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Embryo implantation and proteinase activities in a marsupial (Macropus eugenii)

II. Preliminary characterization of proteinases

H.-W. Denker

Abteilung Anatomie der RWTH, Aachen (West Germany)

Note added at the time of website publication

This is an unpublished manuscript and has been scanned as it was when completed in 1986. Notice that at that time point molecular biology data were not available, nor had any biochemical isolation and characterization of these marsupial enzymes been done yet. For that reason a very preliminary first classification was attempted in this manuscript using inhibitor profiling and pH optima as criteria.

The companion paper by H.-W. Denker and C.H. Tyndale-Biscoe [Embryo implantation and proteinase activities in a marsupial (Macropus eugenii): Histochemical patterns of proteinases in various gestational stages], cited here as "submitted", was published in: Cell Tissue Res 246: 279-291 (1986).

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SUMMARY

High activities of certain proteinases were previously found at implantation sites in the tammar wallaby (Macropus eugenii, Macropodidae) (Denker and Tyndale Biscoe, in press). Since an important role of proteinases in embryo implantation initiation has been established for eutherian mammals, classification of the marsupial enzymes is of interest in the context of hypotheses on evolution of viviparity in mammals. In this investigation, a series of proteinase inhibitors of known specificity is used as probes for classification of tammar wallaby proteinases.

The proteinase present at the interface between the trophoblast of the bilaminar yolk sac and the uterine epithelium (only around day 19, i.e. implantation initiation) is characterized as an unusual alkaline thiol (SH) proteinase. It is strongly inhibited by E-64, antipain, leupeptin, chymostatin, elastatinal, Z-Phe-Ala-CHN_2 and iodoacetamide, but not by typical serine or metal proteinase inhibitors. This is of particular interest since this enzyme has many other features in common with blastolemmase, the alkaline proteinase which was previously found to play a key role in the initiation of embryo implantation in the rabbit, and which is a serine enzyme, showing a clearly different spectrum of inhibition.

On the other hand, the alkaline proteinase found in tammar wallaby stroma cells (mainly in the myometrium) is classified as a serine enzyme. The predominant acid proteinase activity found in this material (in the bilaminar yolk sac wall, endo-

metrial stroma cells) was more difficult to classify since inhibitor effects were much less pronounced in this case. It appears possible that several different enzymes contribute to gelatinolysis at these sites, and / or that activation of a latent form of enzyme by another proteinase may play a significant role here. While unequivocal identification of these proteinases as well as of their possible interaction would depend on biochemical isolation, the main acid proteinase activity seems to be due to an intracellular thiol enzyme.

Possible functional roles of the described enzymes are discussed in the context of findings about eutherian implantation-associated proteinases. Of particular interest is the alkaline proteinase present at the interface between trophoblast and uterine epithelium in the tammar wallaby. Striking analogies between this enzyme and the eutherian counterpart, rabbit blastolemmase, include location and time of appearance and disappearance (i.e. association with implantation initiation) and may suggest partially comparable functions. However, completely different enzymic properties (different catalytic class) seem to exclude any homology, and suggest that convergent evolution has occurred. This is considered an argument, on the molecular level, for independent evolution of viviparity in the two groups of mammals, the marsupials and the eutherians.

KEY WORDS:

Nidation - proteinases - development, phylogenetic - cytochemistry - Marsupialia

INTRODUCTION

Considerably elevated proteinase activity is found at implantation sites in the marsupial, Macropus eugenii, as shown in a previous communication (Denker and Tyndale-Biscoe, in press). Using a histochemical gelatin substrate film test, main proteinase activity was localized in the yolk sac wall, at the interface between trophoblast and uterine epithelium, in endometrial crypt cells and in endometrial and myometrial stroma cells. On the basis of pH dependence, this activity appears to belong to two distinct classes of enzymes, a proteinase(s) with pH optimum in the acid range (localized in the yolk sac wall with maximum activity in the bilaminar yolk sac wall, BYS, at the basis of endometrial crypts and in endometrial stroma cells) and another type of proteinase(s) with pH optimum in the alkaline range (found at the interface between trophoblast and uterine epithelium around day 19, and in stroma cells predominantly of the myometrium).

Acid proteinase activity (cathepsin type) is elevated at implantation sites also in certain eutherian mammals (see DISCUSSION). Of particular interest is an alkaline proteinase which appears to play a central role in the process of implantation initiation in the rabbit. As shown in in vivo inhibitor experiments, it is essential for disolution of the extracellular blastocyst coverings, a prerequisite for trophoblast attachment to the uterine epithelium (Denker, 1977a).
In the tammar wallaby, an alkaline proteinase is found at a somewhat comparable extracellular site, i.e. at the interface

between trophoblast and uterine epithelium in the BYS region (abembryonic pole) where it is active for only about 1 day. During the same period of time, the processes of removal of extracellular embryo coverings and contact formation between trophoblast and uterine epithelium are going on in this region, although in the TYS region (embryonic pole) this contact is formed without comparable proteinase activity ever appearing.

In the present communication, a preliminary classification of the marsupial enzymes is attempted. A series of proteinase inhibitors of known specificity is used as probes as it has been done before in the rabbit (Denker, 1976, 1977a). As will be shown, evidence suggests that the predominant alkaline proteinase of the marsupial, although its location and time of appearance are analogous to the situation in the rabbit, does not belong to the same class as the Eutherian enzyme but must have evolved independently. Functional aspects will have to be discussed in the context of these phylogenetic considerations.

MATERIALS AND METHODS

Conceptus-containing uteri were obtained from tammar wallabies (Macropus eugenii) and were processed for cryostat sectioning as described previously (Denker and Tyndale-Biscoe, in press. Stages around the time when extracellular embryo coverings (shell membrane) disappear and contact is established between trophoblast and uterine epithelium, i.e. stages between day 17 and 20 after removal of pouch young (RPY), were selected for the present study from the material described in the previous

communication (Denker and Tyndale-Biscoe, in press , focussing on day 19 RPY when alkaline proteinase activity as well as maximal acid proteinase activity are found.

The highly sensitive variant of the gelatin substrate film test (Denker, 1974) was used for localization of proteinases. For the identification of different classes of proteinases, inhibitors of known specificity were employed according to the guidelines given previously (Denker, 1977b). In brief, one half of the slide with the gelatin film was soaked with inhibitor solution, while the other half was treated only with buffer (and, in case of water-insoluble inhibitors, with the solvent alone). Native cryostat sections taken from conceptus-containing uteri were mounted on these films and were incubated in moist chambers at 37°C for 105 min., fixed and stained as described before (Denker, 1974, 1977b).

