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HISTOLOGICAL AND HISTOCHEMICAL ANALYSIS OF THE FORMATION OF IMPLANTATION CHAMBERS IN THE MOUSE UTERUS

C. A. FINN AND J. R. HINCHLIFFE

Department of Biological Sciences, Wye College (University of London), Ashford, Kent

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Summary. It has been found that in the majority of cases the injection of oil into the uterus of mice on the 4th day of pseudopregnancy results in the formation of several discrete decidual swellings along the length of the uterus. Histochemical and histological examination on the 6th day of pseudopregnancy reveals that a structure closely resembling an implantation chamber is formed in the middle of the swellings. There is a down-pushing of the lumen from the mesometrial side into the centre of the swelling and breakdown of epithelium on the antimesometrial side of the chamber. The decidual tissue around the base of the chamber contains both glycogen and PAS-positive diastase-fast material, similar to that found around an implanting blastocyst.

The lumen of the artificial implantation chamber contains relatively large quantities of a secretion rich in protein and a diastase-fast PAS-positive material, probably a neutral mucopolysaccharide–protein complex. This secretion is also found in natural implantation chambers on the 6th day of pregnancy, though in much smaller quantities. It is suggested that it is a nutritive substance secreted by the uterus as part of the implantation reaction and normally taken up by the blastocyst.

It is concluded that the 4-day pseudopregnant mouse uterus has the competence to react to a natural or unnatural decidual stimulus with the formation of implantation chambers.

INTRODUCTION

Study of the implantation of the blastocyst can be approached from three main directions. Firstly there is the necessary endocrine environment of the uterus, secondly the reaction of the uterus to the stimulus provided by the blastocyst and thirdly the activity of the blastocyst. In normal implantation, of course, all three factors are operating together. Under experimental conditions in the mouse, however, it is possible, to some extent, to study them separately. Thus, the capacity of the blastocyst for growth and invasion has been widely studied by transplantation of blastocysts to extra-uterine sites (Fawcett, Wislocki & Waldo, 1947; Kirby 1963). The capacity of the uterus for morphological change in the absence of a blastocyst has been investigated by stimulation of

the uteri of pseudopregnant mice with certain artificial stimuli which can evoke the decidual cell response (Krehbiel, 1937).

The original and commonest method for producing a decidual response is traumatization of the endometrium (Loeb, 1908). This method is, however, unsatisfactory from two points of view. In the first place, it cannot reasonably be expected to mimic the physiological stimulus, because from histological study it is clear that the blastocyst stimulates a decidual response well before there is any overt damage to the uterine epithelium. In the second place, the damage inflicted on the uterus makes interpretation of histological material difficult and uncertain.

Recently, Shelesnyak & Kraicer (1961) have suggested that the intraperitoneal injection of the drug Pyrathiazine provides a 'physiological' stimulus. However, de Feo (1963) has pointed out that any stimulus which is not acting in the same position as the blastocyst, i.e. in the uterine lumen, cannot be considered to mimic the blastocyst. The mere absence of operative trauma does not make a stimulus physiological. Several workers, Orsini (1963), de Feo (1963) and Finn (unpublished), have found, moreover, that this method gave a very poor percentage of positive responses. Furthermore, the description by Shelesnyak and his colleagues of the extremely troubled condition of the animals after the injection (Marcus, Kraicer & Shelesnyak, 1963), which has been confirmed by one of us (C.A.F.), combined with the widespread tissue damage shown *post mortem*, raises grave doubts about the 'physiological' nature of the stimulus.

From the foregoing it is apparent that a suitable stimulus, where histological work is to be carried out, must be one that is placed inside the uterine lumen, does not cause any overt damage to the endometrium and will reliably induce decidual cell formation. The intra-uterine injection of oil probably provides such a stimulus. Finn & Keen (1963) have reported that it is a reliable inducer of decidual changes in rats and this has now been confirmed in mice. It has been shown also that it causes no damage to the endometrium which can be detected by histological means (Finn & Hinchliffe, 1964).

