

Proteases and implantation in the rabbit: role of trophoblast vs. uterine secretion

Zur Bedeutung von Proteasen für die Implantation beim Kaninchen: Abhängigkeit vom Trophoblasten oder vom Uterussekret?

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Abstract

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Two hypotheses have been proposed for the origin of the gelatin dissolving enzyme involved in the dissolution of blastocyst coverings in the rabbit: trophoblastic vs. maternal origin. In a series of experiments described in the present communication, egg coverings without living blastocyst tissue (mucoprotein-covered Sephadex beads, morulae and unfertilized eggs) were used as models which lack trophoblastic factors. These models were placed, for variable intervals, in pseudopregnant or pregnant uteri. Enzyme activity was studied using a highly sensitive gelatin substrate film technique. In contrast to the high activity of control blastocysts, most of the models did not show any detectable amount of enzyme. It is concluded that the protease activity normally depends on blastocyst (trophoblastic?) factors, which might be proenzyme or activator in nature. In a few cases very low activity developed when the beads were placed *in utero* for 4 days. Therefore uterine proteases (proenzymes?) possibly adsorbed by the models may also be involved but are obviously insufficient in the absence of trophoblastic tissue.

Introduction

Around the time of implantation, the rabbit blastocyst exhibits a remarkable activity of an enzyme which dissolves gelatin membranes [5]. In a histochemical substrate film test, this activity is more prominent than the activity of uterine secretion, and it seems

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to differ from the latter in such properties as optimal pH. At 7.5 days post coitum (p. c.) maximal activity is found in the abembryonic part of the blastocyst, where at this stage, the extracellular blastocyst coverings are in the process of dissolution and the trophoblast starts to penetrate the uterine epithelium [2, 6, 7].

The blastocyst coverings, as well as the surface of the uterine epithelium, are rich in negatively charged glycoproteins containing sialic acid and sulfate ester groups [6]. These substances may form a "barrier" between trophoblast and maternal tissues, and may also be involved in adhesion due to changes in physicochemical properties prior to implantation. The gelatin dissolving enzyme is probably involved in the breakdown of these substances or in the increase in stickiness exhibited by the coverings and by the surface of trophoblast cells. The enzyme was presumed to be a protease, secreted by the trophoblast [5, 9]. Initiation of implantation and concomitant rise of protease activity are both controlled by ovarian progesterone but not estrogens [8].

KIRCHNER et al. [15], on the other hand, reported that uterine secretion proteases may be responsible for the dissolution of rabbit blastocyst coverings. The protease(s) of the blastocyst coverings and those of the uterine secretion were found to migrate differently in agar gel electrophoresis [13]. Although these findings could suggest that uterine and blastocyst enzymes might be different entities, and that the blastocyst enzyme itself might be a product of the blastocyst, KIRCHNER proposed that the blastocyst-bound enzyme may be derived from maternal genital tract secretions. The enzyme could be incorporated into the blastocyst coverings in an enzymatically inactive form, and would be activated around implantation time by unknown factors.

The purpose of this investigation is to test whether or not the embryo plays a major role in the production of this protease activity. We therefore exposed to the uterine milieu egg coverings which did not contain a living embryo. If the embryo itself normally plays a passive role and does not contribute anything, these coverings should gain enzyme activity also in absence of embryonic tissue.

Materials and methods

Two types of models were used as follows:

- (a) Sephadex G-100 beads, coated with mucoprotein layer in the oviduct [4]. In addition, beads may have acquired a gloiolemma (BÖVING [2]), at least in those experiments in which they stayed in the uterus for sufficient time (see below and Fig. 1, Exp. A).
- (b) Morulae and unfertilized eggs with their zona pellucida and surrounded by a mucoprotein layer. In some experiments they possibly also acquired a gloiolemma as in (a).

These models were exposed to the uterine milieu as follows: New Zealand White female rabbits, weight 3.4 to 5.2 kg, were housed in constant light and fed standardized pellet diet and water ad lib. They were mated each to two fertile bucks, or injected intravenously with 50 I.U. of HCG (A. P. L. AYERST) to induce pseudopregnancy. Laparotomy was performed, under pentobarbital anesthesia, using a midline incision. A small volume of freshly made and autoclaved suspension of Sephadex G-100 (Pharmacia, Uppsala, Sweden; particle size according to manufacturer: 40 to 120 μm) in 0.9% NaCl solution was deposited into the fimbrial end of the oviduct before or after ovulation. Some 30 to 40 beads were pipetted into each oviduct.