Chemicals:

Proteinase inhibitors: antipain: art.-no. BAY f 7412 provided by Dr. W. Wirgender, Bayer AG Elberfeld, or Protein Research Foundation (Peptide Institute, Inc.), Osaka, Japan, No. 4062; aprotinin (Trasylol®): Bayer AG Elberfeld (6600 KIU/mg); chymostatin: Protein Research Foundation, Osaka, Japan, No. 4063, or Sigma No. C 7268; DL-dithiothreitol: Sigma No. D 0632; E-64: Cambridge Research Biochemicals No. 16525; EDTA (ethylenediaminetetraacetic acid, disodium salt; Titriplex III): E. Merck, Darmstadt, No. 8418; elastatinal: Protein Research Foundation, Osaka, Japan, No. 4064; iodoacetamide: Serva, Heidelberg, No. 26710; leupeptin: Protein Research Foundation, Osaka, Japan,

No. 4041, or Bachem AG, Bubendorf, Switzerland, No. 9355; phosphoramidon: Protein Research Foundation, Osaka, Japan, No. 4082; SBTI (soybean trypsin inhibitor): E. Merck AG, Darmstadt, No. 24020, or Serva, Heidelberg, No. 37329, or Worthington No. LSOO 03570; Z-Phe-Ala-CHN_2 (Z-phenylalanyl-alanyl-diazomethyl ketone): Enzyme Systems Products, Livermore, CA, No. DK-3; Z-Phe-Phe-CHN_2 (Z-phenylalanyl-phenylalanyl-diazomethyl ketone): dto., No. DK-2.
Gelatin: E. Merck AG, Darmstadt, No. 4070.

RESULTS

The results of the inhibitor experiments are summarized in Tab. 1. The main proteinases that are demonstrable with the gelatin substrate film test at various histological sites show a characteristic and different pattern of interaction with the used series of inhibitors.

The alkaline proteinase present around day 19 RPY at the interface between trophoblast and uterine epithelium in the BYS region (cf. the previous communication, Denker and Tyndale-Biscoe, in press) is strongly inhibited by the microbial inhibitors antipain, leupeptin, chymostatin, elastatinal and E-64, by the synthetic inhibitor Z-Phe-Ala-CHN_2 and by the SH reagent iodoacetamide. The metalloproteinase inhibitor phosphoramidon is without any effect, and the combination of the chelating agent EDTA plus the SH protecting compound dithiothreitol shows either no effect on the enzyme activity or slight activation. The serine proteinase inhibitors, aprotinin and SBTI, likewise remain without any clear-cut effect on this enzyme.

The alkaline proteinase activity present in stroma cells (predominantly in the myometrium), although likewise strongly inhibited by the microbial inhibitors antipain, leupeptin and chymostatin, differs distinctly from the former by not being affected by E-64 and by suffering only weak and varying inhibition by iodoacetamide; only questionable effects of Z-Phe-Ala-CHN_2, no activation by EDTA + dithiothreitol and slight although varying inhibition by aprotinin were observed.

The acid proteinase(s) predominantly present at the BYS wall, at endometrial crypts and endometrial stroma cells shows again a different spectrum of interactions with inhibitors, but relatively similar at all 3 histological sites. There is weak inhibition by the microbial inhibitors antipain, leupeptin, chymostatin and elastatinal, strong inhibition by iodoacetamide and mostly strong activation by EDTA + dithiothreitol. No effect is observed with the thiol proteinase inhibitor E-64 (except for questionable effects at the BYS wall), and also no inhibition by the serine proteinase inhibitors aprotinin and SBTI. Inhibition by the diazomethyl ketones Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 is weak at the best. Slight differences in reactivity with the inhibitors were noted between the 3 histological sites, however: Effects of the microbial inhibitors are generally more clear-cut at the BYS wall than at the two other sites, which holds also true for the diazomethyl ketones (although the results are more varyable here). The activation by EDTA + dithiothreitol is most pronounced at the BYS wall and in endometrial stroma cells, but more varyable at endometrial crypts.

DISCUSSION

Classification of tammar wallaby proteinases

Proteinases can be assigned to the various catalytic classes, i.e. serine, thiol, carboxyl, and metalloendopeptidases, on the basis of their pH dependency and their interactions with specific proteinase inhibitors, as summarized in Table 2. There is some overlap in the specificities of certain of the inhibitors, however, e.g. the microbial inhibitors antipain, leupeptin and chymostatin are active against certain serine as well as thiol proteinases. It is advisable, therefore, to use more than one inhibitor for each class (as done here) which, in addition, offers the possibility to attempt a certain degree of sub-classification at the same time. In a histochemical study like the one presented here, emphasis has to be put on qualitative effects of the various inhibitors but not on the concentrations used, since the latter are somewhat misleading: For technical reasons, the gelatin substrate films must be impregnated with a relatively concentrated inhibitor solution (as indicated in Tab. 1), while the actual concentration as present during the test is much lower but cannot be determined accurately (Denker 1977b).

Alkaline proteinase of the trophoblast-endometrial interface

The gelatinolytic proteinases found at tammar wallaby implantation sites can be classified on this basis preliminarily when the results summarized in Table 1 are compared with Table 2. The alkaline proteinase present on day 19 RPY at the

BYS-endometrial interface is clearly classified as a thiol (SH) proteinase. The strongest argument for this conclusion is the clear-cut inhibition by E-64 and the diazomethyl ketones (Z-Phe-Ala-CHN_2 being more inhibitory here than Z-Phe-Phe-CHN_2) both of which are reported to be highly specific for thiol proteinases (Hanada et al. 1978, 1983; Green and Shaw 1981; Shaw and Green 1981). An additional argument for this is the inhibition by the microbial inhibitors antipain, leupeptin, and chymostatin, as well as by iodoacetamide, although the specificity of these inhibitors is less restricted (the three microbial inhibitors being active also against certain serine proteinases). There is no argument for classification of this marsupial enzyme as a serine proteinase, on the other hand, since there are no clear-cut effects of aprotinin (Trasylol) and SBTI. The proteinase does certainly not fall into any of the remaining two classes of proteinases, carboxyl and metalloendopeptidases, since there is no inhibition by phosphoramidon and also no inhibition but activation by EDTA + dithiothreitol. The strong inhibitory effect of elastatinal may appear to contradict literature if this enzyme is indeed a thiol proteinase, since this microbial inhibitor has been reported to be highly specific for pancreatic elastase which is a serine enzyme (Umezawa and Aoyagi 1977, 1983). However, all the inhibitors with aldehyde groups (including elastatinal) do inhibit thiol enzymes (Fritz, personal communication). We conclude, therefore, that the alkaline proteinase active at the BYS-endometrial interface in the tammar wallaby around day 19 RPY is a so far unknown alkaline thiol (SH) proteinase.