In this paper Finn & Hinchliffe described the changes in the distribution of alkaline phosphatase occurring during implantation and during the artificial oil-induced decidual cell reaction in the mouse uterus. The appearance of alkaline phosphatase in the uterine stroma was shown to provide a very good indicator of decidual cell formation. After the intra-luminal injection of oil on the 4th day of pseudopregnancy there appeared to be foci of activity along the length of the uterus on the 6th day, with changes in the uterine epithelium resembling those found near an implanting blastocyst.

In the present experiments, these foci have been studied in more detail to gain information on the ability of the uterus to form implantation chambers in the absence of a blastocyst, and to compare the artificial with the natural chamber. In addition, because of the close association of alkaline phosphatase with neutral mucopolysaccharides (Moog & Wenger, 1952), uteri have been examined using the periodic acid–Schiff technique during the development of both types of chamber and during pseudopregnancy (the latter as a control). In the mouse, blastocysts are present in the uterus on Day 4 of pregnancy and

have nidated into implantation chambers by Day 6. The changes occurring over Days 4, 5 and 6 have, therefore, been studied.

MATERIALS AND METHODS

Mice of the CF1 strain randomly bred in the college's animal house were used. Females were placed overnight with entire or vasectomized males and checked for vaginal plugs the next morning. The day a copulation plug was found was taken to be Day 1 or the first day of pregnancy or pseudopregnancy. To induce the artificial decidual cell reaction 0·03 ml of arachis oil was injected through the utero-tubal junction on Day 4 of pseudopregnancy. The mice were killed by dislocation of the neck and the uterus removed and placed in fixative.

Staining for neutral mucopolysaccharides was carried out using the periodic acid–Schiff (PAS) procedure of Hotchkiss (as described by Gomori, 1952). Uteri were fixed in Carnoy's fluid and 10 μ paraffin sections prepared.

Glycogen was demonstrated by comparison of adjacent sections, the first, stained directly with the PAS reagents, the second after digestion for 1 hr at 37° C in 1% diastase made up in distilled water. Sites containing glycogen stain positively in only the first of the pair of sections.

Staining for acid mucopolysaccharides was carried out by the metachromatic staining method, using toluidine blue boiled with aluminium sulphate as described by Heath (1961).

TABLE 1

SUMMARIZED DESCRIPTION OF MATERIAL EXAMINED

	Day 4	*Day* 5	*Day* 6
Pregnant	6	11	14
Oil-injected pseudopregnant	—	21	24
Pseudopregnant	4	4	6

Staining for the presence of proteins in secretions was carried out on Carnoy fixed sections using the Hartig-Zacharias method (as described by Gomori, 1952) and the bromophenol blue method, described by Mazia, Brewer & Alfert (1953). Alkaline phosphatase activity was demonstrated by a slight modification of the Gomori (1952) calcium-cobalt method on material fixed in 80% alcohol (Finn & Hinchliffe, 1964). The number of animals examined using the PAS technique are shown in Table 1. In addition other mice were used for specific purposes as detailed in the text.

RESULTS

PSEUDOPREGNANCY

Sections of uteri stained with the PAS procedure on the 4th day of pseudopregnancy show that the glands contain positive material in their lumina, and that there are small quantities of a PAS-positive material on the luminal surface of the epithelial cells. This PAS-positive material persists to the 5th day of

pseudopregnancy, as does the material in the glands. On the 6th day, PAS-positive material is still found in the glands, but only in very scant amounts in the uterine lumen. The disappearance of PAS-positive material from the lumen over this period is associated with the disappearance of alkaline phosphatase from the uterine epithelium (Finn & Hinchliffe, 1964). This PAS-positive material presumably corresponds to the polysaccharides found in uterine washings from pseudopregnant rabbits (Killingbeck, Haynes and Lamming, 1963).

PREGNANCY

On the 4th day of pregnancy, the blastocyst is surrounded by a thick PAS-positive coat which is, presumably, the zona pellucida. In most cases the blastocyst is either firmly adherent to the uterus or strands of PAS-positive material are found sticking to the uterine epithelium, indicating that the blastocyst before fixation was attached to the uterus by means of the PAS-positive coat. The uterus stains in a similar way to the uterus in pseudopregnancy, with small quantities of positive material in the lumen and positive material in the lumina of the uterine glands.