Four experiments (A, B, C1, C2) were conducted using two recipient animals in each group, and a similar number of donor animals as needed.

Experiment A (duration of stay in the uterus: 4 days; blastocysts present): Beads were transferred to both oviducts of normally mated does 14 to 15 hr p. c., i. e. after fertilization of ovulated eggs (Fig. 1). Beads were left in the genital tract together with the normally developing eggs until 7 days p. c., when the animals were sacrificed.

Experiment B (duration of stay in the uterus: 4 days; no blastocysts present): For studying

whether the results might be influenced by the presence of blastocysts in the same uterine "horn", beads were transferred in the oviducts of unilaterally pregnant does. Rabbits were mated, laparotomy was performed 9 hr p.c. (1 hr before ovulation). In one ovary, the preovulatory follicles were pricked with a needle and the eggs were aspirated with a pipette. The eggs were counted under a dissecting microscope to make sure all of them were removed. Beads were transferred to the oviduct of that side. The other side was left intact to serve as control, but one of the animals also received beads at this side. The animals were autopsied 7 days p.c.

Experiment C (duration of stay in the uterus: 1 day): Donor and recipient animals were used (Fig. 1). The donor animal, being normally pregnant, received Sephadex beads deposited into the oviduct 13 to 17 hr p.c. Some 61 to 65 hr p.c. the animals were autopsied and the oviducts flushed with sterile physiological saline to recover the mucoprotein-coated beads and morulae. Beads and eggs were quickly sorted out under a dissecting microscope and transferred separately to the left and right uterine horn of recipient animals: pseudopregnant does 6 (experiment C 1) or 7 (experiment C 2) days after injection of HCG. The does were autopsied 24 hr later.

Two further *control* experiments were performed, comprising only one donor and one recipient animal each. These experiments were performed like experiment C, but the stage or duration of stay in the uterus were different. In *control 1*, beads and eggs were transferred to the uterus of a pseudopregnant recipient 3 days after HCG injection and the animal was sacrificed 24 hours later. In *control 2*, beads and eggs were transferred to a 7 day pseudopregnant uterus which was quenched with liquid nitrogen immediately after transfer. Normal control blastocysts and uteri were obtained from one normally mated untreated doe autopsied at 7 days p.c.

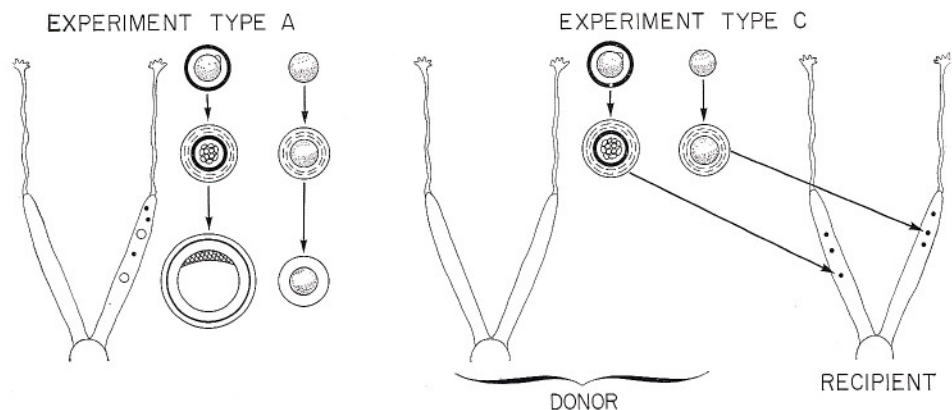


Fig. 1. Schematic representation of the design of the experiment. The two main models used are fertilized eggs and Sephadex beads. Both collect a mucoprotein layer during their stay in the tubes. They are then transferred to an implantation stage uterus (experiment C), or are left in the same genital tract until the morulae have expanded into blastocysts which start implantation (experiment A).

