AN ABSTRACT OF THE THESIS OF

Arie Robert Cornelius Dyk for the degree of Master of Science in Animal Science presented on February 9, 1990.

Title: Electrophoretic Characterization of Plasminogen Activators Produced by Early Bovine Embryos.

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Sodium dodecyl sulfate - polyacrylamide gel electrophoresis and zymography were used in two separate experiments to determine the tissue source and type of plasminogen activator (PA) produced by bovine blastocysts. Twelve-14 d blastocysts were collected at slaughter from estrous synchronized, superovulated and artificially inseminated Holstein cows. In Experiment I, blastocysts were cultured for 24 h in Ham's F-12 with 15 mg/ml bovine serum albumin under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 37°C. Following culture, blastocysts and media were recovered and stored separately at -20°C until further analysis. In Experiment II, embryonic discs were separated from trophoblast by microdissection, and undissected blastocysts, embryonic discs and trophoblast were cultured for 24 h, recovered and stored as in Experiment I. In both experiments, embryonic tissues and media were thawed and co-electrophoresed with urokinase standards and molecular weight markers. Polyacrylamide gels were laid onto casein-agar gel plates (zymography) and incubated at room temperature for 24-48 h. Lytic

zones in casein-agar gels containing human plasminogen were evidence of the presence of PA. In Experiment I, bovine blastocysts contained and secreted both urokinase-type (47.0 ± 1.0 kD) and tissue-type PA (86.1 ± 0.7 kD). In Experiment II, undissected blastocysts and trophoblast produced both urokinase-type (41.5 ± 1.5 kD) and tissue-type PA (92.2 ± 2.7 kD). Plasminogen activators were not detected in embryonic disc tissue or the respective culture medium. The results suggest that 12-14 d bovine blastocysts produce both urokinase-type and tissue-type PA, and the tissue source of PA is the trophoblast.

ELECTROPHORETIC CHARACTERIZATION OF PLASMINOGEN ACTIVATORS PRODUCED BY EARLY BOVINE EMBRYOS

by

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Typed by Paula Hird for Arie Robert Dyk

DEDICATION

To my parents, Alle and Bernice Dyk, who, as hard-working Dutch immigrants, have always stressed to instill in me the fire-eating will to succeed in whatever I do. Through good times and hard times, I owe to them a lifetime of loving, caring, and serving. Whatever our future holds, my educational accomplishments are, in part, a result of their support, patience, and love. God bless you both.

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Preface

A problem many can agree on is the task of feeding a rapidly expanding world population. Although methods for intense cultivation of farmland will continue to improve, increasing efficiency of food animal production will play a vital role as the demand for meat and milk continues. Dairy and beef cattle operations in the United States are aware of several management concepts including nutrition and feeding, environment and housing, herd health, and genetics and breeding. Basic laboratory research results play an often subtle but vital role on the path to economic efficiency and, as available technologies improve, contribute to efficient food production. Before an applicable technique, such as embryo transfer in cattle, can be utilized to its full potential, detailed information about bovine embryo structure and function must be made available. Plasminogen activator production may be involved in early developmental events in the bovine and studies of this phenomenon may provide insight into measures aimed at improving reproductive efficiency. This research was conducted in an effort to provide at least one more byte of information to the ever-expanding field of bovine embryo physiology.

A.R. Dyk
1990

ELECTROPHORETIC CHARACTERIZATION OF PLASMINOGEN ACTIVATORS PRODUCED BY EARLY BOVINE EMBRYOS

Introduction

Plasminogen activators (PA) are serine proteases that convert the plasma zymogen, plasminogen, to plasmin, which is an enzyme active in clot lysis (Christman et al., 1977). Plasminogen activators are classed into two groups based on molecular weight (MW): urokinase-type PA (uPA) and tissue-type PA (tPA). Urokinase-type PA exhibit MW between 30 and 55 kiloDaltons (kD), whereas tPA are approximately 72 kD (Dano et al., 1985). Plasminogen activator is produced by mouse embryos in a biphasic manner in which the first phase corresponds to implantation and the second phase to embryonic cell migration during tissue remodelling (Sherman et al., 1976; Strickland et al., 1976). Using a technique known as zymography, Marotti et al. (1982) observed that mouse parietal endoderm produced a tPA whereas visceral endoderm and extraembryonic mesoderm produced an uPA. In bovine embryos, PA production is undetectable until the blastocyst stage, increases during blastocoelic expansion and hatching and remains elevated after hatching (Menino and Williams, 1987). Evaluation of the type of PA produced by bovine blastocysts may provide information about biochemical events associated with embryo development in this species. Therefore, the objective of this research was to identify the tissue source and type of PA produced by bovine blastocysts.

Review of Literature

Historical Perspective of Plasminogen Activator

Plasminogen activators (PA) are serine proteases that convert zymogen plasminogen to protease plasmin. Historically, the roles of plasminogen, plasmin and PA are traced to blood clot lysis when early researchers noted that under certain conditions blood clots dissolved spontaneously and could not be reformed (as reviewed by Christman et al., 1977). In the circulatory system, thrombin converts fibrinogen to fibrin, which is then deposited as fine filamentous proteins entangling red and white blood cells and platelets, hence, forming a clot. Plasmin is the protease responsible for the degradation of fibrin in blood and renal tubule clots (Guyton, 1986).

By the turn of the twentieth century, scientists realized blood clot lysis was due to fibrinogen or fibrin being proteolytically converted to a form no longer able to clot blood, but at that time enzymes responsible for this conversion could not be identified. As early as 1933, Tillet and Garner identified streptokinase, a streptococcal enzyme, as having fibrinolytic activity in human plasma. In 1945, Christensen demonstrated that plasma contains the inactive serum zymogen, profibrinolysin or plasminogen, which can be converted to the active protease, fibrinolysin or plasmin, by streptokinase. These initial observations inspired research to investigate systemic PA and the possibility of an alternative mechanism for regulating fibrinolysis via plasminogen activation to plasmin. Some of the early reports on fibrinolysis in blood clotting

were contradictory due to the difficulties involved in isolating and purifying components of the plasmin system.

Plasminogen activators have been identified in almost all tissues and tissue extracts, in vascular endothelium, in blood and in many body fluids, including urine (Christman et al., 1977; as reviewed by Dano et al., 1985). Such a wide distribution of PA led to the hypothesis that fibrinolysis was crucial to many aspects of cellular maintenance in the intact organism, including: vascularization during wound healing, inflammation and tumor infiltration. As early as 1947, it was suggested that cultured cancer cells released an enzyme that activated plasminogen to plasmin. However, due to the later-discovered broad specificity of PA and plasmin and the failure to make a distinct connection to malignancy, interest in fibrinolytic proteolysis and plasminogen activation in cancer waned (Dano et al., 1985). The 1970's saw a renewed interest in PA and cancer when it was shown that transformation of cultured cells by a variety of oncogenic viruses consistently induced a drastic increase in extracellular proteolytic activity, due mainly to the release of PA from the transformed cells (Ossowski et al., 1973a,b, 1974; Quigley et al., 1974; Unkeless et al., 1973, 1974). Since then, many new purification techniques have been developed and the results reaffirm that PA plays a role in tissue degradation and maintenance in the normal organism as well as in cancer (Dano et al., 1985).

With an historical synopsis of PA and the plasmin system in mind, the biochemical components and mechanisms involved in plasminogen activation can be discussed. Such a review will provide the basic biochemistry needed to understand the physiologic functions of PA in

classical clot lysis and cancer, as well as its role in reproduction and embryonic development.

Classification of Plasminogen Activators

According to Christman et al. (1977) vertebrate PA were formerly divided into several groups: circulating plasminogen activators found in blood, tissue plasminogen activators, urinary plasminogen activators (urokinase) and tissue culture plasminogen activators. More recently, however, PA are classed into two main groups based primarily on MW observations: urokinase-type PA (uPA) and tissue-type PA (tPA; Dano et al., 1985). Other notable differences between the two PA types may be made according to exact function or immunological reactivity; however, the present focus will concentrate on the MW categorization of PA. They are respectively termed uPA and tPA because they were originally identified in urine and tissue extracts.

The molecular weight of uPA falls in the 30-55 kD range because urokinase exists in two proteolytically active forms with MW of 31.5 and 54.7 kD as determined by sedimentation-equilibrium methods (White et al., 1966); whereas the molecular weight of tPA is approximately 70 kD (Binder et al., 1979; Dano et al., 1985). Differences in amino acid and corresponding complementary deoxyribonucleic acid (cDNA) nucleotide sequences have shown both types of PA to be separate products of independent genes (Dano et al., 1985). Advances in isolation and purification of mammalian plasmin system components have allowed more detailed studies of the enzymology and characterization of plasminogen activation.

Biochemistry of Plasminogen Activation

Plasminogen and plasmin biomolecules are for the most part homologously conserved across at least several mammalian species in regard to terminal amino acid sequences and MW, suggesting similar proteolytic cleavages are involved in the activation of all plasminogens (Robbins et al., 1973; Summaria et al., 1973; Christman et al., 1977). Human plasminogen activation has been quite extensively studied because it plays an important role in clinical medicine. Isolated, intact and undegraded human plasminogen, termed Glu-plasminogen, is a single polypeptide chain with an approximate molecular weight of 92 kD having a glutamic acid (Glu) residue at the amino (NH_2-) terminus and an asparagine (Asn) residue at the carboxyl (COOH-) terminus (Figure 1; Robbins et al., 1967, 1973; Christman et al., 1977). Plasminogen activator catalyzes the cleavage of a sensitive single arginine-valine peptide bond in Glu-plasminogen to form the active protease Glu-plasmin, consisting of two polypeptide chains held together by a single disulfide bond. Alternatively, it has been suggested Glu-plasminogen can be converted to another one chain form called Lys-plasminogen by the cleavage of a lysine-lysine bond, resulting in liberation of a 7 kD "activation peptide." Lys-plasminogen is then converted to two-chain Lys-plasmin by hydrolysis of the same arginine-valine bond as in Glu-plasminogen. Autocatalysis by plasmin allows for the conversion of Glu-plasmin to Lys-plasmin (Dano et al., 1985).

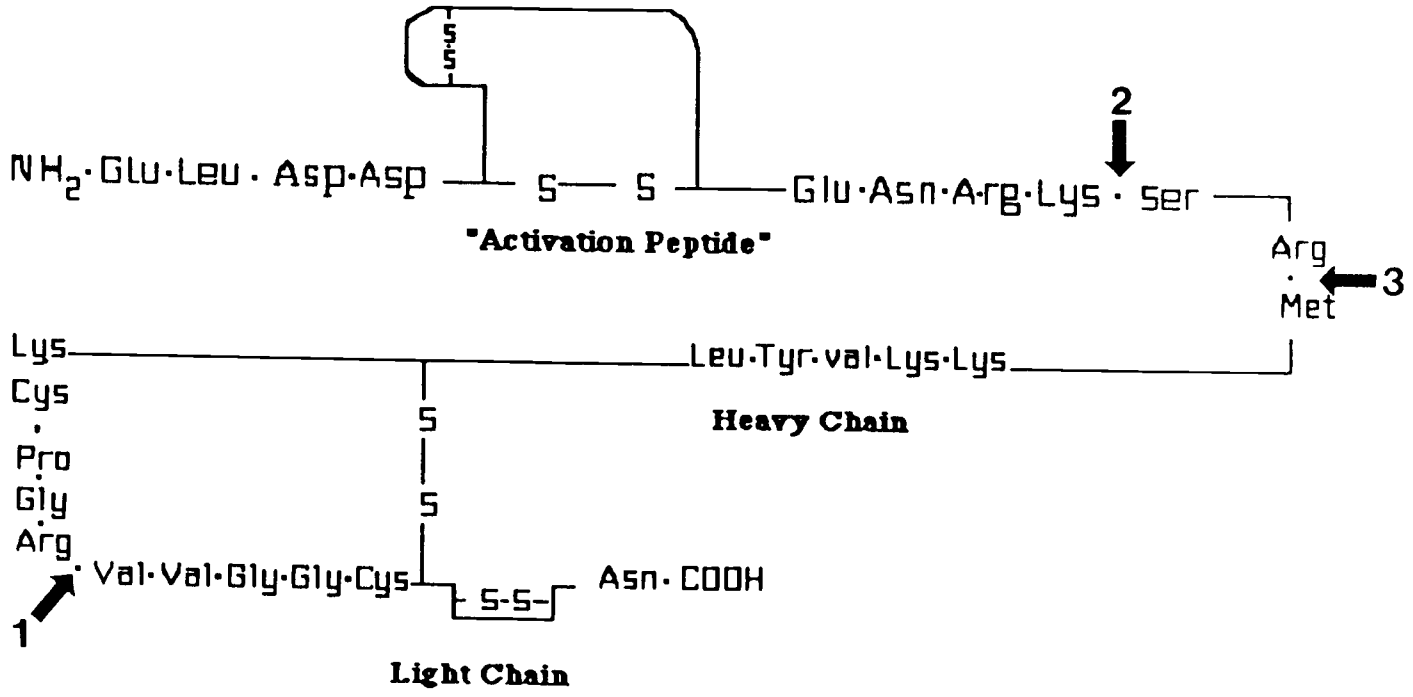


Figure 1. Representative drawing of human Glu-plasminogen. Arrow 1 indicates site of bond cleavage to form plasmin. Arrows 2 and 3 show points of possible activation peptide removal.

