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Structural Dynamics and Function of Early Embryonic Coats

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Key Words

Embryonic coats · Blastocyst coverings · Development · Implantation · Eutherian mammals

Abstract

The extracellular embryonic coats (embryo/blastocyst coverings) that surround early mammalian embryos are most often still referred to as zona pellucida. Accumulating evidence from a number of earlier and recent studies clearly indicates that this is an oversimplification which cannot be defended anymore, at least in many species. Structural modifications of the coats occur during cleavage and blastocyst stages; these are most obvious in a number of species with the central type of implantation and, related to this, a high degree of blastocyst expansion, notably the rabbit and the horse. In this contribution, formation and transformation of the various layers of coats (zona pellucida, mucoprotein layer, neozona and gloiolemma in the rabbit, capsule in the horse) will be reviewed, as will be comparable structures seen, e.g., in the fur seal (subzonal layer) and the baboon. These phenomena will be discussed in the context of structurally more subtle changes found in other species, including those with small blastocysts and other types of implantation, in particular with biochemical modifications which may be physiologically quite important. The molecular mechanisms of deposition of coats and their transformation and shedding/dissolution will be briefly addressed. The possible functional significance of the coats and their transformation will be discussed (mechanical, morphogenetic and immunological role, molecular transport control, blastocyst positioning, implantation, trap and reservoir function for signalling molecules).

Introduction

The extracellular embryonic coats (embryo/blastocyst coverings) that surround the early embryo of eutherian mammals (usually up to the blastocyst stage) are most often referred to as *zona pellucida*. In recent years evidence has accumulated which shows that this is an oversimplification which cannot be defended anymore, at

Abbreviations used in the paper

p.c.	post coitum
IVF-ET	in vitro fertilization-embryo transfer
PVM	perivitelline space matrix
ECM	extracellular matrix

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least with respect to many species. Chemical modifications occur in the zona pellucida after fertilization, and often even structural transformations take place which continue during cleavage and blastocyst stages. These changes are most obvious in a number of species with the central type of implantation and, related to this, a high degree of blastocyst expansion. Changes may remain structurally more subtle, however, particularly in species with small blastocysts and other types of implantation; these changes on the other hand include biochemical modifications which may be physiologically quite important.

It appears remarkable that the molecular and structural dynamics of the embryonic coats of mammals has up to this point received little attention in experimental investigations. Indeed, even the nomenclature proposed to describe the various new layers of coat material which appear on the embryos during development, e.g. in the rabbit (to be reviewed below), is rarely being used, and many authors still stick to the term zona pellucida even in this case. The main reason why so little attention has been paid to blastocyst coats is most probably that interest is focussed on the important role that the original zona pellucida plays during fertilization, being a receptor for sperm adhesion, eliciting the acrosome reaction, serving as a matrix for sperm penetration and forming a barrier to polyspermy after the cortical reaction has occurred (to be discussed further below) [Prasad et al., 2000]. The blastocyst coats (coverings as they were called earlier) have predominantly been regarded just as a potential physical impediment for proper blastocyst expansion and for attachment of the trophoblast to the endometrium at implantation initiation: for example, when early rabbit embryos are cultured in vitro, the normal transformation of the coats (to be described below) does not occur, and during attempted blastocyst expansion, breaks appear in the relatively rigid nondissolved zona pellucida so that the trophoblast protrudes (herniates) into the surrounding *mucoprotein layer* (fig. 1b). This phenomenon has received attention since it seems to lead to disturbances in normal blastocyst development [Lewis and Gregory, 1929; Adams, 1970; Kane, 1975; Nozawa, 1976; Fischer et al., 1991; Maurer and Denker, unpublished]. That the blastocyst coats/coverings can be an impediment for implantation is shown impressively when the hatching proteinase system (blastolemmase) is blocked by intrauterine infusion of appropriate non-toxic proteinase inhibitors in the rabbit: in this case contact formation of trophoblast and uterine epithelium and subsequent invasion are effectively inhibited (fig. 1c). Interestingly, development/differentiation of the embryo can continue for quite a while in these unattached post-blastocyst stages (thus showing a high selectivity of the treatment) [Denker, 1977; Meinshausen and Denker, 1983]. It has long been well known that in the guinea pig the trophoblast develops specialized pseudopodial protrusions in order to penetrate the zona pellucida at implantation initiation thus demonstrating morphologically that here is indeed some type of barrier [von Spee, 1901; Blandau, 1971; Enders and Schlafke, 1969].

In in vitro fertilization and embryo transfer (IVF-ET), zona hardening is of concern, and the techniques of zona drilling and assisted hatching are being discussed vividly with respect to their value [De Vos and Van Steirteghem, 2000; Magli et al., 1998]. Surprisingly, however, relatively little effort has been invested into basic research on the chemistry of zona hardening and its possible prevention and/or reversal, and the limited work that has been done has usually concentrated on the original zona, not including material that is added to it during tubal and uterine passage in vivo. However, I feel that such studies could well profit from comparison with investigations on coat formation and transformation at later stages (to be discussed below).

Apart from these considerations with respect to fertilization and implantation, the extracellular egg or embryo coats have received relatively little interest. It is realized that the zona pellucida has a morphogenetic role in holding blastomeres of early cleavage stages together, thus preventing monozygotic twinning. Also, some observations suggest a so far unexplained influence of the zona on the rate of cell division and also the distribution of cells to the trophoblast and embryoblast [Eckert et al., 1997]. The morphogenetic role may indeed deserve more careful consideration (see below). An immunological role and a function in molecular transport control have also been discussed for the coats, but available information remains very limited. Overall, the coats of early mammalian embryos appear as a largely neglected research topic. Recently, however, this seems to be changing since reports on the presence of growth factors and cytokines and related modulating molecules in these coats are eliciting special interest [Herrler and Beier, 2000].

This review will focus on evidence suggesting that blastocyst coats are indeed different from the original zona pellucida in many (if not all) species, on mechanisms of coat transformation and on aspects of embryo coat function.

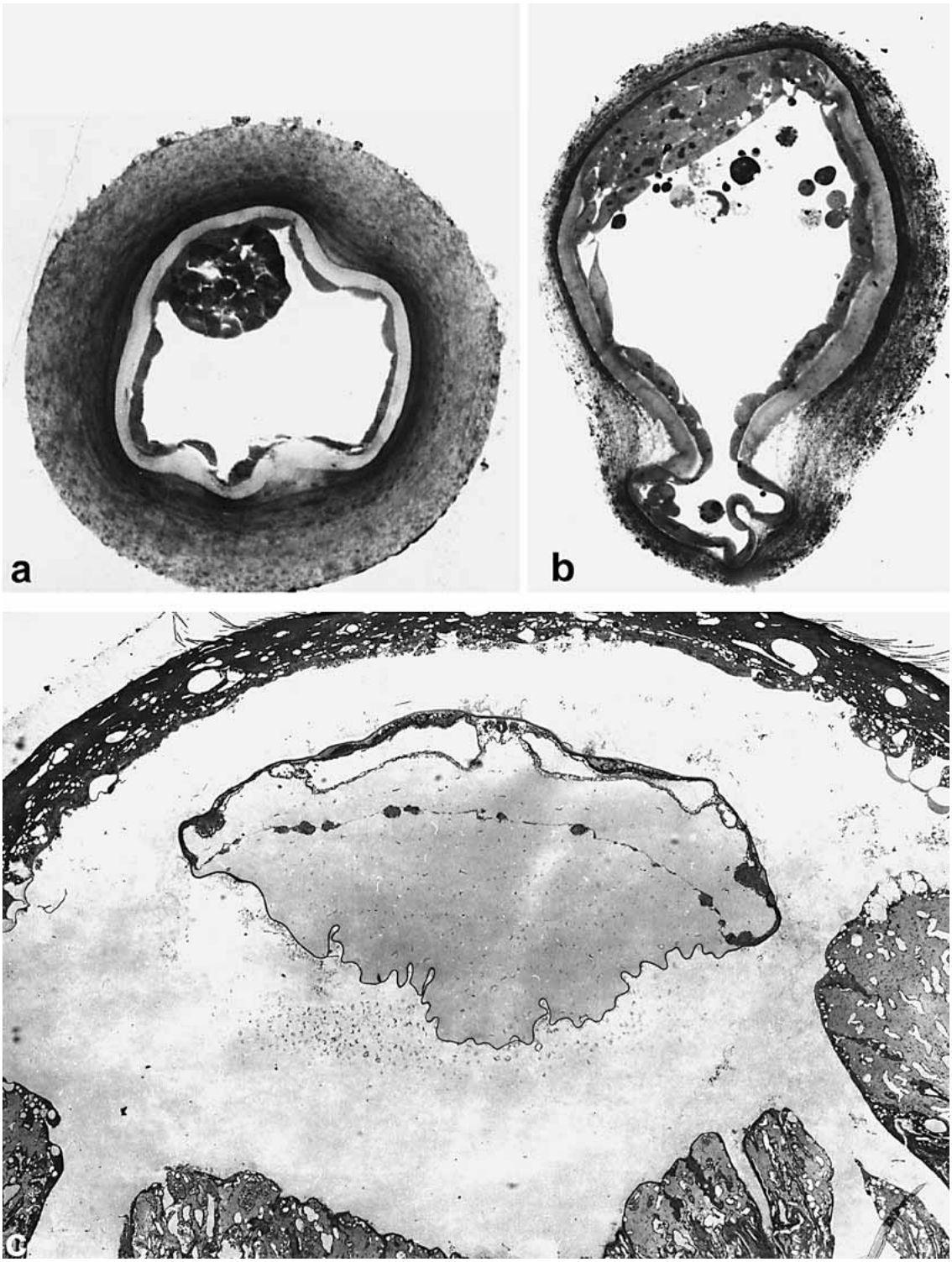

Fig. 1. Embryonic coats as an impediment for blastocyst expansion and implantation. **a** A normal early blastocyst of the rabbit (3.5 days p.c.), developed in vivo, shows two layers of coats: a thick mucoprotein layer (outside) surrounds the zona pellucida (bright band). Semithin section, alkaline toluidine blue staining. × 280. From Denker and Gerdes [1979]. **b** During in vitro culture, rabbit blastocysts have difficulties in expanding properly, in part due to insufficient transformation of the coats. This blastocyst was cultured in vitro for 48 h from the morula stage on (66 h p.c.) in conventional, BSA-containing medium. Regular dissolution of the zona pellucida has not taken

Transformation of Early Embryonic Coats: Morphological and Biochemical Evidence

Transformation of Embryonic Coats (Blastocyst Coverings) in the Rabbit

Tubal eggs and embryos of the rabbit have for a very long time been known to be surrounded not only by the *zona pellucida* but also by tubal secretion-derived mucinous coat material, the *mucoprotein layer* (also called mucin coat, mucoid coat, albumen layer, or mucolemma) [Cruickshank, 1797; Barry, 1839; von Baer, 1837; Bischoff, 1842; Böving, 1954, 1957]. Most authors tend to assume that these two coat layers persist during blastocyst expansion until they are shed at implantation. However, even simple direct observation shows that the physical properties of the coats (blastocyst coverings) change considerably during blastocyst expansion. Everybody who manipulates rabbit blastocysts is impressed by the strength, even rigidity of rabbit blastocyst coats at, e.g., 5–6 days p.c. During my early work I observed that the coats of such middle and late blastocyst stages become very brittle after formaldehyde fixation and paraffin embedding (which is a nuisance for the histologist), whereas at earlier stages they do not. Histochemical studies then showed that this is accompanied by or due to chemical changes: so, e.g., neuraminic acid which is not present in the mucoprotein coat (although it is in the zona pellucida) appears in most if not all the various layers of late rabbit blastocysts [Denker, 1970a, b].

place, but there are local defects in it at the embryonic and the abembryonic pole, and the trophoblast herniates into the mucoprotein layer (below). Semithin section, alkaline toluidine blue. × 440. From Maurer and Denker (unpublished). **c** When the hatching enzyme system (specifically, blastolemmase) is blocked by intrauterine infusion of appropriate proteinase inhibitors, dissolution of blastocyst coats (coverings) does not take place and thus trophoblast attachment to the endometrium and implantation are prevented. In this case, aprotinin (Trasylol®) was infused into the rabbit uterine lumen at a rate of 84 µg/h from 5.5 days p.c. on, using an Alzet® minipump. At 9.5 days p.c. (i.e. 2.5 days after implantation had started in the controls) this blastocyst still lies free in the uterine lumen, completely surrounded by its coats (dark, waved line; the abembryonic trophoblast with many trophoblastic knobs has retracted from the coats due to fixation and embedding). Note that this blastocyst is improperly oriented in the uterus, with its embryonic pole facing the antimesometrial endometrium (above). Interestingly, it has continued developing at an only slightly reduced rate, in spite of the fact that it has not implanted. It shows a neural tube, somites, somatopleure, splanchnopleure, exocoelom and the thickened syncytiotrophoblast of the embryonic pole. Semithin section. ×22. From Meinshausen and Denker [1983]; for details see also Denker et al. [1989].

