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Hugo Eiler, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
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# THE ROLE OF FETAL SEROTONIN (5-HT) IN BOVINE PLACENTA DETACHMENT

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Kellie A. Fecteau

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# DEDICATION

This dissertation is dedicated to you, Mom, for all that you are to me. I love you heart and soul.

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# ABSTRACT

The enzyme collagenase is involved in degrading uterine collagen during postpartum involution. It has been reported that cultures of rat and human myometrial smooth muscle cells produce collagenase when grown in medium containing fetal bovine serum (FBS) but not in medium containing newborn bovine serum (NBS). The substance in FBS that induces the cells to produce collagenase is 5-hydroxytryptamine (5-HT, serotonin). The concentration of 5-HT in FBS is between 30 μM-50 μM. In contrast, NBS has a concentration of approximately 1 μM or less. It has been proposed that 5-HT serves as a signal to initiate uterine collagen hydrolysis. Furthermore, a 5-HT transporter exists in the mouse and human placenta. The function of the transporter is not known, however it is speculated that it may act in growth and development of the fetus. Five-HT is known to act as a growth factor for many cell types yet its effect on placental cells is not known. In addition, the source of 5-HT found in placental tissue is unclear. It may be that the source for placental 5-HT is the fetal intestine since 95% of the body's 5-HT is located in the gastrointestinal tract and intestinal resection causes a drop in blood concentration of 5-HT.

The working hypothesis of this research was that 5-HT from the fetal intestine is a "proliferation factor" that supports placental cell growth and inhibits activity of matrix metalloproteinases (MMPs) during pregnancy. It is suspected that withdrawal of fetal 5-HT during partum causes placenta separation by cessation of cell proliferation and stimulation of MMP secretion. The objectives of this research were to: determine 5-HT concentrations in blood and tissues of bovine fetuses and neonates; determine whether or not 5-HT acts as a proliferation factor for cultured placental cells; determine whether 5-HT stimulates or inhibits placental MMP activity in cultured cells, isolated placentomes, and pregnant cows; and immunolocalize 5-HT and collagenase in placental tissue.

Blood, intestine, cotyledon, caruncle, and muscle were collected from mid-term and

full-term gestation fetuses at an abattoir and from 24 hour and 48-72 hour old calves obtained from The University of Tennessee Dairy Farm. Blood was collected from the umbilical cord of cesarean section fetuses and from pregnant and non-pregnant cows. Samples were analyzed using an enzyme immunoassay procedure. Fetal blood 5-HT concentrations remained elevated from mid (54,111 nM) through full (51,640 nM) pregnancy, declined ($P \leq 0.05$) during delivery (13,625 nM), and stayed low in 24 hour old calves (24,460 nM), 48-72 hour old calves (18,400 nM), and in cows (8,004 nM). Five-HT concentration in intestine of mid (5,321 ng/g) and full (7,059 ng/g) gestation fetuses and 48-72 hour old calves (6,618 ng/g) was significantly higher ($P \leq 0.05$) than 24 hour old calves (1,410 ng/g) and cows (3,049 ng/g). Concentration of 5-HT in placenta of mid (4,570 ng/g) and full (5,788 ng/g) gestation fetuses was significantly higher ($P \leq 0.05$) than postpartum placenta (1,176 ng/g). Concentration of 5-HT in muscle of mid gestation fetuses (1,412 ng/g) and 24 hour old calf (1,759 ng/g) were significantly lower ($P \leq 0.05$) than in full gestation fetuses (4,941 ng/g).

Cotyledon and caruncle cells were cultured from primary explants of cow placenta. Ten thousand cells were plated in each well of a 96-well plate. Cells were either used as control or treated with 5-HT ranging in concentration from 2.5 μM to 10 μM. The proliferative effect of 5-HT was determined by incorporation of $^{3}$H-thymidine into DNA of cells, a tetrazolium-based colorimetric proliferation assay, and cell count. Increasing concentrations of 5-HT were shown to stimulate incorporation of $^{3}$H-thymidine into DNA of cells; however, 5-HT was inhibitory of the reaction in the colorimetric proliferation assay. Five-HT had no effect on cell number when counted on a hemacytometer or by a Coulter counter.

Cultured cotyledon and caruncle cells were grown in serum-free medium, and medium supplemented with either FBS or NBS and stimulated with 5 μM 5-HT. Media samples were analyzed for MMP activity using a fluorometric technique. Five-HT did

not stimulate MMP activity; however, when all 5-HT samples were pooled (2.05 nM), there was significant ($P \leq 0.05$) inhibition of MMP activity compared to control (3.81 nM). Isolated placentomes were: (1) perfused for 4 hours with blood containing 50 μM 5-HT then incubated for 4 hours with 5-HT, and (2) infused with 5 μM 5-HT and incubated for 4 hours to 11 hours. Matrix metalloproteinase activity was determined using a manometric technique and hydroxyproline and total protein analysis. There were no differences ($P \geq 0.05$) in manometric pressure (force needed to separate cotyledon from caruncle) between control (118 mm Hg) and 5-HT (116 mm Hg) treated placentomes nor in the amount of total protein released in control (1.70 mg/dL) and 5-HT (1.61 mg/dL) treated placentomes. However, amount of hydroxyproline released was discretely higher ($P \leq 0.05$) from 5-HT (2.06 μg/mL) treated placentomes than from controls (1.57 μg/mL). Fourteen near-term cows were induced to deliver with injections of dexamethasone and $PGF_2\alpha$. These injections also cause approximately 70% of cows to retain their placenta. Twenty-four hours later, 7 cows were injected with 50 μg/kg of 5-HT and 7 cows were injected with saline every 12 hours until delivery or for a total of 3 days. In each group, all but 1 cow retained placenta. Results of these experiments indicated that 5-HT does not stimulate MMP activity in the induced model.

Cotyledons and caruncles from mid and full gestation and naturally delivered placentae were fixed for light microscopy and electron microscopy study. Using light microscopy, 5-HT was localized to the surface membrane of epithelial cells and connective tissue of prepartum cotyledons and caruncles and postpartum cotyledons. Using electron microscopy, 5-HT was localized in close proximity to collagen fibers of postpartum cotyledons. We were unable to localize collagenase in the bovine placenta.

The results of this research support the following: (1) a pattern of 5-HT concentration change during pregnancy and parturition; (2) 5-HT may be a proliferation factor for placenta; (3) 5-HT did not stimulate MMP activity in placenta; (4) 5-HT is present in the bovine placenta during pregnancy and postpartum.

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# PART I

# INTRODUCTION

## STATEMENT OF PURPOSE

Justification

Fetal membrane rupture and placenta detachment are critical issues for physiological termination of pregnancy. Placental detachment is proposed to be part of uterine involution and can be considered as one of the earliest expressions of uterine involution. Placenta detachment implies a gradual turn off of physiological interactions and separation of anatomical connection between the fetus and the mother. In the cow, placenta detachment is usually completed within 3 to 6 hours after the calf has been expelled, which is contrary to most domesticated species including humans which expel their placenta at delivery or shortly thereafter. The reason for physiological retention in the cow is not known.

Fetal membrane rupture, placenta detachment and uterine involution are essential in order to deliver the fetus and return the cow to fertile life. The basic mechanism of the process of rupture, detachment and involution is activation of uterine-placental proteolytic enzymes known as matrix metalloproteinases (MMPs). The best studied MMP is collagenase. Activity of collagenase increases markedly during the peripartum period in the cervix and in the early postpartum period in the uterus. The hydrolytic capability of collagenase on placental tissues of the cow, mare and woman was investigated in our laboratory. Our research led to the development of a specific treatment for retained placenta based on the injection of collagenase into retained placenta via the umbilical cord. Incidence of pathological retention (over 12 hours) is a frequent occurrence in the cow. In this country, the average incidence of placental retention in cows is 7%. Often retention may last between 2 and 10 days, impacting the dairy industry with significant economic losses.

Etiology of placental retention has been related to a lack of MMP activity during partum. Factors regulating production and activation of collagenase in the uterus have not been fully elucidated. It has been reported that gonadal steroids, relaxin, and selected growth factors have a role in regulating collagenase. Most recently, 5-hydroxytryptamine (5-HT, serotonin) present in fetal blood in high concentrations (30-50-fold maternal blood) was found to induce collagenase production/activation by cultured rat and human myometrial smooth muscle cells. Moreover, 5-HT is a proliferation factor in many tissues and neoplasias. The above information suggested the possibility that 5-HT may be involved in the control of activation of MMPs and/or proliferation of placental cells. If 5-HT is a collagenase secretagogue and proliferation factor, it may participate in placenta detachment and uterine involution. From a practical perspective, injections of 5-HT may be useful in the treatment and prevention of retained placenta by inducing uterine collagenase. On the other hand, if 5-HT is a placenta proliferation factor, it may in part control placental growth and placental attachment during pregnancy. With these ideas in mind, experiments were designed to attempt to answer selected aspects of our working hypothesis. We hypothesize that high fetal blood 5-HT concentration may originate from the bovine fetal intestine and act as a "proliferation factor' to support placental cell growth during pregnancy. Partial withdrawal (by decreased secretion and/or increased inactivation) of fetal blood 5-HT may be a cause for both (1) arrest of placental cell proliferation and (2) inactivation of MMPs. Activation of MMPs and arrest of placental cell proliferation would lead to postpartum placenta detachment.

Objectives

The objectives of this research were:

1) Determine concentration profile of 5-HT in intestine, placenta, muscle and blood of bovine fetuses at different times during gestation and delivery, and in calves up

to 72 hours old.

2) Assess growth stimulatory effect of 5-HT on bovine placental cells by direct cell count, incorporation of $^{3}$H-thymidine into DNA, and a tetrazolium-based colorimetric proliferation assay.

3) Determine effect of 5-HT on MMP activity in bovine placental cells in culture, in isolated placentomes, and in the whole animal.

4) Localize 5-HT and collagenase in bovine placenta.

## LITERATURE REVIEW

### History of 5-HT

Serotonin, a low molecular weight (176 dalton) biogenic amine, was first known to mammalian physiologists as a vasoconstrictor that appeared in serum when blood was allowed to clot. It was considered a nuisance in perfusion experiments in which defibrinated blood was used and was later considered to play a part in arterial hypertension. This vasoconstrictor was finally isolated in 1948 and given the name serotonin. A year later serotonin's active moiety was identified as 5-hydroxytryptamine (5-HT). Further research in the 1950's led to the discovery that serotonin was also located in the gastric mucosa and in the brain (Goodman and Gilman, 1975). Today it is known that serotonin is widely distributed in the animal and plant kingdoms, present in vertebrates to fruits and nuts (Sanders-Bush and Mayer, 1996).

### Biosynthesis of 5-HT

The chemical structure of 5-HT is: 3-(ß-aminoethyl)-5-hydroxyindole (Figure 1.1). Five-HT is synthesized from the essential amino acid tryptophan (Figure 1.2). Approximately 2% of dietary tryptophan is converted to 5-HT. Tryptophan is hydroxylated by the enzyme tryptophan-5-hydroxylase to 5-hydroxytryptophan (5-HTP), then decarboxylated by the enzyme aromatic L-amino acid decarboxylase to

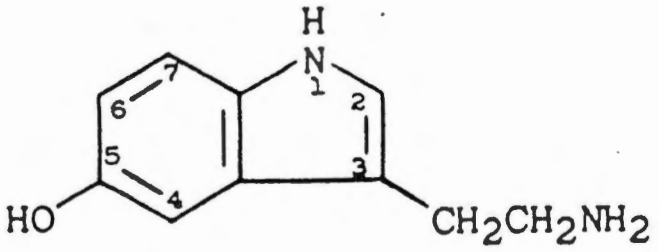



**Figure 1.1.** Chemical structure of 5-HT.

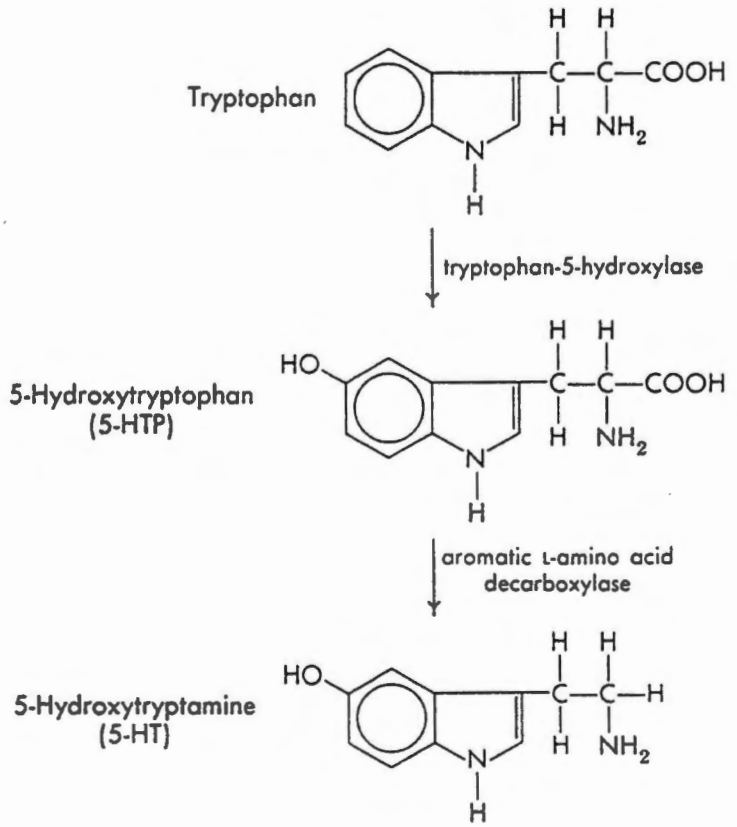



**Figure 1.2.** Biosynthetic pathway of 5-HT.

5-hydroxytryptamine. The rate-limiting enzyme in 5-HT synthesis is tryptophan-5-hydroxylase (Goodman and Gilman, 1975).

