Embryonic axis orientation in the mouse and its correlation with blastocyst relationships to the uterus

Part 1. Relationships between 82 hours and $4\frac{1}{2}$ days

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SUMMARY

Up until at least $7\frac{1}{2}$ days p.c., the orientation of all axes in the mouse conceptus, embryonic axis included, is directly correlated with two orientations of the 82 h blastocyst within the lumen. These two are: the almost horizontal position of the blastocyst's inner cell mass–abembryonic pole axis and the fixation of its abembryonic pole to either the right wall of the uterine horn (type-R orientation) or to the left (type-L orientation). At 88 h and at $4\frac{1}{2}$ and $6\frac{1}{2}–7\frac{1}{2}$ days equal numbers of conceptuses are found in type R and L orientations. Axes of symmetry also are recognizable in some developmentally advanced 82 h blastocysts and in all older conceptuses. For example, when the inner cell mass–abembryonic pole axis has become vertically oriented within the lumen by $4\frac{1}{2}$ days, the inner cell mass is oblique to this axis. It is concluded that the 82 h blastocyst's orientation to the uterine walls and floor provides it with positional information used in the location of its axes.

INTRODUCTION

When considering the factors involved in mammalian development, the uterus is usually assigned only the role of providing the environment necessary for embryonic growth and development, i.e. a permissive role. The possibility that the association between uterus and trophoblast might be instructive, that it might provide positional information directly or indirectly to the embryo comparable to that provided by some cell–cell interactions within the embryo is generally ignored. However, if the experiments are correct which suggest that mammalian eggs together with those of acoel turbellarians are unique among metazoans in possessing no morphogenetically significant and stable heterogeneities of their cytoplasm (Davidson, 1976) then the environment external to the developing mammal egg may very well play such an instructive role.

A mechanism involving a cell's position within the mouse morula (inside or outside) appears to be responsible for the determination: inner cell mass or

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trophoblast (Tarkowski & Wróblewska, 1967; Hillman, Sherman & Graham, 1972; Wilson, Bolter & Cuttler, 1972). However, it is difficult to imagine how such a mechanism, in the absence of environmental heterogeneity, can generate a cytoplasmic state which leads to embryonic axis orientation except as the result of stabilization of random fluctuations within the cells of the determined layers. If this is the case, the orientation of the axis would then be expected to show no particular relationship to any fixed structures outside the embryo. This is not what has been reported; instead the embryonic axis of the mouse embryo has been found to have a distinctive orientation to the walls of the uterus: more or less perpendicular to them (Snell & Stevens, 1966).

Data will be reported here which suggest that embryonic axis determination and a sense of right and left are the result of specific blastocyst-uterine relationships. The uterus appears to be supplying positional information to the developing mouse conceptus in a manner which can be considered to be an extension of the mechanism postulated to bring about polarity or localization of morphogenetic determinants within the oocyte. Just as substances under the control of the oocyte genome may be polarized or localized by the relationship of oocyte to ovary, substances under the control of either the mouse oocyte’s genome or of the embryonic genome may be polarized or localized by specific relationships of embryo to uterus.

MATERIALS AND METHODS

The conceptuses come from two sources: those 82–96 h old and many of the others are F2s from crosses between Balb C or Falconer’s J strains and strains heterozygous for T/t12 or T/t1. The rest are from the outbred Q strain. Mating was arbitrarily assumed to have occurred at 1 a.m. of the day a vaginal plug was found and the timing used here is the time of sacrifice of the female after mating. Females from which the younger conceptuses were obtained were present with males for a 2 h period centering around midnight: those from which the older ones came were with males continuously or for 15 h.

Before fixation the uterine horns were stimulated causing them to shorten, then pinned flat on paraffin wax. Most were fixed in Carnoy’s fluid without chloroform, a few in Bouin’s fluid. They were cut in transverse, frontal or sagittal section (Fig. 1) and stained with Azure B by the method of Flax & Himes (1952), by the PAS technique or with haematoxylin and eosin. Camera lucida drawings were made of every section of the 82 h blastocysts illustrated and their surrounding uterine walls. At least five sections of all older conceptuses were drawn.

Frontal sections of horn stained with Azure B proved to be the most useful. Azure B brings about much better discrimination between trophoblast and uterine cells and allows for easier determination of the implantational state of the horn than does haematoxylin and eosin. Because there is also a clear difference in the intensity of the stain in the polar trophoblast and inner cell mass (ICM) cells in implanting blastocysts and this difference persists until at least
Embryonic axis orientation in mouse. I

Fig. 1. Diagrams showing 4½-day blastocysts in type-R (upper) and type-L (lower) orientations as they would appear in the transverse (A), frontal (B) and sagittal (C) planes of the uterine horn. The location of the anterior end, right and left walls and dorsal and ventral surfaces of the horn is indicated. The direction of all putative blastocyst axes is given for Diagram (A). For (B) and (C) only the anterior (a) and posterior (p) sides and either the right (r) or left (l) half of the ICM, and by extension, the blastocyst are identified. The dorsal (d) surface of the ICM faces the polar Trophoblast; the ventral (v) surface, the blastocoel.