This has prompted more detailed studies [Denker and Gerdes, 1979] from which the following conclusions were drawn concerning the dynamics of rabbit blastocyst coat/coverings formation and transformation (fig. 2a): at the outer surface of the zona pellucida a thick mucoprotein layer is deposited during tubal passage. This material is very rich in sulfate ester groups and therefore strongly negatively charged. It can still be seen clearly at early blastocyst stages around 4 days p.c., but there is evidence that it loses part of its sulfate ester groups, beginning from the interior, from around 3 days p.c. on [Denker, 1970a, b; Iwaki and Nozawa, 1978]. Already during earlier cleavage stages, changes in protease sensitivity of the coats were observed [Ziomek et al., 1990]. During blastocyst expansion, which attains considerable degrees in the rabbit (the diameter of the blastocyst around implantation at 7 days p.c. when the coats are shed is about 5 mm), the mucoprotein layer and the zona pellucida are naturally distended and thinned out. The mucoprotein layer remains visible, however, at least in the electron microscope (fig. 2a, b). With respect to the zona pellucida it has been calculated that stretching thins it out to a degree that it is not visible anymore in the light microscope [Böving, 1954, 1957, 1972]. Electron microscopy showed, however, that it is dissolved away completely from the inside of the mucoprotein layer at the early blastocyst stage, around 4.5 days p.c. and, even more interestingly, it becomes replaced by new material deposited at this place immediately thereafter. This new material accumulates to form a new innermost layer of the blastocyst coats which we called *neozona*. Finally a fourth type of material is deposited on the outside of the mucoprotein layer, the 'gloiolemma' (following the terminology proposed by its original describer, Böving). The gloiolemma appears to be derived from uterine secretion material, and its thickness is quite variable from one blastocyst to the other. The coats of a late blastocyst of the rabbit are, therefore, composed of three layers, i.e. from the inside to the outside the neozona, the mucoprotein layer and the gloiolemma (fig. 2a, b).

These three layers are of different origins. The middle layer appears to represent remnants of the tubal secretion-derived mucoprotein layer. The outer layer (the gloiolemma) seems to be derived from uterine secretion material, as suggested by the timing of its deposition and by its morphological similarity to concretions of mucous material that may be found here and there in the uterine lumen. The innermost layer, the neozona, could theoretically be derived from trophoblast secretion material or from molecules that are secreted by the endometrium and that diffuse through the outer layers and are deposited at this spe-

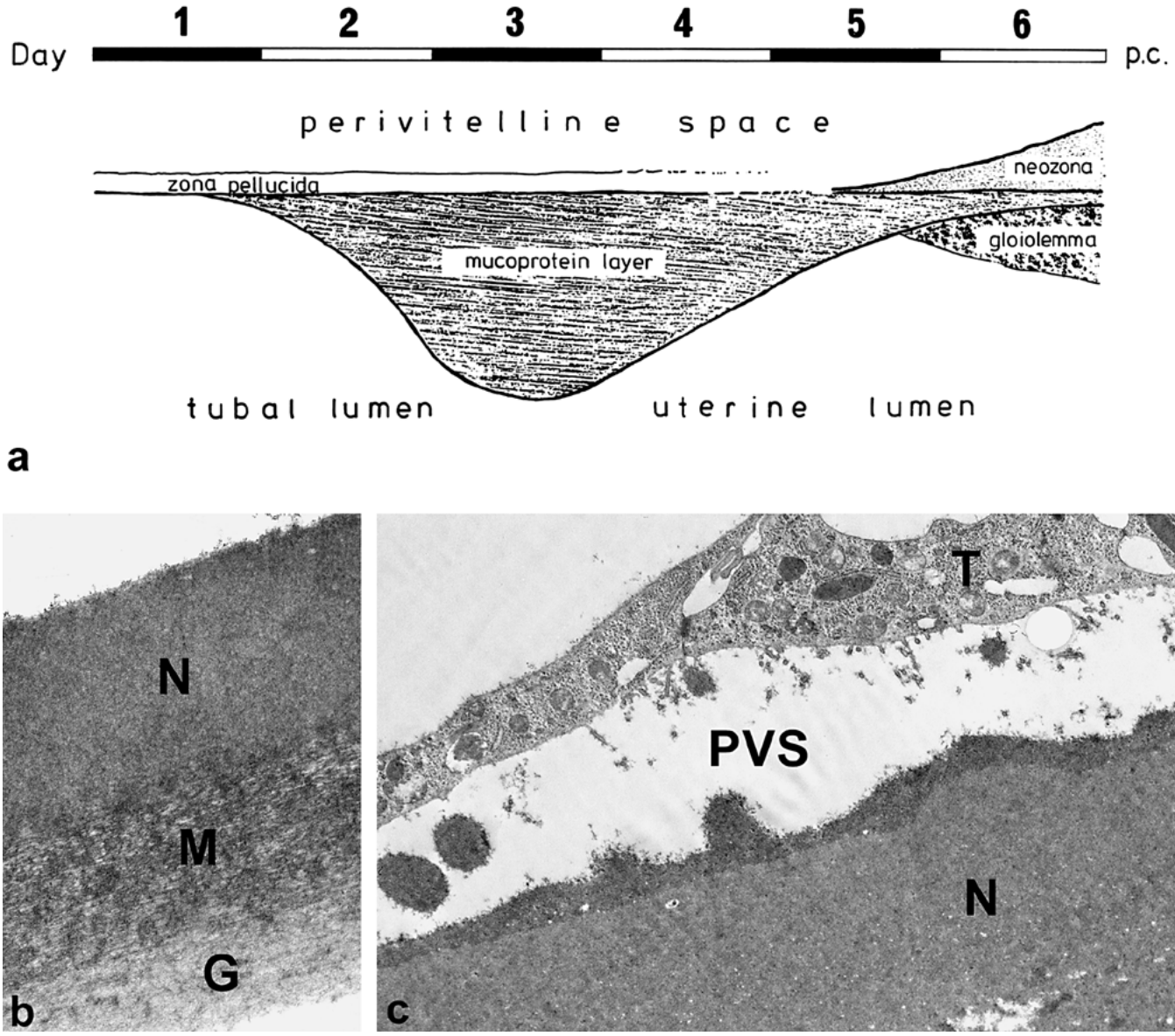

Fig. 2. Transformation of embryo coats in the rabbit. **a** Schematic summary of the formation and removal of individual layers (see text for a description). Tubal passage lasts until 3 days p.c.; thereafter blastocyst expansion starts in the uterine lumen. **b** TEM of mature blastocyst coats at 6.5 days p.c. N = Neozona; M = mucoprotein layer; G = gloiolemma. × 11,500. **c** Snowball-like, globular concretions are seen in the perivitelline space (PVS) of a rabbit blastocyst, but only in an experimental situation when proteinase activity was inhibited in vivo by aprotinin (fig. 1c). These seem to merge with the neozona (N) where they form a dense innermost layer not seen in untreated controls. The question whether they may be produced by the trophoblast (T) cannot be answered on the basis of this observation. × 5,700.

cific location by putative factors contributed by the trophoblast. Morphological evidence [Leiser and Denker, 1988] suggests that the trophoblast indeed plays an important role in producing the neozona: from about 6 days p.c. on, the neozona tends to be considerably thinner in those parts of the coats that are adjacent to the embryonic disc as compared to the other regions. At this timepoint, the part of the trophoblast overlying the embryonic disc (Rauber's layer) is already degenerating and would thus not be expected to be able to contribute any secretion material anymore. This fits the observation that from this time on the thickness of the neozona continues to grow in

the other parts of the blastocyst except for this specific region. Morphological signs of a secretory activity of the trophoblast, although demonstrable, are however normally not very conspicuous. Only in a special experimental situation (blockage of the proteinase system involved in degradation of the coats around implantation) did we see globular concretions in the space between the abembryonic trophoblast and the neozona, and these snowball-like structures seemed to merge with the neozona (fig. 2c). On the other hand, there is experimental evidence (from in vitro culture experiments and synchronous/asynchronous embryo transfer) that uterine secretion components are also essential for neozona formation [Fischer et al., 1991]. Such uterus-derived components were also found essential for dissolution of the zona pellucida within the coats at the early blastocyst stages (this will be discussed below). What needs to be pointed out clearly when describing the various layers of rabbit blastocyst coats at late blastocyst stages is that the borders between them are never completely sharp, so that not only precipitation of new material onto preexisting layers but also diffusion into those and chemical interactions in the border zone may take place.

Let us briefly compare this picture just drawn about formation and transformation of the various layers of coats in the rabbit with concepts presented by other authors.

The most detailed earlier observations on rabbit embryonic coats were made by Böving [1954, 1957, 1972]. He used the term 'coverings' and proposed a consistent nomenclature for the various layers, i.e. 'oolemma' for the zona pellucida, 'mucolemma' for the mucoprotein layer, and 'gloiolemma' for the outermost, uterine secretion-derived layer. He measured the thickness of the various layers in the different developmental stages, calculated the volumes and argued that expansion of the blastocyst would cause stretching of the zona pellucida (oolemma) to such a degree that it finally could not be seen anymore with the light microscope. Also these calculations clearly showed that new material must have been added to the coats during blastocyst stages (i.e. the gloiolemma). He also attributed a function to the gloiolemma with respect to orientation of the blastocyst in the uterine lumen, i.e. the immobilization of the blastocyst in the developing implantation chamber and the alignment of the embryonic-abembryonic axis with the mesometrial-antimesometrial axis. These physiological implications will be discussed further below. Böving considered the coats of late preimplantation stage blastocysts of the rabbit to be composed largely of only two layers: the mucoprotein layer (mucolemma) and the gloiolemma. He did not describe the neozona, obviously due to the fact that his studies did not include electron microscopy or histochemistry.

Most close to our own studies come investigations by Okada/Nozawa (maiden name) including electron microscopy, autoradiography, S-content analysis and observations on solubility properties and in-vitro culture phenomena [Nozawa, 1976; Iwaki et al., 1975; Iwaki and Nozawa, 1978; Okada, 1979]. The main conclusion of this author was that the coats of late blastocysts were clearly different in their composition and properties, had a much reduced S content, and that uterine secretion material must have been added. The neozona was not described, however.

Observation by other authors remained more fragmentary and mostly quite contradictory with respect to the composition of the mature blastocyst coats. Gothié [1958] considered the latter to consist of just the mucoprotein layer; Bacsich and Hamilton [1954] addressed the coats at 5.5 days p.c. as just intact zona pellucida without mucoprotein layer; Adams [1965] imagined that the zona pellucida persists until implantation; Kirchner [1972] addressed the neozona as the zona pellucida but very nicely demonstrated the gloiolemma with immunohistochemistry. Shapiro et al. [1974] presented some immunohistochemical observations using an antiserum against an acidic glycoprotein from rabbit oviductal fluid and concluded that the '4-day-old eggs had lost most of their "mucin" coat'. Data from Morgan and Kane [1993] on the protein content of rabbit blastocyst coats clearly showed that some additional material is taken up by the coats between days 5 and 7 and that the resulting thickness of the coats cannot be explained by assuming a swelling process alone.

In summary, regardless of differences between authors with respect to the denomination of the various layers and their origins, it can be concluded that there is ample evidence from the literature showing that the coats of late preimplantation stage rabbit blastocysts are completely different, structurally and chemically, from not only the zona pellucida but also the mucoprotein layer, and that complex transformations have occurred, including removal of the zona pellucida and addition of new material to the inner and outer aspects of the mucoprotein layer.