Metabolism of 5-HT

Five-HT is principally metabolized by monoamine oxidase (MAO). Monoamine oxidase is localized in mitochondria and has two isoforms, A and B. Monoamine oxidase-A preferentially metabolizes 5-HT and norepinephrine, whereas MAO-B preferentially metabolizes β-phenylethylamine and benzylamine. Monoamine oxidase deaminates 5-HT to form 5-hydroxyindole-acetaldehyde which is degraded to the main metabolite 5-hydroxyindoleacetic acid (5-HIAA) by the enzyme aldehyde dehydrogenase. Five-HT is also degraded to the minor metabolite 5-hydroxytryptophol (5-HTOL) by the enzyme aldehyde reductase (Sanders-Bush and Mayer, 1996) (Figure 1.3).

Many tissues have the enzymes to metabolize 5-HT, however the main tissues that degrade 5-HT are lung, liver, and brain. Following degradation, the principal metabolite 5-HIAA is excreted in the urine. Okatani et al. (1990) reported plasma free 5-HIAA increased in pregnant women after 30 weeks of gestation and reached its peak at 37 weeks. In the fetus, 5-HIAA concentrations in umbilical cord plasma were found to be significantly higher than in maternal plasma (Okatani et al., 1990). This increase in fetal 5-HIAA appears to correspond with the increase in deaminating activity of MAO in the placenta near the end of pregnancy (Kirkel et al., 1992) and in fetal tissues (Benedetti et al., 1992). The half-life of 5-HT varies according to tissue and species. In rabbit, half-life of the following tissues were determined using $^{14}$C-tryptophan and $^{14}$C-hydroxytryptophan: intestine, 11 hours; stomach, 17 hours; platelets and spleen, 33-48 hours; brain (determined by use of harmaline, a monoamine oxidase inhibitor), minutes (Udenfriend and Weissbach, 1958). Half-life of 5-HT in human gastrointestinal tract was determined to be 7 to 12 hours and 6 to 8 hours in dogs. In the brain, half-life was

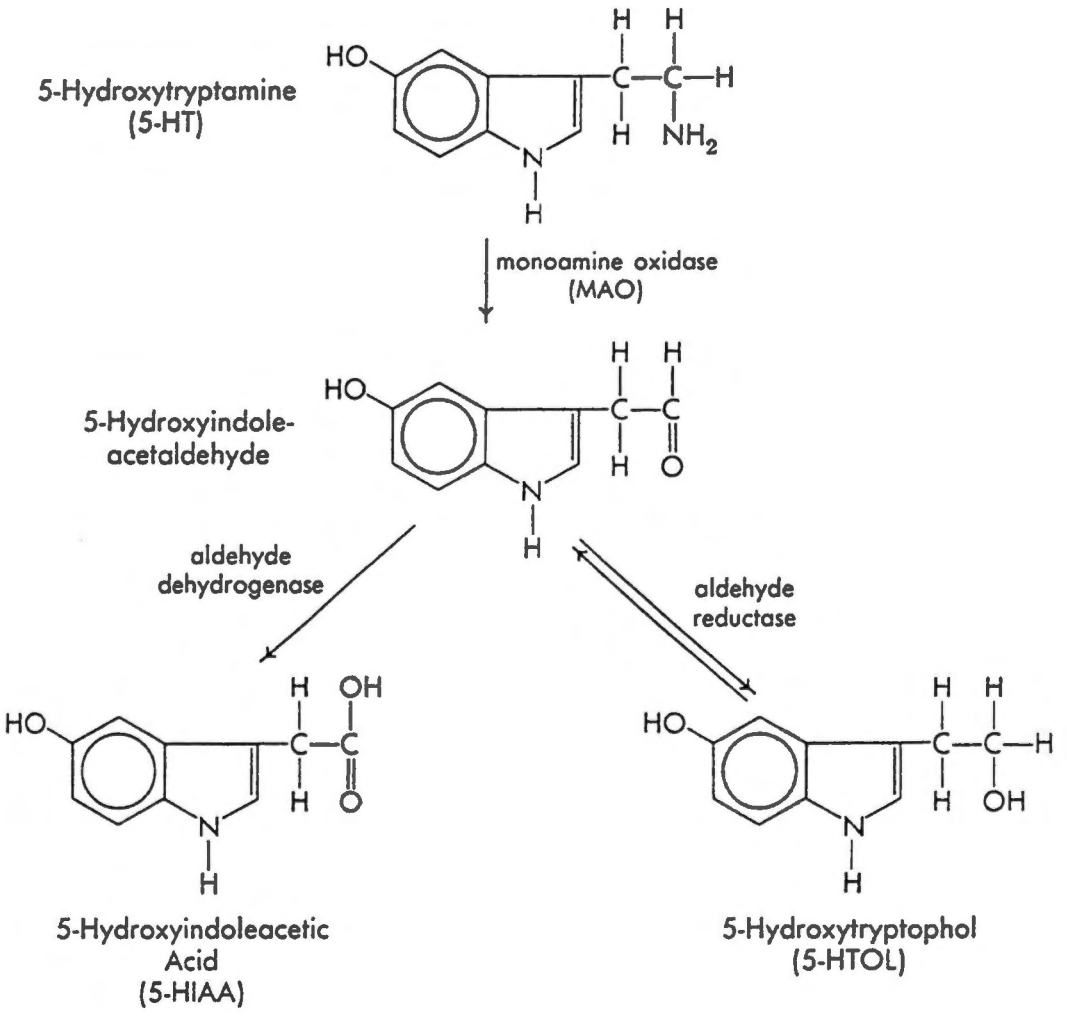



**Figure 1.3.** Metabolic degradation of 5-HT.

found to be between 10 to 30 minutes (Garattini and Valzelli, 1965).

Distribution of 5-HT

In mammals, approximately 95% of 5-HT found in the body is located in the enterochromaffin cells of the gastrointestinal mucosa (Tyce, 1990). The concentration of 5-HT in different parts of the gastrointestinal tract varies according to species. For example, in the mouse a higher concentration of 5-HT is found in the large intestine (3.1 μg) compared to the small intestine (1.6 μg). The development of the enterochromaffin system begins early in gestation. Enterochromaffin cells appear in the 3 month old bovine fetus (Totzauer, 1991), and an immunohistochemical study in human fetal intestine showed 5-HT immunoreactivity as early as 8 weeks of gestation (Facer et al., 1989). The appearance of enterochromaffin cells correlates with first gastrointestinal movements that commence in the first third of gestation (Totzauer et al., 1991).

Five-HT has also been located in placenta by immunohistochemical methods (Huang et al., 1998) and its concentration directly quantitated (Garattini and Valzelli, 1965; Fecteau and Eiler, 1996). In a preliminary study of bovine placenta, Fecteau and Eiler (1997) measured concentrations of 5-HT in prepartum placenta at 3.1 μg/g and at 1.1 μg/g in postpartum placenta. In human placenta, prepartum 5-HT concentration was much lower at 0.13 μg/g (Garattini and Valzelli, 1965) than bovine placenta. There is disagreement, however, to whether or not the placenta can synthesize 5-HT. Huang et al. (1998) stated that trophoblast cells cultured in serum-free medium and syncytiotrophoblasts and stromal cells showed 5-HT immunoreactivity suggesting that human placental villi may produce 5-HT. However, Yavarone et al. (1993) stated that no evidence of 5-HT synthesis by the mouse placenta was found and that 5-HT present in the placenta is due to uptake, not synthesis. It is interesting that no literature is available concerning the enzyme tryptophan hydroxylase in the placenta. In order for the placenta to synthesize 5-HT, it must have the hydroxylase and decarboxylase enzymes.

The purpose of 5-HT in the placenta is not known.

Five-HT can be found in a variety of other body tissues, in blood platelets and in central nervous system (CNS) neurons. The role of platelet 5-HT is to promote platelet aggregation and act as a constrictor at the site of blood vessel injury (Goodman and Gilman, 1975). Platelets cannot synthesize their own 5-HT because they lack the decarboxylase enzyme. Instead, platelets sequester 5-HT that is released into the blood by enterochromaffin cells. Platelets take up 5-HT by passive diffusion and active transport. In the cell, 5-HT is bound to ATP and divalent cations and stored in vesicles. In the CNS, 5-HT acts as a neurotransmitter and influences sleep, appetite, and temperature regulation as well as many other functions (Sanders-Bush and Mayer, 1996).

Pharmacological Actions of 5-HT

The actions of 5-HT are varied and often species dependent. For example, administration of 5-HT caused renal vasoconstriction in rabbits, however dogs responded with an initial increase and later a decrease in renal blood flow (Hindle, 1994). Not only are the actions of 5-HT species dependent, they are also receptor dependent. 5-$HT_2$ and 5-$HT_1$ receptors mediated the increase and decrease of dogs' renal blood flow, respectively. Currently, there are 7 5-HT receptors (5-$HT_1$ to 5-$HT_7$) (Peroutka, 1994; Lucas and Hen, 1995) with more than 16 receptor subtypes (ex. 5-$HT_{2A}$) (Saxena, 1995). All 5-HT receptors belong to the G-protein superfamily, except the 5-$HT_3$ receptor which forms a part of cation channels (Saxena, 1995).

In the gastrointestinal tract, 5-HT can either stimulate or inhibit gastric and smooth muscle motility and contraction depending on which receptor subtype is involved in the action. Five-HT can facilitate gastrointestinal peristalsis by causing constriction of smooth muscle through direct action on the muscle and by stimulating ganglion cells in the intestinal wall (Burkhalter and Frick, 1984). Many studies demonstrated the

involvement of 5-$HT_4$ receptor in peristalsis (Foxx-Orenstein et al., 1998; Sanger et al., 1998). In one study, 5-$HT_4$ agonists along with a delta-receptor antagonist increased the velocity of propulsion in isolated colonic segments of guinea pig intestine (Foxx-Orenstein, 1998). Sanger et al. (1998) studied the ability of a new 5-$HT_4$ receptor antagonist (SB-207266) to reduce the symptoms of irritable bowel syndrome. They used human isolated intestine, guinea pig isolated ileum and conscious mice. The antagonist did not affect normal patterns of intestinal motility however it did, in a concentration-dependent manner, antagonize the ability of 5-HT to sensitize the peristaltic reflex and lower the distention threshold at which peristalsis was evoked.

Five-HT acts on smooth muscle elsewhere in the body. Five-HT, Substance P, neuropeptide Y and VIP were localized by immunocytochemical staining methods in mouse lung. There was widespread distribution of isolated endocrine cells and neuro-epithelial bodies containing 5-HT-like immunoreactivity. Substance P immunoreactivity was observed in nerve fibers located in smooth muscle of the airways. Neuropeptide Y was visualized in nerve fibers in airway smooth muscle and surrounding the blood vessels while VIP- like immunoreactivity was located in nerve fibers and ganglia around blood vessels and in bronchial smooth muscle. It was suggested that these substances play a role in the regulatory function of the mouse respiratory tract by endocrine, paracrine or neurosecretory pathways or a combination of the three (Verastegui et al., 1997).

Five-HT has been shown to have excitatory effects on upper airway motoneurons, which is reduced during sleep. Veasey et al. (1996) studied the effects of two 5-HT antagonists on upper airway dilator-muscle activity and diaphragm activity in an animal model of sleep disordered breathing. The results showed reduced dilator muscle activity and lesser reductions in diaphragm activity along with inspiratory collapse suggesting that 5-HT is important in the maintenance of patent upper airways in obstructive sleep apnea. Desmecht et al. (1992) observed various reversible adverse effects when 5-HT

(0.05 mg/kg) was administered to calves either intravenously (IV) or in bolus form. Response to 5-HT consisted of bronchoconstriction, pulmonary vasoconstriction, and congestion of conjunctiva, lacrimation, and tachypnea. In our own experience, injecting cows with 5-HT at a dose of 50 μg/kg in bolus (IV) form caused an almost immediate response of increased respiratory rate, defecation and lacrimation that was short-lived (Fecteau and Eiler, unpublished). A lower 5-HT dose of 5 μg/kg did not elicit the same response. In blood vessels, 5-HT can cause vasoconstriction or vasodilatation. In the rat, rabbit and mouse, 5-HT caused placental, uterine and umbilical vessels to constrict, and in some laboratory animals caused necrosis of the renal cortex due to the extreme sensitivity of renal vessels to 5-HT. The vasodilatation effect of 5-HT occurs in vessels of skeletal muscle as demonstrated by increased blood flow in areas such as calf and forearm (Goodman and Gilman, 1975).

In the mammalian nervous system, 5-HT modulates numerous sensory, motor and behavioral processes (Tecott et al., 1995). Tecott et al. (1995) generated mutant mice lacking functional 5-$HT_{2C}$ receptors that were proposed to mediate numerous central nervous system actions of 5-HT. They found that the mutant mice were prone to spontaneous death from seizures, suggesting that 5-$HT_{2C}$ receptors mediate tonic inhibition of neuronal network excitability. In addition, the mutant mice were twice as heavy as their wild-type siblings. A non-selective serotonergic agonist with high affinity for 5-$HT_{2C}$ receptors was administered to both mutants and wild type to act as an appetite suppressant. Food intake was reduced in the wild-type mice but not in the mutant mice, and it was determined that mice were not obese due to a metabolic disorder. The mice were determined overweight as a result of abnormal control of feeding behavior, establishing a role for 5-$HT_{2C}$ receptor in serotonergic control of appetite.

Five-HT is involved in neuroendocrine control and has been shown to facilitate the

release of prolactin (PRL) and adrenocorticotropin releasing hormone (ACTH) and under certain conditions inhibits release of luteinizing hormone. Di Sciullo et al. (1990) determined, using 8-Hydroxy-2-(di-n-propylamino)tetralin and ipsapirone, that $5\text{-HT}_{1A}$ receptors are involved in regulation of PRL, ACTH and β-endorphin secretion. However, ipsapirone behaved as a partial agonist on ACTH and β-endorphin secretion suggesting that different neuronal targets are involved in the stimulation of the three hormones by 5-HT.