Alkaline proteinase of myometrial stroma cells

The alkaline proteinase of tammar myometrial stroma cells shows superficially some features that are similar to those of the former enzyme but turns out to be a different entity, nevertheless. Similarities are the strong inhibition by antipain, leupeptin and chymostatin and, on the other hand, lack of inhibition by phosphoramidon. On the other hand, inhibition by iodoacetamide is only weak and there is no activation by EDTA + dithiothreitol. The strongest argument for the enzyme being different from the above-mentioned extracellular proteinase of the BYS-endometrial interface is the complete lack of inhibition by E-64 and the diazomethyl ketones. This makes it very improbable that this enzyme is a thiol proteinase. The clear-cut effects of antipain, leupeptin and chymostatin as well as the pH optimum exclude the possibility that this is a carboxyl proteinase. The lack of any effects of phosphoramidon and the combination of EDTA + dithiothreitol exclude the group of metalloendopeptidases. On the other hand, there are slight although somewhat varying effects of aprotinin (Trasylol) and SBTI. We conclude, therefore, that we are dealing here with a serine proteinase. Since a large number of serine proteinase inhibitors with varying specificity is available and has been successfully used for classification of mammalian implantation-associated proteinases with the gelatin film test before (Denker 1977a) it can be expected that stronger and more clear-cut effects of certain typical serine proteinase inhibitors could be detected in future more extensive studies.

Acid proteinase(s)

Classification is much more difficult for the acid proteinase activities than for the alkaline enzymes, in this marsupial material. Classification as a serine endopeptidase seems to be excluded due to both lack of any effects of the appropriate inhibitors as well as to the pH range of activity. Also excluded seem to be the classes of metalloendopeptidases (lack of clear-cut effects of phosphoramidon, no inhibition by EDTA + dithiothreitol), although phosphoramidon had (slight and varying) effects in case of the endometrial crypts so that a contribution of a metalloendopeptidase cannot be completely excluded for this particular histological site. The carboxyl endopeptidase class seems excluded since there are inhibitory effects of certain microbial inhibitors which would not inhibit this group of enzymes (see below). On the other hand, the strong effect of iodoacetamide suggests that we are dealing here with a thiol proteinase(s). In agreement with this, there is activation by EDTA + dithiothreitol. Also, there are inhibitory effects of the microbial inhibitors, antipain, leupeptin and chymostatin, but they are only slight and somewhat different at the three histological sites. Very surprising, however, is the lack of clear-cut effects of E-64 and the generally non-impressive effect of the diazomethyl ketones. The clearest effects are seen with the diazomethyl ketones at the BYS wall (where also E-64 may show some questionalbe inhibition); however, in cannot be excluded that this as well as the effects of the above-mentioned microbial inhibitors may, at the BYS wall, be due to the alkaline proteinase discussed above which

is present at this site, which is strongly inhibited by these inhibitors and which may not be completely suppressed when the test is run at pH 5 (cf. also Figs. 1 + 4).

On the basis of these data, classification of the acid proteinase(s) must remain somewhat speculative for the present time. Most probably we are dealing with intracellular proteinases of a cathepsin type (like cathepsin B, H or L or the "collagenolytic cathepsin", Barrett 1977b), which are SH enzymes and which may, in the marsupial, show a number of properties that differ from those reported for eutherian mammals. In particular, kinetics of complex formation with the used inhibitors may differ. Obviously, biochemical work would be needed in order to resolve open questions of identity. For definitive classification it would appear very important to achieve biochemical separation of the various proteinases present in this material in order to avoid overlapping effects of different proteinases present at the same histological sites which is always a possibility in a histochemical study. In this case, mutual activation/inactivation processes may play a significant role in such a histochemical test system, which may be the reason for the observed difficulties in interpreting results in case of the acid proteinases.

Eutherian proteinases related to implantation

Alkaline proteinase of the trophoblast-endometrial interface

The implantation-associated proteinase which has been studied most extensively so far in eutherian mammals is rabbit blasto-

lemmase (Denker 1977a, 1982). This enzyme is found at the extracellular space between trophoblast and uterine epithelium during the phase of development when the blastocyst coverings are being dissolved and contact is being established between trophoblast and the endometrium. Remarkably, the activity is high only as long as these processes are going on, i.e. for only about one day (around 7 d p.c.). Blastolemmase was classified as an alkaline serine endopeptidase of the trypsin family. It shows very restricted substrate specificity (highly specific for arginyl bonds and also recognizing the neighbouring amino acids in the positions P_2 and P_3 so that it most probably causes limited proteolysis). Blastolemmase is strongly inhibited by most of the typical serine proteinase inhibitors (including aprotinin = Trasylol and SBTI used also in the present study) and by the microbial inhibitors antipain and leupeptin. Chymostatin is inhibitory only at very high concentration but was tested only in the histochemical system so that contribution by other proteinases (like cathepsins or a chymotrypsin-like enzyme) cannot be excluded. Iodoacetamide and EDTA are without any effect on this enzyme.

In the cat, which is another example of the central type of implantation (like the rabbit and the tammar wallaby), however, no major activity of any trypsin-like enzyme could be detected at implantation sites, although pH-dependency and inhibitor studies (effect of chymostatin) suggested the presence of an alkaline proteinase (Denker et al. 1978). It should be mentioned in this context that chymotrypsin-like enzymes

cannot be detected well with gelatin substrates as used in these investigations due to paucity of aromatic amino acids in gelatin. No definitive information on chymotrypsin-like enzymes can, therefore, be derived from studies employing the gelatin substrate film test which holds also true for the investigations on the tammar wallaby communicated here. With gelatin substrate films, only very little alkaline or neutral proteinase activity is detected in blastocysts or uteri in the rat, mouse and hamster, although with different techniques evidence for such activities has been obtained also in these species (Dabich and Andary 1976; Glass et al.1983 ; Kubo et al. 1981; Mintz 1971; Pinsker et al. 1974; Rosenfeld and Joshi 1977).

We will omit here a discussion of the proposed role of the plasminogen activator-plasmin system in implantation, since 1.) this system does not seem to be detected with the gelatin substrate film test, 2.) it was not found to show elevated activity at in situ implantation sites (although in vitro the mouse trophoblast does show elevated activity at equivalent stages), and 3.) inhibitor studies have failed to give clear evidence for its functional role in implantation (for a discussion and for literature, see Denker 1983).