Histochemical techniques

The does were sacrificed with an overdose of sodium pentobarbital. The uteri were quickly removed and three segments (oviductal end, midpiece and cervical end) of the uterus were frozen with liquid nitrogen. Tissues were stored in sealed plastic bags at -25°C for up to 5 months without apparent loss of protease activity. Unfixed sections were cut on a cryostat at a thickness of $14\mu\text{m}$ and tested for protease activity using an improved histochemical substrate film technique [10]. Two different incubation periods (4 hrs and 24 hrs) were used throughout the study. No statistical analysis of the results was attempted to avoid the false impression of an accuracy which can never be reached with a histochemical substrate film test.

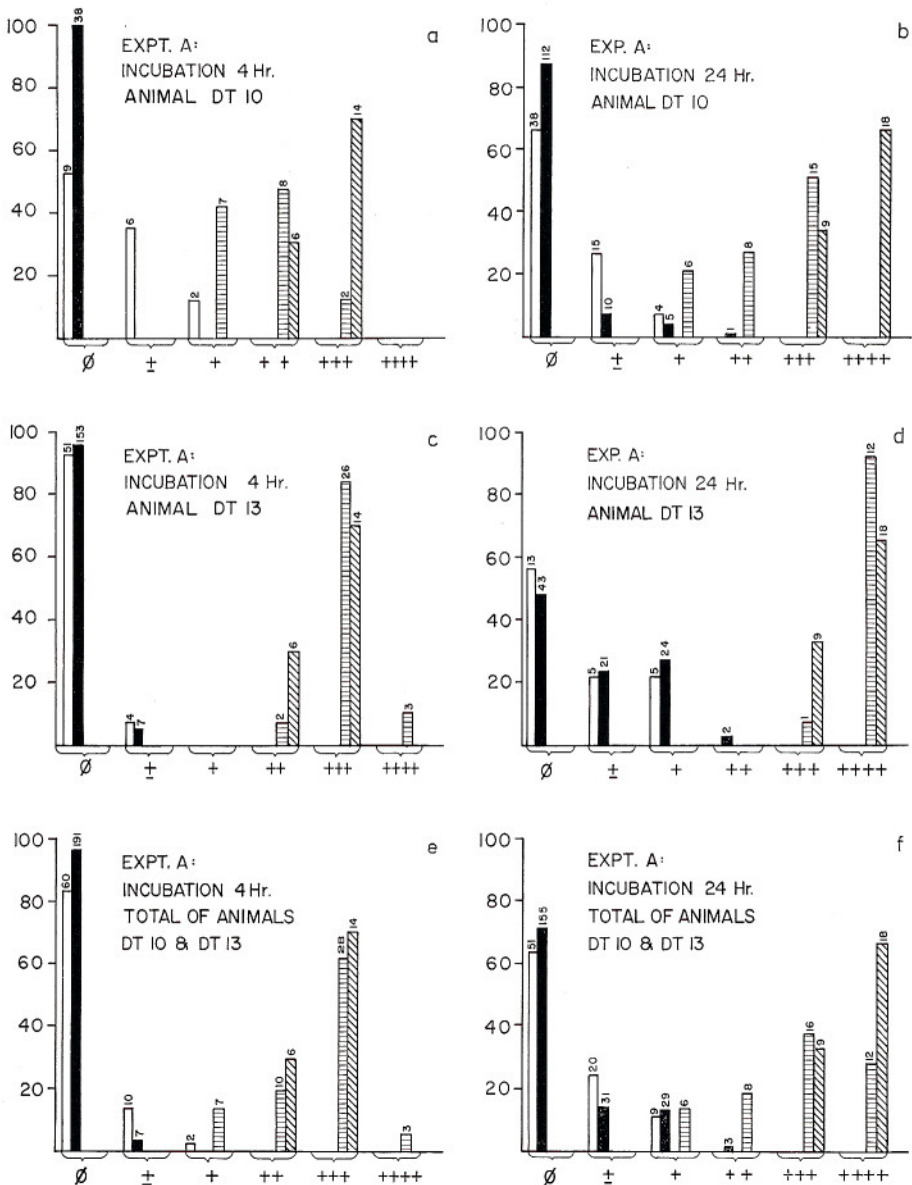
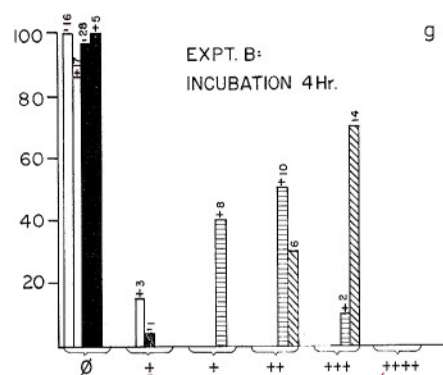
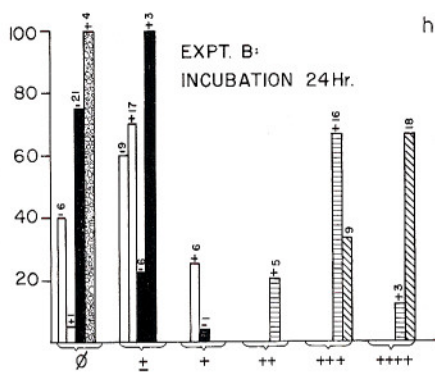


