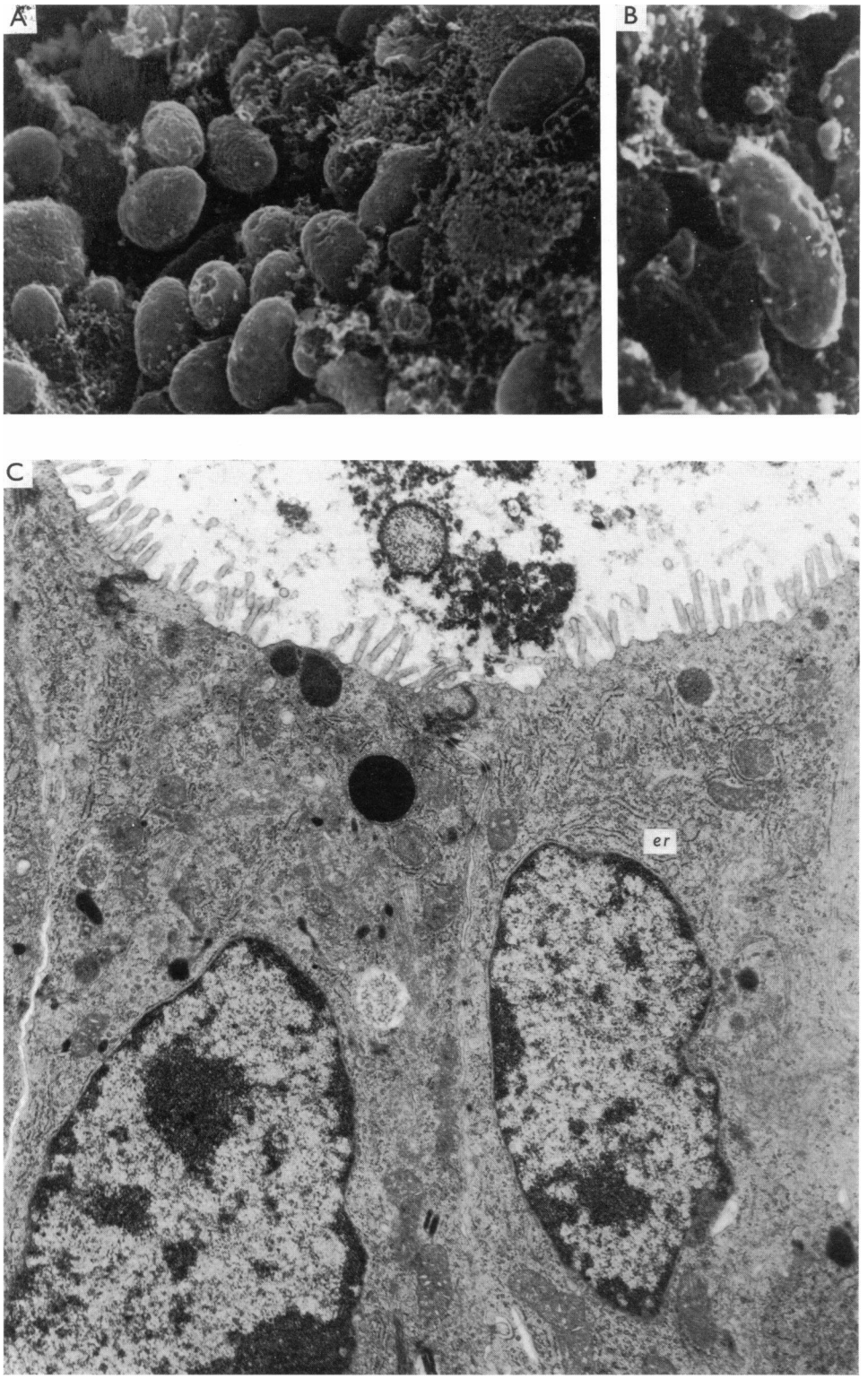


Fig. 6. The endometrium during the phase of rapid embryonic growth. A, Scanning electron microscopy of the luminal epithelium during embryonic elongation (animal 72/33). Note the bulging microvillous border of an epithelial cell and adjacent smooth protrusions. × 5315. B, Transmission electron microscopy of the endometrial ducts during placental attachment (animal 71/61). Note the hypertrophied granular endoplasmic reticulum (*er*). × 6960.


A
B
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er

Large amounts of a moderately electron-dense material were observed distending both the cisternae of the endoplasmic reticulum and the continuous perinuclear space. Although lipid deposits were now absent from these cells, numerous mitochondria, free ribosomes and occasional lysosome-like granules could be seen. In addition, a number of membrane-bound granular inclusions were present, particularly in the vicinity of the apical cell membrane, where they appeared to be discharging their contents into the lumen (Fig. 7). The development of the endoplasmic reticulum was also associated with a marked increase in the number of apical microvilli, while large irregular projections from the surface of some cells suggested the continued release of an apocrine secretion.

The number of ciliated cells had increased during this stage but their ultrastructural appearance was unchanged.

During this phase of development, the lumina of the ducts and glands contained large amounts of an electron-dense granular material and some cell debris.

Smooth apical protrusions were still observed projecting from several luminal epithelial cells in both the caruncular and intercaruncular regions of the uterus during the initial stages of placental attachment. The bulging apical membranes of the remaining luminal epithelial cells were covered with long simple microvilli. During the more advanced stages of placental attachment (animal 72/41) the number of apical protrusions seemed to decline.