Urokinase was extensively studied and commonly used in the first plasminogen activation studies. Kinetic studies have shown a possible sequence of plasminogen activation by urokinase as depicted in Reaction Sequence I:

I

(1) Glu-plasminogen –> Lys-plasminogen + "Activation Peptide"

92 kD 86 kD 7 kD

(2) Lys-plasminogen —urokinase——> Lys-plasmin

63 kD ——— 25 kD
(heavy chain) (light chain)

Controversy existed as to whether Reaction (1) could be catalyzed by both urokinase and plasmin or only by plasmin, and as to whether Reaction (1) occurs before enzymatically active plasmin is formed. Alternatively, Reaction Sequence II suggested another pathway:

II

(3) Glu-plasminogen —urokinase——> Glu-plasmin

92 kD 70 kD —— 25 kD

(heavy chain) (light chain)

(4) Glu-plasmin ——————> Lys-plasmin + "Activation Peptide"

63 kD —— 25 kD 7 kD
(heavy chain)(light chain)

However, Reaction Sequence II had never resulted in detectable amounts of Glu-plasmin. In Reaction Sequence I, once any plasmin is formed either at low efficiency by urokinase or existing as a plasminogen contaminant, plasmin removes the activation peptide. It was suggested Glu-plasmin in Reaction Sequence II may exist transiently and could catalyze activation peptide removal from Glu-plasminogen to form the predominantly found Lys-plasmin. Conversion of Lys-plasminogen to Lys-plasmin seemed to occur much more rapidly than that of Glu-plasminogen to Lys-plasmin (Christman et al., 1977)

Although it had been concluded that Reaction Sequence I was most likely the case for plasminogen activation by urokinase, more recent evidence indicates otherwise for in vivo systems. Holvoet et al. (1985) described a murine monoclonal antibody specific for Lys-plasminogen and Lys-plasmin-α_2-antiplasmin complex, but not specific for Glu-plasmin and Glu-plasmin-α_2-antiplasmin. Using this antibody, it was shown under physiological conditions that plasminogen activation yields the Glu-plasmin-α_2-antiplasmin complex, indicating the direct cleavage of the arginine-valine peptide bond in Glu-plasminogen and refuting the concept of Lys-plasminogen intermediate formation. Thus, Reaction Sequence II may be the correct sequence for the activation of human plasminogen.

Plasmin

Regardless of activation sequence, once active plasmin is formed it consists of two polypeptide chains held together by a disulfide bond. Plasmin resulting from activation of either Glu- or Lys-plasminogen

consists of a heavy chain (63 kD) and a light chain (25 kD; Sodetz et al., 1972; Christman et al., 1977). The light chain contains the active site and has amino acid sequence homologies similar to other serine proteases such as trypsin and chymotrypsin (Dano et al., 1985). Plasmin has broad trypsin-like specificity hydrolyzing lysine and arginine peptide bonds in several proteins, some of which include casein (Remmert and Cohen, 1949), cell membrane proteins (Lazarowitz et al., 1973), immunoglobins and blood-clotting factors V, VII and XIIa (Christman et al., 1977).

Two seemingly important inhibitors of plasmin are α_2-macroglobulin and α_2-antiplasmin. Other strong plasmin inhibitors include bovine pancreatic trypsin inhibitor, lima bean trypsin inhibitor and soybean trypsin inhibitor (Dano et al., 1985).

Urokinase-Type Plasminogen Activator

Besides urine, uPA has been isolated from human blood and seminal plasma, malignant prostate tissue and from several sarcoma cell line culture fluids (Dano et al., 1985). Human urokinase and uPA have been collectively categorized as far as MW is concerned. Each form of urokinase has a specific MW (31.5 and 54.7 kD), and any uPA in general is categorized as exhibiting MW between both human urokinase forms. Thus, the terms urokinase and uPA will be used synonomously in this section.

Urokinase-type PA exists in a single-chain form (scuPA) and in a two-chain form (tcuPA). In 1979, scuPA was identified as having a

molecular weight of about 54 kD, and it was indicated to be the actual precursor to active tcuPA with little intrinsic activity. However, the catalytic efficiency of scuPA against plasminogen as a substrate was later reported to be kinetically equivalent to that of tcuPA (Gurewich, 1987). Hence, scuPA was not considered to be a true zymogen (Collen et al., 1986). More recent evidence supports the contrary. Petersen et al. (1988) have reinvestigated this issue and have legitimately concluded that scuPA is indeed a proenzyme form of tcuPA with little or no intrinsic acitvity. Nonetheless, tcuPA contains an "A-chain" or "light chain" (20 kD) and a "B-chain" or "heavy chain" (30 kD); the chains are bridged by a disulfide bond. Molecular weights of both chains vary among species and sample preparation techniques. The A-chain contains a "kringle" structure and an "epidermal growth factor domain", whereas the B-chain is actually the structurally conserved region of the molecule possessing the serine protease active site. Limited proteolysis by plasmin can cause cleavage of scuPA between the A- and B-chains into active tcuPA, and is followed by part of the A-chain being removed. The complete amino acid sequence of human tcuPA has been elucidated, and the nucleotide sequence of porcine uPA strongly suggests close homology among species. Human, pig and mouse cDNA and messenger ribonucleic acid (mRNA) for uPA have been identified and characterized (Figure 2; Dano et al., 1985).

Although plasminogen is the most documented substrate for uPA, studies have shown uPA to hydrolyze low MW substrates with amide and ester derivatives of arginine and lysine and an extracellular matrix

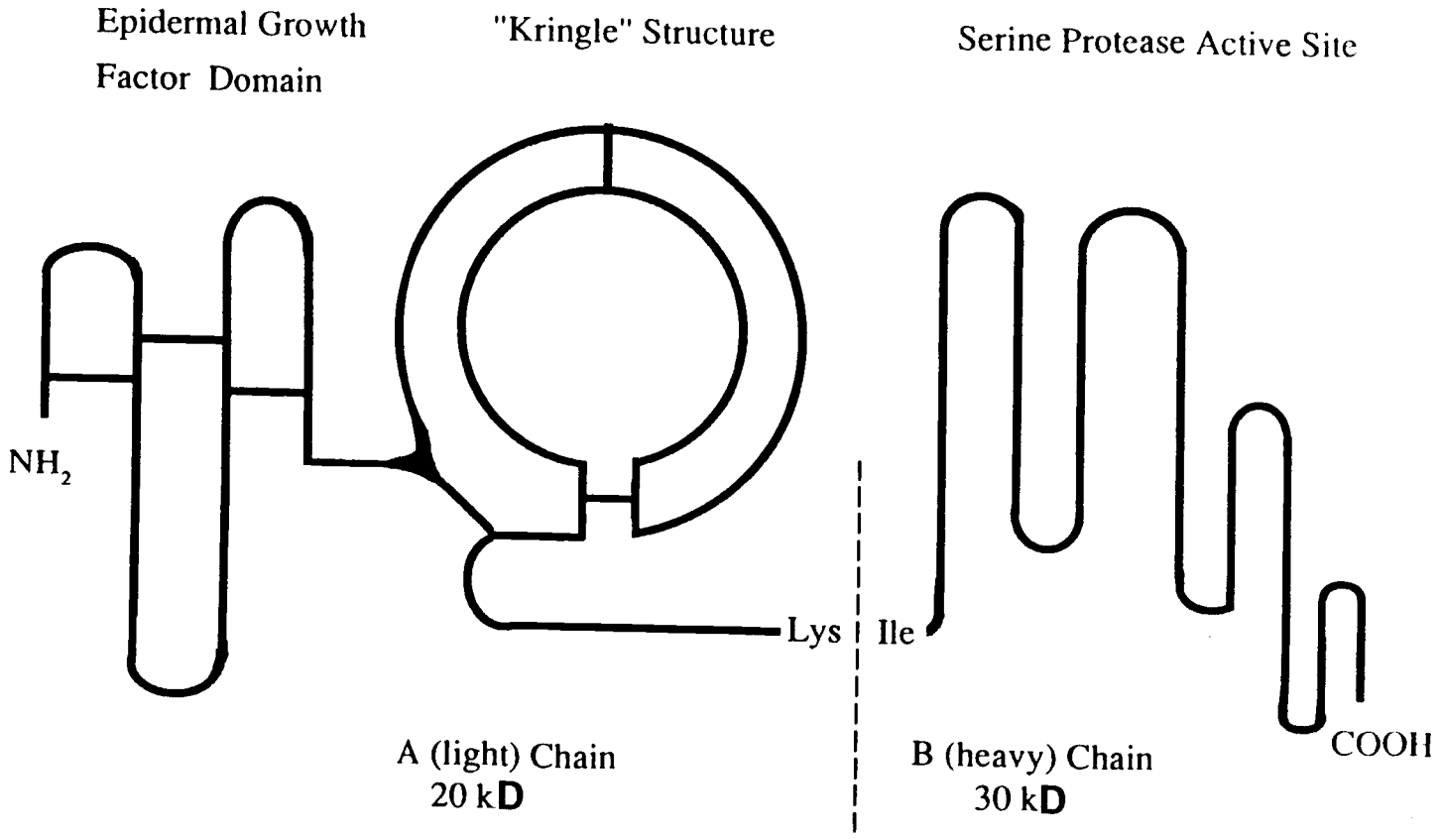


Figure 2. Skeletal molecular sketch of single-chain urokinase-type plasminogen activator. Dashed line indicates point of limited proteolysis to yield uPA.

protein. Species specificity of uPA exists to some degree with respect to plasminogen. For example, chicken uPA has no activity on human plasminogen, but human uPA may inefficiently activate chicken plasminogen (Dano et al., 1985).

Inhibitors of uPA include: diisopropyl flourophosphate (DFP) which irreversibly binds to the serine active site; zinc (2+); some rare earth metals; and α-tocopherol. Some plasmin inhibitors, such as bovine pancreatic trypsin inhibitor and soybean trypsin inhibitor do not inhibit uPA. Urokinase-type PA specifically catalyzes arginine-valine bond hydrolysis in plasminogen, hence, arginine and L-arginine methyl esters are effective uPA inhibitors. Physiological concentrations of sodium chloride have been demonstrated to inhibit uPA, the effect being exerted through a suggested inhibitor of PA and plasminogen. Sodium chloride does not inhibit plasmin activity (Dano et al., 1985).

A major breakthrough was the determination of uPA amino acid sequences in comparison to those of tPA, and the resulting indication that both types of vertebrate PAs are definitely different gene products.

Tissue-Type Plasminogen Activator

Tissue-type PA has been purified from various tissue extracts including: porcine heart, kidney and ovary, human uterine tissue, blood, plasma and cell culture fluids, and several tumor cell lines (Dano et al., 1985). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has revealed the mean MW of human tPA to be approximately 70-75 kD (Binder et al., 1979; Dano et al., 1985). Thus, for classification

purposes, PA exhibiting MW significantly higher than the range for uPA are classed as tPA.

Similar to uPA, tPA also exists in a single-polypeptide chain form (sctPA) and in a two-polypeptide chain form (tctPA). The tentative conclusion that sctPA may be an inactive zymogen should be approached with caution because some reports on the enzyme activity of sctPA, like that of scuPA, are conflicting. Two-chain tPA contains an "A" or "heavy" chain (40 kD) and a "B" or "light" chain (30 kD), both linked together by a disulfide bond. The A-chain contains two "kringle" structures, an "epidermal growth factor domain", and a "fibronectin finger domain", whereas the B-chain contains the serine protease responsible for its plasminogen-activating capacity. Single-chain tPA is converted to tctPA by limited plasmin proteolysis or other contaminating proteases during assay which cleave an arginine-isoleucine peptide bond between the A- and B-chains (Figure 3; Dano et al., 1985; Lijnen and Collen, 1987). Complete amino acid and cDNA nucleotide sequences for human tPA have been determined (Edlund et al., 1983; Pennica et al., 1983; Degen et al., 1986).

The only known substrate for tPA is plasminogen; unlike uPA, however, tPA has a strong affinity for fibrin which actually stimulates plasminogen activation by tPA. Arginine chloromethylketone, p-nitrophenol-p-guanidinobenzoate and DFP are inhibitors of tPA. As with uPA, bovine pancreatic trypsin inhibitor and soybean trypsin inhibitor do not inhibit tPA activity. Because different in vitro assay techniques have been used among uPA and tPA, it should be noted that complications exist when comparing the two PA types (Dano et al., 1985).

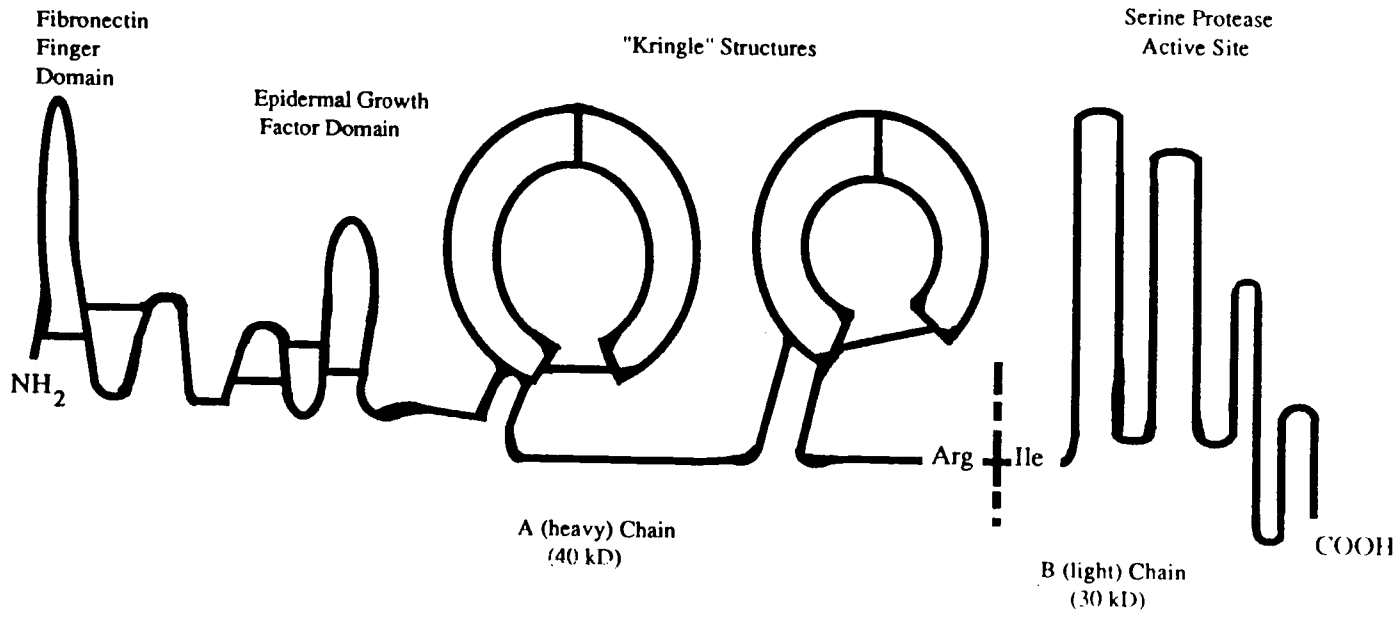


Figure 3. Skeletal molecular sketch of single-chain tissue-type plasminogen activator. Dashed line indicates point of limited proteolysis to yield tPA.