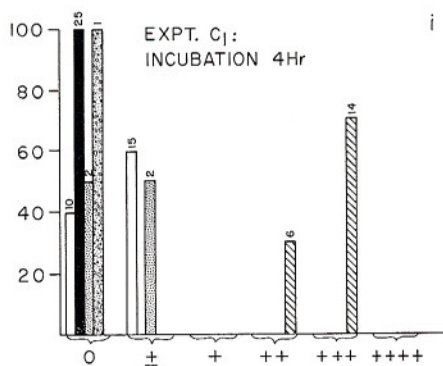
Fig. 2 a to f. Incidence and intensity of protease activity of the entities tested (*abscissa*), calculated in percentage of the total number of sections examined for each entity (*ordinate*). On top of the bars, the absolute number of sections representing the indicated percentage is given. In g and h, + and - indicate uteri with (+) or without (-) blastocysts. **a and b** and **c and d** respectively indicate the results obtained from one animal each; **e to f** give the totals of the two animals studied in each experiment.



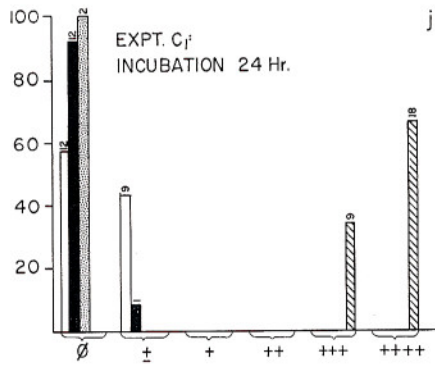
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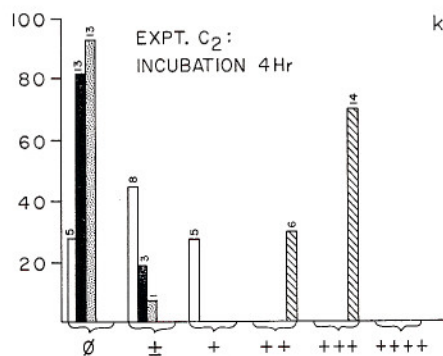
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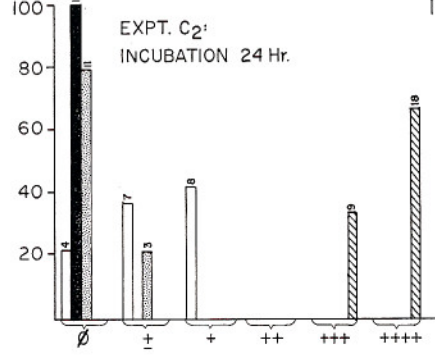
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l

- uterine secretion
- mucoprotein layer surrounding Sephadex beads
- ▨ transferred morulae
- ▩ unfertilized eggs
- ▧ blastocysts of experimental animal
- ▦ control blastocysts

Results

The number of beads, eggs and uteri which were tested for protease activity is given in Table 1. The enzyme activity of uterine secretion, beads, eggs and blastocysts is indicated in Figure 2. The percentage of the sections which exhibited a certain level of protease activity is given. Counting the number of sections rather than the number of beads etc. seemed to be the best way: It was not always possible to calculate the number of beads (eggs) from the sections, especially in crowded segments. Furthermore there is always some variation in the results of substrate film tests: e. g. in different areas of a single slide, adjacent sections of the same specimen may show slightly different reactions. Studying a sufficient number of sections is therefore essential.