Comparative Aspects of the Transformation of Embryonic Coats

Is the described complex transformation of embryonic coats just another document that the rabbit is bizarre and

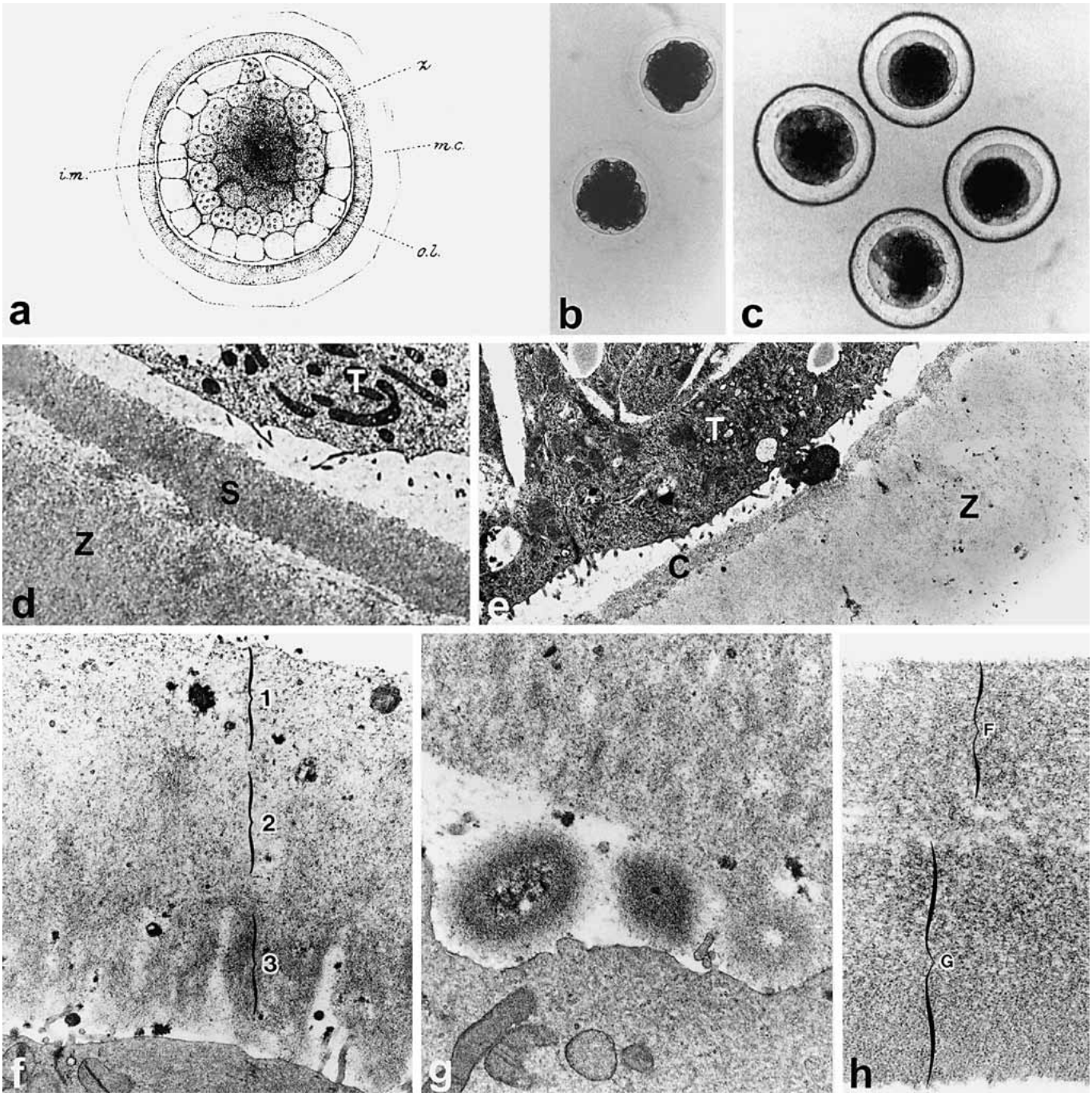

Fig. 3. Comparative aspects of embryonic coat transformation in various species. **a** An early depiction of a mucoprotein coat (mc.) deposited around the zona pellucida (z) of a morula of the mole *(Talpa europaea)*. From Heape [1886]. **b, c** In the cat, material appears to be deposited around the zona since the coats become thicker during passage down the genital tract. Note that the outer layer appears more clearly defined (more refractile) in uterine (**c**) than in tubal (**b**) embryos. × 160. From Swanson et al. [1994]. **d** The 'subzonal layer' (S) of a fur seal blastocyst. Z = Zona pellucida, T = trophoblast. × 6,200. From Enders [1971]. **e** Horse. The beginning of the deposition of the capsule (C) on the inner surface of the zona pellucida (Z) in a 6.5-day-old blastocyst. T = Trophoblast. The subzonal layer of the fur seal (**d**) and the capsule of the horse are quite comparable to the neozona of the rabbit. × 5,500. From Flood et al. [1982]. **f** Differential texture in the various layers that can be discerned in the coats of a baboon blastocyst (in this case exposed to cationized ferritin). × 9,200. From Enders et al. [1989]. **g** Globular concretions ('spherules') of material similar to that seen in the inner layer of the coats are found in the perivitelline space of the baboon blastocyst. They seem to merge with the inner parts of the coats, and depressions in

has 'odd habits' in its reproductive biology, as Finn [1980] put it?

Reports that tubal and uterine secretion material is added to the zona pellucida during passage down the genital tract in several species including the horse, badger, cat and dog can already be found in the very old literature, not to mention monotremes and marsupials [for references, see Bacsich and Hamilton, 1954; Amoroso, 1966]. Already Heape [1883, 1886] described 'an irregular layer of hyaline gelatinous material derived from the uterus' that enclosed the zona pellucida of uterine embryos of the mole (*Talpa europea*; fig. 3a).

With respect to the coats, the *horse* appears to be relatively close to the rabbit. This has received considerable attention in recent years thanks to the re-discovery of the 'capsule' and its systematic investigation by Betteridge and coworkers [Betteridge, 1989; Betteridge et al., 1982; Bousquet et al., 1987; Flood et al., 1982; Oriol et al., 1991, 1992, 1993a, b]. Using specific antibodies and an immunohistochemical approach, evidence was presented that the mucin-like capsular glycoproteins may be secreted, at least in part, by the trophoblast. On the other hand there is clear evidence for addition of uterine protein to the capsule [Stewart et al., 1995; Crossett et al., 1998].

In an excellent review, Betteridge [1989] compared coat formation in the horse, in particular the capsule, with the situation in various mammals and specifically in the rabbit. The capsule of the horse appears indeed to be closely comparable to the neozona of the rabbit at least in the early stages of capsule formation, and to me it would appear reasonable to unify the terminology here. A difference exists with respect to the timing of shedding of the zona pellucida: the horse capsule begins to appear at a time when the zona pellucida is still intact, so that it is clearly deposited on the inside of the zona pellucida (fig. 3e). In the rabbit, deposition of the neozona begins when dissolution of the zona pellucida (inside the mucoprotein layer which is serving as a support) is already under way and locally even finished. In the horse the zona is shed from the outside of the then well-developed capsule at a later stage. The horse also has a mucoprotein layer like the rabbit (and some other species) as mentioned in the beginning of this chapter, but it is much thinner here than in the rabbit [reviewed by Betteridge, 1989; the mucoprotein layer can also be seen clearly in illustrations provided by Grøndahl and Hyttel, 1996, see their fig. 4, 5, 10]. The fate of the equine mucoprotein layer is unknown. A structure similar to the gloiolemma of the rabbit cannot be distinguished morphologically in the horse, but uterine secretion-derived protein components can be detected in the capsule (see above). The difference to the rabbit would then again be a gradual one: since it is known that large molecules can penetrate through the coats (see below), it is no wonder that uterine secretion-derived proteins can be found even in deeper areas of the coats. In the horse, complexing and insolubilization of such molecules may take place thoughout the whole thickness of the capsule, while in the rabbit such processes may dominate on the outside of the (modified) mucoprotein layer thus forming a visible outer layer, the gloiolemma. Direct evidence that also the neozona of the rabbit may contain uterine secretion-derived material is lacking, but at least uterine factors are involved in its deposition as already mentioned.

While in most species morphological stratification of blastocyst coats is not as obvious as in the rabbit, there is on the other hand ample evidence for chemical modification during cleavage and blastocyst stages. Best known are tubal secretion-derived glycoproteins (oviduct-specific glycoproteins) that not only penetrate through the zona pellucida into the perivitelline space but also seem to be complexed with the zona material in many species including various *primates* and the *human* [Buhi et al., 2000; Boice et al., 1990; O'Day-Bowman et al., 1996; Jaffe et al., 1996; Verhage et al., 1997]. Morphological evidence for addition of material to the zona pellucida and for a transformation of the coats is also available for various primates. In the baboon, 2–3 morphologically distinct layers have been described electron microscopically in the blastocyst stage (fig. 3f) [Enders et al., 1989]. Here even globular concretions ('sperules') were seen in the space between the trophoblast and the innermost layer of the 'zona' with which they seemed to merge (fig. 3g). The morphological similarities as compared to the structures seen after proteinase inhibition in the rabbit (fig. 2c) are striking. It appears entirely possible that this innermost layer is directly comparable to the neozona of the rabbit (fig. 2b). In a later report Enders et al. [1997] have stressed the fact

the surface of trophoblast cells (below) suggest that they could be produced by the latter. Compare with neozona formation in the rabbit, and with somewhat similar structures in figure 2c. × 14,400. From Enders et al. [1989]. **h** Blastocyst coats of the western spotted skunk *(Spilogale putorius latifrons)*. Note the different texture of the various layers which is not unlike the situation in the rabbit [fig. 2b; in Denker and Gerdes, 1979]. × 14,300. From Enders et al. [1986].

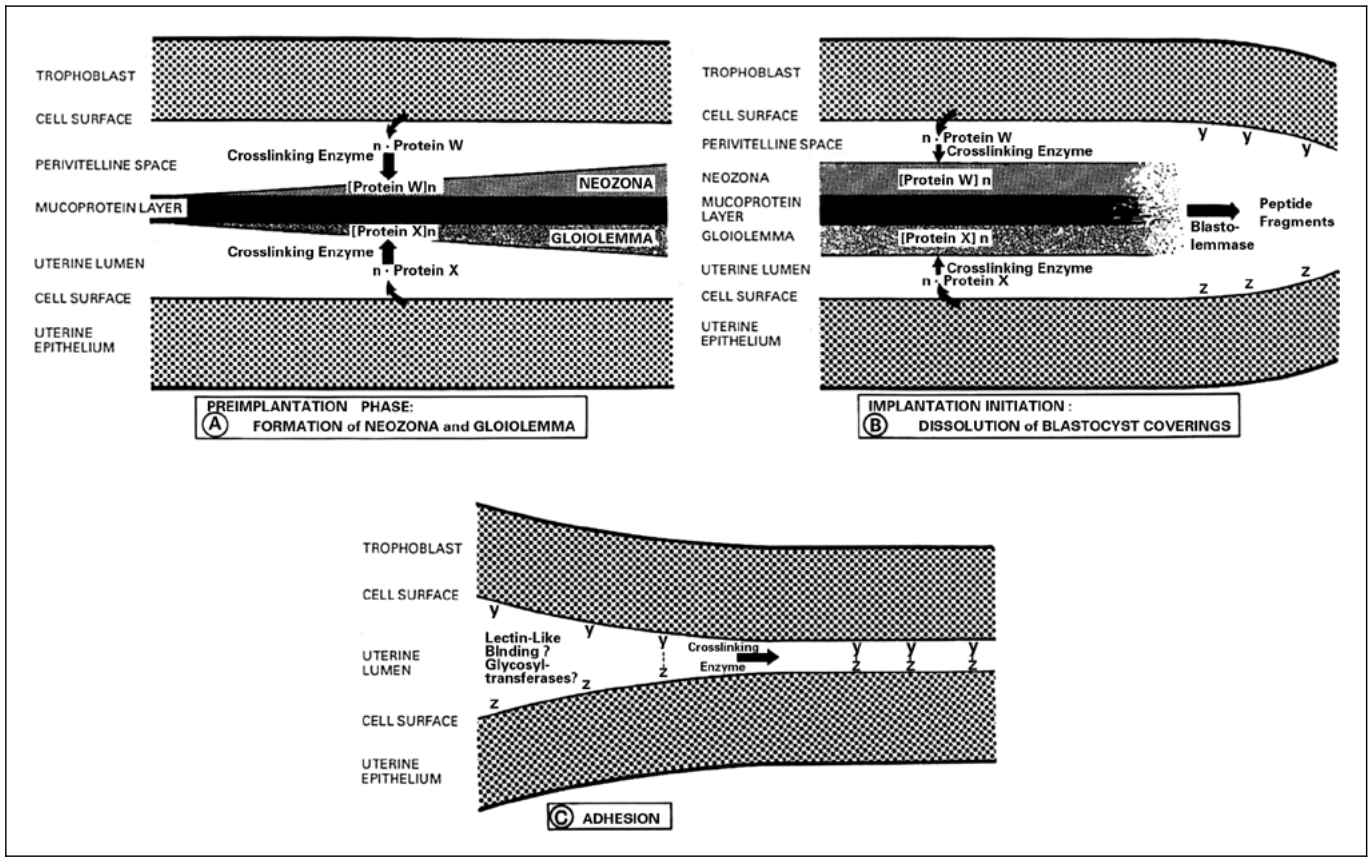

Fig. 4. Simplified diagram to show molecular processes that may be involved in deposition and dissolution of blastocyst coats in the rabbit. Deposition of the mucoprotein layer in the tube, and dissolution of the zona pellucida inside this layer at early blastocyst stages are not depicted. For simplicity, it is assumed that only one neozona precursor glycoprotein (W) is secreted by the trophoblast, and another such protein (X) is contributed by the uterine epithelium as a precursor for the gloiolemma; however, multiple molecules may be involved and could even permeate more deeply into the preexisting layers and be deposited there in addition. One possibility is that lectin-like molecules diffuse against the neozona precursor(s) and the gloiolemma precursor(s), respectively, and may cause the initial precipitation at the two surfaces of the mucoprotein layer (the same may take place during precipitation of the mucoprotein layer in the tube). Enzymatic cross-linking (e.g. by a transglutaminase) may subsequently stabilize the newly formed layers (see text; fig. 5). From Denker [1983].

that tails of supernumerary sperm can be found exclusively in the superficial layer of baboon blastocyst coats. My interpretation is that this would well fit the ideas that the innermost layer is comparable to the neozona, and/or that the outer layer represents an equivalent to the mucoprotein layer or gloiolemma, i.e. tubal secretion or uterine secretion deposited here together with dead sperm.