6½–7½ days, the development of the derivatives of these cells too can be more readily followed.

Until this study deviations of the plane of section from the perfect transverse plane (or irregularities of fixative or paraffin wax penetration) appear to have been considered to be responsible for any observed asymmetries of the blastocyst around its ICM–blastocoel axis or in its attachment to or implantation into the walls of the horn. Underlying this conclusion are the assumptions that the blastocyst is radially symmetrical and implants symmetrically. Although perfect frontal sections of the horn are no easier to cut than are perfect transverse sections, the angle of deviation of the sections can be more readily recognized. When the lumen is not symmetrical anterior and posterior to the conceptus, the sections...
incline toward the transverse plane; when the lumen is off center in the section, the sections incline toward the sagittal plane. If the ICMs of adjacent transversely sectioned blastocysts face toward different ends or walls of a frontally sectioned horn and the angle of cut of the sections containing the blastocysts is identical, the possibility that this is due to sectioning error is remote. If the mural trophoblast cells at the abembryonic pole of a blastocyst whose ICM faces toward the posterior end of the horn are always attached to a different wall of the horn than are the abembryonic cells of an adjacent blastocyst whose ICM faces the anterior end of the horn, these asymmetries cannot be explained on the basis of deviations from the perfect frontal section. Instead these differences must be due to asymmetries in the shape of the blastocyst around its ICM–blastocoel axis and in its behavior and orientation toward the ends and walls of the horn. The consistent correlation: ICM toward one end of the horn, then abembryonic mural trophoblast attached to a specific wall; ICM toward the other end of the horn, abembryonic pole cells attached to the other wall also eliminates the possibility that these differences are due to fixation or embedding-induced artifacts. That the ICM of the 4½-day blastocyst is tilted toward either the anterior or posterior end of the horn as is reported here can also be seen by examination of sagittal sections of the horn. That it is tilted toward the right or left walls can also be seen in transverse sections.

Terms of location used

Uterus. The dorsal surface of the lumen is also called its roof; the ventral surface, its floor. The median wall of the right horn is its left wall; the median wall of the left horn is its right wall. Wall is used only in reference to the horn; side, to the conceptus.

Conceptus. The ICM (and by extension the blastocyst as a whole) is described here as having poles of symmetry which correspond as closely as possible to the same poles in 6½- to 7½-day embryos as determined by retrograde analysis. Before differentiation of the epiblast and hypoblast, the ICM surface closest to the polar trophoblast is considered to be dorsal; the surface closest to the blastocoel, ventral. After their differentiation, the epiblast is considered to be located dorsally; the hypoblast, ventrally. The ICM’s putative anterior and posterior sides are the sides toward the wall of the blastocoel (yolk cavity) that the head process and primitive streak respectively face at 6½–7½ days. A right and left side is determined once the location of dorsal–ventral and anterior–posterior axes is known.

For easier visualization of the orientation of the blastocyst to the axes of the uterine horn, all descriptions here relate to what can be seen in blastocysts in horns sectioned frontally. All photographic illustrations are from horns cut in frontal section with the dorsal surface of the section toward the viewer. If the sections were viewed from the ventral surface of the section, the blastocysts would appear to be in mirror image orientation to the horn. The photographs
are oriented on the page as in Fig. 1B. From 88–90 h on, only blastocysts in the so-called type-L orientation to the horn are shown. Reversing the illustrations 180° will show how similarly aged blastocysts in type-R orientation are seen.

RESULTS

82 h

The blastocysts are clustered within the uterine horn lumen in three of the eight 82 h litters studied. In three, some are still clustered and in the remaining two, all are distributed along the length of the lumen. In spite of these differences in the spread of the 82 h blastocysts along the lumen, all have a distinctive orientation within it: their ICM–blastocoel axis is parallel to the floor of the lumen. This characteristic position is evident in Figs. 2, 4, 5 and 6 and indicates that blastocysts normally pass down the lumen with this axis almost horizontal. An additional orientation toward the ends and walls of the horn is also either suggested or clear. For example, the abembryonic pole of the upper blastocyst in Fig. 2A faces the right wall of the horn; the abembryonic pole of the other, the left wall. Furthermore, the polar trophoblast–inner cell mass (PT–ICM) complex of the upper blastocyst appears to be tilted toward the posterior end of the uterus while that of the lower, in other sections, appears to be tilted toward the anterior end. The two distinct orientations (type R and type L) to the ends and walls of the uterine horn in which all implanted blastocysts were found are shown in Figs. 1 and 3. The position of the upper blastocyst in Fig. 2A is suggestive of a blastocyst in type-R orientation; that of the lower, of a blastocyst in type-L orientation.