Alkaline proteinase of stroma cells

An alkaline proteinase like the one found in (predominantly myometrial) stroma cells in the tammar wallaby has previously been described in the cat, here in both the endometrium and the myometrium (Denker et al. 1978). The cat enzyme is inhibited by antipain, aprotinin (Trasylol) and SBTI but not by EDTA, and it is in this respect quite similar to the tammar wallaby enzyme although the effects of aprotinin and SBTI were more pronounced in the cat. A difference lies in the lack of an effect of chymostatin in the cat. In both species, these stroma cell proteinases appear to be serine endopeptidases. In the rabbit, no alkaline proteinase activity was detected with the gelatin substrate film test in stoma cells (Denker 1977a) although acid proteinase was found (see below). These cells which are probably macrophages either lack major activity of an alkaline proteinase in the rabbit uterus, or they possess an enzyme which is chymotrypsin-like and cannot be detected easily with the gelatin film test.

Acid proteinases

Considerable activity of acid proteinases (cathepsins) was found with the gelatin film test at implantation sites in a number of species. Cathepsin activity is particularly high in the cat where it is found to increase from 12 d p.c. until 14 d p.c. (implantation) at the trophoblast (trophoblast-endoderm complex) and in the lumina of endometrial glands.

Activity is highest between trophoblast and endometrium in the invasion zone at 14 d p.c. At all these sites, enzyme activity can be inhibited by iodoacetamide and chymostatin while effects of antipain are only marginal. There is no clearcut inhibition by aprotinin, SBTI or EDTA. Cathepsin activity is also detected with the same technique at somewhat comparable sites, particularly at the trophoblast-endoderm complex, in the pig (day 16, Denker and Heap unpublished) and the horse (day 25 - 30, Denker and Betteridge in preparation).

In the rabbit, acid proteinase activity was detected with the gelatin film test in part of the extraembryonic endoderm (after 7 d p.c.), in scattered endometrial stroma cells (less frequent in the myometrium) and in endometrial crypts (after 9 d p.c.) (Denker 1977a,1982). This acid proteinase activity can be suppressed by iodoacetamide, antipain and chymostatin, while aprotinin, SBTI and EDTA remain without any effect. The enzyme was not completely characterized, but on the basis of properties known so far it was proposed that it may be related to cathepsin B, cathepsin L or the "collagenolytic cathepsin".

In contrast to the rat, the mouse and the hamster, where gelatinolytic proteinase activity is low at implantation sites (Blandau 1949; Bergström 1970; Denker 1983), high such activity was found in implantation stage guinea pig blastocysts (Blandau 1949). Only very little work has been done on biochemical characterization of this enzyme(s) including only early postimplantation stages; an acid pH optimum was found, and EDTA showed no inhibition (Owers and Blandau 1971).

Functional and phylogenetic aspects

Acid proteinases, typically compartimentalized in lysosomes, serve important functions in intracellular protein breakdown (Barrett 1977a). It is no surprise, therefore, to find very high activities of enzymes of this type in tissues with high metabolic rate, e.g. in the endoderm, the invasive trophoblast, in endometrial glandular epithelia and in macrophage-like stroma cells. There is uniformity in most of the species examined insofar as considerable acid proteinase activity is indeed found in the mentioned tissues. Acid proteinase activity is particularly high in species which are representatives of the central type of implantation, i.e. the cat, horse, pig and rabbit, while it is much less pronounced in the rodents (eccentric or interstitial implantation). Since the former mode of implantation involves considerable expansion of the conceptus before implantation, there is need for high protein transport and turnover rates. The yolk sac endoderm has been found to take particularly active part in protein metabolism serving metabolic functions as an early embryonic equivalent to liver tissue (Beck and Lowy 1982; Freeman and Lloyd 1983; Freeman and Brown 1985). Notwithstanding minor differences in detail (like different sensitivity to various inhibitors) the acid proteinases found at the named sites in the various species appear to be of the type of lysosomal thiol endopeptidases with acid pH optimum. Biochemical work would be needed in order to analyze existing differences further. It is also quite probable that proteinases belonging to other classes,

e.g. the carboxylproteinase cathepsin D (known to be very active in the rat uterus, Moulton and Koenig 1983) contribute significantly to proteolysis at some of these sites. Due to the central role of all these enzymes in protein metabolism it may be expected that these are relatively conservative proteins whose structure as well as tissue distribution has undergone only modest changes during phylogeny.

While histochemical substrate film tests are of great value for screening for unknown proteinases in general, they are certainly not optimal for localizing intracellular proteinases at this cellular level. An essential element of substrate film tests is that the enzyme must be diffusible in order to be able to penetrate the insoluble substrate (Denker 1977b). This precludes the localization of insoluble enzymes, and localization at the subcellular level is rendered very difficult by the mentioned diffusion processes which, on the other hand, are essential for the test. For intracellular, e.g. lysosomal proteinases, conventional histochemical tests using diffusible, low molecular weight substrates have been developed and are being continuously improved (Dolbaere and Vanderlaan 1979; Gossrau 1985). In this case more precise intracellular localization can be achieved by immobilizing enzymes through fixation.

The major advantage of histochemical substrate film tests lies in localizing extracellular diffusible enzymes that are usually lost into the incubating medium if a conventional histochemical test system (diffusible substrate) is employed. Some of these

enzymes are of particular functional significance, however. An example for this are the alkaline proteinases present at the interface between trophoblast and uterine epithelium at implantation sites, i.e. blastolemmase in the rabbit and the alkaline proteinase newly described in the marsupial in this communication. Rabbit blastolemmase is the only enzyme for which a specific role in embryo implantation has been demonstrated by inhibitor experiments in vivo (Denker 1977a). This serine endopeptidase is essential for dissolution of the blastocyst coverings, a thick extracellular glycoprotein coat that surrounds the preimplantation stage blastocyst and that needs to be removed in order to bring the trophoblast in contact with the uterine epithelium. As mentioned above, blastolemmase is a highly specific enzyme and seems to cause limited proteolysis. It is considered, therefore, to cooperate with other proteinases that are also present at these sites for complete dissolution of blastocyst coverings material, blastolemmase probably playing a regulatory role in this complex proteolytic process. The central role of blastolemmase is impressively shown if non-toxic proteinase inhibitors (like aprotinin = Trasylol or SBTI) are administered into the uterine lumen in vivo. In this case dissolution of the blastocyst coverings can be completely blocked.