In *carnivores* which show the central type of implantation like the rabbit and the horse, blastocysts expand to a considerable degree, and it has long been recognized that, by some unknown mechanism, the volume of the coat increases considerably during this process in order to compensate for the effects of stretching [Amoroso, 1966; Enders, 1971; Denker et al., 1978]. Enders originally pointed out that there is no morphological evidence for any addition of material to the zona, at least in the ferret, and favored the idea that swelling due to hydration was the mechanism behind the increasing volume of the zona. On the other hand, Enders [1971] has also presented very clear and impressive ultrastructural evidence for the formation of a 'subzonal layer' in the fur seal (fig. 3d). To me this layer appears to be entirely comparable to the neozona of the rabbit. In a more recent publication, Enders et al. [1986] showed a very clear distinction of 2–3 layers of different texture in the coats of western spotted skunk blastocysts (fig. 3h). Morphological similarities to the situation in the rabbit are quite striking, and it appears perfectly possible that the innermost layer is comparable to

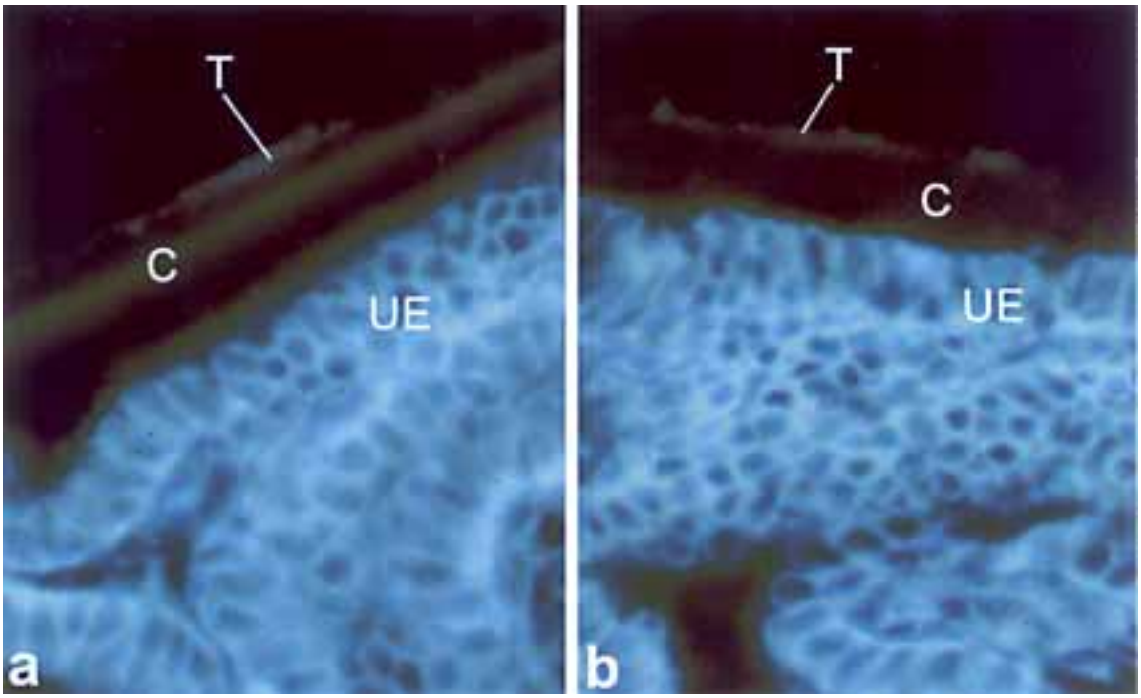

Fig. 5. Histochemical demonstration of transglutaminase activity in the coats of a rabbit blastocyst at 6 days p.c., using the methodology of Buxman and Wuepper [1978] based on dansyl cadaverine binding. Cryostat sections through implantation chamber, trophoblast (T), coats (C), uterine epithelium (UE). The signal in the coats (C) seen in **a** can be inhibited by iodoacetamide (**b**) or EDTA and is thus clearly attributable to transglutaminase activity. It appears to have a maximum in the innermost parts of the coats (neozona). × 800. Denker (unpublished).

the neozona of the rabbit. In the space between the trophoblast and the coats, an accumulation of material that stained metachromatically with Azure B was usually seen, again quite similar to observations in the rabbit ('sublemmal material' of Denker and Gerdes, 1979). Also the morphology of dissolution of the coats during the initial phase of implantation is strikingly similar to the situation in the rabbit [Enders and Mead, 1996; compare with Denker, 1977]: The coats are (probably enzymatically) dissolved preferentially by the syncytial trophoblast, less by the cytotrophoblast, and least or not at all by the epiblast of the embryonic disc. Enders and Mead [1996] conclude with respect to the trilayered structure of the coats that it seems likely that these are not solely an expanded zona pellucida but include materials added during blastocyst enlargement. However, they feel that 'until the exact composition of this membrane has been determined, it remains convenient to refer to it as a zona pellucida'. To me it appears that it is time now to be more explicit in this respect and to adopt a new terminology which considers the 'model' investigations on the rabbit and the horse.

Here and there in the literature, very fragmentary information can be found about other carnivore species. Bonnin and Canivenc [1980] described the coats of the blastocyst of the badger *(Meles meles)* to be composed of two parts, a superficial spongy part increasing in thickness during the diapause and a subjacent lamellar part, but they did not provide an illustration. Marshall and Enders [1942] reported that trophoblast cells were embedded in 'crypts formed in the zona', i.e. that projections of the inner parts of the zona existed in the blastocyst of the marten during diapause. One would speculate today that these crypts are depressions within neozona-like material, but this cannot be substantiated since the reported observations were made with the light microscope on suboptimally fixed, collapsed blastocysts. Quite conspicuous coat material of variable thickness is deposited around blastocysts in experimental diapause in the ferret [R. Mead, personal commun.; for experimental procedures, cf. Foresman and Mead, 1978]. In the mink, such material accumulating around the zona pellucida was also reported and, since the layer was absent from eggs or blastocysts

which had only recently entered the uterus, was considered to be of uterine origin [Adams 1973]. Clear evidence for a morphological transformation of the coats including addition of uterus-derived material has also been presented for the cat, but its morphology and chemistry have not been studied in detail [Roth et al., 1994; Swanson et al., 1994] (fig. 3b, c). With respect to tubal secretion-derived material, there is evidence for diffuse impregnation of the zona pellucida with such a glycoprotein (oviductin) in the cat [Robitaille and Bleau, 1993] in quite the same way as mentioned above for primates and as found also in many other (if not all) groups of mammals (see also below).

In most *ungulates* (except e.g. the horse, see above) the blastocyst hatches from the zona pellucida before it expands considerably. Perhaps for this reason there is no need for addition of much trophoblast- or uterus-derived material to the zona, and consequently no major morphological alterations of the zona have been reported. However, from a great number of recent publications, there is ample evidence for chemical modifications that occur in the glycoprotein composition of the 'zona' during tubal and early uterine passage, and for an impregnation of the zona with glycoproteins derived from the genital tract secretions that in part seem to form complexes with the zona matrix and in part penetrate to the perivitelline space [Buhi, 2000; sheep: Gandolfi et al., 1989, 1991; Murray, 1993; pig: Brown and Cheng, 1986; Hedrick et al., 1987; Buhi, 1993; cow: Duby et al., 1995; Staros and Killian, 1998].

Basically the same holds true for the *rodents* as for ungulates. In these species, which also show only very little expansion of the blastocysts, morphological evidence for a transformation of the coats, in particular for any addition of material, is lacking. However, here again ample evidence is available for chemical modification of the zona and, in particular, for an uptakte of tubal secretion glycoproteins (oviductin) [hamster: Abe and Oikawa, 1991; Fox and Shivers, 1975a, b; Kan et al., 1989, 1993; Léveillé et al., 1987; Malette et al., 1995, Robitaille et al., 1988; Suzuki et al., 1995a; mouse: Sendai et al., 1995].

Another type of chemical modification of the zona pellucida is caused not by tubal, uterine or trophoblast secretions but by the contents of the *cortical granules* that are released from the oocyte at fertilization. This modification is thought to be the basis of the 'zona reaction' and 'hardening' and part of the block to polyspermy, and will be discussed in more detail elsewhere in this volume [Prasad et al., 2000; De Vos and Van Steirteghem, 2000) and will be referred to again further below. Relevant for the present discussion is, however, that the cortical granules which are exocytosed into the perivitelline space contain not only enzymes that may modify the zona matrix molecules but also macromolecules (glycoproteins) that may themselves constitute some type of matrix within the perivitelline space or on the inner aspect of the zona pellucida or may even permeate the latter (see further below).

While we are concentrating in this review on the situation in eutherian mammals, a few remarks are in place with respect to a comparison with the situation in *marsupials* (dealt with in more detail by Selwood [2000]). Traditionally three coat layers are described to be discernible in marsupial embryos: zona pellucida, mucoid coat (mucoprotein layer), and shell membrane (from the inside to the outside). The thickness of these various layers differs considerably between species (see Selwood [2000]; for comparison with non-mammalian species, cf. also Hughes [1977]). During expansion of the blastocyst, the zona pellucida and the mucoid coat are dissolved inside the persisting shell membrane by mechanisms (enzymes) that remain to be defined (which is also true for the enzymes responsible for dissolution of the rabbit zona pellucida within the mucoprotein layer, see also below). The rabbit is often said to present, with its various layers, some archetypical features already found in marsupials, although it does not have a shell membrane but instead the rather soft and irregular gloiolemma. Would the marsupials also have a neozona like the rabbit? This possibility, discussed before [Denker and Tyndale-Biscoe, 1986], contradicts the traditional view that only the uterus-derived shell membrane surrounds late blastocyst stages in marsupials. However, some evidene does point to the possibility that neozona-like material is either deposited onto the inside of the shell membrane or permeates into it during blastocyst expansion, as suggested by the different layers of the shell membrane that can be recognized with the electron microscope in certain marsupial species [for literature see Denker and Tyndale-Biscoe, 1986]. For example, figure 20 of Hughes [1984] shows a 'granular layer' that was formed inside the 'shell membrane' after the zona pellucida and the mucoid coat had disappeared. It is also remarkable that in certain marsupial species the ultrastructural appearance of the 'shell membrane' changes considerably during blastocyst expansion from a granular to a fibrous state [Roberts and Breed, 1996].

A feature that has recently been receiving interest in marsupial embryos is material called extracellular matrix (ECM) which is extruded from the oocyte and blastomeres and is deposited on the inside of the zona pellucida and in the perivitelline space [Selwood, 2000]. This material seems to have a morphogenetic role in influencing the

polar organization/differentiation of trophoblast vs. embryoblast cells, an aspect that will be discussed together with other functional aspects further below. It needs to be compared with the glycoprotein-rich material found at the same location in eutherian animals including the rabbit ('sublemmal material' of Denker and Gerdes, 1979) which has not been given a specific name but which we will refer to as *perivitelline space matrix* (PVM; see below).

Much more data on coat formation and transformation are available for *non-mammalian vertebrates*, and here excellent reviews have been published which I can recommend to the readers [Hedrick and Nishihara, 1991; Larabell and Chandler, 1991; Yamagami et al., 1992]. An interesting fact is that certain precursor molecules for the coats are synthesized in the liver and transported via the blood stream, e.g. choriogenin H (which shows homologies to the zona pellucida protein ZP2) in the fish [Murata et al., 1997].

In summary, quite a number of data, although widely spread in the literature, strongly suggest that, like other vertebrates, eutherian mammals show considerable transformation of their coats during embryonic development, and that these changes might deserve being investigated more systematically. In a number of species these changes cannot easily be recognized, in particular not with conventional morphological techniques, but may include considerable chemical modifications. Morphological changes are predominantly seen in certain species in which blastocysts expand to a considerable degree before they implant, i.e. some (not all) of the species with the central type of implantation, including certain primates.