A portion of uterine horn containing two blastocysts that are plainly oriented is shown in Fig. 4 and the blastocysts in more detail in Fig. 2 (B and C). Portions of a zona are evident around one and both are surrounded by a heavy PAS-
Fig. 3. Diagrams showing suggested mechanism for the specific orientation of blastocysts within the horn. The horn is shown as if in frontal section. (A) The abembryonic pole becomes 'stuck' to the right or left wall at random. (B) The left wall responds by bulging into the lumen between the blastocyst and the posterior end of the horn and the ICM is carried toward the anterior end. The response of the right wall is reversed 180° and the ICM is carried toward the posterior end of the horn. (C) Continued reorganization and then decidualization of the stromal cells produces implantation chambers with asymmetries complementary to those of the blastocyst.

positive layer presumably zonal material although an acid fixative was used (Carnoy's). In Fig. 4 the 180° difference in the blastocyst–uterine wall orientation of the two blastocysts is evident. The left wall of the horn, which is in contact with the abembryonic pole of the upper blastocyst whose ICM is directed toward the anterior end of the horn, bulges into the lumen between the blastocyst and the posterior end of the horn. The right wall of the horn, which is in contact with the abembryonic pole of the lower blastocyst whose ICM is directed toward the posterior end of the horn, bulges into the lumen between it and the anterior end. The opposite wall is straight next to both ICMs. These two blastocysts then are in the orientations to the uterus called type L and R respectively even though a zona is still present. The basophilia and size of the subliminal stromal cells next to oriented 82 h blastocysts seems no different from that of more distant subluminal stromal cells suggesting that the bulging of the uterine walls into the lumen is due to reorganization of the stroma and not to differential decidualization. The difference in the orientation of longitudinally sectioned structures such as capillaries and glands in the two walls also suggests that stromal reorganization has occurred. Also illustrated in Fig. 4 is the regular zig-zag pattern of the lumen characteristic of all horns of this age in this study. Since all blastocysts that are distributed along the length of the lumen are located in or close to the point where the lumen changes direction it seems likely that the intersections are where the blastocysts lodge before implantation.

Note that three axes at right angles to each other can be drawn through these
Fig. 4. Frontal section of horn showing 82 h blastocysts in type-L (upper) and -R (lower) orientations at intersections where the zig-zagging lumen changes direction. PAS: Section 10 µm thick.
Fig. 5. Sections through six 82 h littermates. All except (F) found in good frontal sections of horn. Azure B. Sections 6 μm thick.
two blastocysts (Fig. 3B). Axis 1, approximately parallel to the anterior–posterior axis of the horn, passes from the embryonic to the abembryonic pole of the blastocysts. Axis 2, parallel to the dorsal–ventral axis of the horn, goes from the blastocysts' surface facing the roof to the surface facing the floor of the lumen. Axis 3 is approximately parallel to the median–lateral or right–left axis of the horn, this terminates at the sides of the blastocysts facing the right and left walls of the horn. If the latter sides of the blastocysts are designated a and b, the same side (a) of both blastocysts is against the uterine wall to which the abembryonic pole is attached.

Sections of six of nine blastocysts from one frontally sectioned horn which appear to be the most developmentally advanced of the 82 h blastocysts in this
study are shown in Fig. 5; a seventh in Fig. 6. As is evident, the sections of all except that in Fig. 5F are sections showing the same pattern of asymmetry not radial symmetry. The blastocoel of these blastocysts is wider and rounded on one side (Figs. 5A–C, E and 6B) and narrower and pointed on the other. The latter characteristic is particularly well shown in Fig. 6C, where the trophoblast cells at the pointed end look as if they may have been attached to the floor of the lumen. Furthermore, the ICM is wedge-shaped with the blunt side of the wedge toward the rounded side of the blastocoel (Figs. 5A, B, E and 6B) and the polar trophoblast cells over the blunt side seem to be more cuboidal (Figs. 5A, B and 6B).