Other Plasminogen Activators

In addition to uPA and tPA, other known PA include: 1) Streptokinase, a streptococcal protein that has a mechanism of action much different from that of vertebrate PA; 2) serum kallikrein; and 3) blood coagulation factors XI and XII, the latter two exhibiting very little activity. A PA identified as a serine protease contained in outer membranes of two Escherichia Coli strains has been identified (Dano et al., 1985). In 1982, Harvey and coworkers reported a lung cancer cell line that secreted a PA having a MW of over 1,000 kD. This PA, consisting of disulfide-linked oligomers, also had the capacity to bind anti-uPA antibodies.

Although most of the literature and research on PA is directly linked to clinical medicine and hematology, several reports have alluded towards a role for PA in reproduction (Strickland, 1980). Ovaries from the pregnant pig were identified as a source of tPA as early as 1969 (Kok and Astrup, 1969). The involvement of plasminogen activation in ovulation was later described by Beers (1975), and in the cow nonetheless!

Plasminogen Activator in Male Reproduction

A variety of cells and tissues produce PA, some of which are directly involved in reproduction. In rats, Sertoli cells that line seminiferous tubules and nourish developing spermatids produce and secrete PA (Strickland, 1980). Lacroix et al. (1977, 1981) have postulated PA involvement in localized proteolysis during seminiferous

tubule restructuring when early prophase spermatocytes are translocated from basal to adluminal compartments, and in spermiation during stages VII and VIII of the cycle of the seminiferous epithelium. Marzowski and coworkers (1985) demonstrated the existence of a Sertoli cell membrane-associated PA (38-40 kD) that may function to restructure membrane components at cell - cell interfaces, whereas the secreted PA form may act fibrinolytically in the seminiferous epithelium. Cultured Sertoli cells will secrete increased amounts of a tPA (70 kD) in response to dibutyryl cyclic adenosine monophosphate (dbcAMP) and follicle-stimulating hormone (FSH; Lacroix and Fritz, 1982), whereas under basal conditions an uPA (45-48 kD) is primarily secreted (Hettle et al., 1986). These findings led Hettle et al. (1986) to postulate the association of PA with migration of Sertoli cell cytoplasmic extensions between the basal lamina and preleptotene spermatocytes. Immunohistochemical and zymographic studies enabled Vihko et al. (1988) to detect both uPA and tPA activity at different stages in the cycle of the seminiferous epithelium. Tissue-type PA activity has also been demonstrated in seminal plasma, but no correlation exists between PA and fertility (MacGregor et al., 1987).

Plasminogen Activator in Female Reproduction

In the mammalian female, PA, plasmin and collagenase are involved in ovulation, where plasmin is thought to participate in follicle wall breakdown (Beers et al., 1975; Strickland, 1980; Reich et al., 1985). However, Shimada et al. (1983) indicated that prostaglandins were not involved in the preovulatory synthesis of PA and PA was not involved in

follicular wall rupture. They suggested alternative roles for PA during ovulation, such as cumulus cell detachment and granulosa cell proliferation. Gonadotropins stimulate release of a tPA (80 kD) from cultured rat ovarian granulosa cells, whereas uPA (40 kD) release is not affected. In addition, an identified inhibitor of fibrinolysis is suppressed by FSH and luteinizing hormone (LH; Ny et al., 1985). At ovulation, hormonally stimulated rat granulosa cells produce only tPA whereas thecal cells are the major source of uPA. A hypothesis explaining the ovulatory mechanism is that thecal cell uPA may be involved with follicle wall rupture, and tPA from granulosa cells may be essential for immediate blood clot fibrinolysis and follicular fluid proteoglycan degradation (Canipari and Strickland, 1985). In the mouse, mRNA probes for uPA have been used to demonstrate that granulosa cells produce mainly uPA rather than tPA (Canipari et al., 1987). Interestingly, equivalent ovarian follicular cells from very closely related species express two perhaps functionally similar yet genetically different enzymes.

Fibrinolytic activity in uterine flushings has been attributed to a PA (Kwaan and Albrechtsen, 1966) which appears to be hormonally regulated in the rat (Katz et al., 1976). Estrogen stimulates plasminogen uptake by the mouse uterus suggesting a mechanism by which uterine plasminogen levels can be increased (Finlay et al., 1983). Plasminogen in the porcine uterus exists presumably as a serum transudate and may serve as a substrate for PA produced by pig blastocysts (Mullins et al., 1980; Fazleabas et al., 1983). Although a specific role for

uterine PA has not been established, the enzyme may be involved in maternal-embryonic interactions.

Soon after ovulation, the female gamete, or oocyte, becomes a free-floating entity within the Fallopian tube or oviduct. Although the oocyte remains surrounded by an acellular glycoprotein matrix, the zona pellucida, prior to and after fertilization, a number of mRNA transcripts will remain stored until specific developmental events require expression. Mouse and rat primary oocytes and zona pellucida-denuded fertilized eggs do not contain PA, whereas ovulated secondary oocytes contain a tPA (Huarte et al., 1985). After the resumption of meiotic maturation in primary oocytes, tPA mRNA undergoes a structural change referred to as "polyadenylation" at the 3′ end of the molecule (Huarte et al., 1987). This allows for the possible translation and production of tPA at fertilization; however, tPA activity is not expressed again until after Day 6 of mouse embryo development (Strickland et al., 1976; Huarte et al., 1985, 1987). Although the exact role(s) of tPA in the oocyte has not been completely defined, Huarte et al. (1985, 1987) have suggested its involvement in fertilization and in the zona reaction, which blocks the phenomenon known as polyspermy. Liu and Hsueh (1987) have suggested tPA activity may be related to cumulus cell expansion and dispersion.

A review of early mammalian embryo development is included mainly with reference to the bovine; however, other species are included for comparison or contrast when necessary.

Aspects of Early Mammalian Embryo Development

If after ovulation the oolemma is penetrated and the oocyte is fertilized by a viable spermatozoan, the "activated" oocyte undergoes maturation and completion of meiosis with the release of polar bodies into the perivitelline space. Fertilization normally takes place in the ampullary-isthmic junction of the oviduct, after sperm have had ample time for capacitation. Any remaining corona radiata cells are shed from the zona pellucida. The resulting one-cell embryo or zygote undergoes a series of mitotic divisions, or cleavages, and morphological changes leading up to establishment of pregnancy and fetal development (Bazer et al, 1987; Betteridge and Flechon, 1988).

First cleavage of the zygote into two separate embryonic cells, called blastomeres, occurs approximately one day post-fertilization for most mammals. Several cellular features characteristic of mammalian embryo cleavage distinct from lower animal forms include:

1) cleavage occurs at a much slower rate;
2) "rotational cleavage" exists in which the first cleavage is a normal split, but remaining cleavages occur in different patterns between the first two blastomeres;
3) Although initial cleavages occur simultaneously, synchronization is eventually lost and blastomeres begin dividing independently of each other; and,
4) the phenomenon of "compaction" where after the eight-cell stage (third cleavage) blastomeres huddle together into a mulberry shape, called the "morula" stage (Gilbert, 1985).

The zona pellucida plays an important role in livestock embryo development through the morula stage, which is approximately Day 5 of development in the bovine. The embryo enters the uterus at about this time. In addition to keeping blastomeres together, the zona pellucida may function to conserve the microenvironment of the perivitelline space, the space between the zona pellucida and the oocyte (Betteridge and Flechon, 1988). The zona pellucida is a very important component in the block to polyspermy immediately following the cortical reaction observed during fertilization (Bazer et al., 1987).

While yet contained within the space of the intact zona pellucida, blastomeres of the compacted morula develop tight intercellular junctions followed by fluid accumulation into a central cavity called the blastocoele. This marks conversion to the early blastocyst stage on approximately Day 7 of bovine development. With asynchronous cleavage blastomeres become unequal in size and differentiate into two distinct cell populations. Larger blastomeres comprise the majority of cells forming the outside peripheral cuboidal layer termed trophoblast or trophectoderm. The outer portion of the placenta, the chorion, is later formed from trophoblast. A group of smaller blastomeres residing within the embryo and surrounded by trophectoderm forms the inner cell mass. The inner cell mass will later develop into three primary germ layers (ectoderm, mesoderm, endoderm) during the process of gastrulation. The distinction between trophectoderm and inner cell mass represents the first differentiative event in mammalian embryo development. On approximately Day 8, endoderm cells spread out from beneath the inner cell mass and by Day 10 completely line the trophectoderm surrounding the

blastocoele, forming the trophoblast (Gilbert, 1985; Bazer et al., 1987; Betteridge and Flechon, 1988).

The bovine blastocyst will divest itself of the zona pellucida during the process of hatching between Days 9 and 10 of development. In rabbits, zona pellucida dissolution occurs via blastolemmase, a trophoblastic enzyme. The mouse blastocyst rhythmically expands and contracts (Bazer et al., 1987), and Perona and Wassarman (1986) suggested limited proteolysis by a trypsin-like proteinase, strypsin, is involved in hatching. A uterine or embryonic enzymatic factor may be involved in changes in the integrity of the porcine zona pellucida, and hence may contribute to the hatching process (Menino and Wright, 1982; Broermann et al., 1989). Expansion and contraction caused by hydrostatic pressures in the bovine blastocyst play a major role in hatching as the zona pellucida distends and weakens (Bazer et al., 1987). However, bovine embryos can activate plasminogen to the active enzyme plasmin, which may represent a mechanism by which hatching is facilitated (Menino and Williams, 1987).

Following hatching, the bovine blastocyst continues to expand spherically while blastocoelic fluid accumulation continues. By Day 12, the inner cell mass ruptures through to the outside of the sphere and is identified as embryonic disc. Between Days 12-14 the bovine blastocyst begins to undergo a rapid logarithmic elongation phase. The shape of the embryo changes from spherical to ovoid during this time, and by Day 14-16 mesodermal cells from the embryonic disc begin to migrate between the trophectoderm and endoderm. The bovine blastocyst will have undergone the morphological change from a 3-mm spherical shape on Day 13 to a 25-

cm filamentous form on Day 17. By Day 18 of gestation, the blastocyst has extended into the contralateral uterine horn. This occurs via hyperplasia of the trophectoderm and endoderm. Days 16 to 19 have been defined as "critical" for the maternal recognition of pregnancy, presumably due to embryonic signals aimed at the uterus (Bazer et al., 1987; Betteridge and Flechon, 1988).

Unlike rodents and primates in which blastocysts penetrate the uterine mucosa invasively, implantation in ruminants occurs through interdigitation of trophoblast and endometrial microvilli. Placental attachment begins on approximately Days 28-30, and is completed between Days 40-45 of gestation in the cow (Bazer et al., 1987).

Studies of early embryonic development using mammalian models have been increasingly focused at the biochemical and molecular levels. Within the last two decades, roles for plasminogen, PA and plasmin in the embryo have been speculated and will be discussed in this literature review.

Plasminogen Activator in Embryo Development

Rodent Embryos. Fibrinolytic activity due to a PA was first linked to embryo development by Leidholm and Astedt (1975) in the rat. Fibrinolytic activity was exhibited by Day 1-4 rat embryos during oviductal passage; however, fibrinolysis decreased when embryos entered the uterus on Day 5 and disappeared at implantation. Rat oviduct and endometrium also exhibited fibrinolytic activity. Leidholm and Astedt (1975) postulated that PA prevented the adherence of embryos to fibrin deposits lining the mucosa, and the subsequent disappearance of

fibrinolysis following entry into the uterus may be a prerequisite for the embryo to implant.

Strickland et al. (1976) and Sherman et al. (1976) characterized a biphasic pattern of PA production in cultured mouse embryos. Using a fibrin-agar overlay assay that allowed direct visualization of fibrinolysis, PA was first detected in Day 6 embryos. Production of the protease peaked on approximately Day 8-9 then decreased, representing the first phase. Second phase of PA production by egg cylinder stage mouse embryos began on Day 11, peaked on approximately Day 12, and was maintained until at least Day 15 of in vitro development. Level of peak PA activity of the second phase was fivefold higher than that of the first phase. Trophoblast cells were largely responsible for PA secretion during the first phase, which closely correlated to invasiveness of the trophoblast in vivo, suggesting a possible role for PA in implantation. The second phase of PA secretion was mainly due to parietal endoderm, suggesting that PA may be a contributory mechanism in embryonic endodermal cell migration and tissue remodelling. Sherman et al. (1976) suggested PA may also serve as a useful biochemical marker in identifying certain cell types within the developing mouse embryo; however, Bode and Dziadek (1979) reported PA secretion to be more widespread in the mouse embryo, rather than specifically limited to certain tissues. Utilizing casein and fibrin agar overlays containing purified plasminogen, Bode and Dziadek (1979) indicated that all tissues (parietal endoderm, visceral yolk sac endoderm and mesoderm, and amnion) in the Day 7-10 post-implantation mouse embryo eventually secrete PA, and they concluded that

PA is consistent in playing a role during rapid embryonic growth and morphogenetic changes.

Sherman (1980) reported that fibrinolytic activity due to PA in preimplantation mouse embryos was associated with the zona pellucida. Sherman (1980) concluded that PA secretion in mouse blastocysts was not directly associated with hatching or the acquisition of trophoblast cell adhesiveness and proposed that PA was involved with trophoblast invasiveness. Kubo and Spindle (1980) concluded that at least two types of proteolytic activity are present in mouse blastocysts at the time of implantation:

1) trypsin-like activity primarily involved in attachment; and
2) fibrinolytic activity primarily involved in trophoblastic outgrowth.

Plasminogen activator production was one of several enzymatic parameters investigated in mouse embryos cultured in various media. Blastocysts cultured in suboptimal medium produced less PA than blastocysts cultured in enriched medium (Sellens and Sherman, 1980).

Marotti et al. (1982) investigated the distribution of the PA types in Day 12 mouse embryonic tissues. Marotti et al. (1982) used three criteria to distinguish between the two types of PA produced by mouse embryonic cells:

1) MW;
2) interaction with serum inhibitors; and
3) antigenic properties.