The alkaline proteinase present at the tammar wallaby trophoblast-endometrial interface has some remarkable features in common with rabbit blastolemmase as discussed earlier (Denker and Tyndale-Biscoe, in press): maximal activity at an extracellular site, i.e. the interface between trophoblast and

uterine epithelium; restriction to the abembryonic part of the conceptus (BYS in the tammar wallaby, but no anatomically demarkated border in the rabbit); present during only a very restricted time period of about one day, i.e. the phase when the extracellular embryo (blastocyst) coverings are being shed and the trophoblast makes initial contact with the uterine epithelium in this area; correlation with an increase in maternal circulatory progesterone levels in both species (an essential role for maternal progesterone has been shown by ovariectomy and hormone replacement therapy experiments in the rabbit).

On the basis of these similarities it may be supposed that both the eutherian and the marsupial enzyme may perhaps serve comparable functions. In both species, embryos are surrounded by thick extracellular embryo (blastocyst) coverings which show some resemblences in composition, although the "shell membrane" of the marsupial embryo appears to be more rigid and rich in disulphide bonds (for discussion and literature, see Denker and Tyndale-Biscoe, in press). It could be argued, therefore, that the alkaline proteinase may play a role in degradation of blastocyst coverings material in the marsupial like in the rabbit. However, morphology of removal of embryo coverings shows also some discordant features when both species are compared as described in detail before (Denker and Tyndale-Biscoe, in press). In particular, it remains quite unclear how the coverings are being shed in the TYS region which usually becomes free of them before the BYS does, but which never develops a comparable alkaline proteinase activity.

Also the remnants of embryo coverings (which are usually found at the BYS region) do not have as smooth contours as in the rabbit. This may have to do with the above-mentioned apparent greater mechanical strength of embryo coverings in the marsupial, but it may also indicate that mechanical rupturing of these structures may play a more significant role in the marsupial than in the rabbit. Alternative physiological functions for the marsupial alkaline proteinase should, therefore, be considered, e.g. processing of proteins ("histiotrophe") on their way from the maternal tissues to the yolk sac in concerted action together with subsequent processing by the acid intracellular proteinase(s) (see above and Denker and Tyndale-Biscoe, in press). Such a function can also not be excluded to play an additional role in case of rabbit blastolemmase, particularly since after proteinase inhibitor treatment embryos (which were more or less completely prevented to implant but continued to develop for some days) were found typically hypoplastic (Denker 1977a).

In the context of these functional considerations it is tempting to speculate that, during phylogeny, an ancestral enzyme secreted by the embryonic membranes may have undergone changes in its physiological role, e.g. being involved in processing of albumen (and equivalent material) in oviparous species, and of histiotrophe in primitive viviparous species, whereas acquiring more specialized morphogenetic functions in embryo implantation in the eutherian mammal. Such a conversion could well have involved a narrowing of catalytic specificity in the course of evolutionary changes in the enzyme molecule, i.e. the conversion

from an enzyme with relatively broad substrate specificity cleaving many peptide bonds in a given protein ("digestive type") to an enzyme which causes only limited proteolysis (like blastolemmase).

While the mentioned similarities could suggest a homology between the eutherian and the marsupial alkaline proteinase, the biochemical properties reported here clearly exclude such an interpretation, since both enzymes are members of two completely different catalytic classes, i.e. serine and the thiol (SH) endopeptidases, respectively. These two classes of endopeptidases are usually considered to show only little sequence homology. To the author's knowledge it has never been shown that a thiol endopeptidase is converted into a serine enzyme (or v.v.) in nature by a single point mutation. Obviously it would be most interesting to have amino acid sequence data available for both enzymes. However, such data are not even available at the present time for the better investigated rabbit enzyme, and only very recently has partial purification from uterine flushings material been achieved (Tisljar and Denker 1985). Keeping in mind the difficulties in obtaining sufficient amounts of material from pregnant tammar wallabies, it becomes clear that for the present time we have to rely on data from non-purified tammar material as presented here. It remains uncertain so far whether the tammar wallaby trophoblast perhaps produces a blastolemmase-like serine proteinase in addition to the alkaline thiol enzyme, v.v. the trophoblast of the rabbit. If we are dealing with a proteinase system (which is probably

the case) in which one component activates or inactivates the other, considerable changes can be brought about by slight modifications in the activation mechanisms. On the other hand, generalization is certainly premature as long as only few eutherian and one marsupial species have been investigated appropriately, and there is urgent need for additional data from other species (preferably of the central type of implantation, see above). Conclusions or phylogenetic aspects should, therefore, be drawn with caution at this stage. However, the preliminary data from the present study do suggest that the predominant alkaline proteinases present at the interface between trophoblast and uterine epithelium at implantation initiation in the rabbit (blastolemmase) and the tammar wallaby, possibly serving partially somewhat comparable functions, are analogous and not homologous proteins, and that convergent evolution may have occurred. If future studies including larger series of eutherian mammals as well as marsupials show that this enzymologic difference can be generalized, this may be another interesting argument for the hypothesis that marsupial and eutherian mammals have developed viviparity independently after taking different routes of evolution from a common oviparous ancestor in the early Cretaceous (Sharman 1970; Tyndale-Biscoe 1973).

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Legends to Figures

Fig. 1. Acid proteinase, BYS wall and uterus, day 19 RPY. Gelatin substrate film test, pH 5, inhibitor experiment with Z-Phe-Ala-CHN_2, x 42.

a: Control. High proteinase activity (bright lysis zone) around the trophoblast-endoderm complex of the BYS wall (above) and at the interface between it and the endometrium where the disintegrating embryo coverings (dark line) are located. Note that the lysis zone extends slightly beyond the trophoblast-endoderm complex into the BYS cavity. Strong activity also around deep parts of endometrial crypts and certain endometrial stroma cells.

b: Z-Phe-Ala-CHN_2. Proteinase activity is not or only slightly inhibited at the (trophoblast-) endoderm (above) and at endometrial crypts and stroma cells. On the other hand, activity is completely blocked at the interface between trophoblast and uterine epithelium (i.e. around the embryo coverings, dark line) (cf. Fig. 1a). This is interpreted as a result of inhibition of the alkaline proteinase activity present at this site (cf. Figs. 2a+b)(which is very high at this stage and cannot be completely suppressed by pH 5 buffer) while the acid proteinase is resistant to Z-Phe-Ala-CHN_2.

Fig. 2. Alkaline proteinase, BYS wall and uterus, day 19 RPY. Gelatin substrate film test, pH 8, inhibitor experiments with Z-Phe-Ala-CHN_2, x 42.

a: Control. High proteinase activity at the interface between trophoblast and uterine epithelium where remnants of embryo coverings (dark band, partially doubled) are also found. Note that in contrast to Fig. 1 there is no activity, at this

pH, at the inside of the yolk sac wall (endoderm, above) nor at endometrial stromal cells and crypts. Alkaline proteinase present, however, in scattered stroma cells in the myometrium and the subserosa.

b: Z-Phe-Ala-CHN_2. Proteinase activity at the interface between trophoblast and uterine epithelium is completely inhibited (remnants of embryo coverings present here only in the right hand part of the photograph). The alkaline proteinase of myometral and subserosal stroma cells (below), on contrast, is resistant to this inhibitor.