On the 5th day of pregnancy (Pl. 1, Fig. 1) the blastocyst has lost its zona pellucida. The uterus still contains PAS-positive material in the lumen and the glands, which are disappearing from the stroma around the blastocyst, still contain positive material. There is no sign of PAS-positive material appearing in the stroma around the blastocyst.

On the 6th day of pregnancy sections containing a blastocyst and stained with haematoxylin and eosin show a differential response of the epithelium on opposite sides of the uterus. On the antimesometrial side the nuclei are pycnotic and the cells are breaking down. Often, during fixation, they become detached from the basement membrane, while on the mesometrial side the cells remain cuboidal. Compared with the previous day the distribution of PAS-positive material shows several changes (Pl. 1, Fig. 2). The basement membrane adjacent to the blastocyst is much thicker than it is mesometrially. Around the base of the implantation chamber the stroma contains PAS-positive material. Under high power magnification this material is found to consist of two components: first, deposits of glycogen which are removed by diastase digestion and which are found intra-cellularly, and second, material which appears to lie between the cells, which is not sensitive to diastase and which may be continuous with the basement membrane. The area containing this material appears to correspond with the alkaline phosphatase-free area found at this time. In the lumen, on the surface of the epithelial cells, is a PAS-positive secretion which resists diastase digestion. This secretion is found only in sections actually containing a blastocyst and in a few sections on either side of it. There is no secretion in inter-implantation areas of the uterus and none even in the outermost sections taken through a decidual swelling. The total amount of secretion is never very great. Usually it is found mesometrially to the blastocyst and never actually adjacent to it.

Longitudinal or transverse serial sections of the uterus show that, apart from the implantation chamber which reaches down to the centre of the decidual swelling, the lumen is pushed up against the mesometrial side.

Oil-injected group

After the injection of oil into the uteri of pseudopregnant mice the decidual response can be clearly seen macroscopically on the 7th day. The type of response varies a little. Usually several discrete swellings develop along the length of the uterus. Sometimes, however, the response is more or less uniformly distributed along its entire length to produce a sausage-like appearance. An analysis of some earlier work showed that the first type of response occurred in approximately 75% of twenty-four uteri that reacted. The decidual response is not visible macroscopically on Day 5.

Histological sections of the uterus at this time, stained with the PAS technique, appeared similar to those from 5-day pregnant animals, though, of course, there is no blastocyst. Slight amounts of PAS-positive substance are present in the uterine lumen and in the glands, but there is no strongly positive material in the stroma. The epithelium is intact and cuboidal and no difference can be detected between the mesometrial and antimesometrial sides.

The position of the decidual swellings can just be seen on the 6th day, especially after fixation and clearing, when they appear as opaque areas. Serial sections through these opacities show that they possess a very interesting internal structure. In sections taken from the area just outside the swelling, the lumen is pushed up against the mesometrial side of the uterus (Pl. 1, Fig. 3). Alkaline phosphatase-positive decidual tissue occupies most of the stroma and the epithelium is intact and devoid of the enzyme. As the sections get nearer to the centre of the swelling, the uterine lumen extends towards the antimesometrial side to form a chamber (Pl. 1, Fig. 4). The size and shape of the chambers vary considerably. The epithelial cells on the antimesometrial side are very low and give positive reactions for alkaline phosphatase. At the base of the lumen there is a stromal area free of alkaline phosphatase very similar to that found at the base of the implantation chamber.

Similar sections stained by the PAS technique (Pl. 2, Figs. 5, 6 and 8) show that the area around the base of the lumen contains PAS-positive material in the stroma, which, like that found at the base of the implantation chamber, consists of two components: diastase-sensitive granules within the cells and diastase-resistant material which appears to be between them. Within the lumen there are large quantities of a PAS-positive substance. This substance appears to be similar to the secretion found in the pregnant animals, although in the oil-stimulated uteri it is usually present in large quantities and not so clearly localized to the centre of the decidual swelling. By employing specific staining procedures, such as those of Hartig-Zacharias and the bromphenol-blue method of Mazia *et al.* (1953), protein has been demonstrated in the secretion. With toluidine blue there is no metachromatic reaction.