Mechanisms and Regulation of Coat Formation and Transformation

Molecular Mechanisms

The molecular mechanisms of deposition of coat material are largely unknown for the mammalian system, as are the mechanisms behind the structural transformations that we have just described. A hypothetical simplified scheme has, however, been presented earlier in order to stimulate the search for the molecular mechanisms involved [Denker, 1983] (fig. 4). The scheme does not depict chemical changes like the decrease in sulfate ester groups already referred to above, nor does it illustrate that tubal secretion or uterine secretion macromolecules (like proteins) may penetrate deeply into preexisting layers of the coats and may become complexed there (as already discussed for various species above). The point that it tries to make is that we have to look for mechanisms that would allow genital tract secretion material or trophoblast secretion material to be precipitated down onto the mucoprotein layer (in case of the rabbit) or onto the zona pellucida (in case of certain other species) in such a way that a new layer of considerable physical strength is finally formed.

The following molecular mechanisms are to be discussed: (i) carbohydrate group-based mechanisms – modification of carbohydrate side chains by glycosyl transferases or glycosidases, precipitation by lectin-like molecules, and (ii) chemical modification of protein backbones and cross-linking, e.g. by transglutaminase, peroxidase.

Evidence for the presence of a carbohydrate-binding molecule, probably a *lectin* (with galactose specificity) in the epithelia and the secretions of the tube and the uterus of the rabbit was presented by Biermann et al. [1997], and it was proposed that this lectin could be involved in precipitating coat material, in the tube (mucoprotein layer) and the uterus (gloiolemma, neozona). Differences with respect to reports by other authors who had detected certain lectins in the stroma but not the epithelium are probably due to differences in methodology (discussed by Biermann et al. [1997]). *Glycosyltransferases* show impressive changes in activities in the tubal and uterine fluid of the hamster during the estrous cycle [Tulsiani et al., 1996]. Glycosyltransferases had previously been considered candidate molecules possibly involved in attachment of trophoblast to the uterine epithelium during implantation, according to the Roseman hypothesis [Chávez, 1990]. Any involvement in the process of coat deposition and transformation has so far not been shown and is just a theoretical possibility but might be worthwhile investigating. The same holds true for various glycosidases that can be found in the secretion material [Denker, 1971a, b].

Any glycoprotein precipitate produced due to enzymatic modification of carbohydrate side chains (and/or by complexing with lectins) would not be expected to have much physical strength. So the known toughness of early embryonic coats suggests that additional processes of cross-linking should be involved. In studies on the coats of lower animals, the involvement of *peroxidases* and *transglutaminases* has been demonstrated [for review, see Larabell and Chandler, 1991; Yamagami et al., 1992]. Surprisingly little work has been done with respect to these enzymes in the mammalian system, in particular with respect to embryo coat formation/transformation (we will come back to this point briefly with respect to zona hardening below).

It may be particularly worthwhile investigating transglutaminases in this respect. In preliminary experiments we found evidence that transglutaminase activity appears to be present in rabbit blastocyst coats during the time when the neozona and the gloiolemma are being deposited, with more signals at the location of the former than the latter (fig. 5) which fits the fact that the neozona is physically stronger. Transglutaminases cause covalent cross-linking of proteins by formation of ε-(γ-glutamyl)lysine bonds and are involved, for example, in the stabilization of the fibrin clot (factor XIII) and in formation of the cornified envelope during skin keratinization [Candi et al., 1998; Tyler, 1972]. Transglutminases are also discussed to play a role in matrix cross-linking in cartilage, in lens capsule formation and metastatic tumor cell adhesion [Aeschlimann et al., 1995; Hidasi et al., 1995; Menter et al., 1992].

There is good evidence that transglutaminases play an important role in coat formation and stabilization (hardening) in sea urchins and fishes [Battaglia and Shapiro, 1988; Yamagami et al., 1992]. To my knowledge no data (except for those presented in fig. 5) are available on a potential role of transglutaminases in mammalian embryonic coat formation/transformation. On the other hand, transglutaminase has been shown to be present in the endometrium (although predominantly in the stroma and decidua), trophoblast and seminal vesicles (playing a role in coagulation of the ejaculate) [Chung, 1972; Pelusco et al., 1994; Thomázy and Fésüs, 1989]. That rabbit blastocyst coats are indeed covalently cross-linked appears highly probable to anyone trying to dissolve them for electrophoretic analysis using, e.g., SDS or reducing agents. Nozawa has pointed out that rabbit blastocyst coats are quite resistant to mercaptoethanol and high urea concentrations, while the original zona pellucida is dissolved easily [Nozawa, 1976; Iwaki et al., 1975]. Similar observations on resistance of the capsule but not the zona to these agents have been made in the horse [Bousquet et al., 1987; Oriol et al., 1993a] (for literature concerning the solubility of the zona pellucida in SDS and dithiothreitol in various species, see Bedford [1998]). From these data it appears quite probable that cross-linking by a transglutaminase could be involved in providing the physical strength to blastocyst coats, perhaps in particular to the neozona, and that this enzyme as well as peroxidases should indeed be investigated in this respect.

In addition to the above-mentioned mechanisms, *limited proteolysis* could be involved in deposition and transformation of coat material. The latter has been extensively discussed in the context of sperm penetration through the zona pellucida and the block to polyspermy (see below). Interesting details are available on the effects of limited proteolysis on coat formation/transformation (fertilization envelope formation) in non-mammalian species [Hedrick and Nishihara, 1991; Larabell and Chandler, 1991].

The ECM material that is found in the perivitelline space after cortical granule exocytosis (PVM), which will be discussed more in detail below (morphogenetic role), may differ somewhat with respect to the mechanisms of precipitation. This substance appears not to get much physical strength but to remain more gel-like. It may be related to the hyalin of sea urchins (see the reviews cited above).

The views just discussed about the mechanisms involved in embryonic coat formation and transformation may be quite simplistic due to our lack of knowledge. In investigating these in further detail, it may be warranted to take side views to systems not related to reproduction in which a great deal of information is already available on perhaps related mechanisms. For example, the tectorial membrane of the inner ear is also a product of epithelial cells, as are the otoconial membranes of the utricule and saccule and the cupula of the semicircular canals. Here, specific proteins, otogelin and the tectorins play an important role. Interestingly, the tectorins have domains that are homologous to the zona pellucida proteins ZP2 and ZP3; α-tectorin has a region homologous to zona adhesin. Some information is available on how these proteins interact with each other during precipitation and fibril formation [Cohen- Salmon et al., 1997; Killick et al., 1995; Legan et al., 1997]. It may be worthwhile to compare the molecular mechanisms playing a role in these systems with what takes place during deposition of egg and embryo coats.

Physiology of Coat Formation and Transformation

The role of the tubal or uterine mucosa/secretions on one hand and any factors contributed by the egg and embryo on the other hand in coat formation/transformation has attracted some limited interest in the past.

Coats of a similar appearance to the mucoprotein layer are also deposited in the rabbit around foreign bodies, e.g. glass beads [Greenwald, 1958] or Sephadex beads [Denker and Hafez, 1975; illustrated in Denker, 1982]. Discharge into the tubal lumen (and subsequent deposition) appears to depend on progesterone and is inhibited by estrogens, and Greenwald [1958] concluded that the egg/embryo plays a passive role in the process. However, it has not been determined whether the mucoprotein layer

collected around foreign bodies has exactly the same composition as that formed around rabbit embryos. Yoshinaga and Adams [1967] observed that no mucoprotein layer is deposited around rat blastocysts transferred to the rabbit tube, suggesting that the embryo is not necessarily completely inert and passive in this respect.

With respect to neozona formation in the rabbit, circumstantial and experimental lines of evidence suggest a participation of dual factors provided by the trophoblast and by the uterine secretion, as already mentioned above. The fact that after degeneration of Rauber's layer deposition of neozona material seems to cease at this particular location was used as an argument for a role of the trophoblast [Leiser and Denker, 1988]. In in vitro culture media no formation of a regular neozona was seen; it was, however, not possible to compensate for this apparent lack of a uterine secretion factor by adding uterine flushing material from various phases [Fischer et al., 1991]. In keeping with this, formation of the capsule has not been observed during in vitro culture in the horse, although somewhat conflicting observations have also been reported [discussed by Betteridge, 1989, and Oriol et al., 1993a, b]. All this argues for a role of components of the uterine fluid that still need to be defined.

Dissolution of the rabbit zona pellucida inside the mucoprotein layer is naturally expected to depend on hydrolytic enzymes, probably proteinases. However, this postulated proteinase system must clearly be different from the system dominated by blastolemmase (to be discussed below) that plays a central role in dissolution of the transformed blastocyst coats at implantation initiation, since blastolemmase activity rises much later and cannot yet be detected at these stages when the zona pellucida is being dissolved [Denker, 1971c, 1983]. (We will come back to this question further below when discussing the uterine secretion-derived 'proteinase X'; cf. also fig. 10.) Attempts at blocking that dissolution of the zona pellucida at these early stages with blastolemmase inhibitors in vivo have failed so far [Denker, unpublished observations]. Experiments on the identification of possible candidate enzyme (proteinases, glycosidases) in an in vitro model system have of course not been able to really clarify the situation with respect to the in vivo situation [Kane, 1986]. In this and other studies concentrating on the plasminogen activator-plasmin system [Grobner and Menino, 1994], the behavior of the coats was unfortunately not monitored in detail. Interestingly there is a clear difference between the tubal and the uterine milieu with respect to rabbit zona dissolution: the process is greatly disturbed in tube-locked embryos but does occur in the uterine lumen; it fails to occur in regular in vitro culture media as already mentioned above, but can be restored at least partially with added uterine secretion. However, no stage-dependent differences could be observed in these uterine secretion materials with respect to this ability, in contrast to the grossly changing general protein composition, and the postulated factors did not seem to be uterus-specific since peritoneal fluid was found to do the same. The tubal secretion obviously lacks these factors or contains an inhibiting principle [Fischer et al., 1991].

Functional Aspects

Fertilization

The zona pellucida of the freshly ovulated oocyte is known to play an important role as a sperm receptor, in inducing the acrosome reaction, and (to a degree that varies between species) in establishing the block to polyspermy. The important role of the zona pellucida in this respect is impressively documented by the observed infertility of mice with disrupted ZP3 gene which lack a zona pellucida [Liu et al., 1996].

The composition of the zona pellucida and related functional aspects are discussed elsewhere in this volume [Paterson et al., 2000; Prasad et al., 2000]. Central events in sperm-zona pellucida interactions are adhesion of sperm to the zona matrix and induction of the acrosome reaction. These events are covered by a number of excellent reviews which can be recommended to the readers [Aitken, 1995; Florman et al., 1998; Myles and Primakoff, 1997; Saling, 1989; Snell and White, 1996; Wassarman, 1999; Yanagimachi, 1994; with emphasis on the role of carbohydrates: Sinowatz et al., 1998; Tulsiani et al., 1997]. The traditional view has been that after binding to the zona pellucida and undergoing the acrosome reaction, sperm traverse the zona pellucida by the aid of hydrolytic enzymes, of which the proteinase acrosin is thought to play an essential role. This view has recently been questioned, in particular for eutherian mammals [Bedford, 1998].

After fertilization, the zona pellucida is one of the locations where the block to polyspermy is established, the importance of the zona block as compared to the block at the egg plasma membrane varying depending on the species. Usually the release of the cortical granules upon penetration of the first sperm is thought to cause modifications in the zona pellucida; in species like the rabbit, in which a mucoprotein layer is formed, deposition of this material on the outside of the zona is also thought to con-

tribute to (a late phase of) the zona block [McCulloh et al., 1987]. The composition of the zona pellucida has long been known to undergo changes from the unfertilized oocyte to zygote/cleavage stages [Brown and Cheng, 1986; Hedrick et al., 1987]. These changes could be due to the cortical granule material released into the perivitelline space and diffusing into the zona pellucida, or to tubal secretion material diffusing into the zona from the outside (we will come back to the latter point below). Release of the cortical granules can be induced by various stimuli, including osmotic shock, cooling and various chemicals that alter the properties of membranes including dimethylsulfoxide, and this process differs from activation [Johnson et al., 1988; Vincent et al., 1990, 1991; Denker, 1970a, b]. It was observed early that this release of cortical granules is associated with a process of hardening of the zona pellucida which is thought to be related to the block to polyspermy, but excessive hardening as observed in in vitro culture may pose a problem for hatching of blastocysts and is of concern in IVF-ET procedures [De Vos and Van Steirteghem, 2000]. It will have to be clarified whether this phenomenon is related solely to the process of cortical granule release.