Marotti et al. (1982) utilized SDS-PAGE in combination with a casein-agar underlay technique known as zymography to demonstrate that mouse

parietal endoderm produced mainly a tPA (79 kD) and visceral endoderm and extra-embryonic mesoderm produced an uPA (48 kD). Parietal endoderm tPA was partially affected by acid-treated serum and inhibited by anti-tPA antibodies. Urokinase-type PA produced by visceral endoderm and extraembryonic mesoderm was inhibited by acid-treated serum and immunoprecipitated by anti-urokinase antibodies. Marotti et al. (1982) suggested that these diagnostic procedures which were capable of distinguishing between the two types of PA could be used as biochemical markers for tracing parietal endoderm during development. As this work dealt with quite an advanced embryonic stage, Marotti et al. (1982) suggested that both types of PA may play roles in cell migration during mouse embryogenesis.

Menino and O'Claray (1986) reported more mouse embryos completed hatching, attached to the plastic culture dish substratum, and generated trophoblastic outgrowth in plasmin- and plasminogen-supplemented Whitten's medium than in medium lacking the enzyme or its zymogen. A caseinolytic agar gel assay validated PA production in plasminogen-supplemented medium. A proteolytic as well as a trophic effect may be enhancing in vitro embryo development in Whitten's medium supplemented with either plasmin or plasminogen, as compared to medium supplemented with serum or other proteases, such as pronase and trypsin (Menino and O'Claray, 1986).

Much of the research investigating the role of PA in embryo development has been performed in the rat and mouse. These animals serve as useful mammalian models since they are relatively inexpensive to maintain on experiments and large numbers of embryos can be collected for

meaningful statistical analysis. However, differences do exist in embryo and fetal development among the mammalian species. During the 1980's several research laboratories investigated the involvement of PA in embryo development in the livestock species.

Porcine Embryos. Mullins et al. (1980) established that expanding Day 12 porcine blastocysts release significant quantities of PA into the culture medium. Pig conceptus attachment to the uterine wall occurs gradually beginning on Day 12 with trophoblast becoming firmly fixed in position on about Day 18; yet placentation in the pig is not considered invasive. Pig trophoblast when transplanted to an ectopic site during the period of uterine attachment becomes invasive (Samuel, 1971; Samuel and Perry, 1972). These observations led Mullins et al. (1980) to investigate the existence of a PA inhibitor secreted by the endometrium. Mullins et al. (1980) reported that the porcine uterus produced a progesterone-induced PA inhibitor which may be involved in damping blastocyst proteolysis, thereby preventing invasive implantation.

Fazleabas et al. (1983) also reported the production of PA by porcine embryos. Utilizing an ^{125}I-fibrin plate assay, Fazleabas et al. (1983) observed that pig embryos released PA in a biphasic pattern similar to that found in the mouse embryo. The first phase of porcine embryo PA production on Days 10-12 corresponds to the time of blastocyst elongation, while the second phase on Days 14-16 corresponds to a period at which embryonic DNA content is increasing markedly. Estrogen production by the elongating Day 12 pig blastocyst may also trigger the release of a plasmin inhibitor by the endometrium, hence, restricting embryonic proteolytic activity due to PA (Fazleabas et al., 1983).

Plasminogen existing as a serum transudate in the pig uterus may also be a result of local embryonic estrogen production (Fazleabas et al., 1983). Yolk sac development begins on approximately Day 13 in the pig blastocyst, and by Day 16 the yolk sac extends along most of the length of the conceptus (Perry, 1981). With these observations, Fazleabas et al. (1983) postulated the first phase of porcine blastocyst PA production is associated with a period of intense tissue remodelling whereas the second phase is involved with tissue proliferation.

Bovine Embryos. Bovine embryos cultured with various plasminogen concentrations were evaluated for developmental stages in vitro, and the extent of PA production and plasminogen activation was quantified (Menino and Williams, 1987). Morphologically normal 16-cell embryos and early morulae were cultured in Ham's F-12 medium with 15 mg/ml bovine serum albumin (BSA) containing levels of 0, 15, 30, 60, or 120 μg/ml plasminogen (Figure 4). The percentages of bovine embryos developing to the initiating hatching blastocyst, hatched blastocyst, attached blastocyst, and attached blastocyst with trophoblastic outgrowth stages were not significantly different between the five levels of plasminogen. However, initiation and completion of hatching accelerated as plasminogen concentration increased in the culture media. Plasminogen activator and plasmin levels in the culture media were determined utilizing a caseinolytic assay. Plasminogen activator production was low for the first 48 h of culture until the blastocyst stage, increased between 48-120 h during blastocoelic expansion and initiation of hatching, and tended to remain elevated throughout and after hatching. Plasminogen

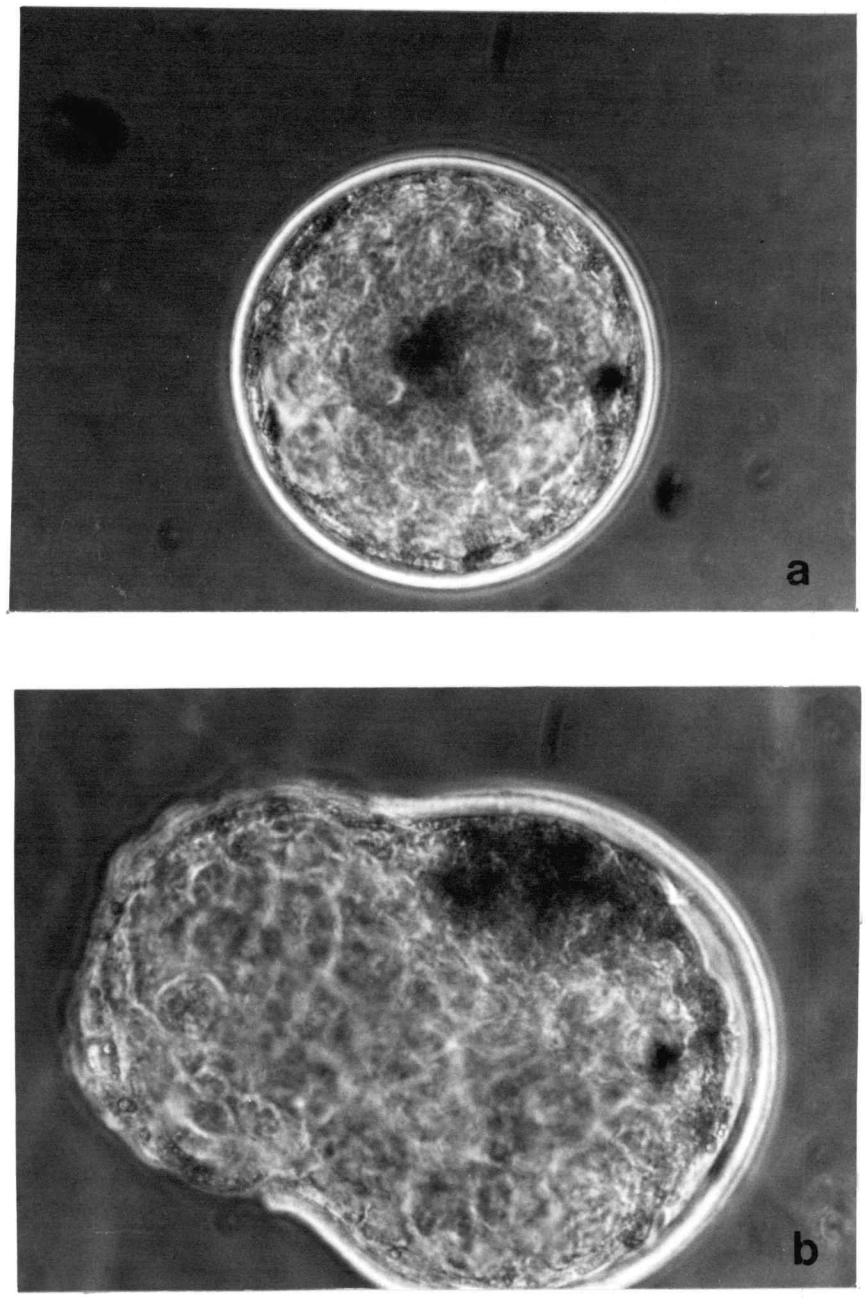
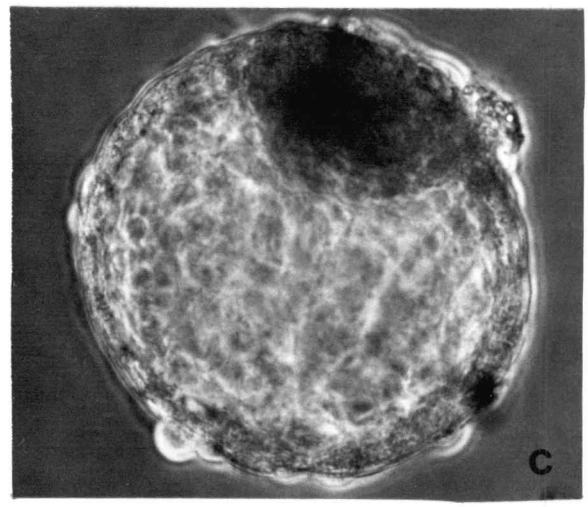


Figure 4. Bovine embryos cultured in Ham's F-12 with 15 mg/ml BSA (x 360). a) Expanding blastocyst cultured for 72 h in medium with 15 μg/ml plasminogen. b) Hatching blastocyst after 120 h in medium with no plasminogen.



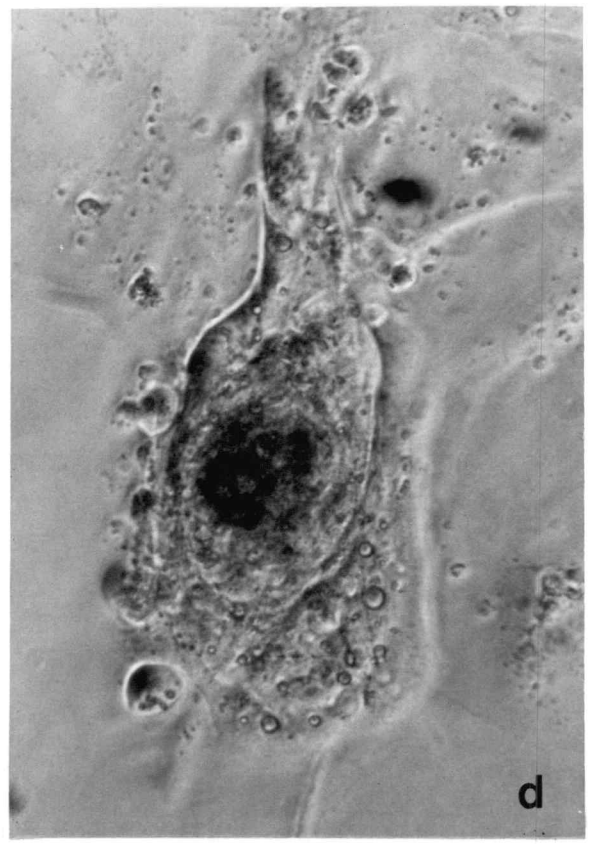


Figure 4. (ctd.) c) Hatched blastocyst cultured for 120 h in medium with 60 μg/ml plasminogen. d) Attached blastocyst with trophoblastic outgrowth after 21 d in medium with 15 plasminogen μg/ml plasminogen.

activation, measured indirectly as the plasmin concentration in a microdrop of medium, followed PA production and was consistently low for the first 48-72 h of culture. Embryonic activation of increased sharply thereafter, and also plateaued after 120 h (Figure 5; Menino and Williams, 1987).

Although the mouse embryo does not exert a dose-dependent response between plasmin or plasminogen level in the culture medium and the percentage of embryos attaining a particular cell stage (Menino and O'Claray, 1986), bovine embryos indeed exhibit accelerated rates of initiation and completion of hatching at 120 μg/ml plasminogen than at the lower experimental concentrations. These accelerated rates of initiation and completion of hatching may be due to plasmin-mediated effects on the zona pellucida. Menino and Williams (1987) suggested the activation of plasminogen to plasmin may be a mechanism utilized by the bovine blastocyst to "effect a sublysis of the zona pellucida" and thereby facilitate hatching. Interestingly enough, bovine embryos cultured in medium containing 120 μg/ml plasminogen hatched at a comparative gestational age of 10 to 12 days (Menino and Williams, 1987), closely approximating the observed in vivo range of 7 to 10 days (Betteridge and Flechon, 1988).

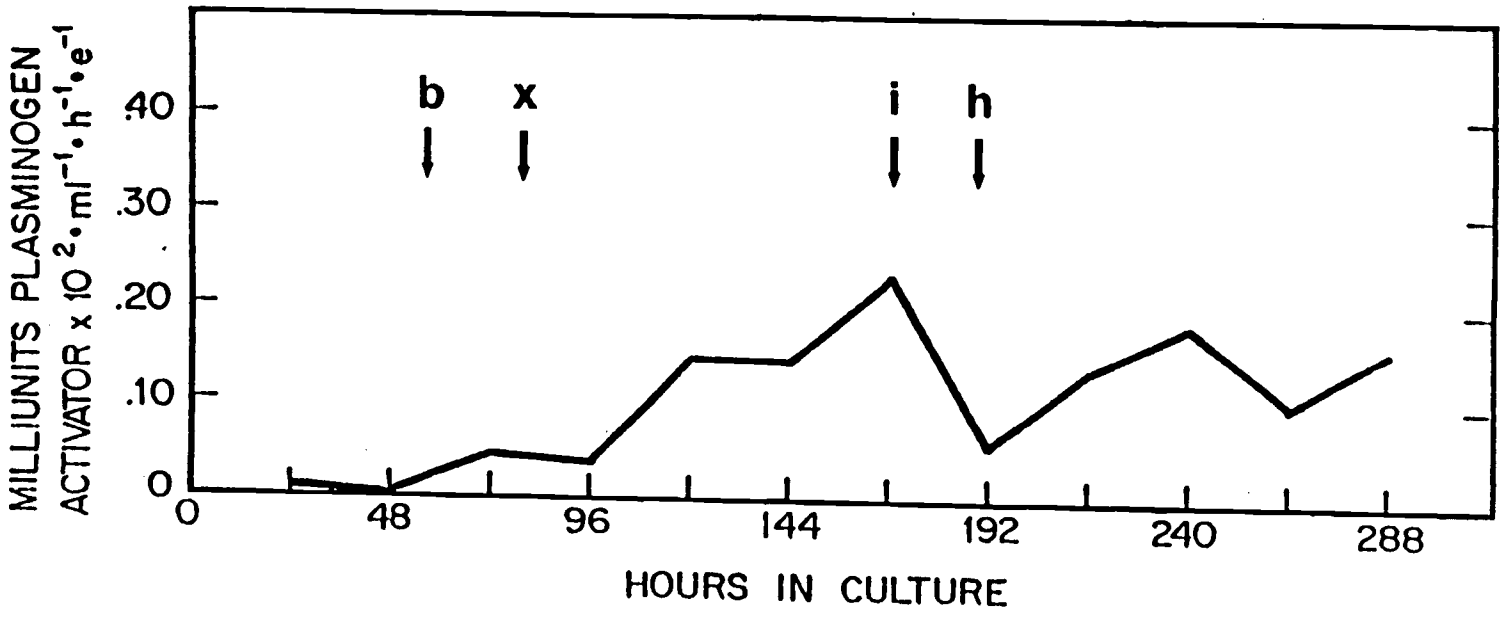


Figure 5. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 15 mg/ml BSA (taken from Menino and Williams, 1987; used with permission; b= blastocyst, x=expanding blastocyst, i=initiating hatching blastocyst, h=hatched blastocyst).