In *experiments A and B* (duration of stay in the uterus: 4 days), the beads remained in the genital tract in the presence (A) or absence (B) of the developing eggs, until 7 days p. c. Whereas the blastocysts exhibited the high protease activity typical for this stage, most of the sections of beads and unfertilized eggs did not show any activity (Fig. 2). Only a minority of beads gave a clear positive reaction after 24 hours incubation. Very few showed an activity as high as ++, which is higher than observed in the uterine secretion. In experiment B, there was no obvious difference between beads in the blastocyst-containing and in the nonpregnant uterus.

Tab. 1. Material tested for protease activity.

Experiment	No. of animals	Incubation hrs	No. of uteri	No. of beads	No. of transferred eggs	No. of unfertilized eggs	No. of own blastocysts
Experiment A	2	4	3	58	-	-	4
		24	4	71	-	-	4
Experiment B (uterus without blastocysts)	2	4	2	8	-	-	-
		24	2	9	-	-	-
		4	2	2	-	-	2
		24	2	2	-	1	3
Experiment C 1	2	4	3	7	1	1	-
		24	3	7	1	-	-
Experiment C 2	2	4	3	5	3	-	-
		24	3	5	3	-	-
Control 1	1	4	2	2	3	1	-
		24	2	2	3	1	-
Control 2	1	4	2	12	1	1	-
		24	2	12	1	1	-
Control blastocysts 7 d p.c.	1	4	2	-	-	-	3
		24	2	-	-	-	3

There is some overlap between the figures given for 4 hours and 24 hours incubation respectively: usually sections taken from the same uteri (beads or eggs) have been used for both sets of slides.

In *experiment C* (duration of stay in the uterus: 1 day), mucoprotein-covered Sephadex beads and eggs exposed to the uterine milieu for one day failed to develop clearly detectable enzyme activity (Fig. 2). The reaction of the uterine secretion was, in some cases, even more obvious than that of the beads and eggs. This was in clear contrast to the intense reaction of control blastocysts.

In *control 1* (not included in the histograms Fig. 2), morulae and beads were transferred into three day - pseudopregnant uteri for 24 hrs. In the histochemical protease test, beads and eggs did not show any obvious activity, the individual observations being as follows: After 4 hours incubation neither sections of the beads (9 sections of 2 beads tested) nor of the young blastocysts (13 sections of 3 eggs) nor of an unfertilized egg (2 sections) exhibited any protease activity. After 24 hours of incubation, none of 7 sections of 2 beads showed any reaction; of 17 sections taken from 3 young blastocysts, 15 were negative and 2 were \pm ; the only section of an unfertilized egg was \pm . The blastocysts, which had developed from the transferred morulae, exhibited all features of normal 4 days p. c. embryos.

In *control 2* (not included in the histograms Fig. 2) the uterus was frozen immediately after transfer of beads and morulae at 7 days of pseudopregnancy. Beads, one transferred morula and one unfertilized egg did not exhibit any protease activity (4 hours of incubation: 72 sections of 12 beads, 2 sections of 1 morula and 3 sections of 1 unfertilized egg; 24 hours of incubation: 57 sections of 12 beads, 1 section of 1 morula and 2 sections of 1 unfertilized egg).

In all experiments, the thickness of mucoprotein layer collected around the beads was less than that of the morulae.

Discussion

For the origin of rabbit blastocyst protease, we shall discuss three possibilities: 1. deriving from the maternal genital tract secretions and not depending on blastocyst factors; 2. deriving from the tubal or uterine secretions but deposited in the coverings in an inactive form, and, during implantation, becoming activated by activators provided either by the blastocyst or by the uterus or by both (Fig. 3 a); 3. deriving from the trophoblast.