Chemical changes elicited in the zona pellucida have been proposed to be due to cortical granule proteinases [Gwatkin, 1976; Gwatkin et al., 1973; Moller and Wassarman, 1989] in analogy to the situation in non-mammalian species [Lindsay and Hedrick, 1995]. However, also the carbohydrate composition of the zona pellucida changes after fertilization [Avilés et al., 1997]. It is of interest that antigens derived from cortical granules can be detected in the perivitelline space of which certainly not all represent enzymes that may modify the zona pellucida matrix [e.g. Pierce et al., 1990]. We will discuss the 'matrix' material found in the perivitelline space again further below. Also of interest in this context is that release of cortical granule material can already be detected before penetration of the first sperm, in vivo and not only under artificial conditions, so that two waves of such extrusions may be present, possibly pointing to the existence of different categories of vesicles/cortical granules with different content, and that there is structural evidence for a modification of the zona pellucida from the inside probably due to the adsorbance of such material [Ducibella et al., 1990; Okada et al. 1993] (fig. 9).

As already discussed in earlier parts of this review, the zona pellucida of zygotes and cleavage stages is also modified by tubal secretion-derived molecules. In the context of fertilization it is a matter of discussion whether and to what extent those tubal secretion-derived molecules may be inhibitory to fertilization in the sense of a block to polyspermy or may even improve sperm binding [O'Day-Bowman et al., 1996; Verhage et al., 1998].

We have discussed before what molecular mechanisms including various enzymes may be involved in deposition of coat material in general. The same mechanisms could be involved in attaching zygote-derived or tubal secretion-derived material tightly to it, or in changing the intermolecular associations between original zona pellucida constituents. Little is known about the role of e.g. ovoperoxidase [Schmell and Gulyas, 1980] and transglutaminase as already discussed earlier. Since zona hardening receives considerable attention with respect to IVF-ET [De Voss and Van Steirteghem, 2000; Cohen et al., 1994], the chemistry of these processes clearly deserves to be investigated further in detail.

Transport Control and Immunology

The question whether the extracellular embryonic coats may serve a function in molecular transport control has been addressed repeatedly over the years. Several authors have been impressed by the finding that there is not a very clear sieve effect nor can a cutoff be defined easily, and even relatively large molecules (proteins) can penetrate [Crossett et al., 1998; Enders, 1971; Guillomot and Betteridge, 1984; Hastings et al., 1972; Hughes and Shory, 1973; Zimmermann, 1965]. Consequently with respect to lower molecular weight compounds including potentially teratogenic agents (drugs), earlier attempts at defining such a cutoff largely failed and gave a very incomplete picture [Austin and Lovelock, 1958; Lutwak-Mann, 1959]. It has become clear, however, that there is a degree of selectivity here even with respect to low molecular weight compounds which cannot be explained easily. Attempts have recently been made to study the physicochemical basis of this selectivity systematically in the mouse, defining molecular parameters of the permeating substances (like hydrophilic or lipophilic properties, the conjugated bond number) that may be of a predictive value. Although differences in permeation properties are noted, it became clear in those model experiments that most biologically active molecules and metabolites can pass through the coats of early mouse embryos largely unhindered [Turner and Horobin, 1997]. On the basis of the modifications of the coats that occur particularly in blastocyst stages in various animals (as discussed above) one should be cautious with rapid extrapolation to other species, and from one stage to the other, and also from in vitro experiments to the in vivo situation. Already Adams et al. [1961] observed that trypan blue did not penetrate

into blastocysts until day 5 in the rabbit, even though it had been present in high concentrations in the uterine secretion. In contrast, it freely entered the blastocyst cavity from the beginning of shedding of blastocyst coats and implantation initiation onwards.

The mentioned permeability of embryonic coats to proteins has already early evoked immunological considerations [Sellens and Jenkinson, 1975]. The picture has not become very clear, but it appears that there is no effective transport control of humoral/secreted antibodies to the blastocyst by the coats and that on this basis the immunologic paradox of pregnancy cannot be explained. What appears most probable is that coats can play a role in physically inhibiting a direct cell-to-cell contact of the blastocyst with immunocompetent cells as well as with microorganisms (potential pathogens) [James, 1969; Schlafer et al., 1987; Vanroose et al., 1998; Warner et al., 1988].

Morphogenetic Role

The embryonic coats (zona pellucida) have long been known to act as a physical scaffold that holds blastomeres together and prevents them from falling apart and forming monozygotic twins, etc., as long as cell-cell adhesion complexes are not yet well developed. That there must be more to this morphogenetic role becomes clear from observations on the transfer of coat-free early embryos to the maternal genital tract: whereas such zona-free embryos develop nicely under in vitro conditions from cleavage stages to blastocysts, development is very poor after transfer of such cleavage stage embryos to the genital tract [Bronson and McLaren, 1970; Modlinski, 1970]. They must have reached the late morula or blastocyst stage in order to develop well after zona-free transfer.

An explanation for this phenomenon is easily found in the context of the polarization hypothesis [Johnson et al., 1981, 1986a, b; Fleming, 1992]. This hypothesis states that differentiation of the trophoblast, the first epithelium in embryogenesis, depends on positional cues, i.e. adhesive cell-cell interactions that must dominate on one pole (the prospective basolateral pole) of the cells, while a free surface must also be present where such interactions do not occur. This free surface will then acquire the properties of an apical plasma membrane domain, so that the typical polar organization of a simple epithelium is attained. Blastomeres can be prevented from polarizing by surrounding them completely with other blastomeres so that a free surface cannot develop; in this case the completely surrounded blastomeres will remain stem-cell-like and nonpolarized (embryoblast). For trophoblast differentiation it is thus essential to provide a nonadhesive environment to the outer blastomeres. This appears to be physiologically the zona pellucida; it can be replaced, in vitro, by the tissue culture medium. It appears that agar, which is also nonadhesive to cells, can also replace the zona, although this has not been studied directly; in experiments published by Willadsen [1980] surrogate zonae were effectively sealed by agar. Blastomeres can attach easily to other cells and to the ECM, e.g. collagen as observed already by Cole and Paul [1965]. Such adhesion to ECMs or other cells could disturb not only trophoblast cell polarization, after loss of the zona, but could also disturb patterns of blastomere arrangements that are normally regular [Gulyas, 1975; Lehtonen, 1980; Suzuki et al., 1995b].

A very impressive experiment which demonstrates the role of the zona pellucida in this respect has been published by Pedersen and Spindle [1980]. It was not performed with the intent to show the role of the zona pellucida but rather to test the 'milieu' hypothesis that was prevalent at that time. When a zona-encased mouse morula was transferred to the blastocyst cavity of a giant host blastocyst (produced by aggregation of multiple embryos), it was able to develop in this 'inner milieu' to a perfectly normal blastocyst, i.e. to differentiate trophoblast in addition to embryoblast. While this was the main message of those studies, contradicting the 'milieu' hypothesis, a control experiment that was also done is much more interesting in the context of the role of the zona discussed here: when the morula was transferred without zona, it did not form a blastocyst, obviously due to the fact that it did not produce polarized trophoblast cells (fig. 6b).

One would conclude that the observations mentioned in the beginning, i.e. that development of morulae is disturbed if they are transferred to the genital tract without a zona pellucida, can be explained in the same manner. Blastomeres need to be confronted with nonadhesive surfaces (like the zona) at one cell pole in order to differentiate into trophoblast. On the other hand the explanation is not quite that trivial: since the tube and the uterus are lined with polarized epithelial cells exposing their (nonadhesive) apical plasma membrane to the lumen, and since this polarity seems to be maintained except for a destabilization during the receptive period of the uterine epithelium [Denker, 1993], it is not completely clear how disturbance of trophoblast differentiation really comes about after morula transfer to the genital tract. Perhaps accidental irregularities like local epithelial defects, or concretions in the lumen of the tube or the uterus, need to be present in order to interfere with the process in this situation. This may be behind the fact

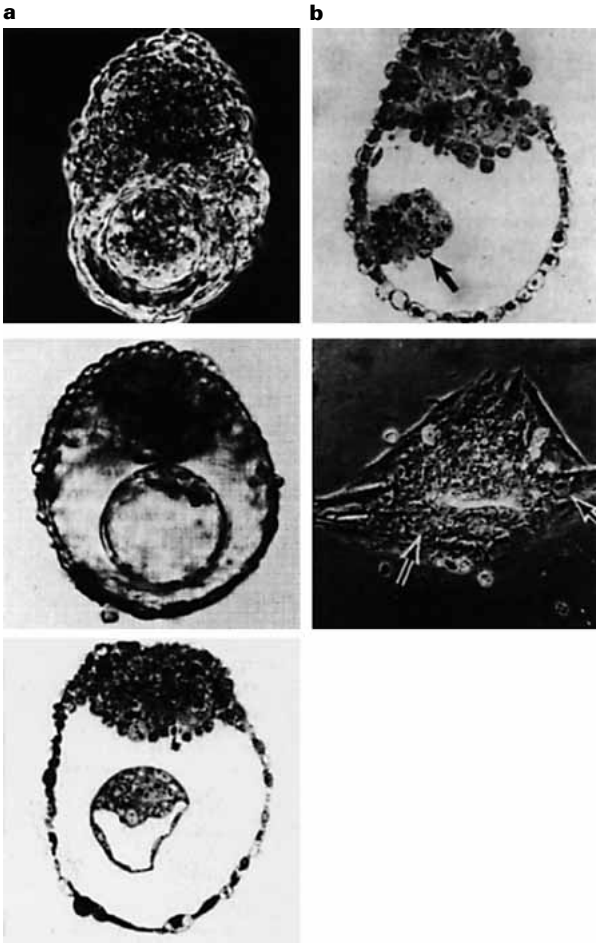

Fig. 6. Morphogenetic role of the coats in the context of trophoblast differentiation. **a** (left column) A mouse morula was transferred, with intact zona, into the blastocyst cavity of a giant chimeric host blastocyst. There it differentiated a normal outer layer of trophoblast, as well as embryoblast (inner cell mass) and was thus able to form a regular blastocyst (within the inner 'milieu' of the host blastocyst). × approx. 170. **b** (right column) When transferred without a zona, the morula (arrowed) attached to other cells of the host (in this case the mural trophoblast), did not differentiate trophoblast and, as a consequence, did not form a blastocyst itself. Cells did maintain the potential to form trophoblast, however, as shown in an outgrowth experiment (below). Above × 220; below × 115. From Pedersen and Spindle [1980].

that disturbance of morula development without a zona pellucida in vivo is not seen in all cases [Bedford, 1998].

Perivitelline Space Matrix

ECM material that can be found in the perivitelline space in *marsupials* (we will refer to it as 'perivitelline space matrix', PVM) has recently received interest because it appears to play an important morphogenetic role [Selwood and Young, 1983; Frankenberg and Selwood, 1998; Selwood, 2000]. Such material can already be detected in the perivitelline space of marsupial oocytes, but more of it (perhaps with different composition) is extruded after fertilization and during cleavage. Differentiation of trophoblast and embryoblast ('pluriblast' according to the new nomenclature proposed by Johnson and Selwood [1996]) appears to be dependent in marsupials to a great deal on cell-matrix interactions, at least during the early phases, in contrast to eutherian mammals where cell-cell interactions seem to dominate these processes (see above). Blastomeres attach to the inside of the zona pellucida and flatten out and migrate here, differing somewhat according to the species [McCrady, 1938; Selwood, 1992, 2000] [fig. 7). Blastomere-zona adhesion typically precedes blastomere-blastomere adhesion by one or two divisions. In species where yolk extrusion occurs at one pole, blastomeres first adhere to the portion of the zona lying nearest the yolk material. This difference in cell behavior can be correlated with differences in the amount (perhaps also the composition?) of ECM material present in the perivitelline space, which also seems to cover the zona pellucida from the inside: the matrix material appears to be extruded from intracytoplasmic vesicles, but this process does not seem to take place at the yolk mass. As a result of this, the PVM (also coating the inner surface of the zona pellucida?) attains a polarity in the egg, and this was proposed by Selwood to provide a mechanism why the blastomeres initially preferentially populate the zona above and around the yolk mass in a number of species. In other marsupial species, e.g. those with a radial emission of yolk, this polarity of PVM is not evident morphologically but may be found with histochemical techniques. Interestingly, a careful electron microscopical study revealed quite complex processes of not only extrusion but also remodelling of matrix material in certain marsupial species [Frankenberg and Selwood, 1998]: club-shaped processes of certain blastomeres which contain (and possibly release) electron-lucent vesicles seem to remove the PVM material locally, and this seems to facilitate cell-zona adhesion. As a result, embryoblast ('pluriblast') cells become exposed directly to the inner surface of the zona pellucida. In contrast, at the opposite (trophoblast) pole the PVM is not only maintained but additional matrix material is even extruded. It was suggested that the polar organization of the marsupial (unilaminar) blastocyst, with embryoblast ('pluriblast') cells differentiating on one pole and trophoblast in the other part, may be influenced by these cell-matrix interactions and in particular the polar deposition of matrix material.