Fig. 3. Acid proteinase, BYS wall and uterus, day 18 RPY. Gelatin substrate film test, pH 5, inhibitor experiment with iodoacetamide, x 70.

a: Control. At this stage (one day earlier than Fig. 1) proteinase activity is clearly restricted to the tissues of the BYS wall (i.e. the trophoblast-endoderm complex, particularly high around the endoderm, above), endometrial (and some myometrial, below) stroma cells and the basal aspects of endometrial crypts. The interface between trophoblast and uterine epithelium (where the intact embryo coverings are located, dark line) is free of activity.

b: Iodoacetamide. Proteinase activity at all sites shown in Fig. 3a is completely blocked.

Fig. 4. Acid proteinase, region of the sinus terminalis (BYS at left, TYS at right) and endometrium, day 19 RPY. Gelatin substrate film test, pH 5, inhibitor experiment with antipain, x 42.

a: Control. Strong proteinase activity at the BYS wall, less at the interface between it and the uterine epithelium (note embryo coverings = dark line), as well as around endometrial crypts and some stroma cells, very little activity at the TYS wall.

b: Antipain has little effect on the activity at the inner aspect of the BYS wall (endoderm) while some degree of inhibition (although incomplete) is seen at endometrial crypts. Complete inhibition at the interface between trophoblast and uterine epithelium, i.e. around the embryo coverings. As in Fig. 1, this effect is due to the very high activity of alkaline proteinase which is found here at this stage (and which contributes to proteolysis even at acid pH) this enzyme being very sensitive to antipain. This is another example for changes in histochemical patterns that can be caused by eliminating (inhibiting) one component, if several enzymes of slightly differing location contribute to a reaction.

Abbreviations

BYS:	bilaminar (avascular) yolk sac membrane = bilaminar omphalopleure
d p.c.:	days post coitum
d RPY:	days after removal of pouch young
EDTA:	ethylenediaminetetraacetic acid
h:	hours
SBTI:	soybean trypsin inhibitor
TYS:	trilaminar (vascular) yolk sac membrane = trilaminar omphalopleure
Z-Phe-Ala-CHN_2:	benzyloxycarbonyl-phenylalanyl-alanyl-diazomethyl ketone
Z-Phe-Phe-CHN_2:	benzyloxycarbonyl-phenylalanyl-phenylalanyl-diazomethyl ketone

Footnote on p. 1:

Preliminary reports on parts of these investigations were presented at the 14th Annual Meeting of the Society for the Study of Reproduction 1981 (Biol Reprod 24 Suppl 1, p 78A, 1981) and at the 3. Arbeitstagung der Anatomischen Gesellschaft 1982 (Anat Anz 153, 268, 1983)

Tab. 1: Results of inhibitor experiments [a]

	Histological site				
	BYS-endometrial interface [b]	BYS wall [c]	endometrial crypts [d]	stroma cells [e]	stroma cells [f]
pH	8	5	5	5	8
Microbial inhibitors:					
antipain	++	Ø-(+)	Ø- +	Ø-(+)	++
leupeptin	++	+	+	Ø-(+)	++
chymostatin	++	+	Ø-(+)	(+)-+	++
elastatinal	++	+	+	(+)-+	
E-64	++	(+)	Ø	Ø	Ø
phosphoramidon	Ø	Ø	Ø-(+)	Ø	Ø
Diazomethyl ketones:					
Z-Phe-Phe-CHN_2	+	Ø-+	Ø-(+)	Ø	Ø
Z-Phe-Ala-CHN_2	++	Ø-+	Ø-(+)	Ø-(+)	Ø
Serine proteinase inhibitors:					
aprotinin (Trasylol)	Ø	Ø	Ø	Ø	Ø- +
SBTI	Ø-(+)	Ø	Ø	Ø	Ø-(+)
Various:					
Iodoacetamide	++	++	++	++	Ø-+
EDTA + dithiothreitol	Ø-↑	↑	Ø-↑	↑	Ø

[a] For literature on the specificity of the inhibitors see Denker, 1976, 1977; for more recent data see Umezawa, 1976, 1979; Umezawa and Aoyagi, 1977, 1983; Green and Shaw, 1981; Shaw and Green, 1981; Hanada et al., 1978, 1983.
++: complete inhibition; +: partial inhibition; (+): questionable inhibition, or results varying; Ø: no inhibition; ↑: activation.
Concentrations of inhibitors used for soaking the gelatin substrate film (higher than actual concentration during the test): antipain, leupeptin, elastatinal, phosphoramidon, SBTI 10^{-3}M; chymostatin 10^{-3} and 10^{-4}M; iodoacetamide, EDTA, ditheothreitol: 10^{-2}M; aprotinin, Z-Phe-Phe-CHN_2, Z-Phe-Ala-CHN_2, E-64: 10^{-4}M.