In the experiments presented here, the experimental models of "egg coverings

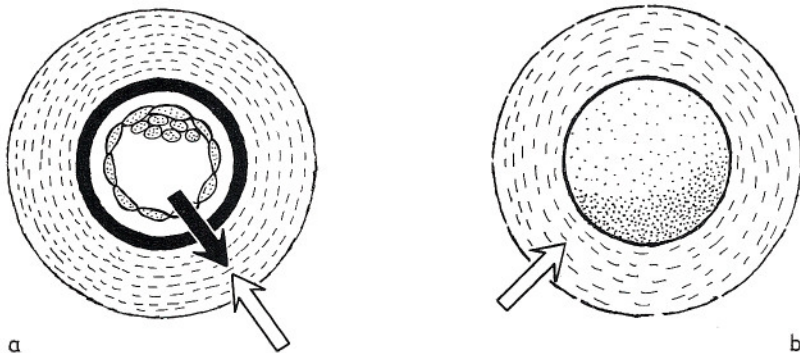


Fig. 3. Hypothetical uterine and blastocyst factors acting on the coverings of a blastocyst (a) or a bead (b).

without living embryonic cells" (mucoprotein-covered beads, morulae and unfertilized eggs) were unable to contribute any factor of blastocyst tissue (trophoblast) origin, so that they were exclusively under the influence of maternal factors (Fig. 3 b). The inclusion of morulae (and unfertilized eggs found incidentally) seems to be significant since the mucoprotein coverings surrounding the beads were thinner than that of normal morulae. In addition, morulae and unfertilized eggs possess a zona pellucida which is absent in the beads. Since the morulae (experiment C) were degenerating after they had been transferred into 2 to 3 days more advanced uterine milieu [1, 3], it is unlikely that they secreted any specific factors. In fact they failed to develop into blastocysts (whereas they did in the appropriate uterine milieu of control 1, showing that a sufficient egg transfer technique was used).

The three experimental models (mucoprotein-covered beads, morulae and unfertilized eggs) failed to develop, in the implantation stage uterine milieu, any protease activity comparable in intensity to that of control blastocysts. This evidently shows that the *protease activity exhibited by implanting blastocysts depends on the presence of blastocyst tissue.*

The presence of blastocysts in the same uterus apparently had no influence on the beads: in both gravid and nongravid uteri most beads completely failed to exhibit protease activity (compare experiments A and B).

In contrast to most of the beads and eggs which showed no protease reaction, a few models did exhibit low activity, but only when prolonged incubation periods of 24 hrs were used during the protease test.

The duration of stay in the uterus also seemed to be important: No enzyme activity was found in control 2, where the uteri were frozen immediately after transfer of tubal eggs and beads at 7 days. Only questionable activity was observed in some beads and eggs of experiment C, where the stay *in utero* was restricted to 24 hours. In contrast, in experiment A, where beads were allowed to stay 4 days in the uterus, occasionally some activity developed. This requirement of a long stay is an argument against the hypothesis that an inactive enzyme precursor is deposited around the egg by the *tubal secretion* so that it would become a constituent of the mucoprotein layer, and that around implantation time this precursor would simply be activated by uterine secretion factors. Such an activation process should require less time than one day. On the other hand, during a long stay in the uterus beads and eggs may acquire an additional covering of *uterine secretion* material ("gloiolemma", as described by BÖVING [2]). Perhaps this material is at least in part incorporated into the mucoprotein layer and/or zona pellucida rather than deposited as a separate covering ([6] p.214, [7] p.292; [14]). In both cases it seems indeed possible that, besides other material, an inactive enzyme precursor derived from the uterine secretion becomes a constituent of the egg coverings. In experiments A and B, the beads were left in the uterus for the full regular preimplantation period of time, and it might be significant that in these experiments some of the beads exhibited protease activity, although far less than the control blastocysts. This might in fact be due to an activation of an inactive form of enzyme deposited around the bead. Since only few of the beads exhibited activity one might speculate that the occasional "activation" in the coverings of the beads could be compared to the "spontaneous" autocatalytic activation of protease proenzymes observed in other biological systems (cf [12]).

Further discussions of these speculations will await the results of biochemical investigations. In the histochemical substrate film test, variables like diffusibility of the enzyme ("bound vs. unbound form") can mimic differences between active and inactive

enzymes. Furthermore, the "activation" phenomenon does not have to be a true conversion of a proenzyme to an active enzyme. A liberation from an enzyme-inhibitor complex might also be possible.

The experiments described here provide evidence that the gelatin dissolving activity of the blastocyst coverings depends on factors derived from blastocyst tissues, probably the *trophoblast*. Neither the blastocyst coverings, nor the surrounding uterine milieu, seem to provide all the necessary factors. Further biochemical data are needed to delineate whether the factor(s) derived from the blastocysts are proenzyme(s) or some type of activator(s).

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