Ovine Embryos. Plasminogen activator production by early ovine embryos has recently been documented by Menino et al. (1989). Menino et al. (1989) speculated that the conversion of uterine plasminogen to plasmin by the secretion of PA may be a mechanism utilized by the ovine blastocyst to cause a sublysis of the zona pellucida and facilitate hatching. Embryonic PA production was quantified and the effects of various levels of plasminogen on in vitro development and zona pellucida integrity were evaluated.

More ovine embryos hatched in medium containing 60 and 120 μg/ml than 0 μg/ml plasminogen. Zona pellucida dissolution time in an acidified phosphate buffered saline was less after incubation in medium with 60 and 120 μg/ml plasminogen than 0 μg/ml plasminogen; however, a solubility change in the zona pellucida occurred in the absence of plasminogen or plasmin. Plasminogen activator production was low until the morula stage, increased during morula-blastocyst transition and remained elevated throughout blastocoelic expansion and hatching. Zona pellucida solubility, PA production and plasminogen conversion to plasmin increased with advancement of embryonic stage; however, PA production and plasminogen conversion to plasmin were poorly correlated with zona pellucida solubility. Menino et al. (1989) suggested that other factors may play a role in changing zona pellucida integrity prior to hatching such as physical forces resulting from blastocoelic expansion or even other as yet unidentified enzymes.

Materials and Methods

Embryo Collection and Culture

Twenty-two Holstein cows from the Oregon State University dairy herd were estrous synchronized with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse®, The Upjohn Co., Kalamazoo, MI) and superovulated with porcine follicle stimulating hormone (pFSH: Sigma Chemical Co., St. Louis, MO). Two 25-mg injections of $PGF_{2\alpha}$ were administered i.m. 12 d apart (Day 0 = first $PGF_{2\alpha}$ injection) to all 22 cows. All cows received twice daily injections of pFSH i.m. at dosages of 6, 5, 4 and 3 mg on Days 10, 11, 12 and 13, respectively. Estrus detection was conducted starting 12 h after the second $PGF_{2\alpha}$ injection and at 12-h intervals thereafter. Visual detection of cows in standing estrus was facilitated by utilizing a vasectomized bull or livestock paint on the tailhead. Cows observed in estrus were artificially inseminated with 1 or 2 straws of frozen bull semen at 0, 12, and 24 h after onset. Twelve to fourteen days after onset of estrus, hatched blastocysts were collected at slaughter by flushing the excised uterus with sterile Dulbecco's phosphate buffered saline (DPBS; Dulbecco and Vogt, 1954) containing 10 ml/l of an antibiotic-antimycotic solution (10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml in 0.9% sodium chloride; Sigma) and either 1% heat-treated bovine serum or 0.5% BSA (Sigma). Uterine flushings were examined with a dissecting microscope and blastocysts were recovered from the flushings by aspiration with sterile glass micropipettes or Pasteur pipettes (Figure 6). Recovered blastocysts were washed in 5 ml of Ham's F-12

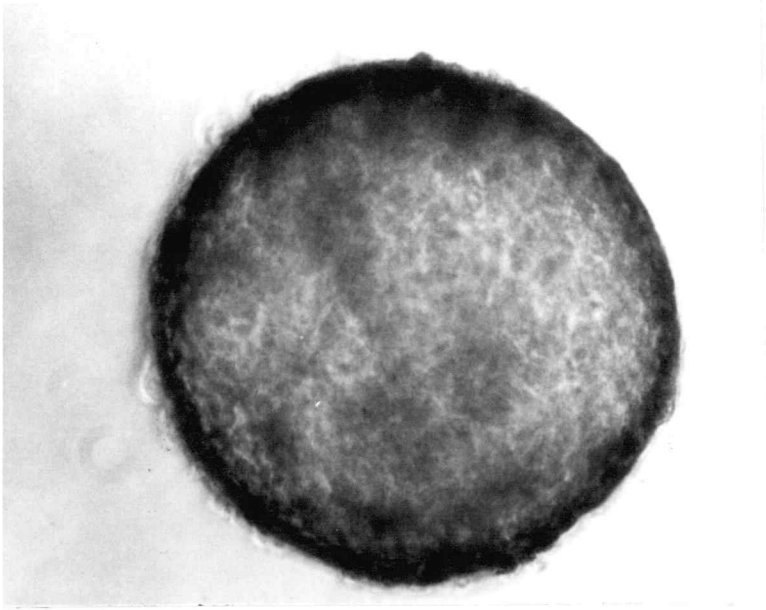


Figure 6. Hatched bovine blastocyst recovered 13 d after onset of estrus (X 390).

(Sigma) containing 15 mg/ml BSA and assigned to either Experiment I or II.

In Experiment I, 12-14 d blastocysts were cultured for 24 h in 100 μl microdrops of Ham's F-12 containing 15 mg/ml BSA under paraffin oil (Fisher Scientific Co., Tustin, CA) in a humidified atmosphere of 5% CO_2 in air at 37°C (Figure 7). At initiation and end of culture, blastocysts were observed for morphology and photographed using an inverted stage-phase contrast microscope at 40-200X. Following culture, embryos and medium were recovered separately from the culture drops by aspiration, placed into 1 ml snap-cap vials, and frozen at -20°C until electrophoresis and zymography. To detect any contamination of plasminogen, PA or plasmin in the culture medium, medium without embryos was incubated under identical conditions and assayed similarly as other medium samples. In Experiment II, blastocysts were either microdissected into component embryonic discs and trophoblast or not dissected (Figure 8). Microdissection was conducted using a microscalpel and microforceps while visualizing the embryo at 70X with a dissecting microscope. Embryonic discs, trophoblast and intact embryos were cultured for 24 h, photographed, and tissues, and media were recovered and stored as described in Experiment I (Figure 9).

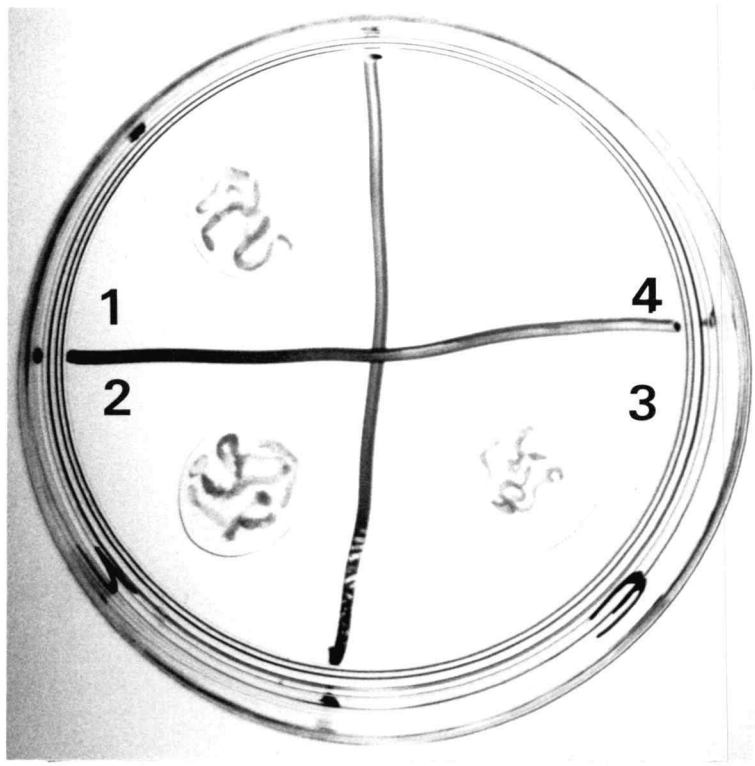


Figure 7. Petri dish containing 14 d bovine blastocysts in 100 μl culture drops (1-3). Culture drop 4 contains medium without embryos (X 1.8).

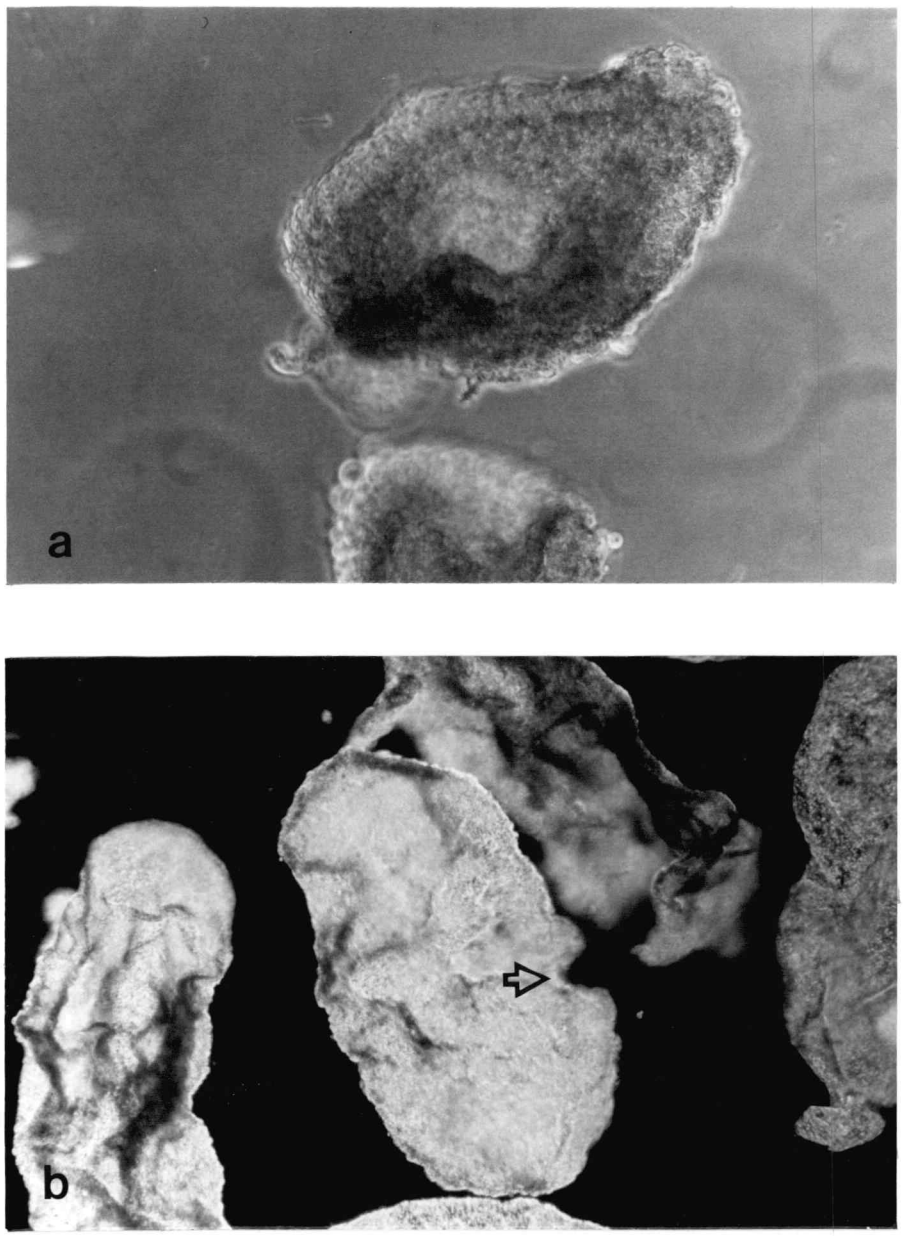


Figure 8. Dissected embryonic disc (a, X 360) and trophoblast (b, X 72) from 14 d bovine blastocysts prior to culture. Arrow indicates site from which embryonic disc was dissected.

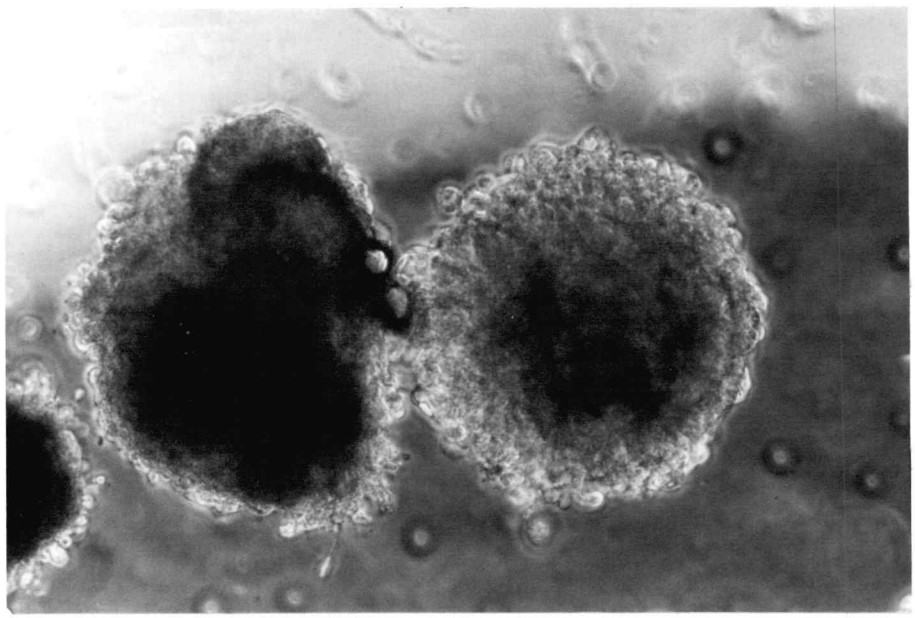


Figure 9. Embryonic discs dissected from 14 d bovine blastocysts cultured for 24 h (X 360).