Although these blastocysts clearly are not attached to the uterine walls like those in Fig. 4 and their ICM–blastocoel axis is more perpendicular than parallel to the anterior–posterior axis of the horn, they too are oriented with respect to its ends and walls. The abembryonic end of the blastocyst in Fig. 6 is toward the left wall of the horn and its pointed side is toward the posterior end of the horn while the blunt and rounded side of its PT–ICM complex faces the anterior end. This is a blastocyst in type-L orientation to the uterine walls as are the blastocysts in Fig. 5A, C and E. The blastocysts in Fig. 5B and D are in type-R orientation. The blastocyst in Fig. 5F and another not shown may be in type-L orientation although, as can be seen, the angle of the sections in which they were found made their classification very difficult. The ninth conceptus of this litter is too poorly developed to classify.

A reconstruction of the 82 h blastocysts in Figs. 5 and 6 is shown in Fig. 7A and a reconstruction of a 4½-day blastocyst in Fig. 7B. The similar asymmetries of their blastocoel wall has been used to identify the same sides and axes in both. The side of the 82 h blastocyst where the ICM is blunter and the wall of the blastocoel is rounded is certainly the same side as the rounded, so-called anterior side of the 4½-day blastocyst; the opposite side where the blastocoel is more pointed is the so-called posterior side. Axis 2 (Fig. 3B), therefore, is the same axis as the so-called anterior–posterior axis of the 4½-day blastocyst. If the dorsal...
Embryonic axis orientation in mouse. I

Table 1

<table>
<thead>
<tr>
<th>Age</th>
<th>No. of litters</th>
<th>Type of orientation to uterus</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>88 h</td>
<td>3 15</td>
<td>16</td>
<td>31</td>
</tr>
<tr>
<td>4½ days*</td>
<td>6 17</td>
<td>21</td>
<td>38</td>
</tr>
<tr>
<td>6½—7½ days</td>
<td>6 33</td>
<td>35</td>
<td>68</td>
</tr>
</tbody>
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* No data for blastocysts in one horn of two uteri.

surface of the ICM is considered to be the one toward the polar trophoblast; the ventral surface, toward the blastocoel; then axis 1 is the same as the dorsal–ventral axis in the 4½-day blastocysts and axis 3 is the right–left axis. Again it should be noted that side a, the right side, is the side of the blastocyst which lies against the wall to which its abembryonic pole attaches whether the blastocyst is in type-L or -R orientation. As is shown in Fig. 6, not only is the ICM–blastocoel axis of each of these blastocysts slightly tilted with respect to the floor of the lumen so that the ICM is closer to the roof but the so-called right–left axis appears to be slightly tilted with respect to the walls of the horn. That is, these sections suggest that if the blastocysts were looked down at through the dorsal side of the horn, more of their putative left than right side would be visible.

88–90 h

Sixty-one blastocysts in six uteri were studied at 88–90 h. All were distributed along the length of the lumen. Three of the uteri, fixed in the Bouin’s fluid with both horns intact and oviducts attached, provided the data in Table 1 on the orientation of the 88–90 h blastocysts. The ICM–blastocoel axis of each is now clearly almost parallel to the long axis of the horn and the PT–ICM complex of 16 of them is directed toward the posterior end (type R) while the PT–ICM complex of 15 is toward the anterior end (type L) of the horn. Only one litter of these three in which there was a distinct decidual reaction was further analyzed. There was no correlation found between the angle of the intersection of the segments of the lumen forming the zig-zag pattern (Fig. 4) (toward the right or left wall of the horn) or between the side of the intersection (anterior or posterior) and where type-L (5) and type-R (5) blastocysts are located in either horn. This appears to eliminate the possibility that there is a lateral versus medial response on the part of the horns rather than a right versus left response involved in the orientation of the blastocyst to the horn. Furthermore, this lack of correlation between the orientation of the blastocysts and the implantation site appears to eliminate the possibility that the blastocysts are somehow physically directed to the ‘proper’ sites.

By 88–90 h, although most blastocysts are still almost spherical, hypoblast
cells are present. The blastocyst in type-L orientation to the horn in Fig. 8A and B is now firmly fastened by abembryonic mural trophoblast cells toward its rounded side to the left wall of the horn. It is also fastened by the mural trophoblast cells of its pointed side (and close to the PT–ICM complex) to a more ventrally situated portion of the right wall. The PT–ICM complex is toward the anterior end of the horn and side a (Fig. 3B) or the so-called right side of this
blastocyst lies against the wall to which the cells of the rounded side are fixed. This is the position the upper blastocyst in Fig. 4 is in. The walls of the developing chamber surrounding the 88–90 h blastocyst strikingly resemble those next to this 82 h blastocyst. The left wall next to both is concave and between the abembryonic ends of both and the posterior end of the horn, the wall bulges into the lumen. The right wall next to both is straight. It is at this time that the first signs of decidualization are seen. By 88–90 h, the subluminal stromal cells adjacent to some blastocysts including this one are enlarged and show increased basophilia of their cytoplasm. In addition, the capillaries in the same region are occluded by hypertrophy of the cells making up their walls (Smith, 1966).