Reproduction and 5-HT

In humans, 5-HT is mostly known for its ability to cause constriction of placental and umbilical vessels during pregnancy (Melmon, 1981). For this reason, many investigators were interested in the relationship between pregnancy induced hypertension (PIH) and 5-HT (Gujrati et al., 1996; Schafer et al., 1996; Ren et al., 1997). Ren et al. (1997) studied the localization and quantification of 5-HT and its receptor (5-HTR) in placental villi of normal and PIH placentae. Five-HT and 5-HTR immunoreactivity was positive in syncytiotrophoblasts and capillary endothelium of normal placentae and weak positive in PIH placentae. The content of 5-HT and 5-HTR was lower in the PIH placentae than in the normal placentae. Ren et al. (1997) concluded that content of 5-HT and 5-HTR in placenta may be related to PIH. Sexton et al. (1996) localized 5-HT, histamine and endothelin in subpopulations of endothelial cells of arteries and veins of late but not early pregnancy human umbilical vessels. They also exposed the vessels to different concentrations of 5-HT (10 nM to 30 μM) which caused sustained concentration-dependent contractions in all vessels from early and late pregnancy. Sexton et al. (1996) concluded that the endothelium may play an autocrine/paracrine role by synthesizing and releasing reactive substances, such as 5-HT, in late pregnancy to influence feto-placental blood flow.

To emphasize how varied the actions of 5-HT can be on different species, it has

been reported (Robson and Sullivan, 1966) that human umbilical vessels *in vitro* are very sensitive to 5-HT, however Robson and Sullivan (1963) observed no such action on mouse vessels *in situ*. Moreover, in rodents 5-HT is known to cause contraction of the uterus *in vitro*, yet Robson and Sullivan (1966) observed little increase in uterine tone in the intact mouse, while oxytocin markedly increased uterine contractions but did not jeopardize viability of the fetus. However, when Robson and Sullivan (1963) injected 5-HT at a dose of 0.5 to 1.5 mg into pregnant mice, the fetuses died within 1 hour, but the mother suffered no acute toxic effects. The average 5-HT fetal content was 0.117 μg/g. In contrast, when the fetuses were directly injected with 1 μg to 5 μg of 5-HT, the fetuses were alive after 1 hour, with no toxic effect, and the average 5-HT fetal content was 1.098 μg/g. They concluded that fetuses died when 5-HT was injected into the mother, and was probably due to impairment of fetal nutrition, which could have been caused by hemodynamic changes or reduction of permeability of maternal and fetal circulation. This conclusion was based on their observation that 5-HT greatly decreased the passage of radio-sodium (indicator of placental function) into the placenta. However, others have reported transplacental transfer of 5-HT from mother to fetus in the early stages of pregnancy (Yavarone et al., 1993; Prasad et al., 1996) without adverse effects to the fetus.

High concentrations of 5-HT in fetal blood compared to maternal or adult blood were reported by several investigators (Garattini and Valzelli, 1965; De Antoni et al., 1980; Okatani et al., 1990; Jeffrey et al., 1991). Using spectrophotometry techniques, Jeffrey et al. (1991) determined the concentration of 5-HT in fetal bovine serum to be between 30 μM to 50 μM, whereas the concentration in newborn bovine serum and cow serum was approximately 1 μM. Their results were consistent with the analyses conducted by a commercial serum supplier. Furthermore, tryptophan levels in human umbilical cord blood collected at birth were high (18.9 μg/mL), then decreased (10.4

μg/mL) significantly 24 hours postpartum, and showed a slight but nonsignificant increase (11.4 μg/mL) 5 days after birth (De Antoni et al., 1980). High tryptophan levels in fetal blood were confirmed by Okatani et al. (1990) when they reported that 5-HT, tryptophan, and 5-HIAA were significantly higher in umbilical cord plasma than in maternal plasma in humans. In addition to having lower concentrations of 5-HT and tryptophan, free maternal plasma 5-HT and tryptophan concentrations did not change during pregnancy. However, free 5-HIAA in plasma increased after 30 weeks of gestation and reached its peak at 37 weeks. This is an interesting finding because it suggests one of two possibilities: (1) 5-HT synthesis and degradation are at a steady state in the mother during this time of gestation, or (2) there is increased degradation of 5-HT in the fetus for preparation of birth. Studies in the rat revealed that in most fetal tissues, the deaminating activity of MAO reaches it peak within a few days prepartum to a few weeks after birth (Benedetti et al., 1992). Lung MAO activity was discovered to be well developed by 10 days postpartum and reached a maximum by day 40 (Benedetti et al., 1992), and in rabbits newborn lungs had greater metabolic capacity than both fetal and adult lungs (Olson et al., 1983). Mantle et al. (1976) found that fetal rat liver MAO was similar to adult values from 3 days prepartum, and human placenta MAO activity increased by 22% at delivery time (Kirkel et al., 1992). The maturation of the 5-HT degrading system supports the idea that the fetus is contributing to maternal excretion of 5-HIAA near the end of gestation.

Five-HT was also reported to be involved in postpartum uterine involution. Collagen content in the pregnant mammalian uterus increases 8- to 10-fold, starting at the end of the first third of pregnancy and continuing until close to parturition. Collagenase is required to catalyze the initial cleavage of collagen that is destined for removal from the postpartum uterus. In the rat uterus, collagenase is detectable only during postpartum involution (Jeffrey, 1991). In order for cultured rat and human

myometrial smooth muscle cells to produce collagenase, they must be stimulated by 5-HT (Jeffrey et al., 1991) which occurs via the 5-$HT_2$ receptor.

Five-HT was also reported to stimulate cultured bovine luteal cells to produce progesterone. Battista and Condon (1986) treated cultured luteal cells with different concentrations of 5-HT ($10^{-6}$M to $10^{-4}$M) as well as varying concentrations of other biogenic amines, such as histamine, γ-aminobutyric acid, melatonin, and the adrenergic agent isoproterenol. Individual treatment of the cells with 5-HT and isoproterenol significantly increased the production of progesterone, whereas the other treatments did not alter progesterone production. The 5-HT stimulated production of progesterone was inhibited by the addition of mianserin, a 5-HT antagonist. It was concluded that in the cow, 5-HT could be involved in the regulation of steroidogenesis by the corpus luteum.

Other roles for 5-HT have been proposed, such as its involvement in neuroembryogenesis. Fluorescence histochemical studies demonstrated 5-HT uptake and synthesis in the notochord, neural tube, and extra-neural regions of the chick embryo during the first few days of incubation (Lauder et al., 1981), and in the rat 5-HT depletion can alter the time of genesis of 5-HT target cells. Furthermore, fetal 5-HT receptors in the forebrain and brainstem were shown to be functional based on their ability to adapt to changes in the level of 5-HT and therefore capable of playing a role in neuronal development (Whitaker-Azmitia et al., 1987).

It appears that the involvement of 5-HT in reproduction is not limited to vertebrates as it has been reported to induce and potentiate parturition in fingernail clams. Fong et al. (1998) exposed clams to selective serotonin re-uptake inhibitors such as fluoxetine, fluvoxamine and paroxetine that increase neurotransmission at serotonergic synapses. A positive parturition response consisted of the release of at least one extra-marsupial larva from an individual clam. It was discovered that of the selective serotonin re-uptake

inhibitors used, fluoxetine was the only one that did not induce parturition, however it did potentiate parturition.

Cellular Proliferation and 5-HT

Five-HT was reported to exert growth stimulatory effects on a number of cell types (Nemecek et al., 1986; Seuwen and Pouyssegur, 1990; Young et al., 1993). Nemecek et al. (1986) found that 5-HT at a concentration above 1 μM maximally increased incorporation of $^{3}$H-thymidine into DNA of bovine aortic smooth muscle cells and stimulated smooth muscle cell mitogenesis to the same extent as human platelet derived growth factor (PDGF) at 12 ng/mL. Boucek and Alvarez (1969) found that 5-HT at concentrations of $10^{-6}$ or $10^{-7}$ M significantly increased fibroblast cell growth by nearly 100%, however at concentrations of $10^{-2}$ and $10^{-3}$ M 5-HT was toxic to the cells and reduced cell numbers by more than 80%. Along with concentration, the time at which 5-HT was added to the cells also affected growth. Five-HT given at 0-time increased the number of cells by 58% within 24 hours. Delaying addition of 5-HT until 1, 2, or 5 days after the cells had been in culture reduced the stimulatory effect. In order to understand the mechanism of action of 5-HT on these cells, Boucek and Alvarez (1969) examined the effect of 5-HT on lag phase of growth. Five-HT increased the number of cells (40%) attached to the culture flask by 18 hours while in control flasks only 25% of cells had attached. When growth up to 24 hours was considered, the increase with 5-HT was greater than 80% while controls were 30%. It was concluded that in the fibroblast cells 5-HT appeared to shorten the lag phase in cell growth. Seuwen and Pouyssegur (1988) also studied the effect of 5-HT on CHL fibroblast cells. They found that 5-HT potentiates the mitogenicity of fibroblast growth factor and to a lesser extent epidermal growth factor and insulin. The mitogenic effect of 5-HT is expressed in the reinitiation of DNA synthesis and is transmitted by 5-$HT_{1B}$ receptors negatively coupled to adenylate cyclase. Studies such as this one aid us in understanding regulation of signal

transduction and the control of cell proliferation. Understanding regulation of cell proliferation is especially important when faced with abnormal cell growth. Five-HT is present in hyperplastic human prostate (Abrahamsson et al., 1986). Abdul et al. (1994) reported the presence of 5-HT binding sites and antiproliferative effects of a 5-$HT_{1A}$ antagonist on prostate carcinoma cells. More recently, Abdul et al. (1995) described the effects of serotonin-uptake inhibitors on different prostate carcinoma cell lines. The inhibitors used were fluoxetine, zimelidine and 6-nitroquipazine. They found that, *in vitro*, the 3 compounds inhibited proliferation of the cell lines studied in a dose-dependent manner. They concluded that serotonin-uptake inhibitors could potentially be used in the treatment of prostate cancer.

Five-HT was also proposed as involved in the growth of embryos. Fluorescence histochemical studies demonstrated 5-HT uptake and synthesis in the notochord, neural tube and extra-neural regions of the chick embryo during the first few days of incubation (Lauder et al., 1981) and in the rat 5-HT depletion can alter the time of genesis of 5-HT target cells. Furthermore, fetal 5-HT receptors in the forebrain and brainstem were shown to be functional based on their ability to adapt to changes in the level of 5-HT and therefore capable of playing a role in neuronal development (Whitaker-Azmitia et al., 1987).

Matrix Metalloproteinases and 5-HT

Matrix metalloproteinases (MMPs) are involved in reproduction in such areas as placentation (Librach et al., 1991; Polette et al., 1994; Marquoi et al., 1997), rupture of membranes (Dieron and Bryant-Greenwood, 1991; Bryant-Greenwood, 1998) and uterine involution (Jeffrey et al., 1991). Collagenase (MMP-1) was shown to be produced by myometrial smooth muscle cells in the involuting rat uterus, but not the pregnant or nonpregnant uterus (Jeffrey, 1991). Jeffrey et al. (1991) reported that cultured rat and human myometrial smooth muscle cells produced collagenase when in

medium containing fetal bovine serum (FBS) but not in medium containing newborn bovine serum (NBS). They isolated the substance in FBS and identified it as 5-HT. They determined that 5-HT was maximally stimulatory of collagenase production at a concentration of 5 μM and production of collagenase began after a lag period of 6 hours. Myometrial smooth muscle cells have an obligatory requirement for 5-HT in order to produce collagenase whereas other cell types such as fibroblasts can produce collagenase without stimulation from 5-HT. Five-HT induces collagenase in smooth muscle myometrial cells by activating the gene for the enzyme (Wilcox et al., 1994) which is mediated by the 5-$HT_2$ receptor (Rydelek-Fitzgerald et al., 1993). Activation of the collagenase gene was studied further to realize that 5-HT induces interleukin-1 mRNAs and that this production by the myometrial cell is necessary for production of collagenase (Wilcox et al., 1994). Recently, it was reported that 5-HT acts as a negative regulator of alpha2-macroglobulin (an anti-protease) in smooth muscle myometrial cells (Huang and Jeffrey, 1998) and it has the ability to down-regulate type I collagen gene expression (Passaretti et al., 1996). Therefore, 5-HT may be a positive or negative regulator of gene expression.

It is worthy to mention combined injections of 5-HT and ergometrine into pregnant cows decreased retained placenta's occurrence from 30% to 10% (Zaiem et al., 1994). The possibility of 5-HT causing a reduction in the number of retained placentas is of interest, because it is suspected that the etiology of retained placenta includes lack of collagenolysis, and it is possible that 5-HT stimulates collagenolysis in the placenta. Moreover, Maj and Kankofer (1997) compared MMP activity in retained and non-retained bovine placenta and found that retained membranes lacked the 64- and 60-kDa active forms of MMP-2 which may have influenced the hydrolysis of collagen and release of fetal membranes. In addition, injections of bacterial collagenase, via umbilical vessels, into cows with retained placenta has been highly effective in detaching the placenta

within 24 hours (Eiler and Hopkins, 1993).

Literature on 5-HT inducing collagenase production in other cell types is limited. Other than the specific myometrial cell type, 5-HT was reported to stimulate phospholipase A2 activity, which enhanced collagenase type II activity in chondrocytes from human osteoarthritic articular cartilage (Richard et al., 1991).

Immunolocalization of 5-HT

Recently, 5-HT and its receptor were localized in human placenta (Ren et al., 1997; Huang et al., 1998). Huang et al. (1998) used light and electron microscopy techniques to localize 5-HT in placentae ranging from 6 to 24 weeks of pregnancy. Five-HT and its receptor were localized in the cytoplasm and on the cell surface of syncytiotrophoblast microvilli. Nuclei were immunonegative. Cytoplasm of maternal decidual cells showed strong 5-HT and 5-HT receptor immunoreactivity. Capillary endothelium, white blood cells in the capillary cavity and stromal cells of placental villi all showed 5-HT receptor immunoreactivity in the cytoplasm but had negatively stained nuclei. Huang et al. (1998) also observed 5-HT immunoreactivity in the cytoplasm of trophoblast cells cultured in serum-free medium, however there were varying degrees of staining intensity among the cells. Huang et al. (1998) concluded that trophoblast cells produce 5-HT and that 5-HT may play roles in placental development and pregnancy maintenance by paracrine and autocrine interactions.