The PVM is not a peculiarity of marsupials. It is widely distributed in vertebrates and invertebrates, and various functions including a morphogenetic role are being discussed (cf. the hyalin of the sea urchin [Larabell and Chandler, 1991; Matese at al., 1997; Hedrick and Nishihara, 1991]). PVM can also be demonstrated in *eutherian mammals.* In the rabbit, it can be shown that a peak of extrusion occurs at fertilization (fig. 8) so that this material appears to be released from the cortical granules or related structures. Interestingly, the amount found in the perivitelline space seems to increase further during cleavage [Denker, 1970a, b, 1971b] so that I always felt it might have some type of morphogenetic role. Extrusion of PVM was stimulated in the rabbit by cold shock and osmotic shock [Denker, 1970a, b] as known for, e.g., hyalin in the sea urchin and cortical granule material in the mouse [Johnson et al., 1988; Vincent et al., 1990, 1991]. This material, which is negatively charged and rich in sialic acids, was seen in extremely exaggerated quantities in experiments in which rabbits had been treated with relatively high doses of estrogen/progesterone combinations; in these cases cleavage was disturbed [Denker, unpublished earlier work].

It is not known whether PVM material is derived exclusively from cortical granules, covers or penetrates the inner aspect of the zona pellucida, coats the egg/blastomere surfaces, and perhaps has a morphogenetic role also in eutherian mammals in addition to any function in the context of the block to polyspermy. It may be worthwhile to investigate this aspect further. Based on electron microscopical studies, Okada et al. [1993] have suggested that PVM may be heterogeneous and possibly derived from various sources in the egg, that part of such material is already extruded before sperm penetration, and that a gradient of extrusion from the animal to the vegetal pole occurs at egg maturation/polar body formation (fig. 9). It has long been known to electron microscopists that the PVM seems to merge with the inside of the zona pellucida and seems to penetrate into it. It is conceivable, therefore, that even in eutherian eggs and early embryos the zona pellucida shows some polarity along the animal/vegetal axis. However, there is no evidence that such a polarity, if present, influences development. As mentioned earlier, formation of trophoblast vs. embryoblast seems to be governed by cell-cell interactions in eutherian embryos, and all the zona pellucida and PVM apparently have to provide here is a nonadhesive surface so that outer blastomeres can form an apical plasma membrane (see polarization hypothesis, above). Morphological evidence for an attachment of blastomeres to the zona is lacking in euthe-

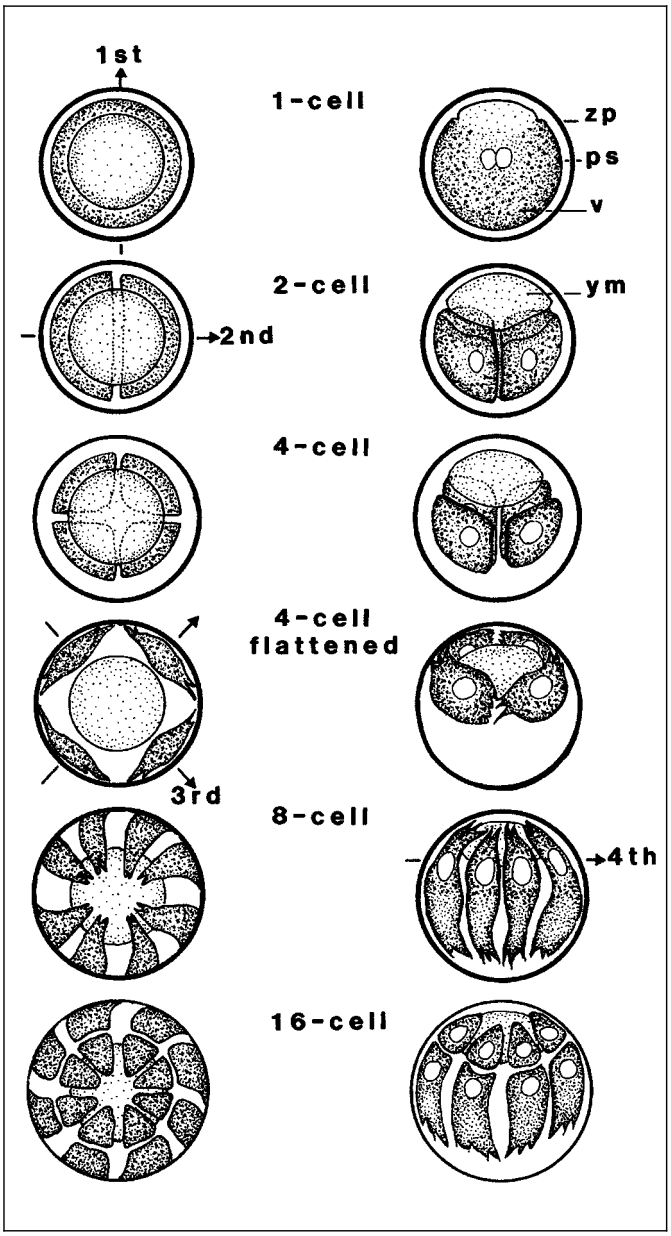

Fig. 7. Morphogentic role of the zona and perivitelline space matrix in a marsupial, *Antechinus stuartii.* Blastomeres flatten and migrate on the inner surface of the zona during cleavage. Note the obvious polarity of the events with respect to the excentric location of the extruded yolk mass (ym); a gradient of perivitelline space matrix material (not indicated here) is aligned with this axis. Left-hand column: viewed from the yolk pole, right-hand side: viewed from the side. zp = Zona pellucida; ps = perivitelline space; v = vitellus; ym = yolk mass. From Selwood and Young [1983].

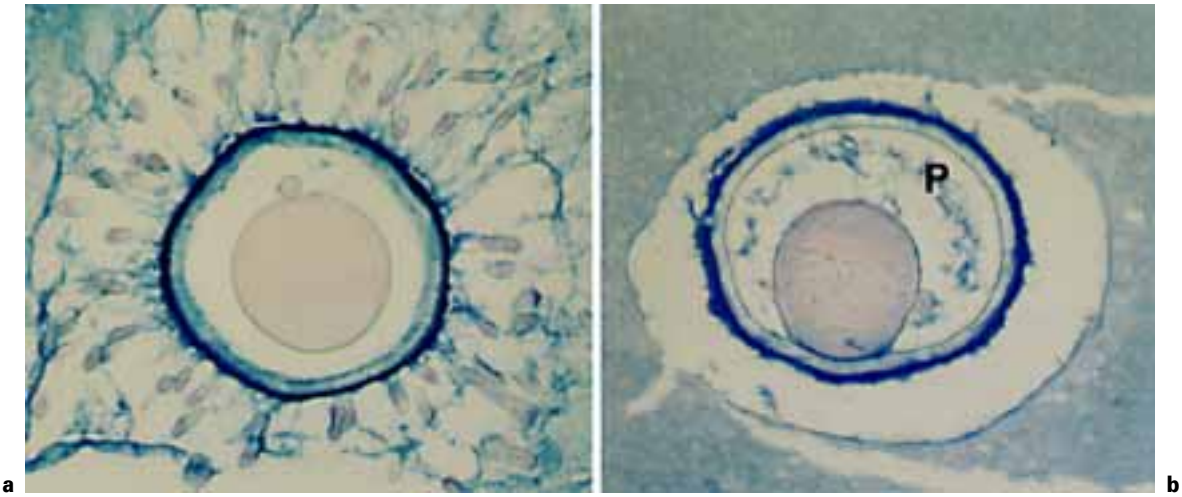

Fig. 8. Perivitelline space matrix (P) in the rabbit, appearing after fertilization (**b**) but not yet demonstrable light microscopically in nonfertilized eggs of the same age (12 h p.c.) (**a**). Does were either normally mated (**b**) or mated with a vasectomized male (**a**). The blue stain marks acid glycoconjugates, their charge being largely due to sialic acid groups (surrounding matrix in **b** is agar). Glutaraldehyde-calcium fixation, paraffin sections, saponification, Hale reaction, nuclear fast red counterstain. × 310. From Denker [1970a].

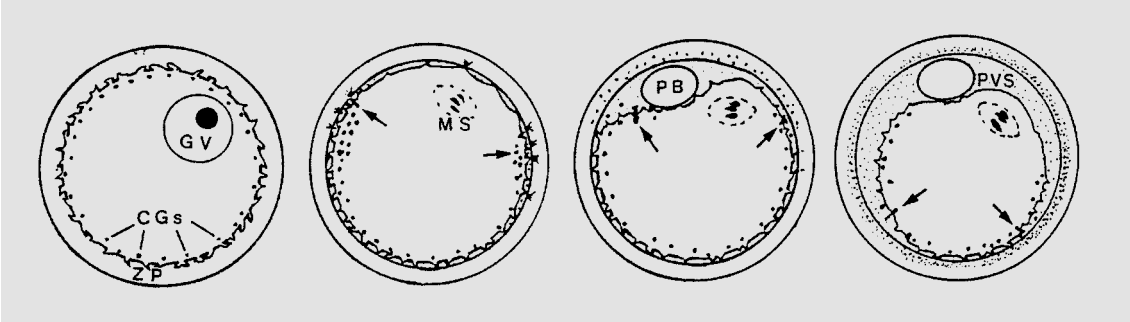

Fig. 9. 'Conditioning' of the inner parts of the mouse zona pellucida (symbolized by the stippling) by material extruded from the egg already during maturation. Possibly a special set of vesicles/cortical granules is involved in this first wave of matrix deposition, i.e. before sperm penetration. Note that a polarity (along the animal-vegetal axis) is established this way on the zona, at least temporarily, which could influence cell behavior and axis formation during cleavage. However, no evidence for such a role has been shown yet in eutherian mammals, in contrast to marsupials (fig. 7). From Okada et al. [1993]. CGs = Cortical granules; GV = germinal vesicle; MS = meiotic spindle; PB = polar body; PVS = perivitelline space; ZP = zona pellucida. Arrows mark sites of active cortical granule release.

rian mammals in contrast to marsupials. In an attempt to inforce such an attachment to matrix onto blastomeres in the rabbit, laminin was injected into the perivitelline space, but this change of composition/properties of the PVM did not disturb regular polarization of presumptive trophoblast cells and early development [Strunck-Kortenbusch, 1991; Strunck-Kortenbusch and Denker, unpublished]. This may show once more that cell-cell interactions prevail over adhesive cell-matrix interactions during trophoblast/embryoblast differentiation in eutherian mammals, although it cannot be excluded that injection of a complex, polymerizing matrix (like Matrigel) might have given different results. In any case, we should be open for the possibility that the polarity of PVM formation along the animal-vegetal axis could influence axis formation, not only in marsupial but perhaps even in eutherian mammals. Recently, there is a great deal of renewed interest in axis determation in mammals [Antczak and

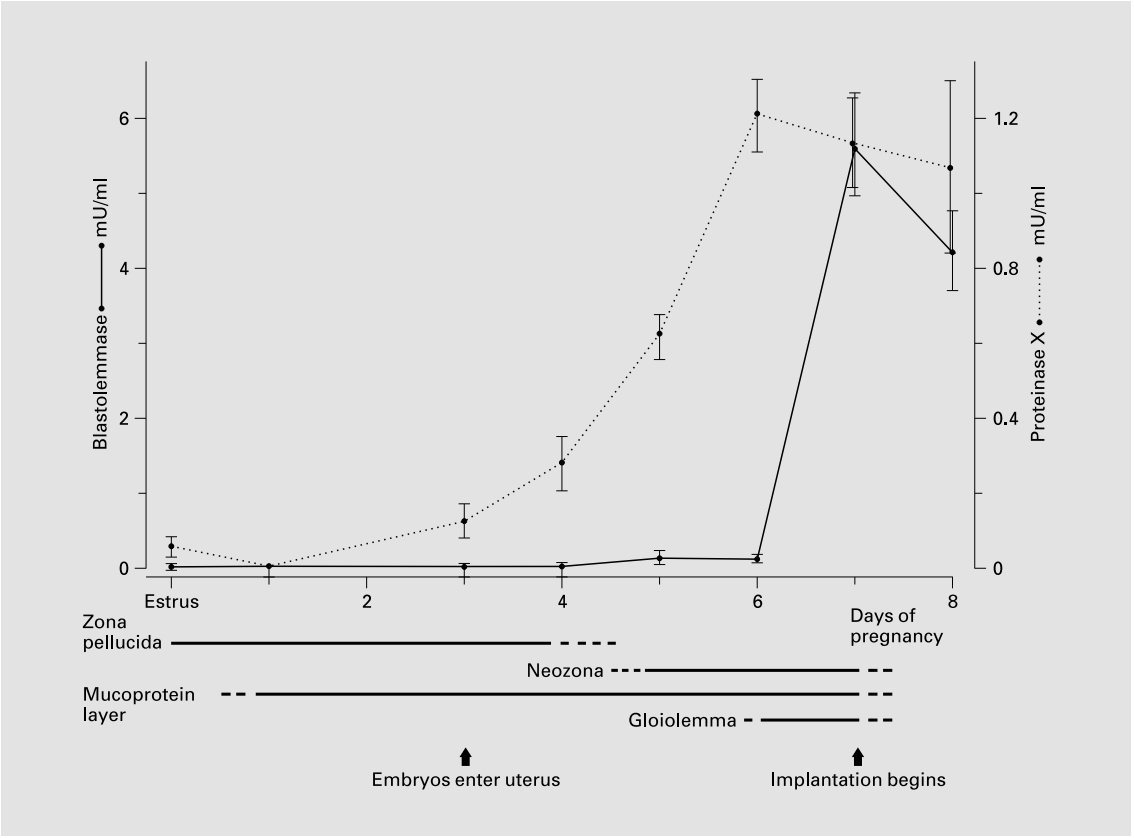

Fig. 10. Correlation of proteinase activities with dissolution of rabbit blastocyst coats (coverings). Blastolemmase is trophoblast-dependent (probably a product of trophoblast cells) and, therefore, only demonstrable in flushings from pregnant (not pseudopregnant) uteri. It is a serine endopeptidase with high substrate selectivity (splitting only certain of the arginyl bonds available) and almost certainly causes limited proteolysis in natural (protein) substrates, potentially producing biologically active large peptide fragments that could play a role in local signalling in the implantation chamber. As shown by inhibitor experiments (fig. 1c), this enzyme is essential for hatching of the blastocyst from its coats at implantation initiation. Proteinase X has not yet been characterized to the same extent. In contrast to blastolemmase, it appears in the uterine lumen even in pseudopregnancy (not shown), although at reduced levels, and has a substrate specifity that is different from blastolemmase (hydrophobic amino acid bonds). The correlation of the appearance of proteinase X in the uterine fluid with the dissolution of the zona pellucida within the mucoprotein layer may suggest a causal connection, but this has not been demonstrated so far experimentally. From Denker et al. [1989].