What appears to be 'transformation' (Dickson, 1966) of the mural trophoblast cells was first seen also in 88–90 h blastocysts. In another blastocyst of this age (Fig. 8C, D) many abembryonic pole cells are enlarged and appear vacuolated. This blastocyst in type-L orientation is somewhat ovoid, the blastocoel enlarged parallel to both the anterior–posterior and dorsal–ventral axes of the uterus. It is (or was) also firmly fixed to the uterine epithelium by its mural trophoblast cells in a pattern of attachment identical to that of the blastocyst in Fig. 8A and B. More of the lumen anterior and posterior to this blastocyst, however, is cut off from the main lumen and the width of the subluminal band of basophilic decidual cells is greater. An additional sign of beginning decidualization is the presence of cells which appear to be lymphocytes in the epithelium making up the floor of the chamber.

4–4½ days

In Fig. 9 is an example of an unusually developmentally advanced 96 h blastocyst in type-L orientation some of whose hypoblast cells now underlie mural trophoblast cells. Its ICM is clearly no longer a symmetrical disc at one end of its ICM–blastocoel axis and this axis is now vertical, i.e. parallel to the dorsal–ventral axis of the frontally sectioned horn. The asymmetry of the walls of the implantation chamber at the level of the asymmetrical PT–ICM complex of this blastocyst again strikingly resembles that of the walls of the lumen next to the 82 h blastocyst in Fig. 4. The concave left wall of the horn arches over one side of the complex and appears to be in intimate contact with the polar trophoblast. Lateral to the complex (Fig. 9A, B), this wall is in close contact with the polar trophoblast on the 'right' side of the complex. The attached mural trophoblast cells seen in A and B seem to hold the blastocyst to this wall. The right wall of the horn dorsal and lateral to the ICM is straight and most of the ICM–PT complex faces this wall and is cleanly separated from it. The polar trophoblast cells and the mural trophoblast cells underlain by distal endoderm facing the straight right wall are flatter and their cytoplasm is more basophilic than is the cytoplasm of the polar trophoblast cells facing the concave left wall (Fig. 9A, D). Furthermore the floor on the right side of the chamber and toward the posterior end of the horn looks as if it had buckled into the chamber (Fig. 9F). The mural
Fig. 9. Dorsal-to-ventral sections from a frontally sectioned horn containing a developmentally advanced 96 h blastocyst. (A) Section 1 of 10 sections of blastocyst. Cuboidal 'anterior' polar trophoblast cells in intimate contact with concave wall of chamber, 'anterior' mural trophoblast cells attached to same wall. (B, C) Sections 3 and 4. Flattened, basophilic 'posterior' polar trophoblast cells face the straight right wall. (D) Section 5. Hypoblast cells underlie flattened, basophilic 'posterior' mural trophoblast cells. (E) Section 7. (F) Section 9, 'posterior' mural trophoblast cells attached to epithelial shelf at right wall of chamber. Azure B. Sections 10 μm thick.
trophoblast cells closest to the floor of the lumen are fixed to the epithelial "shelf" formed by these invaginated cells. These trophoblast cells are larger and more vacuolated than are the other vacuolated mural trophoblast cells.

Two ICM–blastocoel axes can be measured in the sections of this blastocyst, one like Axis 2 (Fig. 3) almost parallel to the dorsal–ventral axis of the horn, the other like Axis 1 almost parallel to the horn's anterior–posterior axis. The longest ICM–blastocoel axis of this blastocyst is parallel to the dorsal–ventral axis of the horn and measures 100 \mu m; that in its littermates, 110, 120 and 150 \mu m. The axis parallel to the dorsal–ventral axis of the horn in 82 h blastocysts measured approximately 50–60 \mu m; that in the 88–90 h blastocysts, 60–70 \mu m. This axis in 4h\frac{1}{4}-day blastocysts is 150–270 \mu m in length. The length of the ICM–blastocoel axis parallel to the anterior–posterior axis of the horn slightly more than doubled between 82 h and 4h\frac{1}{4} days going from 60–70 \mu m in 82 h blastocysts to 110–120 \mu m in 4h\frac{1}{4}-day blastocysts. The wide variation in length of the first axis in the blastocysts of this and other 4- and 4h\frac{1}{4}-day litters suggests that the blastocoel begins to elongate very rapidly as soon as the sides of the blastocoel are securely fastened to the walls of the implantation chamber and the trophoblast cells begin to invade the uterine epithelium.