Unlike the conclusion by Huang et al. (1998), results of a study by Yavarone et al. (1993) led them to conclude that 5-HT present in the mouse placenta is due to uptake not synthesis. Yavarone et al. (1993) localized 5-HT in the ectoplacental cone and placenta of the mouse embryo *in vivo* and in whole embryo cultures. The 5-HT uptake inhibitor fluoxetine and p-chlorophenylalanine, an inhibitor of 5-HT synthesis were used to evaluate the ability of the placenta to synthesize and transport 5-HT. Fluoxetine significantly reduced 5-HT immunoreactivity, however p-chlorophenylalanine had no

effect on 5-HT immunoreactivity. Furthermore, treatment of embryos with L-tryptophan did not enhance 5-HT immunoreactivity in the placenta. It is very possible that there are differences between cells of mouse and human placentae where one has the ability to synthesize 5-HT and the other does not. It would be beneficial to know if the cells contain the enzymes required for 5-HT synthesis. If the cells are immunoreactive to 5-HT but do not have the enzymes for synthesis, then 5-HT may have been synthesized in another location and transported to the cells. For the mouse placenta, it was suggested that the placenta received 5-HT from the maternal circulation. Essman and Cooper (1978) demonstrated transport of $^{14}$C-5-HT from mother to fetus in the rat. Furthermore, there is evidence that a 5-HT transporter exists in the brush-border membrane of the human placenta (Balkovetz et al., 1989; Ramamoorthy et al., 1995; Prasad et al., 1996). The presence of an active mechanism to transfer 5-HT from mother to fetus suggests that 5-HT may play an important role in regulation of placental function as well as in the regulation of growth and development of the fetus (Balkovetz et al., 1989). Transplacental transfer of 5-HT from mother to fetus is effective in early stages of pregnancy but toward the end of pregnancy maternal 5-HT appears to enter the placenta but does not reach the fetal circulation (Prasad et al., 1996). It may be that the fetus is supplying its own 5-HT.

Five-HT appears in endocrine cells of the human intestine as early as 8 weeks of gestation (Facer et al., 1989) and 12 weeks in the bovine (Totzauer, 1991). Facer et al. (1989) reported that 5-HT immunoreactive cells increased throughout gestation in the small intestine but decreased in the large intestine after the second trimester of pregnancy. Five-HT immunoreactive cells were scattered throughout the villi and crypts of the intestine. Griffith and Burnstock (1983) also conducted experiments on localization of 5-HT in the human fetal intestine from 18.5 to 25 weeks of gestation using immunofluorescence techniques. Five-HT-like immunoreactivity was found in the

nerves as well as in the enterochromaffin cells of the intestinal mucosal villi epithelium of all studied fetuses. Totzauer (1991), using light microscopy techniques, studied morphological changes in the bovine fetus duodenal mucosa from 3 months of gestation until birth. At 3 months, intestinal villi and crypts were formed with enterochromaffin cells mainly in the crypts. Totzauer (1991) concluded that the advanced state of development of bovine fetal enteroendocrine cells suggests that they are capable of producing 5-HT, which corresponds to the time gastrointestinal movements are known to begin and may be an inducer of muscular motility of the developing intestine.

# PART II

# CONCENTRATION PROFILE OF 5-HYDROXYTRYPTAMINE IN FETAL AND NEONATAL CALF SERUM AND TISSUES

## INTRODUCTION

Five-HT is a well-known neurotransmitter and multi-functional amine with a broad range of physiological effects including vasoconstriction, bronchoconstriction, platelet aggregation, intestinal motility, and release of pituitary hormones (Goodman and Gilman, 1975). However, the role of 5-HT in pregnancy, placenta detachment, and delivery is not well defined. It is known that 5-HT concentrations in fetal bovine serum (30 μM to 50 μM) are 1.7 to 50 times greater than 5-HT concentrations in newborn calf serum (1.0 μM to 18 μM) (Jeffrey et al., 1991; Fecteau and Eiler, 1997). In addition to cows, high 5-HT concentrations in fetal blood were also reported for goats and women. For example, 5-HT, its precursor tryptophan, and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were significantly higher in umbilical cord plasma than in human maternal plasma (Okatani et al., 1990). Moreover, De Antoni et al. (1980) demonstrated that tryptophan levels were very high in human umbilical cord at birth, decreased significantly 24 hours after birth, and showed a slight but not significant increase 5 days after birth. Also at pregnancy's end, 5-HT degrading enzymes in fetal liver, fetal lung, and placenta become active (Benedetti et al., 1992; Kirkel et al., 1992), which probably contribute to the low 5-HT concentrations in the neonate. However, the source of high blood 5-HT concentrations in the fetus is not known. In the mouse, it was found that 5-HT can be transferred from maternal to fetal circulation in early pregnancy (Yavarone et al., 1993). Yet in late pregnancy, 5-HT no longer reached the fetal circulation (Prasad et al., 1996). We suspect that the source of fetal blood 5-HT may be the fetal intestine, since approximately 95% of 5-HT in the body is found in the enterochromaffin cells of the gastrointestinal tract (Tyce, 1990). Furthermore, it was reported that intestinal resection caused a significant decrease in blood 5-HT.

The physiological purpose of a high set point for fetal blood 5-HT is puzzling. It is interesting that when blood concentrations of 5-HT in 200 kg calves were increased by

IV infusion to roughly fetal concentration, 5-HT became toxic (Aitken and Sanford, 1972; Desmecht et al., 1992). In addition, when Robson and Sullivan (1963) injected 5-HT into pregnant mice in the second part of gestation the fetuses died within 1 hour. However, when the fetuses were directly injected with 5-HT all fetuses were alive and without any toxic effects after 1 hour. The reason for high 5-HT tolerance by the fetus is not known. It is suspected that high concentrations of 5-HT in fetal blood may favor pregnancy. It is speculated that 5-HT may act as a "proliferation factor" to sustain both placental growth and placental cell turnover during pregnancy. Supporting this view is the fact that growth stimulatory effects of 5-HT were described on a variety of cell types (Nemecek et al., 1986; Takuwa et al., 1989; Seuwen and Pouyssegur, 1990; Ishizuka et al., 1992). It was also proposed that 5-HT may have a role in fetal growth processes (Garattini and Valzelli, 1965; Lauder et al., 1981; Whitaker-Azmitia et al., 1987), however, information on 5-HT and placental growth is scarce. It is known that the human placenta possesses 5-HT receptors and 5-HT has been localized in cells of the placenta (Huang et al., 1998). Therefore, based on its proliferative effects on various cell types, its presence in the placenta, and high fetal blood concentrations we hypothesize that 5-HT from bovine fetal intestine acts as a "proliferation factor" to support placental cell growth during pregnancy. In our hypothesis, partial withdrawal (by decreased secretion and/or increased inactivation) of fetal blood 5-HT is a cause for both (1) arrest of placental cell proliferation and (2) activation of matrix metalloproteinases (MMPs). Activation of MMPs contributes to placenta detachment at parturition. A systematic study including the determination of 5-HT in fetal blood and fetal tissues at different stages of pregnancy is lacking. It was our objective to determine the concentration profile of 5-HT in fetal tissues and blood of bovine fetuses at different times during gestation and delivery, in calves up to 3 days postpartum and in adult cows. This information is needed in order to assist in the identification of a potential role of 5-

HT in pregnancy and parturition. If there is a pattern of concentration change in fetal blood 5-HT, physiological correlates can be unfolded.

## MATERIALS AND METHODS

### Blood and Tissue Collection

Intestine (middle jejunum, approximately 1 meter from large intestine), skeletal muscle (semitendinosus), placenta (cotyledon and caruncle), and blood (obtained by heart puncture) were collected from mid and full gestation fetuses at an abattoir within 20 minutes of slaughter. Fetal age was approximated using a fetal development chart (Roberts, 1971). Mid-term pregnancy was considered between 180 days and 240 days and full-term pregnancy was considered ≥ 270 days. Blood and intestine were collected from cows at an abattoir. Intestine and muscle samples and blood were collected from 24 hour old and 48-72 hour old male calves obtained from The University of Tennessee Dairy Farm. Calves were euthanized immediately after blood collection and prior to muscle and intestine collection with a cardiac injection of Beuthanasia-D Special (20 mL) (Schering Plough Animal Health Corporation). Postpartum cotyledons (fetal membranes of placenta) were obtained from cows with hanging membranes after natural delivery at The University of Tennessee Dairy Farm. Blood was collected from the umbilical cord veins of fetuses during delivery by cesarean section and from the jugular vein of cows prior to cesarean section. Blood was collected into vacutainer tubes without anticoagulant. All tissue samples were put into plastic bags and placed on ice for transport to the laboratory.

In the laboratory, blood was centrifuged at 1,700 g for 30 minutes, then serum was removed and placed in a labeled tube and frozen at -20°C until analyzed. Tissues (1 g) were homogenized (Virtis homogenizer) in 0.2 N $HClO_4$ (10 µL/ mg tissue) for 5 minutes, sonicated for 15 minutes, then centrifuged for 5 minutes at 10,000 g. The

homogenates were filtered through a 0.22 μm filter, then the supernatant was neutralized by addition of an equal volume of borate buffer (pH 9.25). The samples were then centrifuged for 1 minute at 10,000 g and placed in labeled tubes and stored at -20°C until analyzed. Samples were analyzed using an enzyme immunoassay kit (Immunotech, Marseille, France).

*Enzyme Immunoassay Performance*

Serum and cotyledon samples were used to determine performance characteristics of the 5-HT immunoassay for bovine samples. The parameters used were intra-assay and inter-assay variation, parallelism, recovery and specificity. In order to determine variation within the assay, each sample was processed in duplicate and the coefficient of variation was calculated by dividing the standard deviation by the mean and multiplying by 100. Parallelism was determined by assaying undiluted and diluted samples. The samples were diluted in the following ratios: 1:2, 1:4, and 1:8. To determine recovery, samples were spiked in a 1:1 ratio with high (200 nM), medium (60 nM) and low (1.6 nM) 5-HT concentration solutions. Percent recovery was calculated by dividing the observed value by the expected value. Specificity (cross-reactivity) was tested by adding norepinephrine (expected released in large quantities during slaughter) to wells at an equimolar concentration to 5-HT when at 50% maximal absorbance.

*Enzyme Immunoassay Procedure*

Serum samples were diluted 1:200 with 1 M borate buffer. Tissue samples were left undiluted or diluted 1:5 with a 1:1 solution of 1 M borate buffer/0.2 M $HClO_4$. For serum samples, the standard curve was prepared using standard supplied with the kit and 1M borate buffer for dilutions. The standard curve for tissues was prepared using standard supplied with the kit and borate buffer / $HClO_4$ as diluent.

Standards (200 nM, 60 nM, 18 nM, 5.4 nM, 1.6 nM, and 0 nM) were prepared, and assay was performed according with manufacturer's instructions (Appendix).

Statistical Analysis

Statistical analysis was performed using the General Linear Models procedure in SAS. Probability of significance was reported at $P \leq 0.05$. Least Square Means were computed and compared using least significant difference techniques.

## RESULTS

Assay Performance

Results of intra-assay and inter-assay variation for serum samples are shown in Tables 2.1 and 2.2, respectively. Results of parallelism and recovery for serum samples are shown in Tables 2.3 and 2.4, respectively. Results of intra-assay and inter-assay variation for tissue samples are shown in Tables 2.5 and 2.6, respectively, and results of parallelism for tissue are shown in Table 2.7.

Specificity (cross-reactivity) of the assay was tested using norepinephrine at equimolar concentration to 5-HT investigated at 50 % maximal absorbance. Cross-reactivity was undetectable. Manufacturer specification reported different degrees of cross-reactivity with analogs. Cross-reactivity was expressed as the ratio of analog concentration/acylated 5-HT concentration at 50% maximal absorbance. Cross-reactivity of analogs, such as N-succinyl 5-HT, acylated tryptamine, acylated 5-hydroxytryptophan and acylated tryptophan varied between 2.8 for N-succinyl 5-HT to 1,000,000 for acylated tryptophan. Sensitivity of the assay is approximately 0.5 nM as determined by the manufacturer.

Assay performance was considered satisfactory. However, variability in tissue samples was larger than for serum samples; that was expected since in tissues there are more interfering substances. Dilution affected tissue samples markedly. Dilution of tissue samples (but not serum dilution) resulted in increased concentrations. Dual homogenization and ultrasound did not decrease variability in tissues compared to homogenization only. In this assay, there was no extraction step, which tends to retain

**Table 2.1.** Intra-assay variation for calf serum assay

| Sample | 5-HT (nM) | Coefficient of Variation (%) |
|---|---|---|
| Pool 1 | 22,000 ± 3,960 | 18.0 |
| Pool 2 | 17,400 ± 1,260 | 7.2 |
| Pool 3 | 9,400 ± 820 | 8.7 |

Abbreviation: 5-HT, 5 hydroxytryptamine
Mean + SD, n=6

**Table 2.2.** Inter-assay variation for calf serum assay

| Sample | 5-HT(nM) | Coefficient of Variation (%) |
|---|---|---|
| Pool 1 | 27,600 ± 3,060 | 11.0 |
| Pool 2 | 13,000 ± 980 | 7.5 |

Abbreviation: 5 Hydroxytryptamine
Mean + SD, n = 6

**Table 2.3.** Serial dilution of calf serum for evaluation of parallelism

| Serum Dilution | 5-HT Observed (O) (nM) | 5-HT Expected (E) (nM) | O/E x 100 (%) |
|---|---|---|---|
| Undiluted | 32,000 ± 848 | | |
| 1:2 | 11,800 ± 1,414 | 16,000 | 74 |
| 1:4 | 7,600 ± 580 | 8,000 | 95 |
| 1:8 | 3,600 ± 320 | 4,000 | 89 |

Abbreviation: 5-HT, 5-hydroxytryptamine
Mean + SD, n = 6

**Table 2.4.** Spiking recovery from calf serum

| Spiking Solution (nM) | 5-HT Observed (O) (nM) | 5-HT Expected (E) (nM) | O/E x 100 (% Recovery) |
|---|---|---|---|
| Sample | 10,000 ± 1,270 | | |
| High | 24,000 ± 800 | 25,000 | 96 |
| Medium | 9,600 ± 580 | 10,000 | 96 |
| Low | 5,660 ± 130 | 7,800 | 72 |

Mean + SD, n = 6
High = 200 nM; Medium = 60 nM; Low = 1.6 nM

**Table 2.5.** Intra-assay variation for cotyledon tissue assay

| Sample | 5-HT (ng/g) | Coefficient of Variation (%) |
|---|---|---|
| Postpartum cotyledon | 1,250 ± 1,405 | 32 |

Abbreviation: 5-HT, 5 hydroxytryptamine
Mean +SD, n = 8

**Table 2.6.** Inter-assay variation for cotyledon tissue assay

| Sample | 5-HT (ng/g) | Coefficient of Variation (%) |
|---|---|---|
| Cow 1 | 845 ± 298 | 35 |
| Cow 2 | 1720 ± 553 | 32 |

Abbreviation: 5-HT, 5 hydroxytryptamine
Mean +SD, n = 6

**Table 2.7.** Serial dilution of cotyledon samples for evaluation of parallelism

| Sample Dilution | 5-HT Observed (O) (ng/g) | 5-HT Expected (E) (ng/g) | O/E x 100 (%) |
|---|---|---|---|
| Undiluted | 1480 + 303 | — | — |
| 1:2 | 1005 + 285 | 740 | 136 |
| 1:4 | 840 + 398 | 370 | 228 |
| 1:8 | 530 + 208 | 185 | 286 |

Abbreviation: 5-HT, 5-hydroxytryptamine
Mean + SD, n= 6

interfering substances. In order to minimize artifacts, samples for a given experiment were analyzed in a single assay. For large numbers of samples, assay kits from the same lot were used. When possible, sample dilution for tissues was avoided. When values were off-scale, samples were diluted and reanalyzed. Concentrations of serum 5-HT in our study were consistent with those reported by Jeffrey et al. (1991) for FBS, however our 5-HT concentrations in NBS were slightly higher.