It is thus apparent that each of the deciduomata which appear as macroscopically visible swellings contains a chamber in its lumen which, histologically and histochemically, closely resembles an implantation chamber. Not surprisingly, however, there is considerable variation in the length of the chamber and those which appear macroscopically as a long sausage have the antimesometrial crypt more or less continuously along their length. This is shown by the fairly

uniform secretion in the lumen and the continuous epithelial thinning and glycogen deposition in the stroma.

It would be very interesting to know more about the origin of the focal response of the uterus. It could be due to the oil stimulating the endometrium only at certain points along its length or, alternatively, it could be due to the uterus organizing itself into definitive structures following a continuous stimulus. Finn & Hinchliffe (1964) showed that the uterine response can be clearly seen on the 5th day as demonstrated by the appearance of alkaline phosphatase in the stroma. It was not clear, however, whether the decidual response was continuous along the length as serial sections were not cut through complete uteri.

Ten pseudopregnant females were injected with oil and serial sections of their uteri examined for alkaline phosphatase on Day 5. Eight of these showed a positive reaction on the antimesometrial side. The sections show a clear response even when viewed with the unaided eye. No signs of foci of development were apparent. There was some degree of variation in the extent of the positive response, but, with two exceptions where a short break in the reaction occurred, the uteri had responded throughout their length.

DISCUSSION

The observations reported here have shown that in the majority of cases the pseudopregnant mouse uterus, after the intraluminal injection of oil, forms discrete chambers along its length which resemble closely the implantation chambers found in pregnancy. This is interesting in view of the finding that the initial reaction to the oil extends more or less continuously along the length of the uterus. It appears that the uterus, under the hormonal conditions of the 4th day of pregnancy or pseudopregnancy, has a certain 'competence' or ability to react to a stimulus causing development in a specific direction. This stimulus can be provided either by the blastocyst or artificially. The response to this stimulus is a sequence of events culminating with the formation of implantation chambers. One of the characteristic features of the artificial chamber is the accumulation in its lumen of large quantities of a PAS-positive substance. A positive response to the PAS technique is usually taken to indicate the presence of polysaccharide material. Nevertheless, the possibility that certain lipids, such as glycolipids and phospholipids, react positively with the PAS reagents has to be considered. Pearse (1960, p. 389) states, however, that 'in paraffin sections they [the lipids] are no longer present, or present in such small quantities that they do not react'. Hence with the technique used they would probably be eliminated.

As the secretion fails to stain metachromatically with toluidine blue, at least under the conditions of fixation used, and as it is diastase-fast, it is concluded that the secretion contains neutral mucopolysaccharides. It is Meyer's view (quoted by Pearse, 1960, p. 229) that such neutral mucopolysaccharides always occur in firm combination with protein and this agrees well with our own findings that the secretion stains positively with specific stains for protein.

The accumulation of the secretion in large quantities in the oil-induced decidual chamber, as compared with the small amounts of a similar secretion found near the blastocyst in the natural implantation chamber, is of interest. It

suggests that the secretion might be nutritive and in pregnancy would be taken up by the blastocyst whereas in the artificial implantation reaction it would accumulate. It might be questioned, however, whether large molecules such as proteins and polysaccharides can pass across the trophoblast into the blastocyst. On the other hand, Brambell, Hemmings & Rowlands (1948) have shown that protein agglutinins can pass unchanged from the maternal circulation into the rabbit blastocyst. This has been discussed by Lutwak-Mann (1963) who concludes that large molecules do pass into the blastocyst from the mother.

There has also been a suggestion by Böving (1963) that PAS-positive material may pass into the rabbit blastocyst about 6 days *post coitum*. The situation in the rabbit is, however, different from the mouse in that the blastocyst at this time is still surrounded by what Böving calls the gloiolemma, in addition to the zona pellucida and the mucous layer, which are all PAS-positive, whereas the blastocyst in the mouse is free of any coating. Böving claims that, originally, the gloiolemma may have been synthesized by the uterine epithelial cells. However, he regards this material as 'uterine fluid' similar to that found earlier in pregnancy and probably similar to the PAS-positive material found on the 4th and 5th days in the uteri of pseudopregnant or pregnant mice. It must be emphasized that the material associated with the implantation chamber which has been described in this paper is not formed in the glands and does not appear in the absence of a blastocyst or artificial stimulation.