The asymmetries of the 4h\frac{1}{4}-day blastocyst in type-R or -L orientation are identical to those of the 4-day blastocyst just described and they continue to be matched by the same complementary asymmetries of the implantation site. (For staging purposes, the histological characteristics of the implantation site can be found in Smith (1966).) The 4h\frac{1}{4}-day blastocyst differs from the 4-day blastocyst in Fig. 9 as follows. Distal endoderm cells underlie the mural trophoblast cells on all sides of its ICM. Its blastocoel is much more elongated along the dorsal–ventral axis of the horn: Some of its mural trophoblast cells have replaced the luminal epithelium toward the floor of the chamber. The trophoblast cells on the side of the blastocyst facing the straight wall of the chamber and attached to or near the shelf of epithelium are usually larger and they replace more epithelial cells. The larger size of the latter trophoblast cells and the greater number of missing epithelial cells suggest that it is these trophoblast cells which first attached to the uterus.

Fig. 7B shows a reconstruction of a 4h\frac{1}{4}-day blastocyst made from sections which are transverse to its longest ICM–blastocoel axis as are those in Fig. 9. Of the 20 blastocysts used for this summary reconstruction, 14 were 200–230 \mu m long; the shortest, 190 \mu m long; the longest 270 \mu m. As is evident, the 4h\frac{1}{4}-day blastocyst is not radially symmetrical but bilaterally symmetrical. Not only is its PT–ICM complex no longer a symmetrical disc at one end of the blastocyst but the sides of the blastocoel bulge asymmetrically. This shape appears to be identical to that of the fully transformed blastocyst flushed from the uterus and photographed live by Dickson (1966, fig. 5).

The orientation of 38 4h\frac{1}{4}-day blastocysts to the anterior and posterior ends of the uterine horn and to its right and left walls is given in Table 1. Most of the
4½-day uterine horns were cut into at least two pieces before sectioning which made it impossible to determine the oviduct end of some of the pieces. These pieces were, therefore, arbitrarily assumed to have their oviduct at the same end and the orientation of the blastocysts in them was determined with respect to this presumed uterine orientation. Since all but one piece contained more than one blastocyst and adjacent blastocysts were found which were rotated 180° from each other, the error induced by this method of classification would not appear to be serious. As Table 1 shows, approximately 50% were oriented in one direction, and 50% at 180° from this direction, i.e. one half were in type-R orientation to the horn; one half in type-L.

6¾–7¼ days

The asymmetry of the 4½-day blastocyst is magnified when the polar trophoblast cells begin to differentiate (unpublished data). These polar trophoblast asymmetries plus those of the implantation site have allowed the development of blastocysts in type-R and -L orientation to be traced to 6¾–7¼ days when the head process and primitive streak can be seen. In Table 1 is a summary of the orientation of the embryonic axis of 68 conceptuses. The head process of approximately one half of them faces the right wall; the head process of the other half faces the left wall. From the asymmetry of both the ectoplacental cone and extra-embryonic ectoderm derivatives of the polar trophoblast of 6¾-day conceptuses and primarily of that of the extra-embryonic ectoderm of 7¼-day conceptuses (the ectoplacental cone is becoming symmetrical between 6¾ and 7¼ days) it was concluded that those embryos whose head process is toward the right wall developed from blastocysts whose abembryonic pole end was attached to the right wall at 82 h in type-R orientation. Those whose head process is toward the left wall develop from blastocysts whose abembryonic pole end was attached to the left wall at 82 h in type-L orientation.

DISCUSSION

Morphological asymmetries

In section the morphological asymmetries of the 82 h blastocyst are obvious. The blastocoel is rounded on one side and pointed on the other. Many of the illustrations of blastocysts flushed from the uterus at 3½ days suggest that these blastocysts share these differences and also the wedge-shaped ICM of blastocysts in section. See, for example, fig. 1C of Handyside (1978). What appear to be axes of symmetry are also present in all five of the blastocysts in this figure and these have the same orientation to the bottom of the culture dish that the axes (1, 2 and 3, Fig. 3B) of the 82 h blastocysts have toward the floor of the lumen. Photographs of other groups of blastocysts show them to be lying on either their ‘left’ or ‘right’ sides (Mintz, 1971, fig. 9) so that a tilted culture dish or other differences in their handling does not appear to be responsible for the asymmetries
seen. These morphological differences are apparent in section only in blastocysts which have reached their implantation site; in blastocysts in vitro they are evident only at more advanced stages of blastocoel formation (Handyside, 1978, fig. 1B). Asymmetry of the blastocoel also seems to characterize blastocysts in lactational delay (McLaren, 1970, fig. 1). This blastocyst, in addition, appears to be oriented toward the uterus in the manner described for 88–90 h blastocysts.