Sample Preparation, Electrophoresis and Zymography (see APPENDIX A for further details).

In both experiments the Protean Dual Slab Cell electrophoresis apparatus (Bio-Rad Laboratories, Richmond, CA) was used for one-dimensional SDS-PAGE under non-reducing conditions with a modified casein-agar gel underlay technique known as zymography (Granelli-Piperno and Reich, 1978; Vassalli et al., 1984). Embryonic tissues were thawed, homogenized by repeated aspiration with a micropipette and combined with equivalent volumes of 2X sample buffer (5.0% SDS; 20% glycerol; 0.0025% bromophenol blue in 0.125 M Tris-HCl buffer) or at least 30 μl of 1X sample buffer if the amount of tissue was small, as was the case with the embryonic discs. Media samples were combined with an equal volume of 2X sample buffer. Urokinase (E.C. 3.4.21.7) standards (Sigma) were prepared with 1X sample buffer to final concentrations of 1.0, 0.5, and 0.1 milli-Units/ml. Tissue-PA standards (Sigma) used in Experiment II were prepared with 1X sample buffer to final concentrations of 1.0 and 0.5 μg/ml. Each 160 X 135 X 1.5-mm thick electrophoretic slab gel included one lane containing the following MW markers (Bio-Rad): phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (42.7 kD), carbonic anhydrase (31.0 kD), soybean trypsin inhibitor (21.5 kD) and lysozyme (14.4 kD). The MW markers were prepared by combining a stock concentrate with 1X sample buffer in a 1:10 ratio. Urokinase and tPA standards as well as MW markers were routinely tested by zymography for activity prior to co-electrophoresis with embryonic tissue and media samples. Samples and standards were vortexed to ensure complete mixing with applied

buffers. Urokinase, tPA and MW markers were aliquoted in 100 μl volumes into castellated wells in a 4.0% acrylamide stacking gel with a 12.0% separating gel. Prepared blastocyst tissue and media were aliquoted onto the acrylamide gels in 30-100 μl volumes. Electrophoresis was conducted under constant voltage at a current of 15 mA/gel through the stacking gel and 30 mA/gel through the separating gel for 2 to 4 h until the dye front reached the bottom edge of the separating gel.

Following electrophoresis, the Protean Dual Slab Cell apparatus was disassembled and the slab gels were gently agitated in 2.5% Triton X-100 (Sigma) for 30 min, rinsed with distilled water 3 times and incubated in DPBS for 30 min. The gel was carefully placed on plastic wrap, excess DPBS blotted gently with tissue paper, and a casein-agar gel containing purified human plasminogen (Sigma) supported on a glass plate was applied to the surface of the polyacrylamide slab gel. The casein-agar gel plates were prepared by dissolving 2 g of nonfat dry milk (Carnation Co., Los Angeles, CA) in 100 ml of buffer containing 0.0013 M $CaCl_2 \cdot 2H_2O$, 0.10 M glycine, 0.038 M Tris and 0.005 M Na azide. Twelve milliliters of the nonfat dry milk were heated to 55°C and combined with 12.0 ml of 2% melted agarose (Sigma) also maintained at 55°C. Purified human plasminogen was added to the warmed mixture in sufficient volume to yield a final concentration of 20-25 μg/ml. The total volume of this mixture was then cast onto a warmed 13.5 x 16.0 cm glass plate and allowed to cool. In Experiment I, electrophoretic gels were run in duplicate so as to apply one casein-agar gel plate containing human plasminogen and the other plate containing no plasminogen. Plasminogen activators, including

urokinase and tPA, which diffused from the electrophoresed slab gels, would show lytic zones in casein-agar gels containing 20-25 μg/ml human plasminogen. The casein-agar gel plate containing no plasminogen and incubated with electrophoretic gels served as a control to detect non-specific proteases. Gel "sandwiches" were incubated at room temperature for 24-48 h and observed for lysis hourly for several hours, and every 4 h thereafter. Protease migration was measured during incubation by measuring, to the nearest millimeter, the distance from the edge of the separating gel to the center of clear lytic bands in each lane. Gel incubation was terminated by separating the gel sandwich, fixing the casein-agar plate with 3% acetic acid for 10 min, and rinsing under tap water. Casein-agar gel plates were dried and stained for permanent storage. Electrophoresed slab gels were stained with 0.05% Coomassie Brilliant Blue (Bio-Rad) in 65/25/10 water/isopropanol/acetic acid for 4-8 h and destained in 80/10/10 water/isopropanol/acetic acid overnight. After destaining electrophoretic polyacrylamide slab gels were dried for permanent storage with a Hoefer Scientific SE-540 Slab Gel Dryer.

Each caseinolytic zone in lanes containing PA from culture medium, blastocyst tissues, or urokinase standards served as an observation. Relative mobilities (Rm) of urokinase standards and bovine blastocyst PA were calculated by dividing the distance of migration from the point of origin on the separating gel to the center of the casein-agar lytic zone by the total length of the slab gel. Molecular weights of PA were determined by log MW-Rm plots derived from the MW markers. Correlation-regression calculations provided the equation of the line and the

correlation coefficients for the log MW-Rm plots (Steel and Torrie, 1980; Sincich, 1985). Unknown MW were calculated from the equation of the line. Differences among MW of PA secreted into the culture medium or contained in blastocyst tissue were detected using the unpaired t-test.

Results

Embryo Collection

A total of 111 embryos of normal morphology were collected from 11 of the 22 cows for a recovery rate of 10.1 embryos per cow. Unfertilized ova or degenerate embryos were collected from two cows and neither ova nor embryos were recovered from the remaining 9 cows.

SDS-PAGE and Zymography

The mean correlation coefficient for plots of log MW by Rm used to calculate the MW of urokinase and tPA standards and embryonic PA was -.99 ± .01 for 27 SDS-PAGE runs. Molecular weights of urokinase standards co-electrophoresed with embryonic tissues and media were 57.5 ± 0.6 and 38.2 ± 0.5 kD for the high and low MW forms, respectively (Table 1). Because no difference ($P>.05$) was detected in MW of high and low urokinase forms between Experiment I and II, the data reported in Table 1 are means pooled from the two experiments. The tPA used as a standard in Experiment II exhibited a MW of 63.6 ± 0.5 kD.

In Experiment I, 12-14 d bovine blastocysts contained and secreted both an uPA and a tPA (Table 2, Figure 10). No difference was seen in MW of uPA between undissected blastocyst tissue and the respective culture medium ($P>.05$). The MW of tPA observed in the medium was greater than that observed for tPA in blastocyst tissue ($P<.05$); however, the number of observations for the tPA MW determination was low.

The MW of uPA and tPA in medium and tissue from intact blastocysts and trophoblast in Experiment II are reported in Table 3. Molecular weights of uPA and tPA did not differ due to source of tissue ($P>.05$). Plasminogen activators were not detected in either embryonic disc tissue or its respective culture medium (Figure 11).

TABLE 1. MOLECULAR WEIGHTS (kD) OF UROKINASE AND TISSUE PLASMINOGEN ACTIVATOR (PA) STANDARDS

Standard	n	Molecular Weight[a]
Urokinase		
High molecular weight form	52	57.5 ± 0.6
Low molecular weight form	48	38.2 ± 0.5
Tissue PA	19	63.6 ± 0.5

[a]Mean ± standard error of the mean.

TABLE 2. MOLECULAR WEIGHTS (kD) OF PLASMINOGEN ACTIVATORS PRODUCED BY BOVINE BLASTOCYSTS

Source	n	Molecular Weight[a]	n	Molecular Weight
Blastocysts	5	48.0 ± 1.1[b]	2	84.5 ± 0.3[b]
Medium	7	46.3 ± 1.6[b]	3	87.3 ± 0.4[c]
Blastocysts and medium	12	47.0 ± 1.0	5	86.1 ± 0.7

[a]Mean ± standard error of the mean.

[b,c]Values in the same column without common superscripts are different ($P<.05$).

TABLE 3. MOLECULAR WEIGHTS (kD) OF PLASMINOGEN ACTIVATORS (PA) PRODUCED BY INTACT BLASTOCYSTS AND DISSECTED TROPHOBLAST IN THE BOVINE

	Urokinase-type PA		Tissue-type PA	
Source	n	Molecular Weight[a]	n	Molecular Weight
Intact blastocysts	15	42.5 ± 1.8	4	91.4 ± 4.0
Trophoblast	10	40.0 ± 2.6	3	93.2 ± 4.2
Intact blastocysts and trophoblast	25	41.5 ± 1.5	7	92.2 ± 2.7

[a]Values reported are means ± standard error of the mean of MW for PA determined from tissue and media samples.

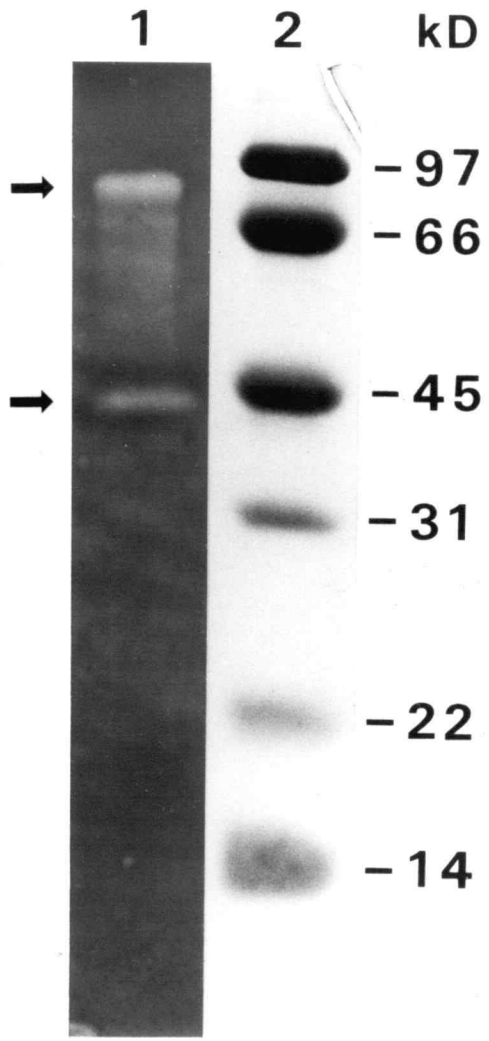


Figure 10. Caseinolytic zymograph of cultured 12-14 d bovine blastocysts (lane 1) and polyacrylamide gel containing MW markers (lane 2). Arrows point to lytic zones caused by embryonic tPA (A) and uPA (B). Molecular weight markers in order of descending MW are: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

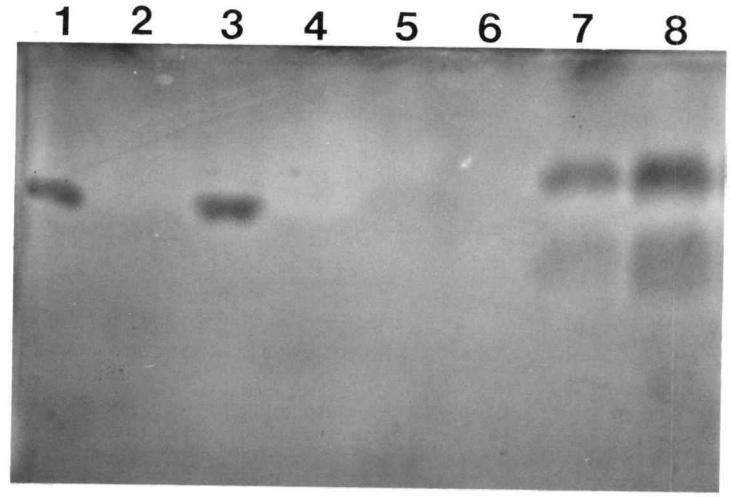


Figure 11. Zymograph of microdissected 12-14 d bovine blastocysts and urokinase standards. Lane 1, intact embryo; Lane 2 and 4, culture medium without embryos; Lane 3, trophoblast; Lane 5, embryonic disc; Lane 6, no sample; Lane 7, 0.1 mU/ml urokinase; and Lane 8, 0.5 mU/ml urokinase.

Discussion

Zymographic analysis for PA revealed that 12-14 d bovine blastocysts contain and secrete both an uPA (41.5 - 47.0 kD) and a tPA (86.1 - 92.2 kD). Molecular weights of urokinase standards observed in the present study were similar to values reported by Christman et al. (1977) for the high (54.7 kD) and low (31.4 kD) MW forms of the protease. The MW of the tPA used as a standard in Experiment II also agrees closely with the 60 kD value reported by Christman et al. (1977) for tPA produced by cultured human melanoma cells. Accuracy of the MW determination for PA may vary accordingly with the precision of Rm measurements, especially with the higher MW proteases (i.e., tPA). Small Rm variations can result in substantial variations in MW calculation, perhaps by as much as several kD. Such variation in Rm measurements may account for the differences in MW observed between Experiments I and II for uPA (47.0 vs. 41.5 kD) and tPA (86.1 vs. 92.2 kD). Likewise, it is improbable that the MW for the tPA in blastocysts and media reported in Table 2 are biologically distinct.

The incidence of detection of the embryonic uPA was approximately threefold greater than that for the tPA. This suggests the embryonic tPA is either more labile than the uPA or not as readily expressed. The latter is indeed possible because tPA and uPA are immunologically distinct and produced by different genes (Dano et al., 1985; Degen et al., 1986). In the present study, embryonic PA activity was associated only with the trophoblast; however, the amount of embryonic disc tissue was considerably less compared to the intact blastocysts and dissected

trophoblast. It is possible that PA activity associated with the embryonic disc was not detected. Sensitivity of the zymographic technique can be adjusted by varying the plasminogen concentration in casein-agar overlays and the incubation time of gel sandwiches (Granelli-Piperno and Reich, 1978). Nevertheless, trophoblastic production of PA accounted for the principal source of embryonic PA.