Although histotrophic nutrition by the blastocyst has been suggested, it has usually been assumed that the nutrient is provided by the decidua as well as by degenerating epithelial cells which are engulfed by the trophoblast (Boyd & Hamilton, 1952, p. 74). Without denying that this may occur, the present authors believe a strong case can be made out for the active secretion of nutrient by the uterus as part of the response to the presence of the blastocyst.

Another characteristic feature of the implantation chamber is the presence of PAS-positive material in the stroma. This occurs in a small circumscribed area around the base of chambers containing a blastocyst but more diffusely around the antimesometrial end of the lumen in the artificial ones. This area corresponds to the alkaline phosphatase-free area found previously (Finn & Hinchliffe, 1964). The positive material contains two components, mucopolysaccharides extra-cellularly and glycogen intra-cellularly. Their function is unknown. In view of the position of this area immediately adjacent to the blastocyst, it is possible that it is a focal point of cellular growth and differentiation and that the mucopolysaccharides are involved in some way in this activity. Relevant to this may be the observation of Moscona (1960) who has demonstrated that disaggregated embryonic cells in tissue culture lay down a sort of template of mucopolysaccharide on which they aggregate. He suggests that this extracellular polysaccharide might, in this case, be responsible for integrating cell activity. It is also possible that the PAS-positive reaction might be due to the presence of lysosomes in this area. Lysosomes have been shown to contain substances, thought to be glycoproteins, which react with the PAS reagent (Novikoff, 1961, p. 443).

The presence of glycogen in this area could be associated with the high metabolic activity. Glycogen is certainly found in areas of high metabolic activity in

the developing embryo (Saxen & Toivonen, 1962, p. 27). Krehbiel (1937) has suggested, however, that the explanation of the presence of glycogen in decidual cells is that it is a food reserve for the embryo.

The other characteristic feature of the chamber is the differential response of the epithelium on the mesometrial and antimesometrial sides respectively. The significance of this was discussed in a previous paper (Finn & Hinchliffe, 1964).

These results demonstrate that the mouse uterus, far from being a simple repository for the blastocyst that limits excessive invasion through decidual transformation, plays a vital role in providing discrete implantation chambers and histotrophic secretions before the establishment of the placenta. The implantation reaction of the uterus, whether natural or artificial, is thus best looked upon as an example of morphogenesis, closely akin to morphogenetic processes occurring during embryonic differentiation. It is of interest to note that, in this case also, the natural stimulus for morphogenesis involves contact of one tissue with another, and that, in both cases, the natural stimulus can be replaced by an artificial one.