DISCUSSION

The most striking feature of the roe deer blastocyst during delayed implantation was the abundance of electron-dense granular inclusions in the cytoplasm of the trophoblast cells. These inclusions were most numerous in the earliest specimens examined but showed a gradual reduction in both number and electron density as diapause progressed. These changes suggested that the inclusions may be an energy reserve which is gradually utilised by the blastocyst during diapause. The blastocysts of many other animals with a delay of implantation show an accumulation of energy reserves; in the mink and fur seal, for example, the stored material is lipid (Enders, 1971). Whether or not the granular inclusions of the roe deer blastocyst have a high lipid content, as suggested in an earlier study (Aitken *et al.* 1973) urgently requires histochemical investigation.

Delayed implantation was also characterised by a marked lack of cytoplasmic organelles in the trophoblast, suggesting a minimum of metabolic activity and cell growth at this time. In the absence of cell growth the changes in the size of the blastocyst as a whole during diapause must have been achieved by the passage of fluid

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Fig. 7. The endometrium during the phase of rapid embryonic growth. A, scanning electron microscopy of the luminal epithelium during embryonic elongation (animal 72/33). Note smooth apical protrusions projecting from the luminal epithelium. × 1555. B, scanning electron microscopy of the luminal epithelium during embryonic elongation (animal 72/33). Several of the apical protrusions are attached to the underlying epithelium by a narrow stalk and resemble the 'fungal-like protrusions' observed by Bergström & Nilsson (1973) in the mouse. × 2865. C, Transmission electron microscopy of the endometrial ducts during placental attachment (animal 71/65). Note the hypertrophied granular endoplasmic reticulum (*er*) and large electron-dense apical granules. × 11 215.

into the blastocoele with consequent distortion of the trophoblast cells. Overall attenuation of the trophoblast cells during diapause was indicated by a gradual decline in cell height and a reduction in the density of microvilli and caveolae on the blastocyst surface. The initial stages of embryonic elongation also appeared to involve the rapid transport of fluid into the yolk sac cavity and a consequent stretching of the trophoblast layer, judging from the low height of the microvilli on three blastocysts measuring 11 mm (Aitken, 1974*a*), 15 mm and 50 mm (Aitken *et al.* 1973) in length. However, in the present study the trophoblast cells of a more advanced embryo (71/44: approximately 30 cm in length) possessed columnar trophoblast cells with a well-developed covering of microvilli, suggesting that much cell growth had occurred during the later stages of elongation.

In confirmation of our earlier findings (Aitken *et al.* 1973), embryonic elongation was accompanied by a dramatic increase in the development of mitochondria, ribosomes, Golgi apparatus and endoplasmic reticulum in the trophoblast cells, presumably in association with a marked increase in cellular activity.

The results also confirmed the existence of a period of intense endometrial secretory activity during the resumption of rapid embryonic growth. In addition to the release of the clear vesicles from the ductal and glandular epithelia, the endometrium appeared to produce an apocrine secretion in the luminal and ductal epithelia and possibly a holocrine secretion in the glands. However, the presence of cell debris in the glandular lumina may have been an artefact of poor fixation. During the more advanced stages of embryonic elongation a fourth type of secretion was synthesized in the ductal epithelium by the hypertrophied granular endoplasmic reticulum. The hypertrophy appeared to start in the vicinity of the enlarged mitochondria shortly after the resumption of rapid embryonic growth, but did not reach maximal development until the time of placental attachment. The timing of the release of this secretion suggests that its formation is a consequence rather than a cause of embryonic growth. Histochemical evidence for the release of a glandular secretion during the post-implantation stage of pregnancy has been obtained in rabbits, rodents (Wislocki, Deane & Dempsey, 1946), women, pigs, cats (Wislocki & Dempsey, 1945) and cows (Yamauchi, Kakishita & Kotera, 1969). In the pig (Dempsey, Wislocki & Amoroso, 1955), sheep (Hoyes, 1972) and mink (Enders, Enders & Schlafke, 1963) ultrastructural studies have revealed that, as in the roe deer, this phase of glandular activity is associated with a marked hypertrophy of the granular endoplasmic reticulum. Hoyes (1972) has also suggested that oestrogen may play a role in stimulating this type of endometrial gland activity. This concept is supported by work on the roe deer, since the release of the endometrial secretions in January is accompanied by a significant rise in plasma oestrogen concentration (Aitken, 1974*a*). It is hoped to test this hypothesis further by examining the endometrial ultrastructure of roe deer given oestrogen implants during delayed implantation.

SUMMARY

Transmission electron microscopy of the trophoblast cells during diapause revealed an abundance of electron-dense, membrane-bound granular inclusions and a marked lack of cytoplasmic organelles. The cells also possessed a well-developed covering

of branched microvilli, numerous caveolae, micropinocytotic vesicles and a lamina of fine fibrillae. The progressive enlargement of the blastocyst during diapause was correlated with a decline in the height of the trophoblast cells and a reduction in the density of microvilli and caveolae associated with the outer membrane. The granular inclusions also declined in number and electron density during the delay phase, suggesting the progressive utilisation of energy reserves. Embryonic elongation was associated with the disappearance of the granular inclusions, a reduction in the amount of fibrillar material and a dramatic increase in the development of cytoplasmic organelles.

During diapause, clear vesicles, apparently derived from the Golgi apparatus, gradually accumulated in the supranuclear region of each gland and non-ciliated duct cell. Embryonic elongation was associated with the sudden release of these vesicles into the glandular lumen and thence into the uterine lumen. Numerous apical protrusions were also observed projecting from the luminal and ductal epithelia at this time, suggesting the formation of an apocrine secretion. Another type of secretion was produced during the early stages of placental attachment by the hypertrophied granular endoplasmic reticulum of the ductal epithelium.

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