Dormant tPA mRNA has been identified in mouse oocytes and tPA is synthesized by mouse oocytes undergoing meiotic maturation (Huarte et al., 1987). Mouse embryonic PA disappears shortly after fertilization (Huarte et al., 1985) and reappears at the time of implantation and subsequent developmental events (Sherman et al., 1976; Strickland et al., 1976). In the cow, Menino and Williams (1987) have suggested a time-dependent mechanism is in effect regulating embryonic PA production because PA is not produced in significant quantity until the expanded blastocyst stage. Although the physiologic significance of PA to early embryo development is not known, Menino and Williams (1987) have suggested that plasminogen activation to plasmin by the blastocyst may be a mechanism utilized by the embryo to effect a sublysis of the zona pellucida and thereby facilitate hatching. Besides a potential role in hatching, PA may also be involved in the series of morphologic changes occurring in bovine embryos between Days 8 to 16. Embryonic endoderm migrates beneath the trophectoderm starting on Day 8 to form trophoblast and on Day 12 the embryonic disc ruptures through the trophectoderm (Betteridge and Flechon, 1988). Mesodermal cells from the embryonic disc also begin to migrate between the trophectoderm and endoderm on Day 14 (Betteridge and Flechon, 1988). Contingent with these cell migrations,

the shape of the hatched blastocyst then changes from spherical to ovoid during a transitory phase preceding elongation that usually begins between Days 12-14 (Betteridge and Flechon, 1988). Marotti et al. (1982) observed that mouse endoderm produced a tPA and visceral endoderm and extraembryonic mesoderm an uPA and speculated a role for PA in cell migration during mouse embryogenesis. Results of the present study suggest 12-14 d bovine blastocysts produce both uPA and tPA, and the possibility of a model in the bovine similar to the mouse, where both types of PA are involved in tissue remodelling and blastocyst elongation.

Conclusion

One goal of researchers in embryo physiology is to link parameters of embryo biochemistry, such as PA production, to applicable reproductive technologies. Embryo transfer has been successfully performed in many mammalian species; however, most research and commercial application of this technology in livestock appears to be documented in cattle (Betteridge, 1977). As biotechnology advances, research in bovine embryo physiology will continue to focus on micromanipulation, in vitro fertilization, nuclear transplantation, cryopreservation, sexing, cloning, and immunology.

Plasminogen activator production may have potential as an indicator of bovine embryo viability prior to transfer and success in pregnancy establishment (Kaaekuahiwi and Menino, 1989). Molecular characterization and aspects of bovine embryonic PA may provide information as to how this biomolecular system functions in vivo and the significance of it during early development.

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APPENDIX

APPENDIX A

Standard Protocol for SDS-Polyacrylamide Gel Electrophoresis for Use in Plasminogen Activator Characterization and Zymography

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August, 1987
Revised July 1988, "2nd Edition"

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I. Solutions, Chemicals and Samples

A. 10% ammonium persulfate solution (must be fresh every 36 h). 0.05 g ammonium persulfate to 500 μl of distilled water. Dissolve in a snap-cap vial and store in refrigerator until needed.

B. 10% (w/v) SDS Solution:

Dissolve 10 g of SDS into 100 ml of dH_2O. Can be stored on lab shelf.

C. "Stock" solutions, as described in Bio-Rad Protean Dual Slab Cell Operating Instructions (Appendix, pp. 31-32):

1. Acrylamide: BIS (30% T, 2.67% C)

29.2 g acrylamide (Note: acrylamide is a neurotoxin and should be handled with utmost care. Use gloves/mask when handling.)

0.8 g N'N'-BIS methylene acrylamide
q.s. 100 ml with dH_2O (i.e. "bring to volume")

Call this "Solution A". No need to filter. Store in dark bottle in refrigerator (30 d maximum).

2. 1.5 M Tris-Cl, pH 8.8
18.15 g Tris base
50 ml dH_2O

Carefully adjust to pH 8.8 with 1 N or conc. HCl.
q.s. 100 ml with dH_2O.

Call this "Solution B" and store in refrigerator.

3. 0.5 M Tris-Cl, pH 6.8
3.0 g Tris base
35 ml dH_2O

Carefully adjust to pH 6.8 with 1 N or conc. HCl. Then q.s. to 50 ml with dH_2O.

Call this "Solution C" and store in refrigerator.

D. Electrode Buffer: Four liters is made up for each gel run. According to Bio-Rad instructions (p. 33), make up 4 l by first adding 12.0 g Tris base and 57.6 g Glycine to approximately 2 l of dH_2O. Stir while mixing. Then add 4.0 g of SDS slowly as a powder. Avoid frothing. q.s. to 4 l with dH_2O.

E. Sample Buffer: per Bio-Rad directions (p. 33):

4.0 ml dH_2O
1.0 ml 0.5 M Tris-Cl, pH 6.8 ("Solution C")
0.8 ml Glycerol
2.0 ml 10% (w/v) SDS
0.2 ml 0.05% (w/v) Bromophenol Blue (Aqueous Solution)

8.0 ml TOTAL. Multiply all components by 2 for 16.0 ml
Multiply all components by 2 (except dH_2O) for 2x sample buffer

Store in bottle on shelf. Note that no 2-mercaptoethanol for PA zymographic analysis is used with this technique.

F. Standard and Sample Preparation

1. Urokinase (E.C. 3.4.21.7) Standards (Sigma, No. U-6876): A serial dilution is normally used in which a frozen stock solution of 0.2 units/ml = 200 mU/ml Urokinase is diluted.

Example:

C_{stock} (mU/ml)	Vol_{stock} (μl)	Vol_{dil} (μl) (Sample buffer)	C_{final} (mU/ml)	Vol_{final} (ml)
200	50	1,950	5.0	2.0
5.0	200	800	1.0	1.0
5.0	100	900	0.5	1.0
5.0	20	980	0.1	1.0
0.5	100	900	0.05	1.0
0.1	100	900	0.01	1.0
0.5	10	990	0.005	1.0
0.1	10	990	0.001	1.0

Volumes are to be combined accordingly into fresh tissue culture tubes and can be stored on lab counter.

2. SDS-PAGE Low Range Molecular Weight Standards (Bio-Rad, #161-0304).

Standards are diluted 1:10 by combining 50 μl of stock concentrate from frozen snap-cap vial to 450 μl of Sample Buffer. 1 μl of Phenol Red can be added as a color dye indicator.

Heat for 3-5 min at 95°C. Prepared Standards can be loaded onto full-length gel wells (50 μl minimum) and can be stored on lab counter.

3. Tissue-type Plasminogen Activator (tPA) stock solutions (Sigma, No. T-7776). Use serial dilution from 10 μg/ml stock solution.

C_{stock} (μg/ml)	Vol_{stock} (μl)	Vol_{dil} (μl) (Sample Buffer)	C_{final} (μg/ml)	Vol_{final} (ml)
10.0	60	540	1.0	0.6
1.0	200	200	0.5	0.4
1.0	50	450	0.1	0.5

Volumes are combined accordingly into separate tissue culture tubes and can be stored on lab counter.

4. Human Plasminogen (hPgn) stock solution at 1200 μg/ml=1.2 mg/ml (Sigma, No. P-5661).

Normally recieved in solid form and must be diluted with sterile DPBS to 1.2 mg protein/ml.

Example: New Vial = 25 Units
84 mg solid
0.32 Units/ml solid
6.0 Units/ml protein

$$25 \text{ units} \times \frac{1.0 \text{ mg protein}}{6.0 \text{ units}} = 4.167 \text{ mg protein}$$

$$\frac{1.2 \text{ mg}}{1 \text{ ml}} = \frac{4.167 \text{ mg}}{x \text{ ml}} \qquad x=3.47 \text{ ml}$$

4.167 mg protein is thoroughly dissolved into 3.47 ml of sterile) DPBS, then dispensed into snap cap vials. hPgn should be aliquoted into various volumes before freezer storage.

5. Embryo Tissue and Media Samples

   a. Tissue samples are homogenized with a micropestle or via aspiration in and out of a micropipet tip, then combined in a 1:1 ratio with 1x or 2x Sample Buffer.

   b. Media samples are measured for volume and combined 1:1 with Sample Buffer.

The highest sample volumes possible should be drawn up in order to complete electrophoresis and allow for maximum PA detection on a caseinolytic agar gel plate "sandwich".

G. Phosphate Buffered Saline (DPBS)* (Dullbeccos and Vogt, 1954, J. Exp. Med. 99:167-82).

	For 1.0 liter	For 4 l DPBS:
1.	8.00 g NaCl	32.0 g
2.	0.20 g KCl	0.8 g
3.	1.15 g Na_2HPO_4	4.6 g
4.	0.20 g KH_2PO_4	0.8 g
5.	0.10 g $MgCl_2$ $6H_2O$	0.4 g

Rinse out each tray as you add to the flask, and bring to volume to 700 ml.

6. Make up $CaCl_2$ (0.10 g) separate to 100 ml with dH_2O. Then slowly add this to the 700 ml volume (0.40 g $CaCl_2$ to 400 ml dH_2O for 4.0 l PBS).

   q.s. to total volume of 1000 ml with dH_2O. q.s. to 4000 ml for 4 l.

7. Store chilled, or conveniently on lab counter. No antibiotics are added for zymography.

H. Triton X-100 (2.5%)* (Sigma or Bio-Rad):

Make up 2 l of this solution by mixing 50 ml of Triton X-100 reagent with 1,950 ml dH_2O.

*Note: DPBS and Triton X-100 solutions are to be used later when rinsing the gel prior to caseinolytic agar plate application.

I. Stains and Destaining Solutions (for use after Section VIII)

1. Coomassie Brilliant Blue (CBB) R-250 (Bio-Rad):

   0.05% in 65/25/10, water/isopropanol/acetic acid (can even be reused after filtering):

   1.0 g CBB R-250 dissolved in:

   1300 ml dH_2O
   500 ml isopropanol
   200 ml acetic acid

   2000 ml (2 l) TOTAL

2. CBB Destaining Solution:

(80/10/10, water/isopropanol/acetic acetic) (Can also be reused if fairly clear.)

3200 ml dH_2O
400 ml isopropanol
400 ml acetic acid

4000 ml (4 l) TOTAL

3. Amido Black Stain, 0.1%:

1.0 g Amido Black q.s. to 200 ml dH_2O
100 ml acetic acid
700 ml methanol

1000 ml (1 l) TOTAL

4. Amido Black Destaining Solution:

700 ml methanol
100 ml acetic acid
200 ml dH_2O

1000 ml (1.0 l) TOTAL

II. Casting a 13 x 10 cm caseinolytic agar gel plate containing 20-25 µg/ml hPgn. This can be done at any time during the course of running an electrophoretic gel. However, it is best to do this after section VII. B., but before section VIII. The following protocol is a common example.

A. 7.375 ml skim milk with azide "stock" mixture
7.375 ml 2% "stock" agarose solution
250 µl hPgn (1.2 mg/ml)

15.0 ml TOTAL volume enough to cover a 10 x 13 cm glass plate.

B. Follow the standard protocol for caseinolytic assay agar gel plate preparation.

1. Buffer preparation:

	Molarity	µg/l
$CaCl_2$ $2H_2O$	.0013	.195
glycine	.10	7.51
TRIS	.038	4.6
NaAzide	.005	.325

2. Dissolve 2 g of nonfat dry milk (Carnation) in 100 ml of buffer (2%). The azide preserves the milk.

3. Dissolve 2 g of agarose (Sigma) in 100 ml of dH_2O (2%). Then autoclave for 30 min to 100°C.

4. These skim milk and agarose mixtures must be made up fresh for each caseinolytic assay.

C. Example: For Plasminogen Activator activity detection, combine equal (1:1) volumes of the heated stock mixtures.

10 ml skim milk
10 ml 2% agarose

20 ml of 1:1 solution at 60°C

D. Take 14.75 ml of above mixture and cool to approximately 55°C in a separate beaker.

E. Immediately add 250 μl of the hPgn, and mix thoroughly. Total volume is now 15.0 ml.

F. Quickly draw up into a pipet and cast onto a warmed 13 x 10 cm glass plate. It is important to do this quickly and evenly in order to get a uniform plate. Then put the cast plate under a cover until later use (i.e., after section VIII).

NOTE: One can always increase or decrease the total volume and hPgn concentrations to meet desired sensitivity specifications for the zymographic assay.

III. Assembly of Protean Dual Slab Cell: (Also in Bio-Rad instructions, pp. 1-33.)

Make sure all plastic equipment and glass plates to be used are very clean and/or tissue-culture (TC) washed. .75 mm or 1.5 mm gels can be made. The casts are to be set before making the gel mixtures.

A. Lay down two 18 x 16 cm glass plates on a clean lab counter. On one, place two "spacers" (grey strips) on each 16 cm edge. Lay the other glass plate on and match up the edges. Keep surfaces clean! Avoid fingerprints!

B. Attach sandwich clamps with one screw slightly tightened on each side (Fig. 1, p. 13).

C. Line up spacers, glass plates, and sandwich clamps squarely. This is very important because the cast may leak if it is not fit together tightly and squarely. Then

uniformly tighten all sandwich clamp screws. (Note: Avoid using metal spatulas to adjust the spacers between the plates and sandwich clamps because the plates chip easily.)

D. Insert the assembled glass plates, spacers and sandwich clamps into the casting stand as shown in Fig. 2 on p. 13. Place a 3 cm wide strip of parafilm between the latex rubber gasket and the bottom edge of the glass plate assembly. In case of any leakage, this parafilm can be stretched to aid in sustaining the leak. Tighten down the assembly using "locking cams" (as in Fig. 3, p. 13). One can also cast a "base seal" onto the parafilm described in section IV.

E. Before making the gels, insert the correct size plastic tubing and needle in between the sandwiched glass plate assembly. Have all equipment and syringes ready for casting the gels, since it gelates rather quickly.

IV. Casting Gels

A. A "gel seal" cast at the base of the assembled glass plates is optional, but highly recommended, since the plates sometimes leak regardless of what is done.