It seems reasonable to suggest that differences in the environmental influences to which its surfaces are exposed by virtue of its horizontal position in the uterus lead to the morphological asymmetries of the 82 h blastocyst. This explanation, however, requires that the surfaces toward the dorsal and ventral walls of the lumen remain in constant orientation to the dorsal–ventral axis of the lumen as the blastocysts are moved along it to their implantation site. Even though the zona enclosing them may rotate, the ICM versus blastocoel difference and a flattening of the blastocyst along one axis which Enders (1971) (mouse) and Huber (1915) (rat) describe may prevent the rotation of the blastocyst itself around its ICM–abembryonic pole axis and gravity may keep this axis parallel to the floor of the lumen.

Contact with the luminal floor may elicit not only a ‘pointed’ response but a ‘posterior’ response from the 82 h blastocyst. At the least, it brings about conditions which will later lead to a posterior response on the part of the ICM cells on this side of the blastocyst. The similar asymmetries of the 82 h to 4 1/2-day blastocysts and the comparable complementary asymmetries of the uterine walls surrounding them when in both type-L and -R orientations indicate that the side of the blastocoel which is pointed and lies against the floor of the lumen at 82 h is the side of the blastocyst which faces toward the straight wall of the chamber at 4 1/2 days. The inclination of most of the 4 1/2-day polar trophoblast toward the straight wall of the chamber and the difference in the development of the polar trophoblast cells facing the straight and concave walls through at least 7 1/2 days, together with the continued asymmetrical shape of the chamber, made it possible to determine that the primitive streak develops on the side of the egg cylinder which is toward the straight wall of the implantation chamber. The portion of the ICM on the pointed side of the 82 h blastocyst is, therefore, its putative posterior part; the portion on the rounded side, its putative anterior part. The similarity in the proportion of blastocysts in type-R or -L orientation at progressively older stages of development supports this conclusion. Since a small proportion of 2-cell-stage eggs can develop in vitro into almost normal-looking 8-day embryos (Hsu, Baskar, Stevens & Rash, 1974), this suggests that it is position in the horns rather than specific information from the horn that the blastocyst is using to locate its axes.

Functional asymmetry at 82 h

The difference in the appearance of the walls of the 82 h horn faced by the ICM and by the blastocoel indicates that by this time the mural and polar tropho-
blast cells are already functionally different even though the blastocyst may still be within the zona. That this difference is due to blastocyst differences and not to uterine wall differences is shown by a comparison of the effects of the 82 h blastocyst on the uterus and the effect of a uniformly irritating object on a pseudopregnant uterus. The effect of the latter is shown in fig. 3 of McLaren (1968) where the apposed non-decidualized luminal walls are perfectly symmetrical around a spherical Diakon bead.

**Blastocyst-uterine orientation**

The two 82 h blastocyst-uterine orientations, type-R and -L, may be explained as follows (Fig. 3). If the right and left walls of the horn respond identically to stimulation by the abembryonic end of the blastocyst but have reversed polarity, the ICM end will become differently oriented with respect to the ends of the horn, depending on the wall to which attachment is made. That is, if stimulation of the left wall by the abembryonic pole causes it to be thrust into the lumen between the blastocyst and the posterior end of the horn, the ICM end no matter what its original orientation to the ends of the horn will be carried toward the anterior end of the horn in type-L orientation. If stimulation of the right wall by the abembryonic pole causes it to be thrust into the lumen between the blastocyst and the anterior end, the ICM will be carried toward the posterior end of the horn in type-R orientation. Blastocysts whose abembryonic poles attach to and stimulate opposite walls of the horn will then become oriented at 180° from each other.

This particular explanation of blastocyst orientation has been selected because it will also account for the finding that it is always the 'right' side of the blastocyst which faces the wall of the horn to which its abembryonic end is attached (Fig. 3). That is, it will explain why blastocysts are not found in the two orientations which are the mirror images of type R and L. An alternative explanation; that the blastocysts which pass down the lumen with ICM toward the anterior end always attach to the left wall and those which pass down the lumen with ICM toward the posterior end always attach to the right wall of the horn, requires that the blastocyst have a sense of right and not-right at this time and that it selects the wall to its right to which to attach. It is difficult to conceive of a basis for such knowledge.