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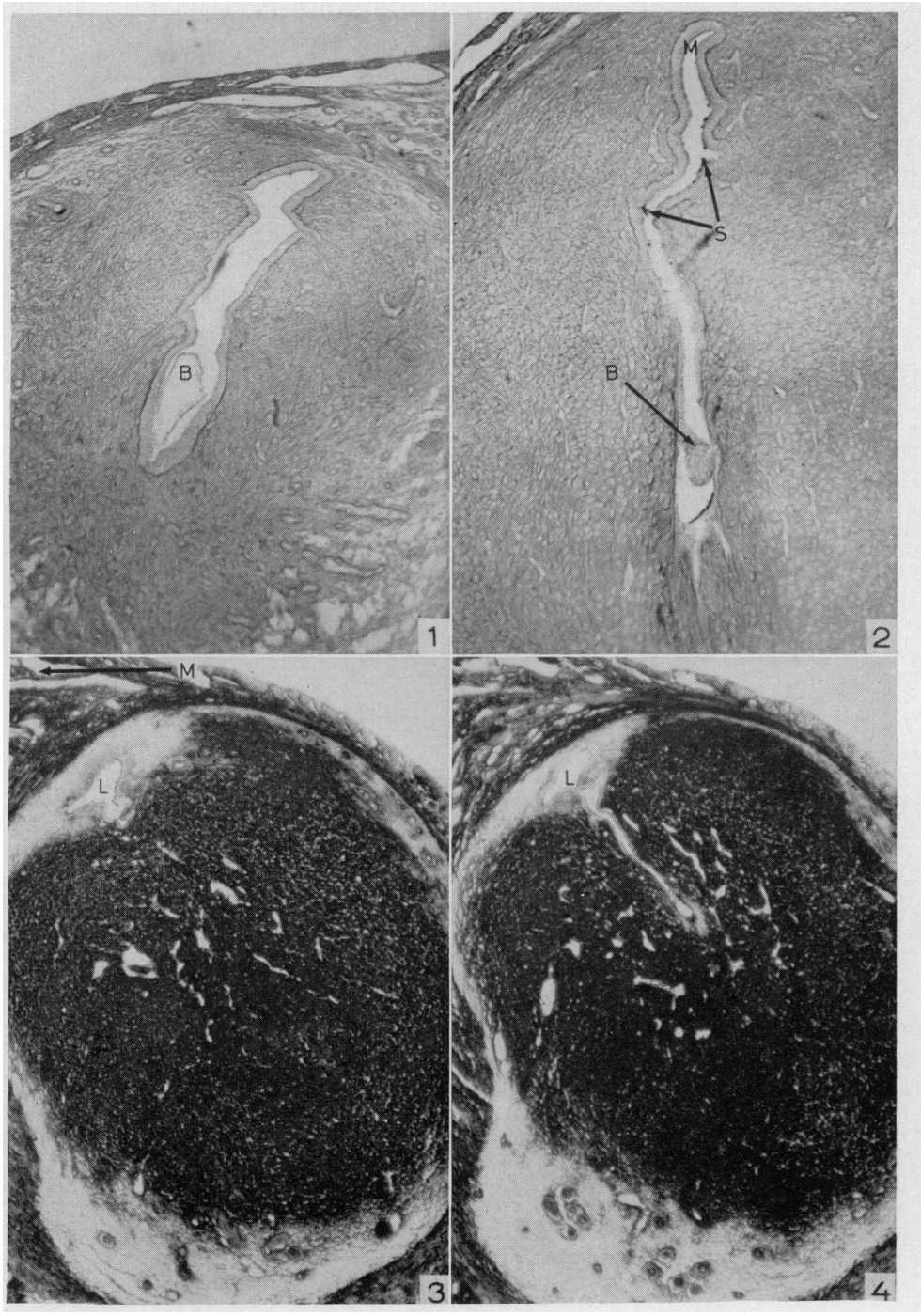
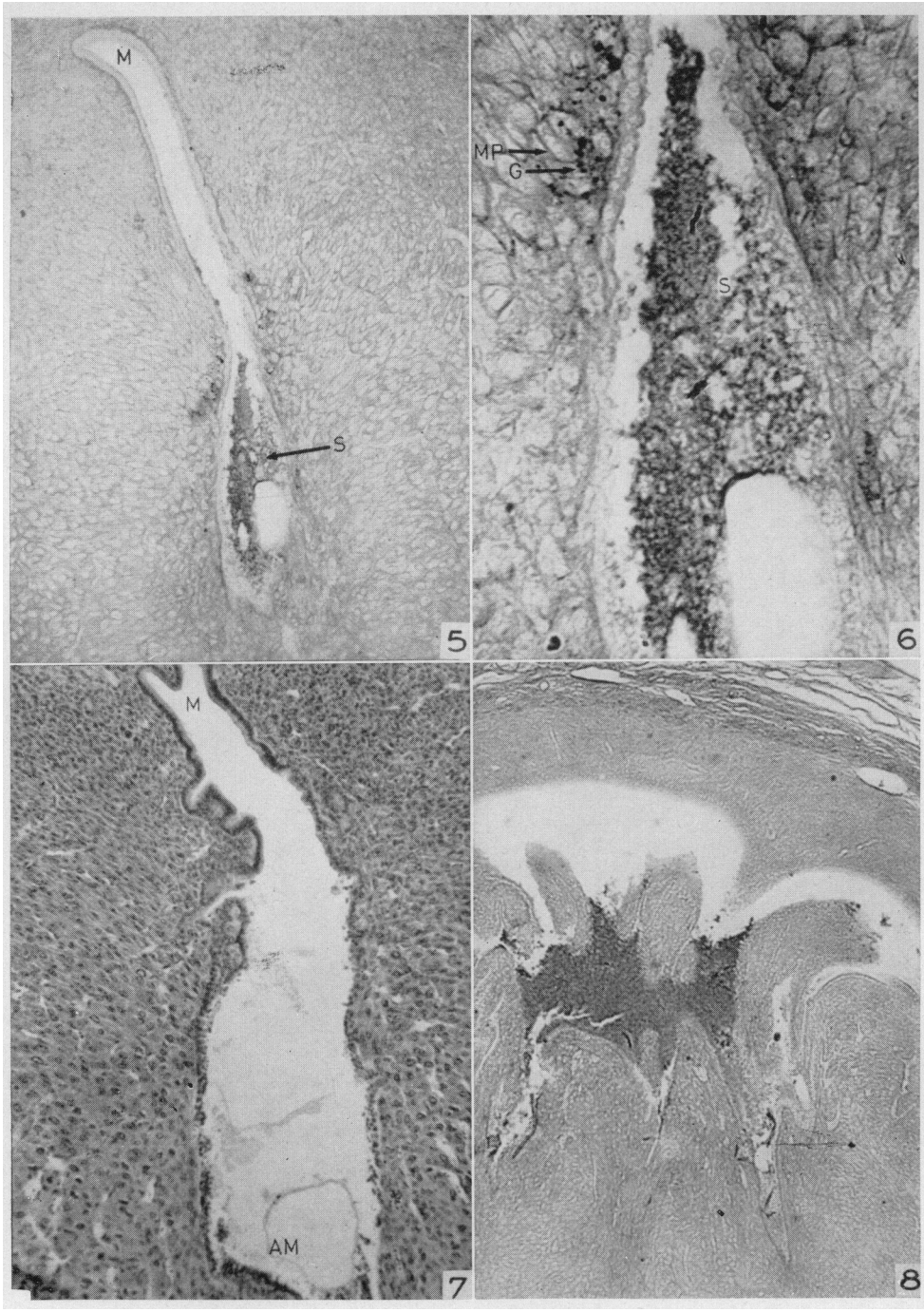
PLATE 1