Enzyme Immunoassay for 5-HT

*Serum 5-HT*

Analysis of serum revealed significant ($P \leq 0.0001$) differences in 5-HT concentration at different stages of gestation and postpartum. The mean ± SEM concentration of 5-HT in blood of mid-term gestation fetuses (54,111 nM ± 6,574 nM) and full-term gestation fetuses (51,640 ± 4,880 nM) was significantly higher than fetuses during delivery by cesarean section (13,625 nM ± 5,576 nM), 24 hour old calves (24,460 nM ± 2,947 nM), 48-72 hour old calves (18,400 ± 2,519 nM) and cows (8,004 nM ± 1,203 nM). Interestingly, blood obtained from fetuses during cesarean section was not significantly different ($P \geq 0.0001$) in 5-HT concentration from blood obtained from newborn calves and adult cow. However, newborn calf blood contained significantly ($P \leq 0.0001$) higher concentrations of 5-HT compared to adult cow (Figure 2.1).

*Tissue 5-HT*

The mean ± SEM concentrations of intestinal 5-HT in mid-term (5,321 ng/g ± 943 ng/g) and full-term (7,059 ng/g ± 1,636 ng/g) gestation fetuses and 48-72 hour old calves (6,618 ng/g ± 430 ng/g) were significantly ($P \leq 0.0001$) higher than concentrations from 24-hour old calves (1,410 ng/g ± 248 ng/g) and adult cow (3,049 ng/g ± 851 ng/g) intestines. There was no significant difference ($P \geq 0.0001$) in 5-HT concentration between 24-hour old calf intestines and adult cow intestines (Figure 2.2).

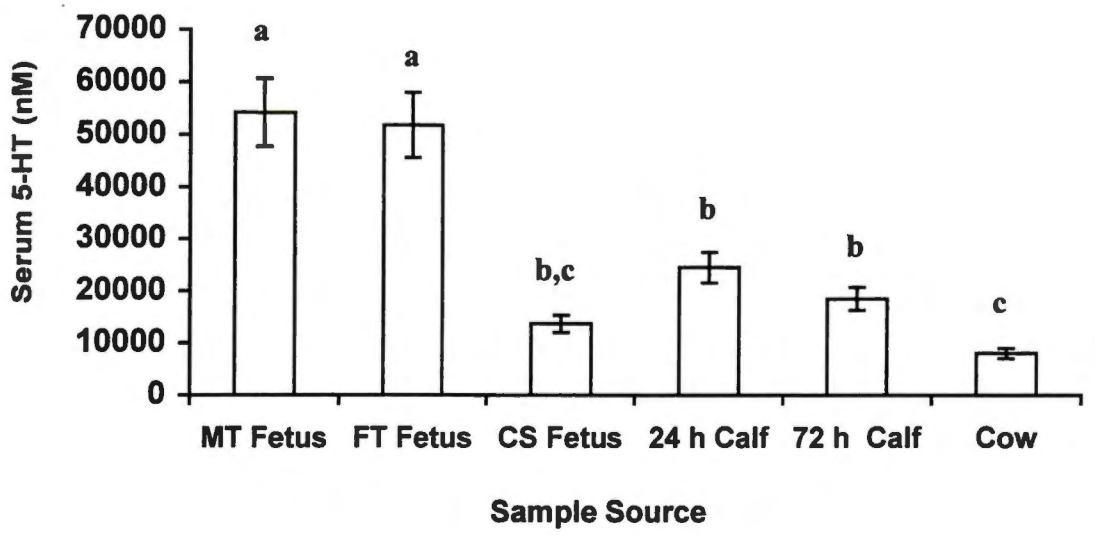



**Figure 2.1.** Concentration of 5-HT in serum of fetuses at mid-term and full-term pregnancy, fetuses during cesarean section, newborn calves at 24 and 72 hours old, and adult cows. Columns are mean ± SE for a minimum of 5 animals per group. Different letters (a,b,c) represent significant difference ($P \leq 0.05$) among groups. Letters MT, FT, and CS indicate stage of fetus as mid-term, full-term, and cesarean section, respectively.

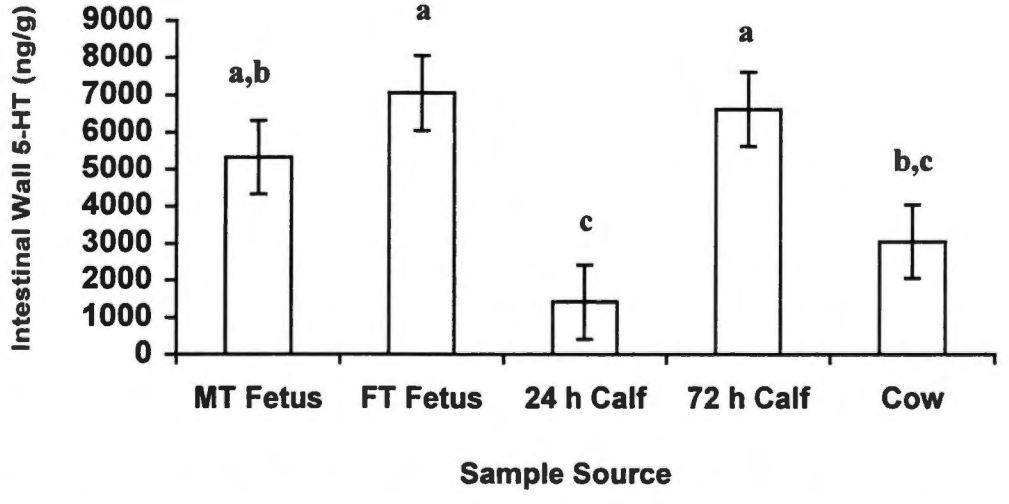



**Figure 2.2.** Concentration of 5-HT in intestinal wall of fetuses at mid-term and full-term pregnancy, newborn calves at 24 and 72 hours old, and adult cows. Columns are mean ± SE for a minimum of 5 animals per group. Different letters (a,b,c) represent significant difference ($P \leq 0.05$) among groups. Letters MT and FT indicate stage of fetus as mid-term and full-term, respectively.

The mean ± SEM concentration of placental 5-HT in mid-term (4,570 ng/g ± 1,710 ng/g) and full-term (5,788 ng/g ± 956) gestational fetuses was significantly higher ($P \leq 0.0001$) than postpartum cotyledon (1,176 ng/g ± 199 ng/g) (Figure 2.3).

The mean ± SEM concentration of muscle 5-HT was high ($P \leq 0.0001$) in full-term gestation fetuses (4,941 ng/g ± 714 ng/g) and low in mid-term gestation fetuses (1,412 ng/g ± 270 ng/g) and 24 hour old calves (1,759 ng/g ± 192 ng/g). There was no difference in 5-HT concentration between muscle from mid-term gestation fetuses and muscle from 24-hour old calves (Figure 2.4).

Comparing changes in 5-HT concentration among tissues at common stages of gestation or postpartum revealed that there were significant ($P \leq 0.0001$) differences between intestine and placenta from mid gestation fetuses, intestine and muscle from full gestation fetuses, muscle and placenta from full gestation fetuses, and intestine, muscle and placenta from 24 hour old calves. An overall view of the relative concentration changes in 5-HT is depicted in Figure 2.5.

## DISCUSSION

The main finding of this work was that concentrations of 5-HT in fetal blood were extremely high in both mid and full gestation fetuses, and was then dramatically decreased to approximately one-fourth of fetal concentration (from 53 μM to 14 μM) during cesarean section delivery. In our experiment, it was not possible to establish the exact time at which 5-HT concentration dropped in fetal blood in relation to delivery time. However, concentrations of blood 5-HT in fetuses delivered by cesarean section were already low. The use of a chronic fetal catheter (Ford, 1995) may aid in determining when 5-HT concentration decreases in the fetus.

Our results using serum samples collected from individual fetuses are in close agreement with data obtained from pooled samples (30 μM-50 μM) obtained from a

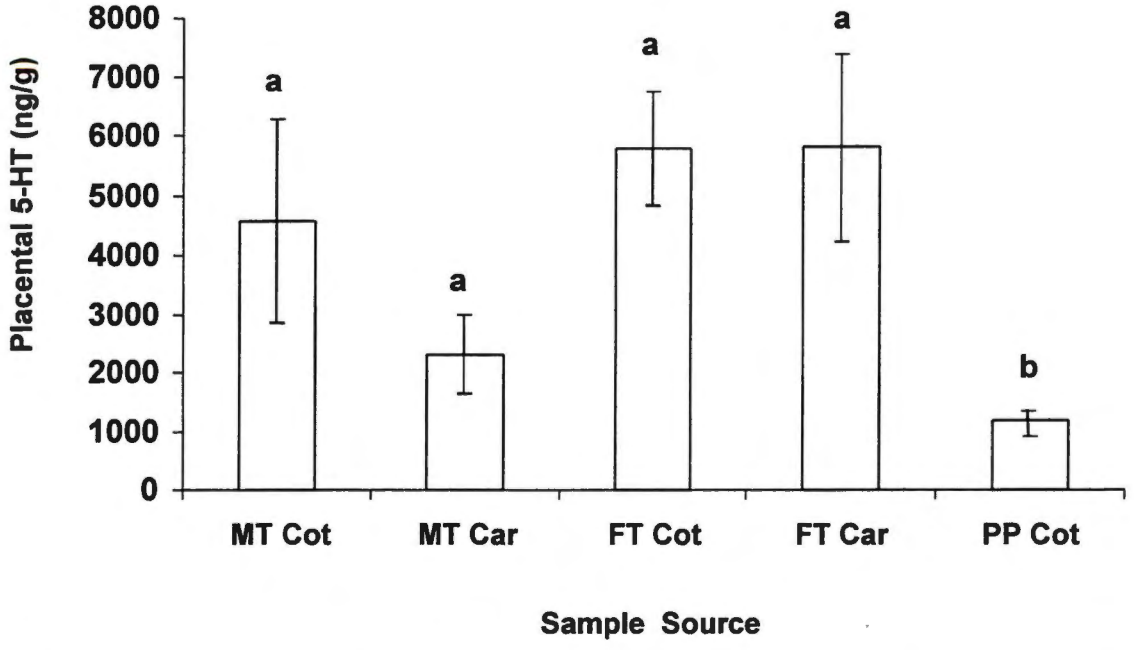



**Figure 2.3.** Concentration of 5-HT in cotyledon and caruncle tissues of bovine placenta during pregnancy and postpartum. Columns are mean ± SE for a minimum of 9 animals per group. Different letters (a,b) represent significant difference ($P \leq 0.05$) between groups. Sample sources were mid-term cotyledon (MT Cot), mid-term caruncle (MT Car), full-term cotyledon (FT Cot), full term caruncle (FT Car), and postpartum cotyledon (PP Cot).