[b] enzyme activity present only around day 19 RPY

[c] less activity of the same type found at the TYS wall

[d] around cell bases

[e] predominantly in the endometrium

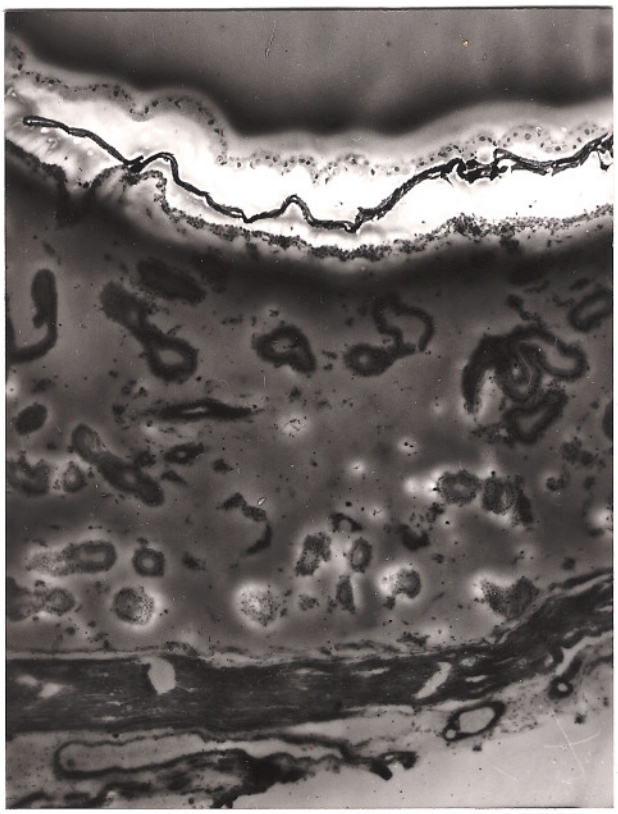
[f] predominantly in the myometrium

Tab. 2: Assignment of proteinases to catalytic classes

	Class of endopeptidase			
	Serine	Thiol	Carboxyl	Metallo
pH	7-9	4-7	2-5	7-9
Microbial inhibitors:				
antipain, leupeptin, chymostatin	+*	+*	Ø	Ø
elastatinal	+*		Ø	Ø
E-64	Ø	+	Ø	Ø
phosphoramidon	Ø	Ø	Ø	+*
Diazomethyl ketones:				
Z-Phe-Phe-CHN_2, Z-Phe-Ala-CHN_2	Ø	+*	Ø	Ø
Serine proteinase inhibitors:				
aprotinin (Trasylol), SBTI	+*	Ø	Ø	Ø
Various:				
iodoacetamide	Ø	+	Ø	(Ø)
EDTA + dithiothreitol	Ø	↑	Ø	+

+: inhibition; Ø: no effect; ↑: activation; *: not all members of this class

Literature: Barrett, 1977b; for further references see footnote a) in Tab. 1



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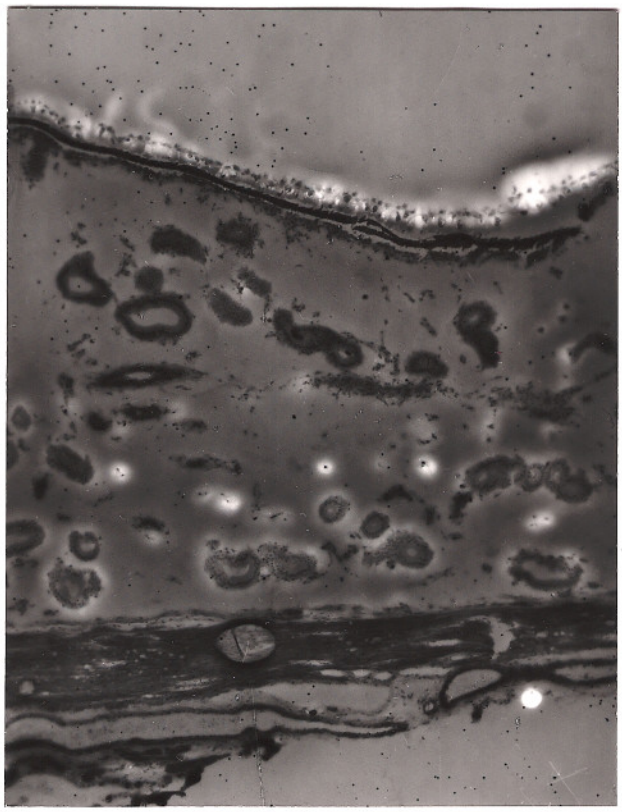

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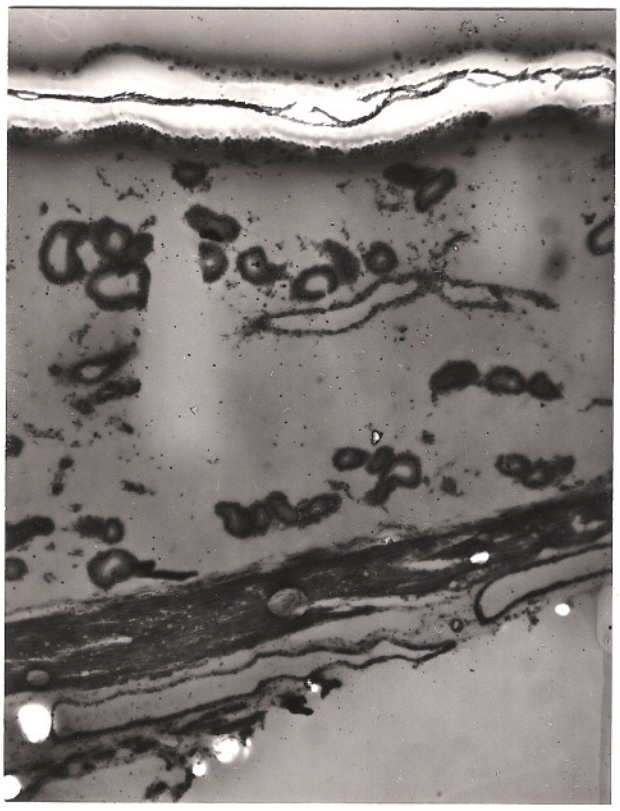

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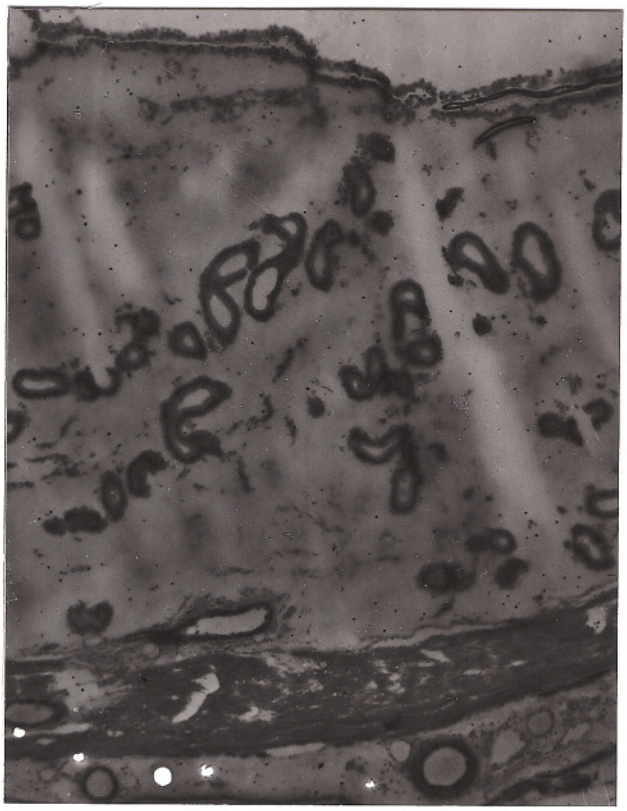