van Blerkom, 1997; Gardner, 1997; Beddington and Robertson, 1999], obviously influenced by the great progress that has been made with respect to axis formation in Drosophila. In the insect system, processes taking place in the perivitelline space and the equivalent of the coats, the chorion, play an important role in axis formation (dorsoventral axis: toll, toll ligand, localized proteinase action [St Johnston and Nüsslein-Volhard, 1992; Nilson and Schüpbach, 1998]). To me it appears very tempting to speculate that Mother Nature may have conserved such principles during phylogeny even though the mammalian egg is, as a rule, round and not elongated like most insect eggs, and thus may be able to rotate inside the zona pellucida. However, according to time-lapse video recordings, rotations of the plasma membrane against the zona do not seem to be very extensive while observable movements may be predominantly intracytoplasmic [Edwards and Beard, 1997; see their fig. 13].

Embryonic Coats, Implantation and Local Signaling

As mentioned in the Introduction, blastocyst coats are a potential impediment for implantation initiation. Discussions are going on as to what extent hardening of the 'zona' should be of concern in human IVF-ET [De Vos and Van Steirteghem, 2000; Magli et al., 1998]. There is ample evidence from studies in various animal systems,

notably the rabbit and the mouse, that proteinase systems (of the trophoblast as well as the uterus) are involved in hatching of the blastocysts by causing weakening and at least partial dissolution of the coats, with most data being available for the rabbit [for review, see Denker, 1977; Denker et al., 1989] and the mouse and rat [Dabich and Andary, 1976; Hoversland and Weitlauf, 1982; Menino and O'Claray, 1986; Mintz, 1972; Perona and Wasserman, 1986; Pinsker et al., 1974; Rosenfeld and Joshi, 1981; Yamazaki et al., 1994; with respect to the unclear role of the plasminogen activator-plasmin system, see Carroll et al., 1993; Liedholm and Åstedt, 1975]. Whether comparable proteinase systems are also involved in human blastocyst hatching is unknown but would appear quite probable. The old controversy whether the blastocyst hatches from the coats just mechanically or whether enzyme systems (of blastocyst or uterine origin or both) are involved seems to be largely solved in favor of the latter possibility, since even in species in which at earlier times mechanical hatching has been assumed to take place normally, careful morphological observations suggest that lytic processes must be involved [e.g. hamster: Gonzales et al., 1996; rhesus monkey: Boatman, 1987].

I do not intend to review the existing data on mammalian hatching enzymes, but it appears appropriate to address briefly two points that are of special interest in the context of implantation initiation: a possible role of the coats and their lysis in positioning the blastocyst within the uterus, and in growth factor regulation and local signalling in the implantation chamber.

Positioning of Blastocysts in the Uterus

In the human the blastocyst is known to prefer certain sites within the uterus for implantation; in polytocous species the blastocysts are spaced more or less evenly over the length of the uterus. In the horse, the (considerably expanding) conceptus is moved from one uterine horn to the other for many days, while its capsule is intact, but even before the capsule is shed it finally becomes immobilized [for literature, see Oriol et al., 1993a, b].

The mechanisms for the positioning are unknown but could involve the embryonic coats at least in those species in which the coats are maintained up to this stage. Little experimental work has been done so far in this respect. Böving [1954, 1972] has pioneered this line of research by providing evidence for a twofold role that the coats may play here: (1) For even spacing of the blastocysts over the length of the uterine horns, the original rigidity of the coats is helpful, and spacing occurs in the same way as with, e.g., glass beads by myometrial activity [Böving, 1972]. (2) For immobilization of the blastocyst and orientation in the implantation chamber (with the abembryonic pole facing the antimesometrial part of the endometrium), he considered it crucial that the coats (coverings) of the rabbit become sticky at the time of implantation initiatition, particularly at the abembryonic pole; indeed he reported that when one tries to pull the blastocyst away from the endometrium, the coats may adhere so firmly to the uterine epithelium that in some places the epithelium is even torn apart or away from deeper tissue. He proposed that the gloiolemma could be specifically involved in this phenomenon, this being the reason why he created the term. Since the stickiness of rabbit blastocyst coats is increased at alkaline pH, since the rabbit blastocyst fluid is a bicarbonate buffer system that shows a considerable increase in pH when CO_2 is lost, and since carbonic anhydrase is highly active in the uterine epithelium at these stages, he proposed that a principle of local cross-talk via this system is at work that leads to local stickiness of the coats due to local alkalinity. The idea was criticized because some of the earlier data on actual pH values in vivo had exaggerated this situation due to excessive artificial loss of CO_2 from the bicarbonate system, but careful measurements done with microelectrodes in vivo have verified that indeed a pH gradient can be measured clearly in the rabbit implantation chamber within the uterine lumen (mesometrial pH 7.32; antimesometrial pH 7.54) [Petzoldt as cited by Denker, 1983].

It was also proposed that the main proteinase involved in weakening and dissolution of rabbit blastocyst coats, blastolemmase, may play a role in this context: this enzyme shows an alkaline pH optimum and appears to be produced by the trophoblast. It appears possible that softening of the blastocyst coats by blastolemmase, which causes a limited proteolysis (see below), could be involved in orientation of the blastocyst with its embryonic-abembryonic axis parallel to the mesometrial-antimesometrial axis of the uterus, since in this position the peak of blastolemmase production in the blastocyst would coincide with the highest local pH value [Denker, 1983]. This is no more than a possibility, and direct experimental proof is lacking, although there is a suggestion from experiments with blastolemmase inhibitors in vivo: in rabbits treated this way the incidence of blastocyst malorientation, which is normally very low [Denker, 1974], is greatly increased [Denker, unpublished].

If a chemical modification of the coats is involved in positioning of the blastocyst/conceptus and in orienting it within the uterus with the attaching part facing the right part of the endometrium, this could of course not only be

Table 1. Relative hydrolysis rates of various p-nitroanilide (NHNp) substrates as caused by rabbit blastocyst proteinase (blastolemmase)

Substrate (notation: P_3 P_2 P_1 NHNp)		Relative hydrolysis rate (I = 100)	Preferably hydrolyzed by
I	TosGlyProArgNHNp	100.0	thrombin
II	DValLeuArgNHNp	130.8	glandular kallikreins
III	DProPheArgNHNp	94.4	plasma kallikrein
IV	DPhePipArgNHNp	68.7	thrombin
V	ZGlyProArgNHNp	51.7	thrombin
VI	BzIleGlu(OR)GlyArgNHNp	16.0	factor Xa
VII	DValLeuLysNHNp	5.6	plasmin
VIII	BzArgNHNp	(2.7)	trypsin
IX	SucAlaAlaAlaNHNp	(2.4)	elastase
X	GlPheNHNp	(1.2)	chymotrypsin

The high substrate selectivity is remarkable. There is not only a preference for arginyl (Arg) over lysyl (Lys) bonds in position P_1, but the neighboring amino acids in positions P_2 and P_3 also strongly influence the hydrolysis rates, meaning that in a natural (protein) substrate only few of the available arginyl bonds will most probably be cleaved (limited proteolysis). Enzymes of this type usually produce biologically active peptides (like the activated forms of blood clotting factors, growth factors, kinins) from high molecular weight precursors. This may also be the case during lysis of the blastocyst coats.

Data from Denker and Fritz [1979].

brought about by proteinases but also by other enzymes, e.g. by glycosidases [Denker, 1971a]. Some of the carbohydrate side chains of glycoproteins can strongly determine the anionic/cationic properties, notably the sialic acid (neuraminic acid) content and sulfate ester groups. It has been reported that the mobility of 5-day rat blastocysts in the electric field is largely influenced by the presence or absence of the zona [Clemetson et al., 1971]. In the horse, the sialic acid content of the capsule was found to decline markedly from about day 16 on, which may be relevant for conceptus fixation in the uterus [Oriol et al., 1993b; Chu et al., 1997]. In the rabbit evidence was presented that sialidase (neuraminidase) and proteinases may cooperate in dissolution of blastocyst coats [Denker, 1970a, 1971a]. Unfortunately, so far very little is known about the proteinases possibly involved in shedding of the capsule in the horse [Denker et al., 1987] and in their possible role in changing the physical properties (stickiness) of this coat.

Growth Factors and Local Signaling

The role of various growth factors and cytokines in the cross-talk between the blastocyst and the uterus and in implantation has recently received a lot of interest [Cross et al., 1994; Graham et al., 1993; Haimovici and Anderson, 1993; Hamilton et al., 1998a, b; Jaber and Kan, 1998; Paula-Lopes et al., 1998; Sharkey, 1998; Surveyor et al., 1998; Tabibzadeh and Babaknia, 1995]. Reports that implantation can be blocked in mice by an IL-1 antagonist [Simón et al., 1994; Karagouni et al., 1998] received particular attention. The EGF-like growth factor family is of special interest since it shows evidence of very local signal exchange within the implantation chamber [Das et al., 1994, 1997; Bush et al., 1998].

Interestingly, recent findings suggest that the embryonic coats may play a role in this context. Herrler et al. [1998a–c] have presented evidence for the presence of insulin-like growth factor binding protein 3 (IGFBP 3) and HB-EGF in the coats of rabbit and horse embryos. It is conceivable that, e.g., IGFBP 3 could act here by regulating the transport and the availability of IGF [Herrler et al., 2000].

There is very good reason to suppose that the biological role of the proteinase system involved in dissolution of the coats (already referred to above) on one hand, and local signaling via growth factors on the other, are interconnected, in particular in the implantation chamber, and I would like to suggest this here. The biochemical properties of blastolemmase are those of a proteinase which causes limited proteolysis (table 1) [Denker and Fritz, 1979]. It has been suggested, therefore, that from its natural substrates within the blastocyst coats blastolemmase cleaves larger fragments that can still be biologically active and would then diffuse from the 'reservoir' (the

coats) towards their potential receptors at either the trophoblast or the uterine epithelium [Denker, 1983; Denker et al., 1989]. Such a mechanism could provide a means of how growth factors/cytokines/binding proteins can be stored around the blastocyst within the coats for local release within the implantation chamber. Due to activation of the proteinase system at implantation initiation, the implantation chamber could literally become 'swamped' with large quantities of such molecules within a short time period, at least in those species in which the coats are retained up to this stage. In species in which the coats are shed before the blastocysts reach their definitive location within the uterus (implantation chamber), one would not think of any local action on the uterus but perhaps rather on the blastocyst. In any case, an interplay between the growth factor and proteinase system is already known from the cell surface-ECM interaction in the stromal space [Nunes et al., 1997]. Interestingly, in this example even the enzyme transglutaminase, for which a function in coat formation was discussed above, seems to play a special and significant role. Somewhat similar processes of regulation may indeed take place within the uterine lumen, since there is evidence that proteinases present there do create biologically active fragments, e.g., of connective tissue growth factor [Ball et al., 1998; Lee at al., 1998]. I would like to suggest that this may be one aspect of early embryonic coats that could be particularly worthwhile for consideration as a 'hot' research topic for the near future.

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