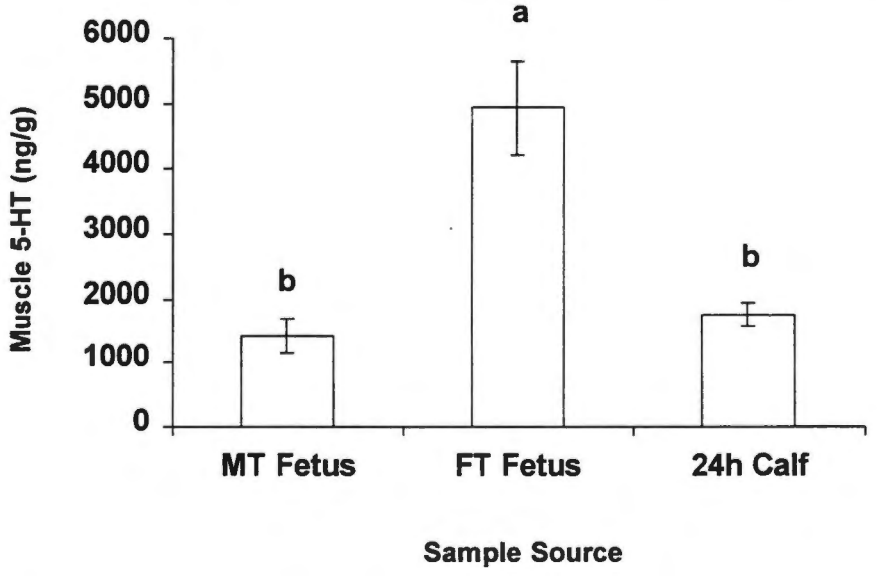
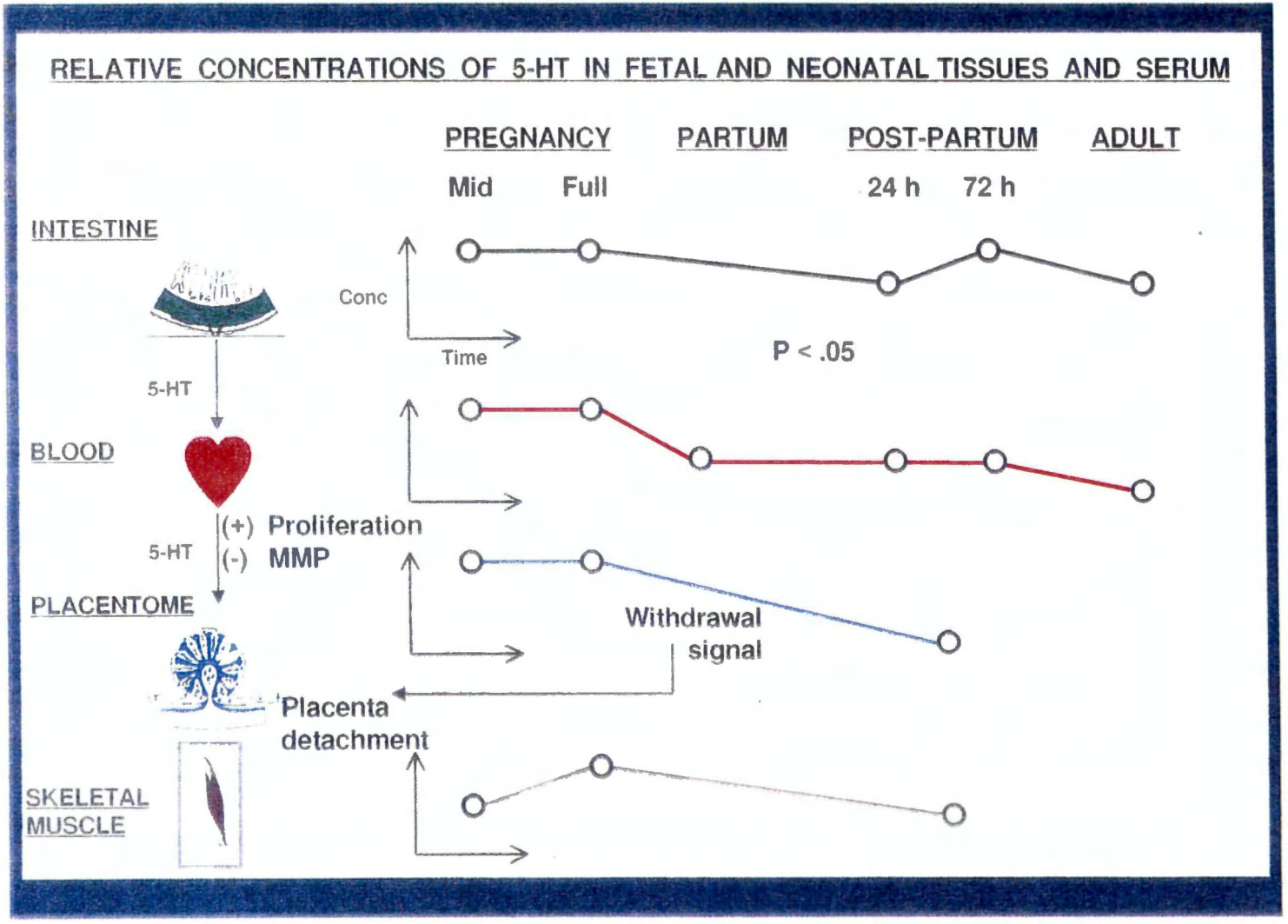



**Figure 2.4.** Concentration of 5-HT in muscles of bovine fetuses and newborn calves. Columns are mean ± SE for a minimum of 12 animals per group. Different letters (a,b) represent significant difference ($P \leq 0.05$) between groups. Sample sources were mid-term (MT) fetuses, full-term (FT) fetuses, and 24-hour old calves (24 h calf).

**Figure 2.5.** Synopsis of relative concentration changes of 5-HT in intestine, blood, placentome, and skeletal muscle of fetuses at mid- and full-term pregnancy, neonatal calves at 24 and 72 hours postpartum, and adult cows. Level of open circles within a line indicates no change (same), decrease (down), or increase (up) of 5-HT concentrations ($P \leq 0.05$). Figure illustrates the hypothesis that 5-HT is secreted by the fetal intestine into fetal blood circulation, bathing placentomes, and fetal muscles. High levels of fetal blood 5-HT stimulate cell proliferation and inhibit MMP activity in the placentome. Decrease in fetal blood 5-HT at partum acts as a withdrawal signal to the placentome causing arrest of cell proliferation and inducing MMP activity, leading to placenta detachment.


RELATIVE CONCENTRATIONS OF 5-HT IN FETAL AND NEONATAL TISSUES AND SERUM
PREGNANCY
PARTUM
POST-PARTUM
ADULT
Mid
Full
24 h
72 h
INTESTINE
Conc
Time
P < .05
5-HT
BLOOD
5-HT
(+) Proliferation
(-) MMP
PLACENTOME
Withdrawal signal
Placenta detachment
SKELETAL MUSCLE

commercial source (Jeffrey et al., 1991). However, in our experiment 5-HT concentration in neonatal calves (18 μM) was moderately higher than the concentration for commercially obtained pooled-newborn bovine serum (1.0 μM) as reported by Jeffrey et al. (1991). The discrepancy in reported concentrations of newborn bovine serum may be explained, in part, by techniques used for analysis of 5-HT and the source of samples.

When concentrations of 5-HT in blood were elevated tissue concentrations were also elevated. High blood and tissue concentrations of 5-HT may have resulted from increased secretion from the intestine and/or low 5-HT metabolism by degradative enzymes. The drop in 5-HT concentrations in the intestinal wall and blood of 24 hour old neonatal calves to one-fourth that of pregnancy concentrations, suggested to us that there may be a decrease in 5-HT secretion from the intestine and perhaps an increase in 5-HT degradation. However, 5-HT concentration in the skeletal muscle in mid pregnancy was low when blood concentration was high. It may be that skeletal muscle is not a primary target tissue of 5-HT in mid pregnancy. Also, 5-HT concentration increased in the intestinal wall at 72 hours postpartum when concentration in blood was low. This transient increase of 5-HT concentration in the intestinal wall at 72 hours postpartum suggested that 5-HT could regulate intestinal motility. The stimulatory effect of 5-HT on intestinal motility is well known (Goodman and Gilman, 1975) and calves that are 72 hours old are capable of nursing regularly, thereby causing stimulation of their gastrointestinal system. During embryonic development, 5-HT is strictly related to the development of the enterochromaffin system (Garattini and Valzelli, 1965). It has been demonstrated through immunohistochemical techniques that enterochromaffin cells appear in the intestine as early as 3 months of gestation in the bovine fetus (Totzauer, 1991) and 2 months in the human fetus (Facer et al., 1989). In experimental animals and humans (Goodman and Gilman, 1975), removal of segments of the small intestine lowers

the level of 5-HT in serum, yet, blood levels are not drastically affected when the large intestine is removed (Garattini and Valzelli, 1965). These facts suggest that blood levels of 5-HT can be adjusted by decreasing or increasing intestinal secretion of 5-HT in the fetus. It should not be ignored, however, that maternal blood is also a potential source for fetal 5-HT. It was reported by Yavarone et al. (1993) that the placenta is capable of transferring 5-HT from the mother to the fetus in early stages of pregnancy. However, near the end of pregnancy maternal 5-HT appears to enter the placenta but does not reach the fetal circulation (Prasad et al., 1996). It has been reported that monoamine oxidase (MAO) activity in human placenta increased by 22% at time of delivery (Kirkel et al., 1992) which may explain, in part, why maternal 5-HT does not reach fetal circulation.

It is interesting that MAO activity in many body tissues, such as lung, brain and kidney, is not well developed until approximately 1 to 2 weeks postpartum, unlike the binary system (MAO-A, MAO-B) in rat liver which is fully developed at least 3 days prepartum (Benedetti et al., 1992). Uptake and degradation by the lung are important in the metabolism of 5-HT. Depending on the rate of infusion 30% to 90% of 5-HT is taken up by pulmonary endothelial cells and metabolized primarily by MAO (Vane, 1969). In sheep, the uptake of 5-HT by the fetal lung is greater than in postnatal stage (Balaguer et al., 1992). It may be that the development of MAO deaminating activity is, in part, responsible for the drop in fetal serum 5-HT concentrations during delivery. At birth human concentrations of 5-HT and its metabolite, 5-HIAA, were significantly higher in umbilical cord plasma than in maternal plasma, which indicates a more rapid metabolism of 5-HT in the fetus than in the mother (Okatani et al., 1990). It is likely that a combination of increased metabolization of 5-HT by fetal tissues and a decreased secretion of 5-HT by the intestine may regulate relative concentrations of 5-HT in fetal blood.

In conclusion, our results of 5-HT concentration change in fetal blood and tissues during gestation and delivery, along with supporting literature, suggest that 5-HT is involved in normal pregnancy and delivery. The role that 5-HT plays in normal pregnancy is not fully elucidated, however we suspect it may be involved in processes that control growth of the placenta and fetus. The unanswered question of the function of 5-HT in pregnancy creates a novel path for future research.

# APPENDIX

**Enzyme Immunoassay Procedure**

40 µL of reconstituted standard plus 1960 µL dilution buffer

300 µL of 200 nM standard plus 700 µL dilution buffer

300 µL of 60 nM standard plus 700 µL dilution buffer

300 µL of 18 nM standard plus 700 µL dilution buffer

300 µL of 5.4 nM standard plus 700 µL dilution buffer

1000 µL dilution buffer

1) Add 100 µL sample or standard to acylation tube (for serum samples also add 50 µL of acylation buffer), vortex to dissolve reagent.
2) Incubate in dark at room temperature for 30 minutes.
3) Add 20 µL of sample or standard, in duplicate, to microtiter wells (leave one well empty for substrate blank).
4) Add 200 µL of enzyme conjugate to each well except substrate blank.
5) Incubate plate in the dark at room temperature with shaking at 350 rpm for 3 hours.
6) Add 200 µL of substrate to all wells and incubate plate as above for 15 minutes.
8) Add 50 µL of stop solution to wells.
9) Read plate at 405-414 nM.
10) Plot absorbance of standards versus concentration of standards to determine concentration of samples.

# PART III

# EFFECT OF 5-HYDROXYTRYPTAMINE ON BOVINE PLACENTAL CELLS: GROWTH STIMULATORY OR INHIBITORY?

## INTRODUCTION

Growth stimulatory effects of 5-HT on a variety of cell types have been described. Five-HT sensitive cells include bovine aortic smooth muscle cells (Nemecek et al., 1986), hamster lung fibroblasts (Seuwen and Pouyssegur, 1990), renal mesangial cells (Takuwa et al., 1989), rat intestinal epithelial cells (Tutton et al., 1987) as well several types of carcinomas (Ishizuka et al., 1992; Cattaneo et al., 1993). Furthermore, it has been proposed that 5-HT may have a role in fetal growth processes (Garattini and Valzelli, 1965), such as in neuronal development in the chick and rat (Lauder et al., 1981; Whitaker-Azmitia et al., 1987). Therefore, 5-HT has been widely documented to be a mitogen in many tissues. However, information on proliferative effects of 5-HT on placental tissues is scarce. Recently, Huang et al. (1998) localized 5-HT and its receptor in human placenta and several researchers have reported the existence of a 5-HT transporter in the maternal-facing brush border membrane of the human and mouse placenta (Balkovetz et al., 1989; Ramamoorthy et al., 1995; Prasad et al., 1996). The purpose of the transporter is not known but may serve to regulate placental function, and/or act in growth and development of the fetus (Balkovetz et al., 1989). Moreover, 5-HT concentrations in fetal bovine serum have been reported to be 30 to 50 times higher than neonatal and cow serum (Jeffrey et al., 1991; Fecteau and Eiler, 1997). The source of this high fetal 5-HT is not known, however it is suspected that the intestine may be the source since approximately 95% of body 5-HT is located in the enterochromaffin cells of the gastrointestinal system (Tyce, 1990). It is our hypothesis that 5-HT from bovine fetal intestine acts as a "proliferation factor" to support placental cell growth during pregnancy. In our hypothesis, partial withdrawal (by decreased secretion and/or increased inactivation) of fetal blood 5-HT is a cause for both (1) arrest of placental cell proliferation and (2) activation of matrix metalloproteinases (MMPs). Activation of MMPs contributes to placenta detachment at parturition. This hypothesis

is appealing because it links unexplainably high concentrations of 5-HT in fetal blood with well-accepted mitogenic effects of 5-HT. Therefore, to determine if our hypothesis was valid in our suggestion that 5-HT was a proliferation factor for placenta, we assessed the growth stimulatory effect of 5-HT on bovine placental cells by direct cell count and two proliferation assays: (1) incorporation of $^{3}$H-thymidine into DNA, and (2) a tetrazolium based colorimetric assay.

## MATERIALS AND METHODS

### Cell Culture Reagents and Materials

Dulbecco's Modified Eagle Media (DMEM)/F-12 (50/50) Nutrient Mixture and trypsin-EDTA (1X) solution were obtained from Fisher Scientific (Pittsburgh, PA); fetal bovine serum (FBS), antibiotic-antimycotic (ABAM, 100X) solution, prepared with 10,000 units/mL penicillin G sodium, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin B, and insulin-transferrin-selenium-X (100 X, ITS) were obtained from Life Technologies/Gibco BRL (Grand Island, NY); epidermal growth factor (100 µg/vial, EGF) was obtained from Becton Dickinson (Bedford, MA); Hank's Buffered Salt Solution (HBSS, 10 X) and 5-hydroxytryptamine hydrochloride were obtained from Sigma Chemical Company (St. Louis, MO); [methyl-$^{3}$H] Thymidine, specific activity 25 Ci/mmol, was obtained from Amersham Life Science (Arlington Heights, IL); self-aligning glass fiber filters were purchased from Packard (Meriden, CT), and CellTiter 96 $AQ_{ueous}$ One Solution Cell Proliferation Assay was obtained from Promega Corporation (Madison, WI).