(*Facing p.* 308)

PLATE 2



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EXPLANATION OF PLATES

PLATE 1

All the photographs of sections stained by the PAS technique have been photographed using a green filter so that the positive pink staining shows up black.

FIG. 1. Transverse section through mouse uterus containing 5-day blastocyst (B) stained by the PAS technique. ×100.

FIG. 2. Uterus containing 6-day implanting blastocyst (B). PAS-positive material around the blastocyst can be seen in the lumen attached to the epithelial cells and in the stroma. (M = mesometrial end; S = secretion.) × 100.

FIG. 3. Transverse section beyond margin of a 6-day oil-induced decidual swelling treated to demonstrate alkaline phosphatase. (L = lumen; M = mesometrium.) ×50.

FIG. 4. Section through middle of a 6-day oil-induced decidual swelling stained for alkaline phosphatase, showing the extension of the lumen (L) antimesometrially to form an artificial implantation chamber. ×50.

PLATE 2

FIG. 5. Section through centre of a 6-day oil-induced decidual swelling, showing secretion and PAS-positive material round the antimesometrial end of the lumen. (M = mesometrial end; S = secretion.) ×100.

FIG. 6. High power view of artificial implantation chamber illustrated in Fig. 5, showing glycogen granules (G) and mucopolysaccharide (MP) in the stroma. ×350.

FIG. 7. Transverse section through a 6-day artificial implantation chamber stained by PAS technique and counterstained with haematoxylin to demonstrate the differential response of the epithelial cells on the mesometrial (M) and antimesometrial (AM) side respectively. ×100.

FIG. 8. Longitudinal section through a 6-day artificial implantation chamber. ×50. (Dark area represents PAS-positive secretion).