If conditions can be achieved *in vitro* which will bring about determination of the embryonic axis and allow almost normal development to the early somite stage (Hsu *et al.* 1974), what is the functional significance of the additional positional information the blastocyst receives by virtue of its specific relationship to the right and left uterine walls? One obvious time at which knowledge of right and not-right is required is when bilaterally symmetrical structures such as the heart become asymmetrical. Much earlier right versus left differences in development have been found, however (unpublished data). The direction of the involution of the extra-embryonic ectoderm into the yolk cavity beginning at
5 days is such that the ICM is not only "pushed" ventrally into the yolk cavity but toward the cavity's 'right'. It is on the side of the egg cylinder toward the 'right' side of the yolk cavity that the head process develops at 6\(\frac{1}{2}\) days.

**The change from the horizontal**

How do the horizontally situated *rounded* and *pointed* sides of the 82 h blastocyst come to be situated vertically by 4\(\frac{1}{2}\) days? Any postulated method whereby this is accomplished must also account for the oblique position of the PT–ICM complex with respect to the ICM–blastocoel axis. Kirby, Potts & Wilson (1967) suggested that the ICM assumes its position toward the dorsal side of the horn by migrating along the walls of the implanting blastocyst. However, Gardner & Johnson (1975) failed to find any change in position of the ICM relative to marked trophoblast cells. Gardner (1977) further states that the position of the ICM relative to melanin-marked trophoblast cells did not change in blastocysts transferred to pseudopregnant females and examined when their long axis was vertical. Based on the data reported here, at least three factors seem to interact to bring about the change in the position of the blastocyst axis. They are: expansion of the blastocoel, the shape of the implantation chamber and the specific mural trophoblast cells that first firmly fix the blastocyst to the walls of the uterus. As was previously stated, the first cells to attach to and begin to invade the uterine epithelium appear to be those mural trophoblast cells forming the pointed side and which face the floor of the lumen (Fig. 8B, D). These attach (closer to the left wall when in type-R orientation; closer to the right wall, in type-L orientation) to the shelf of uterine epithelium (Fig. 9F) which early uterine reorganization has caused to appear toward one wall of the implantation chamber. The axis is already slightly tilted from the horizontal in developmentally advanced 82 h blastocysts (Fig. 6) and may become more tilted at 88–90 h because of the presence of the shelf. While these trophoblast cells are attaching to the floor of the chamber toward one wall, other abembryonic pole trophoblast cells on the rounded and more dorsally situated side of the almost spherical blastocyst become firmly fixed to the opposite wall of the chamber (Fig. 8A, C). This limits the possible future positions of the blastocyst. As is shown in the illustrations and by measurement, the blastocoel then undergoes a very rapid expansion which is much greater along its axis parallel to the anterior–posterior axis of the horn than in a line parallel to its right–left axis. (The length of the latter axis remains almost unchanged even when measured in the longer 4\(\frac{1}{2}\)-day blastocysts.) At the same time, the chamber is being cut off from the lumen anterior and posterior to the blastocyst (Fig. 8) and it is closed off from it entirely ventral to the blastocyst. These facts suggest that the unattached ICM end of the 88–90 h blastocyst is forced to rise upward toward the dorsal side of the lumen and that the rounded and pointed sides of the blastocyst come to be oriented vertically because the fixed blastocyst enlarges and elongates within a limiting chamber. This explanation is consistent with the report (Gardner, 1977) that blastocysts
with ICMs at both ends of the blastocoel tend to implant with their ICMs perpendicular to the dorsal-ventral axis of the uterus. If the first cells to attach to the walls of the horn are those close to the ventrally directed side of the ICM, there is no free end to be carried dorsally by the expansion of the blastocoel. It is also consistent with the finding that rat blastocysts implanted in their normal orientation to the dorsal-ventral axis of the horn even though the dorsal-ventral axis was reversed (Alden, 1945). It is also possible that since the attachment of the mural trophoblast cells to the walls of the chamber appears to occur in the direction—abembryonic pole toward ICM, the blastocyst may help the pull itself upright if the mural trophoblast cells on the rounded side attach one after the other to the closer concave wall. The difference in expansion of the blastocoel seems to be intrinsic since delayed implantation blastocysts in non-decidualized horns are elongated rather than spherical. Expansion seems to occur because the unattached mural trophoblast cells flatten thereby increasing their surface area.

The ICM becomes asymmetrical

The ICM becomes oblique to the long axis of the blastocyst because the side of the ICM complex which is closer to the floor of the lumen at 82 h remains closer to the floor of the lumen than does the other side when the ICM as a whole is carried toward the roof of the lumen as the blastocoel expands. Two factors could account for the difference in the length of the rounded and pointed sides of the blastocyst which keeps the ICM on the pointed side closer to the floor. Since the first cells to attach to the developing chamber appear to be those forming the pointed side, this will limit the number of cells on this side which can be carried dorsally as the blastocoel expands. It is also possible that there is an intrinsic difference between the rounded and pointed sides of the blastocyst in ability to expand.

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