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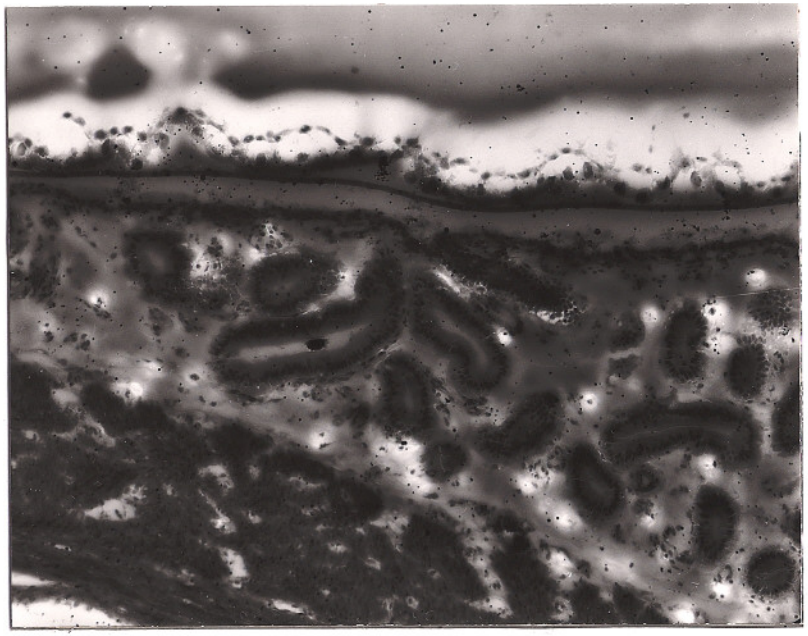

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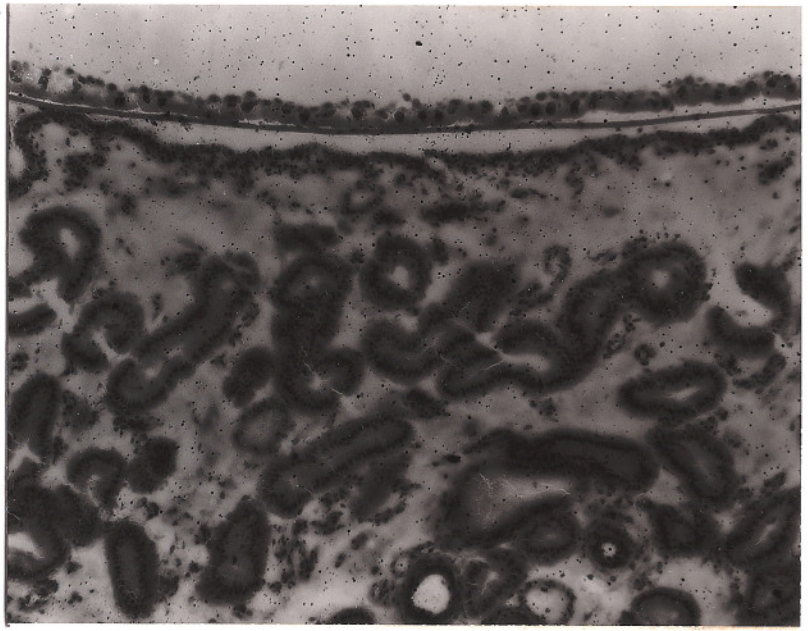

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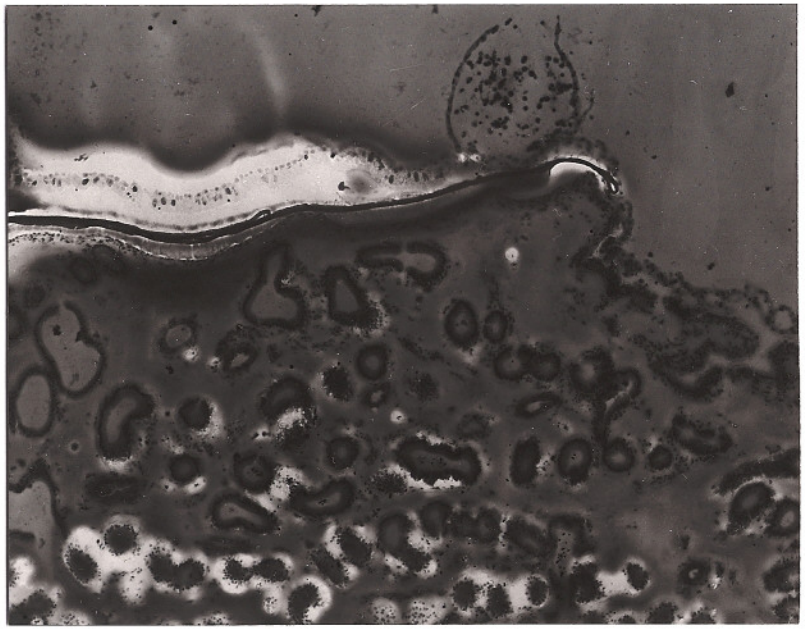

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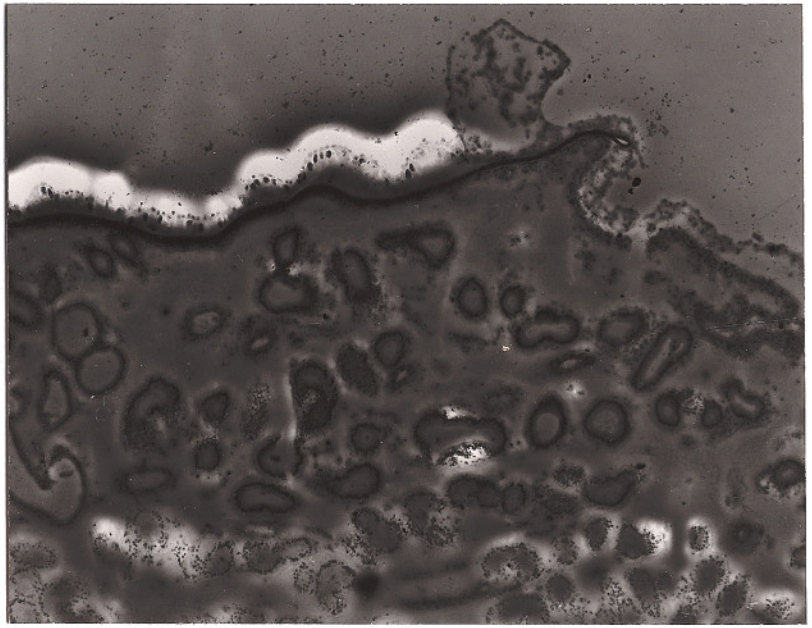

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