### Tissue Collection and Cell Culture Preparation

Primary explants of bovine placenta were prepared in our laboratory and Dr. Linda Munson (Davis, CA) donated characterized trophoblast cells (Munson et al., 1988) for use.

Placentomes were obtained from mid-term (180 days to 240 days) gestation fetuses and full-term (≥ 270 days) gestation fetuses at Brown Packing Company, Gaffney, SC. Fetal age was approximated using a growth development chart (Roberts, 1971). Fetuses were considered full-term gestation if hair coat was complete and incisor teeth were erupted and sharp. Fetal (cotyledon) and maternal (caruncle) tissues of individual placentomes were separated manually. Pieces of cotyledonary villi as well as caruncular tissues were cut with a sterile scalpel and placed in 50 mL sterile centrifuge tubes containing DMEM /F12 cell culture medium, 10% FBS, ABAM, EGF and ITS, and were placed on ice for return to the laboratory. Once in the laboratory, tissues were processed based on a method by Freshney (1994), however slightly modified. Tissue pieces were placed in sterile disposable Petri dishes and minced into small pieces. Minced tissues were placed in 15 mL sterile centrifuge tubes containing HBSS and washed allowing the tissues to settle to the bottom of the tube. Tissue pieces were removed from the tube using a 1 mL pipette and placed in a 25 $cm^2$ Primaria tissue culture flask. Excess fluid was removed and 1 mL of fresh medium was placed in the flask. Flasks were placed in a humidified incubator supplied with 5% $CO_2$ and 95 % air. Tissues were allowed to adhere to the flask for 24-48 hours then 1 mL of media was added to the flask each day until fluid volume equaled 5 mL. The medium in flasks was changed as needed and when the outgrowth of cells reached 50 % confluence or greater, the cells were harvested by trypsinization and passed into 75 $cm^2$ flasks. When cells reached at least 80% confluence, they were used in proliferation experiments.

$^3$H-thymidine Assay

Cells were harvested from flasks by trypsinization, then centrifuged for 10 minutes at 1000 g. Cells were then tested for viability by adding 100 µL of cell suspension to 100 µL trypan blue and counting live cells (dead cells turn blue) on a hemacytometer. Once the number of live cells was determined, different cell densities (500, 1,000, 5,000,

and 10,000) were suspended in 200 µL DMEM/F12 supplemented with 10% FBS, EGF, ITS, and ABAM, then cells were plated in a 96-well tissue culture plate. No cells were plated in 8 of the wells so as to serve as blanks. The plate was then placed in the incubator. Cells were allowed 24 hours to adhere, then were washed with DMEM/F12 and 200 µL DMEM/F12 was added to each well. The plate was placed in the incubator for another 24 hours. After 24 hours, cells were washed with DMEM/F12, and 5-HT at a concentration of 5 µM was added to the wells. Control wells contained cells but no 5-HT. Eight hours after addition of 5-HT, $^{3}$H-thymidine at a concentration of 1 µCi/20 µL was added to all wells except blanks. The plate was left to incubate for a total of 24 hours. The next day, the plate was either placed in a -20°C freezer for 2 days or fluid was aspirated, washed, then 5% trichloroacetic acid was added, aspirated, then 0.2 N NaOH was added to obtain acid-insoluble fraction. After either procedure, the plate was harvested onto a glass fiber filter and disintegrations per minute (dpm) were determined on a beta counter. The same procedure was conducted with 10,000 cells and different concentrations of 5-HT (2.5, 5, and 10 µM) to establish a dose response.

Colorimetric Proliferation Assay

Different cell densities (500,1000, 5000 and 10,000) suspended in 100 µL DMEM/F12 supplemented with 10% FBS, EGF, ITS, and ABAM were plated in a 96-well tissue culture plate. After 48 to 72 hours in an incubator, 20 µL of CellTiter 96 $AQ_{ueous}$ One Solution Reagent were added to all wells. The plate was incubated for 4 hours, then analyzed at a wavelength of 490 nm on a microplate reader (Cambridge Technology, Inc.). A similar procedure was performed with different concentrations of 5-HT. Briefly, 10,000 cells suspended in 100 µL DMEM/F12 supplemented with 10% FBS, EGF, ITS, and ABAM were plated in a 96-well tissue culture plate. No cells were plated in 8 of the wells so as to serve as blanks. The plate was then placed in the incubator. Five-HT, at concentrations of 2.5, 5.0, and 10.0 µM, was either added to

cells when plated or 24 to 48 hours later. Cells were incubated for a total of 4 days. On the 4th day, 20 µl of CellTiter 96 $AQ_{ueous}$ One Solution Reagent were added to all wells. The plate was incubated for 4 hours, then analyzed at a wavelength of 490 nm on a microplate reader.

Hemacytometer and Coulter Counter Cell Count

Cells from primary explants were plated at a density of 20,000 placental cells/75 $cm^2$ flask. Experimental groups consisted of DMEM/F12 plus 5 µM 5-HT, DMEM/F12 supplemented with 10% FBS plus 5 µM 5-HT, and DMEM/F12 supplemented with 10% charcoal-stripped FBS plus 5 µM 5-HT. Control flasks contained no 5-HT. Cells were counted at 24, 48, and 72 hours. To obtain a cell count, cells were harvested from the flasks by trypsinization and centrifuged at 1000 g for 10 minutes. For hemacytometer counts, cells were resuspended in 10 mL of media of which 100 µL was mixed with 100 µL of trypan blue and placed on a hemacytometer. Number of cells/mL was determined using the following formula: average obtained from counting 8 grids x 2 x 10 x 1000. For Coulter counter counts, 250 µL of cell suspension were placed in a beaker with 50 mL of Isoton solution. The machine counted the cells in 2.0 mL increments for an average of 5 counts/beaker. Number of cells/mL was determined using the following formula: $(1/Vc)(1/Vs)(V_I)$, where Vc represented monometer or increment volume, Vs represented sample volume, and $V_I$ represented Isoton and sample volume. Size of cells was also analyzed on the Coulter counter.

Statistical Analysis

Statistical analysis was performed using the General Linear Models procedure in SAS. Probability of significance was reported at $P \leq 0.05$. Least Square Means were computed and compared using least significant difference techniques. In order to

stabilize the variance, the natural log of concentration means and cell number means from the $^{3}$H-thymidine experiments was used.

## RESULTS

### Incorporation of $^{3}$H-thymidine into DNA

As cell number increased so did incorporation of $^{3}$H-thymidine ($r = 0.94$). There were significant ($P \leq 0.0001$) differences among all cell numbers except 5,000 and 10,000 (Figure 3.1). When various concentrations of 5-HT were used to treat a uniform number of cells, incorporation of $^{3}$H-thymidine increased with increasing concentration of 5-HT. All treated groups were significantly different ($P \leq 0.0001$) from control, however 5 μM and 10 μM were not significantly ($P \geq 0.0001$) different from each other but were significantly ($P \leq 0.0001$) different from 2.5 μM 5-HT (Figure 3.2).

### Colorimetric Proliferation Assay

When no 5-HT was added to cells absorbance increased significantly ($P \leq 0.0001$) with increasing cell number (Figure 3.3) indicating that there were more viable cells in a well plated with 10,000 cells versus a well with 500 cells. When different concentrations of 5-HT were added to a uniform number of cells there was decreased absorbance (less colored product formed) suggesting decreased viability of cells. Control cells produced significantly ($P \leq 0.0001$) more colored formazan product than 5-HT treated cells, and 10 μM 5-HT were significantly ($P \leq 0.0001$) more inhibitory than 2.5 μM and 5 μM (Figure 3.4).

### Cell Count and Cell Size

There were no significant differences ($P \geq 0.60$) observed in cell number between cells treated with 5 μM 5-HT and cells used as control. Using a hemacytometer, the mean cell count when 5-HT was added was 68,599 cells/mL and 61,989 cells/mL for

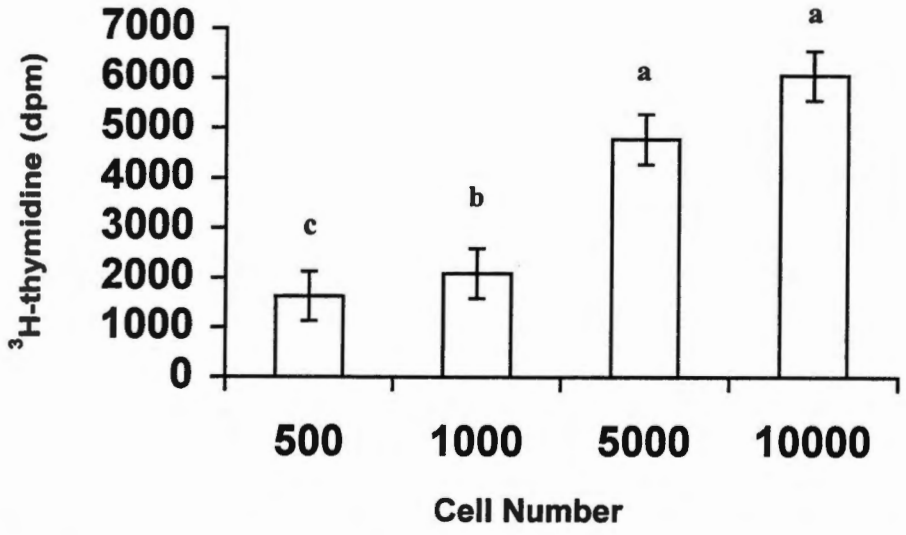



**Figure 3.1.** Incorporation of tritiated thymidine into DNA of bovine placental cells at different cell densities. Columns are mean ± SE for a minimum of 8 experiments. Different letters (a,b,c) represent significant difference ($P \leq 0.05$) among groups.

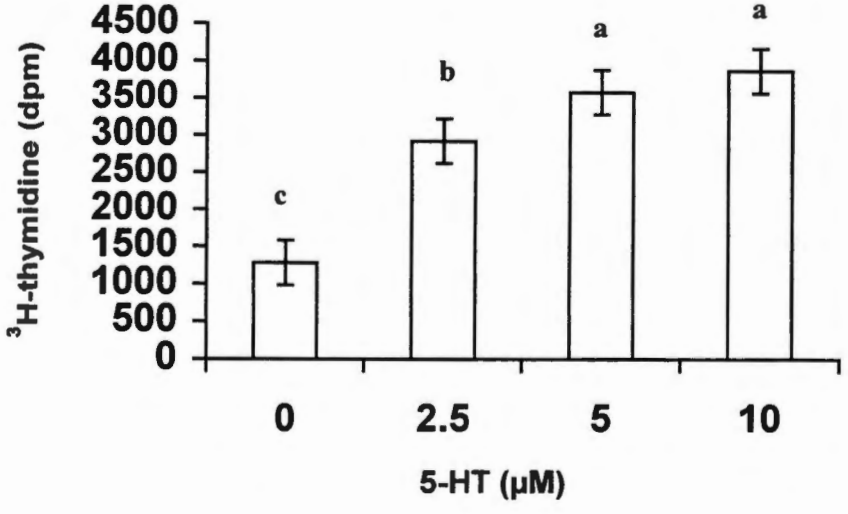



**Figure 3.2.** Incorporation of tritiated thymidine into DNA of bovine placental cells when treated with different concentrations of 5-HT. Columns are mean ± SE for a minimum of 8 experiments. Different letters (a,b,c) represent significant difference ($P \leq 0.05$) among groups.

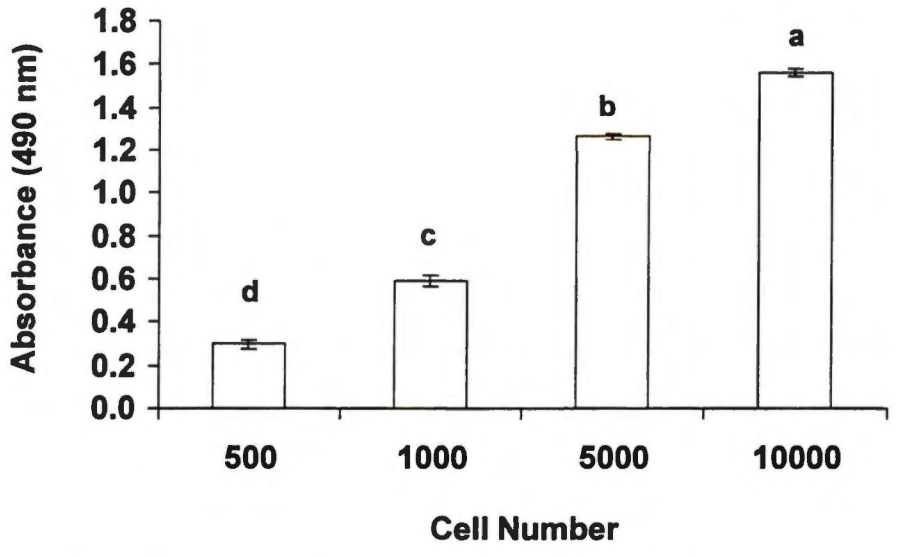



**Figure 3.3.** Color formation (absorbance) in culture medium of bovine placental cells at different densities. Columns are mean ± SE for a minimum of 8 experiments. Different letters (a,b,c,d) represent significant difference ($P \leq 0.05$) among groups.