1. For a total of 5.0 ml:

1.675	ml	dH_2O
1.25	ml	solution "B"
50	μl	SDS
2.0	ml	solution "A"
25	μl	ammonium persulfate
2.5	μl	TEMED (N, N, N′, N′,-Tetramethyl-ethylendiamine)
5.0	ml	TOTAL

2. Cast onto the parafilm at the base of the glass plates and allow to gelate in order to seal any chips or cracks.

B. Separating gel preparation:

1. A 12% acrylamide gel has been routinely used with good resolution. (See Bio-Rad instructions, p. 32).

2. A 1.5 mm separating gel is most sturdy for handling. The formula for one 1.5 mm gel (or two .75 mm gels) is as follows:

13.4	ml	dH_2O.
10.0	ml	1.5 Tris-Cl, pH 8.8 ("Solution B")
0.40	ml	(400 μl) 10% (w/v) SDS
16.0	ml	acrylamide: BIS (30% T, 2.67% C) ("Solution A")
*0.2	ml	(200 μl) 10% ammonium persulfate (fresh - 36 hours)
*0.02	ml	(20 μl) TEMED
40.02	ml	TOTAL

*NOTE: The ammonium persulfate and TEMED are to be added last as catalysts. Swirl gently.

3. With 14 gauge blunt needle on a 30 cc syringe, aspirate the gel mixture. Quickly switch to the 18 gauge needle and tubing already inserted between the sandwiched glass plates. Steadily and evenly drain the gel into the assembly.

4. Fill the assembly up to the level of the inserted solid spacer (approximately 35-40 mm below the top edge of the glass plate assembly). Be sure to rinse all syringes, needles and tubes immediately after use.

5. Let set for 1 min before dripping on a layer of distilled water. Then allow the separating gel to harden for at least 1 hour.

C. Stacking gel preparation: (refer to Bio-Rad instructions p. 32).

1. After the separating gel has set, carefully drain off the layer of dH_2O.

2. Insert a white 1.5 mm 10-well comb between the sandwiched plates above the separating gel. Each sample well can normally contain up to 125 μl of sample.

3. Insert the 1.5 mm tubing between the plates again and have 30 cc syringe with 14 gauge blunt needle ready before mixing stacking gel.

4. The formula for one 1.5 mm 4.0% acrylamide stock gel (or two .75 mm gels) is as follows:

6.1	ml dH_2O
2.5	ml 0.5 M Tris-Cl, pH 6.8 ("Solution C")
0.10	ml (100 μl) 10% (w/v) SDS
1.3	ml acrylamide: BIS (30% T, 2.67% C) ("Solution A")
*0.05	ml (50 μl) 10% ammonium persulfate (fresh)
*0.01	ml (10 μl) TEMED
10.06	ml

*Ammonium persulfate and TEMED to be added last as catalysts.

5. Swirl gently, then quickly aspirate the stacking gel mixture into the 30 cc syringe and switch to the 18 gauge needle with attached tubing.

6. Begin casting by providing an even and constant pressure, and fill up plates to combs until about 1 mm from the top edge.

7. Allow to set for 5 min before applying distilled water. Carefully drip the dH_2O on, then let stacking gel set for at least 0.5 hr to insure complete gelation.

8. After gelation, the comb can be removed very carefully (in order to avoid collapsing the formed wells).

9. Rinse the wells 2x with electrode buffer using syringe and 20 gauge needle. Then fill wells with electrode buffer.

   The sandwich plates and gels are now ready to be attached to the upper buffer chamber and inserted into the lower buffer chamber.

V. Assembly of electrophoretic apparatus (refer to Bio-Rad directions and diagrams, pp. 13-14):

   A. After the wells have been rinsed and filled with electrode buffer, attach the upper buffer chamber with latex rubber gaskets in place to the glass plate asssembly and the buffer dam or "dummy" slab cell (if only 1 gel is being run). Tighten with locking cams (Fig. 3, p. 13).

   B. Remove the assembly from the casting stand, latex rubber gasket, and parafilm.

   C. With the "exchange cooling system" in place in the lower buffer chamber and attached to a tap water faucet draining into the sink, fill the lower buffer chamber with electrode

buffer to approximately 2 l. Make sure the lower buffer chamber sits as level as possible on the stir plate with stir bar in it.

D. Insert the entire assembly of upper buffer chamber, glass plates, sandwich clamps, and cast gels into the lower buffer chamber. (See Fig. 5, p. 14).

VI. Once the apparatus is set up as in Fig. 6, p. 14, the samples can be aliquoted to their respective wells on the stacking gel. It has been recommended not to use wells 1 and 10 due to poorer resolution in those wells.

A. List on a standard gel electrophoresis data sheet the samples to be put into each well along with respective sample volumes.

Example: Run an "embryo sample" in well 2; 0.05 mU/ml Urokinase standard in well 6; 0.1 mU/ml Urokinase standard in well 7; and SDS protein standards in well 9.

It is recommended to skip wells among samples in order to prevent contamination during aliquoting.

B. Procedure for homogenized embryo tissue or media samples. (Consult Dr. Menino.)
Frozen embryo samples should be thawed first. Use a micropestle in a microcentrifuge tube to homogenize any tissue samples, and combine with the proper ration of 1x or 2x sample buffer. Tissue can also be homogenized by aspiration in and out of a micropipette tip.

C. Aliquot standards and samples using a micropipette (P-100) and long flat Multip-Flex pipet tips. Normally, approximately 100 μl of sample can be aliquoted to each well without complications. Avoid bubbling!

D. Once samples have been aliquoted, slowly pour electrode buffer into the upper buffer chamber up to the level of the angled seams. The gel is now ready to run.

VII. Running a polyacrylamide gel.

A. Put the plastic cap with electrodes that is hooked up to the power supply onto the assembled gel apparatus.

B. With voltage button (top) at 500 V and current button at 20 mA (bottom), turn on the power supply. The voltage knob is to be turned clockwise all the way, and adjust the current knob to read 15 mA per gel. Note the time of start-up.

Also refer to Bio-Rad Model 500/200 Power Supply instruction manual. Note safety procedures.

C. Occasionally adjust the current knob back to 15 mA, and check the apparatus to see that it stays cooled during electrophoresis.

D. As soon as the sample tracker dye front has gone from the stacking gel to the separating gel, switch the current button up to 200 mA and adjust to 30 mA per gel.

It normally takes 1-2 h for the dye to run through the stacking gel. The gel will then run for approximately 2-4 h. Check to insure the system is cool and running at 30 mA per gel.

Now, in the meantime, one could cast a caseinolytic agar gel plate. Refer to Section II.

E. Towards the end of 3-4 h, the tracker dye front will approach the bottom edge of the separating gel. Shut the power supply off when it has reached the bottom edge of the separating gel.

Have the caseinolytic agar gel plate ready by this time.

VIII. Processing the Electrophoresed Gel

A. Disassemble the protean dual slab cell and electrophoresis apparatus, and put the used components in a clean tap-water rinse tub for the time being. TC wash later.

B. When separating the glass plates from the gel, be as careful as possible so as not to rip or break the gel itself. Note where the lanes are according to the stacking gel portion, and make notches in the separating gel in order to note orientation for later reference.

C. (Optional) The protein standard lanes can be separated first off by a scalpel blade, and then put into CBB-R250 stain for a minimum of 4 h. This portion of the gel is then put into CBB Destainer solution for several h. Occasionally change the destaining solution. Remember its orientation! Note this aspect of the protocol is optional. We do not do this routinely.

D. Procedures for the Urokinase Standard and Embryo Sample lanes. Handle the gel with care and use gloves.

1. Put the gel into a plastic rinse box containing Triton X-100 for 20-30 min on the shaker. Continue to note orientation.

2. Then run it through a dH_2O rinse 3X.

3. Put into DPBS solution in a plastic rinse box for 20-30 min.

4. Remove the gel from the DPBS onto a clean piece of plastic Saran wrap large enough to completely wrap the gel.

5. Carefully blot the gel dry with tissue paper in order to rid any excess DPBS and moisture.

6. Place the 10 x 13 cm caseinolytic agar plate onto the gel, lining up the top edge of the gel with the top edge of the glass plate. Even though urokinase lanes are normally separated from the embryo sample lanes by several centimeters, the plates can be conveniently placed so as to include both urokinase and embryonic plasminogen activator detection.

E. The acrylamide-caseinolytic gel "sandwich" is now ready for incubation. Wrap this "sandwich" in the plastic wrap and place on the lab counter to incubate at room temperature.

1. The plate is to be checked every h and observed for any lysis.

2. The urokinase standards may show lysis only after 1-2 h of incubation, depending on the concentrations used. Make measurements from the edge of origin to the most accurate assessment of the center of the lytic band.

3. The embryo sample lane may take from 1 to 36 h to show any lysis. (Refer to article by Grannelli-Piperno and Reich, J. Exp. Med. 48:223-24).

4. During incubation and detection of lysis, measure and record distances from the plate's edge to the center of the bands. These measurements are important in calculations and log-MW plots.

F. After incubation, the acrylamide portion of the gel sandwich can be put into CBB R-250 stain and subsequent destainer. Note that it takes longer for the caseinolytic section of the gel to destain than the other part. 4 h CBB, 1-2 d in destainer.

G. After incubation, the caseinolytic agar gel is fixed with 3% acetic acid for 10 min, then carefully rinsed with tap water.

H. Follow up this work with additional measurements, calculations, and log MW plots.

IX. Measurements, Calculations, Log-MW Plots, and Scanning Densitometer Use.

A. Measurements during and after gel "sandwich" incubation.

1. Realizing the correct orientation of the gels and sample lanes, measure the distance (in mm) from the origin edge of the glass plate to the <u>center</u> of each lytic band.

These measurements should be done as soon as the lytic bands are distinctly visible in each lane (approx. 1-2 h of incubation).

2. Calculate Relative Mobilities (Rm) simply by dividing the distance to the center of the lytic band by the total length of the gel.

$$Rm = \frac{\text{distance to lytic band center}}{\text{total gel length}}$$

3. Rm's can also be measured and calculated for the SDS and BSA Protein Standard Lanes after they have been processed and are ready for use on the Scanning Densitometer (optional). A standard curve should also be calculated.

a. Set up the following table for the SDS Protein Standards.

Protein	MW	log MW (X)	Rm (Y)	X·Y
Phosphorylase b	97,400	4.989		
BSA	66,200	4.821		
Ovalbumin	42,699	4.630		
Carbonic anhydrase	31,000	4.491		
Soybean trypsin inhibitor	21,500	4.332		
lysozyme	14,400	4.158		

b. Then calculate: $\Sigma X = 27.422$ $\Sigma Y =$ $\Sigma XY =$

$\Sigma X^2 = 125.801$ $\Sigma Y^2 =$

$\bar{X} = 4.570$ $\bar{Y} =$

c. Finally, follow standard statistical procedures for simple linear regression and correlation using
$Y = a + bX$
where: $Y = Rm$
$X = \log MW$
a = y-intercept = $\bar{Y} - b\bar{X}$
b = slope of the line = $\frac{SSxy}{SSxx}$

and

$$SSxx = \Sigma xy - \frac{(\Sigma x)(\Sigma y)}{n}$$

$$SSxx = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$$

$$SSyy = \Sigma y^2 - \frac{(\Sigma y)^2}{n}$$

and: $r = \frac{SS_{xy}}{\sqrt{SS_{xx}\ SS_{yy}}}$, the correlation coefficient

The slope (b) should be negative, and r should be close to 1.000

d. Now, substitute in the Y (Rm) values from unknown MW species, to calculate X (log MW) and essentially MW.

Example: For Urokinase (with 2 chains showing 2 different lytic bands):

(log MW1) $X_1 = \frac{Y_1 - a}{b}$

Antilog --> MW_1

where Y1 is that band's Rm, a is the calculated y-intercept, and b is the calculated slope of the standard curve.

$$\text{Also, } X_2 = \frac{Y_2 - a}{b}$$

Antilog --> MW_2

(Standard Statistical Procedures taken from Sincich T, 1985. Statistics by Example, Second Edition. San Francisco: Dallen Publishing Co.)

B. Log MW Plots and Scanning Densitometer (optional). Refer to the IBM version of the GS-350 Data System and the Bio-Rad manual for the Model 1650 Transmittance/Reflectance Densitometer Operating Instructions.

X. Photographing gels with the Polaroid MP-4 Land Camera (refer to instruction manual, also) or your own 35 mm camera.

A. The gel is placed on the white fluorescent lamp table under the camera; or using extraneous light sources in the darkroom.

B. Remove lens cap.

C. Adjust with up and down knobs for image focusing.

D. Set Lens Aperture (lower knob) and Shutter Speed (upper knob). These can be adjusted as to desired specifications for conditons pending.

E. Slide the vision box over and remove the lens cap before photographing.

F. "Click" the shutter release on the lens, or the shutter release cord.

G. Remove the film by sliding it out slowly, and then count for approximately 20 sec before beginning to peel chemical package off. (Polaroid B/W Type 107, preferably speed 1000).

XI. Drying a polyacrylamide gel with the Hoefer Scientific SE-540 Slab Gel Dryer

A. Soak the gel to be dried in 5% glycerol for at least 2 h

B. Soak two cellophane sheets in dH_2O for about 10 min (avoid wrinkling).

C. Place two filter paper pads on top of the dryer's sieve screen. Cut off corners first with scissors.

D. Lay down first cellophane sheet on filter paper. Lay gel on, and apply dH_2O.

E. Apply top cellophane sheet; remove air bubbles, using dH_2O squirt bottle.

F. Apply "Mylar Sheet".

G. Fold rubber sheet over entire assembly.

H. Open valves on vacuum oven. Turn vacuum pump on. Seal window shut. Pump to approx. 29 lbs. for 2 min. Then shut off pump. Shut right valve. Re-pump during drying time as needed.

I. Turn vacuum oven on to 80° C.

J. Turn Slab Gel Dryer on by setting timer to 2 h (minimum) for a 1.5 mm polyacrylamide gel.

K. Depressurize by carefully peeling off top rubber sheet.

XII. Permanently Pressing Caseinolytic Agar Gels onto Glass Plate.

A. Lay casein gels on glass on lab counter.

B. Wet filter paper with dH_2O and apply to casein gel on glass. Apply another 8 dry filter sheets on top of that.

C. Apply a large flat surface (i.e. a large glass plate) and a 2-3 kg weight for 15 min. The fragments of casein lysed by the plasmin will absorb.

D. After 15 min, <u>carefully</u> remove filter paper.

E. Put plates in 60°C oven to dry for 5-10 min. Plates must be completely clear.

F. Flood plates in Amido Black staining solution for 3-4 minutes. Do not go over time.

G. Amido Black destain overnight.

H. Rinse with tap H_2O and stand up in oven to dry 5-10 min.