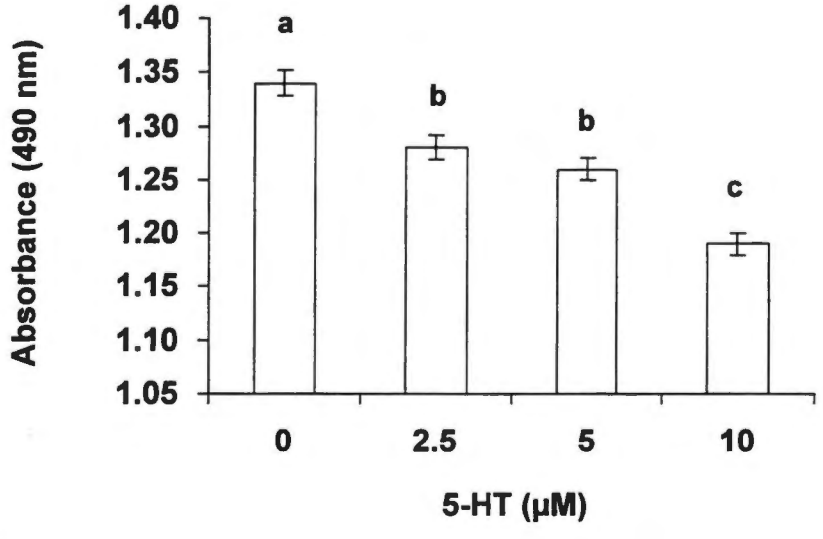



**Figure 3.4.** Color formation (absorbance) in culture medium of bovine placental cells when treated with different concentrations of 5-HT. Columns are mean ± SE for a minimum of 8 experiments. Different letters (a,b,c) represent significant difference ($P \leq 0.05$) among groups.

control cells. Mean cell numbers obtained from use of the Coulter counter were 49,989 cells/mL for 5-HT treated groups and 56,599 cells/mL for control groups. Along with cell number, mean cell size did not differ significantly ($P \geq 0.46$) between 5-HT groups (25.6 μm) and control groups (25.3 μm).

## DISCUSSION

The objective of this research was to determine whether or not 5-HT acts as a "proliferation factor" for bovine placental tissues. The results of these experiments indicated that treatment of bovine placenta cells with 5-HT increased incorporation of $^{3}$H-thymidine into DNA of cells (increased mitogenic activity). However, 5-HT reduced color change in a colorimetric proliferation assay (decreased viability/proliferation), and had no visible effect on cell count and cell size. The dissociation in results between incorporation of $^{3}$H-thymidine and the tetrazolium-based colorimetric proliferation assay was unexpected, since it has been reported that there is less than 5 % difference between the two assays (Piva et al., 1996). The reason for the inconsistency between the results of the two assays is not known.

One should keep in mind that the basis of the two assays is intrinsically different as one assay is based on synthesis of DNA, and the other is based on metabolism of tetrazolium to a colored formazan product. It is generally accepted that cells that incorporate $^{3}$H-thymidine into their DNA will proceed to mitosis. In order to incorporate thymidine into DNA the thymidine must be taken up by the cells and phosphorylated stepwise. The thymidylate kinases which act in the phosphorylation of thymidine are absent from non-growing cells and vary dramatically throughout the cell cycle. Thus, phosphorylation may be a rate-limiting process except in S- and G2-phases of the cell cycle (Adams, 1990).

In the tetrazolium-based assay, the tetrazolium compound is bioreduced by cells into

a colored formazan product that is soluble in tissue culture medium. The conversion of tetrazolium to formazan is thought to be accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (Promega Technical Bulletin, 1996). Metabolically active cells are assumed capable of proliferation. It is of interest though, that not all cell lines are equal in their reduction capabilities to form the colored product. This concern prompted researchers using drug-screening protocols to examine parameters affecting formazan production (Vistica et al., 1991). It was found that culture age and concentration of D-glucose in culture medium affected formazan production. Cell lines that extensively metabolized D-glucose exhibited the greatest decrease in formazan production. These findings indicated that the kinetics of formazan production and degree of saturation vary in a cell line-specific manner (Vistica et al., 1991). Our placental cell cultures were used at early passages but glucose metabolizing capability of these cells that were treated with 5-HT was not known. We conducted a pilot experiment (unpublished and not reported in this manuscript) in which oxygen uptake and radioactive carbon dioxide, that were formed from uniformly-labeled radioactive D-glucose (New England Nuclear, Boston, MA), were measured in cultured placental cells with and without 5-HT. The medium used for treated and untreated cells contained stable glucose and insulin. Results of preliminary data from our laboratory indicated that 5-HT increased decarboxylation of radioactive D-glucose by cultured bovine placental cells. If this is true, then it may be that increasing concentrations of 5-HT caused the cells to metabolize more glucose hence decreasing formazan production by 5-HT treated cells in a dose-related manner. It may be that 5-HT re-directed NADPH, NADH or enzymes that were needed to bioreduce tetrazolium into a different biosynthetic pathway, resulting in decreased color production. Therefore, based on the inherent differences in the two proliferation assays the results should not be considered contradictory.

Since 5-HT had positive effects on placental cell incorporation of $^{3}$H-thymidine, it is puzzling that cell number and size did not increase when cells were treated with 5-HT. It is possible that, *in vitro*, 5-HT inhibited metabolic reactions that were needed to complete proliferation in the placental cell, yet was capable of activating DNA synthesis. Also, it may be that 5-HT requires additional growth factors, as is the case with vascular smooth muscle cells (Nemecek et al., 1986), in order to stimulate cell growth. Further, our primary cultures were a heterogeneous population of placental cells. Since we were interested in placental growth, we thought the logical starting point for cell growth studies would be a mixture of cells representative of the placenta. Perhaps a small population of cells in this mixture was actually affected by 5-HT, and that effect was too small to detect when counting cells or determining cell size. The obvious next step would be to separate cells and determine if 5-HT has a growth stimulatory effect on homogeneous populations.

It is our hypothesis that 5-HT from fetal intestine is a "proliferation factor" that supports cotyledon cell proliferation during pregnancy. It is speculated that 5-HT may operate as a two-function "switch" for the control of two different and sequenced physiological stages. The first stage involves sustaining pregnancy and the second stage involves facilitating detachment of the placenta at partum. It is suspected that high concentrations of 5-HT (50 μM) may stimulate placental cell proliferation to sustain attachment of the placenta during pregnancy, whereas a peripartum drop in fetal blood 5-HT (< 13 μM) may stimulate MMP activity. The proposed stimulation of MMP activity would facilitate detachment of the placenta and uterine regression. Indeed, it has been shown that a low dose of 5-HT (5 μM) stimulates MMP activity in cultured rat and human myometrial cells and may be involved in uterine regression (Jeffrey et al., 1991). Results of the present research support part of our hypothesis by showing that 5-HT increased incorporation of $^{3}$H-thymidine into DNA in a dose-related manner and within a physiological concentration range.

# PART IV

# EFFECT OF 5-HYDROXYTRYPTAMINE ON MATRIX METALLOPROTEINASE ACTIVITY IN BOVINE PLACENTA

## INTRODUCTION

The end of pregnancy is characterized by placental detachment. The mechanism of placental detachment is not clear but appears to involve the activation of matrix metalloproteinases (MMPs), such as collagenase (Eiler and Hopkins, 1993; Maj and Kankofer, 1997). Collagenase-1 (MMP-1) immunoreactivity was reported in amniotic epithelial and interstitial cells, decidual cells, and intermediate trophoblasts in third-trimester human placenta (Vettraino et al., 1996). Ninety-two kDa gelatinase (MMP-9) is produced by cells of the amniochorion during labor, suggesting that proteolysis assists in fetal membrane rupture at birth (Vadillo-Ortega et al., 1995). Furthermore, Maj and Kankofer (1997) reported the presence of proteolytic activity of MMP-2 and MMP-9 in bovine term placenta. This information strongly supports the involvement of MMPs in placenta detachment. It is suspected that in the cow the proteolytic mechanism of placenta detachment may be activated in a short amount of time. For example, spontaneous expelling of placenta that is left attached in the uterus after pre-term cesarean section in cows takes approximately 24 hours, suggesting that the system can be activated during this time (Eiler et al., 1997). Knowing what stimulates the release of MMPs is central to resolving the mechanism of placenta detachment.

Early in this decade, serotonin (5-hydroxytryptamine, 5-HT) present in fetal bovine serum (FBS) was identified as an inducer and activator of collagenase in cultured postpartum rat and human myometrial smooth muscle cells (Jeffrey et al., 1991). It was found that 5-HT at a concentration of 5 μM maximally stimulated the myometrial cells, and that these cells displayed an obligatory requirement for 5-HT to produce collagenase (Jeffrey et al., 1991). Five-HT induces collagenase production in myometrial smooth muscle cells by activating the gene for the enzyme (Wilcox et al., 1992), and this effect is mediated by the 5-$HT_2$ receptor subtype (Rydelek-Fitzgerald et al., 1993). The regulatory effect of 5-HT on collagenase is of special interest, since concentrations of 5-

HT in fetal blood are extremely high (30 μM to 50 μM), dramatically drop at delivery by cesarean section (13 μM) (Fecteau and Eiler, 1998), and stay low in the neonate (Jeffrey et al., 1991; Fecteau and Eiler, 1998). The source of the high 5-HT concentrations is not known, but we suspect it may be the intestine since approximately 95% of 5-HT are located in the enterochromaffin cells in the gastrointestinal tract (Tyce, 1990). The high fetal concentrations of 5-HT may be supporting the growth of the placenta during gestation, since 5-HT was reported to act as a growth factor in various cell types (Nemecek et al., 1986; Tutton et al., 1987; Seuwen and Pouyssegur, 1990), while at the same time inhibiting MMP activation. We hypothesize that 5-HT from fetal intestine acts as a "proliferation factor" to support placental cell growth during pregnancy. In our hypothesis, partial withdrawal (by decreased secretion and/or increased inactivation) of fetal blood 5-HT is a cause for both (1) arrest of placental cell proliferation and (2) activation of MMPs leading to detachment of the placenta. The objective of the current study was to determine if 5-HT stimulates MMP activity in bovine placental cells in culture, in isolated placentomes, and in the whole animal.

## MATERIALS AND METHODS

### Tissue Collection

Placentomes were collected from cows ≥ 270 days of gestation at an abattoir. Gestation age was determined by using a fetus development chart (Roberts, 1971). A minimum of 8 placentomes were obtained from each of 16 cows. During collection, placentomes were cut from the placental tissue to allow excess cotyledon to show umbilical vessels in order to be used for catheterization and perfusion when appropriate. Placentomes were placed in plastic bags moistened with saline and kept in a cooler filled with ice. Placentomes were used within 24 hours once back at the laboratory.

### Reagents

Dulbecco's Modified Eagle Media (DMEM)/F-12 (50/50) Nutrient Mixture and trypsin-EDTA (1X) solution were obtained from Fisher Scientific (Pittsburgh, PA); fetal bovine serum (FBS), newborn bovine serum (NBS), antibiotic-antimycotic (100X, ABAM) solution, prepared with 10,000 units/mL penicillin G sodium, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin B, insulin-transferrin-selenium-X (100 X, ITS) were obtained from Gibco BRL (Grand Island, NY); epidermal growth factor (100 µg/vial, EGF) was obtained from Becton Dickinson (Bedford, MA); Hank's Buffered Salt Solution (10 X), 5-hydroxytryptamine hydrochloride, 5-hydroxytryptamine creatinine sulphate and *Clostridium histolyticum* collagenase type I were obtained from Sigma Chemical Company (St. Louis, MO); fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-$NH_2$ was obtained from Bachem Bioscience, Inc. (King of Prussia, PA).

Cell Culture Preparation

Primary explants of bovine placenta were prepared in our laboratory. Once collected, the two tissues comprising the placentome, which are the cotyledon (fetal part) and caruncle (maternal part) were separated manually. Small pieces of each tissue were cut using a sterile scalpel and placed in individual sterile 50 mL conical centrifuge tubes containing DMEM/F12, 10% FBS, ABAM, EGF and ITS, and placed on ice for return to the laboratory. Once in the laboratory, tissue pieces were finely minced, washed with HBSS, then placed in cell-culture flasks (Primaria, 25 $cm^2$) with up to 5 mL of medium. When cell outgrowths were present and at least 50% confluent, cells were harvested by trypsinization and passed into 75 $cm^2$ flasks.

*Cell Culture Experiment*

The objective of this experiment was to determine if 5-HT could induce MMP activity in cultured bovine placental cells. This was accomplished by directly assessing MMP activity in culture medium of cells treated with 5-HT and cells not treated with 5-

HT using a fluorometric assay for MMPs.

Flasks were set up containing DMEM/F12, DMEM/F12 plus 10% NBS or DMEM/F12 plus 10% FBS, all with and without 5-HT (5μM). Five hundred thousand cells were plated in each flask and media samples were taken at 12, 24, and 48 hours. At each collection time, 2.0 mL were taken from each flask and replaced with 2.0 mL fresh media with or without 5-HT. Once collected, samples were frozen at -70°C until analyzed using a fluorometric assay (Knight et al., 1992). Knight et al. (1992) reported using this assay to determine activity of collagenase, stromelysin, gelatinase and a punctuated metalloproteinase (PUMP).

*Fluorometric Assay*

Matrix metalloproteinase activity was extrapolated from a standard curve (Figure 4.1) that was established using bacterial collagenase from *Clostridium histolyticum* (type I). Standard concentrations used were 1.25 nM, 2.5 nM, 5.0 nM, 7.5 nM, and 10 nM. Collagenase was reconstituted in DMEM/F12 to the concentration of 10 nM, then the 10 nM concentration was diluted in DMEM/F12 to the other standard concentrations. Standards were made fresh for each assay.

An MMP assay buffer (50 mM Tris-HCl, 0.05% Brij 35, 10 mM $CaCl_2$ in 1 L distilled water, pH 7.8) and stop solution (2.0 M sodium formate in 100 mL distilled water, pH 3.7) were warmed to 40°C in a water bath; then 2.45 mL of assay buffer were added to each standard and sample tube plus a tube used as a blank. Standard or sample (25 μL) were added to respective tubes; then 25 μL fluorogenic substrate (reconstituted in 916 μL dimethylsulfoxide) were added to all tubes. Tubes containing the solution were vortexed, then placed in the water bath to incubate for 20 minutes at 40°C. After incubation, the reaction was stopped with 100 μL of stop solution, tubes were vortexed and then samples were analyzed on a recording spectrofluorophotometer (model RF-

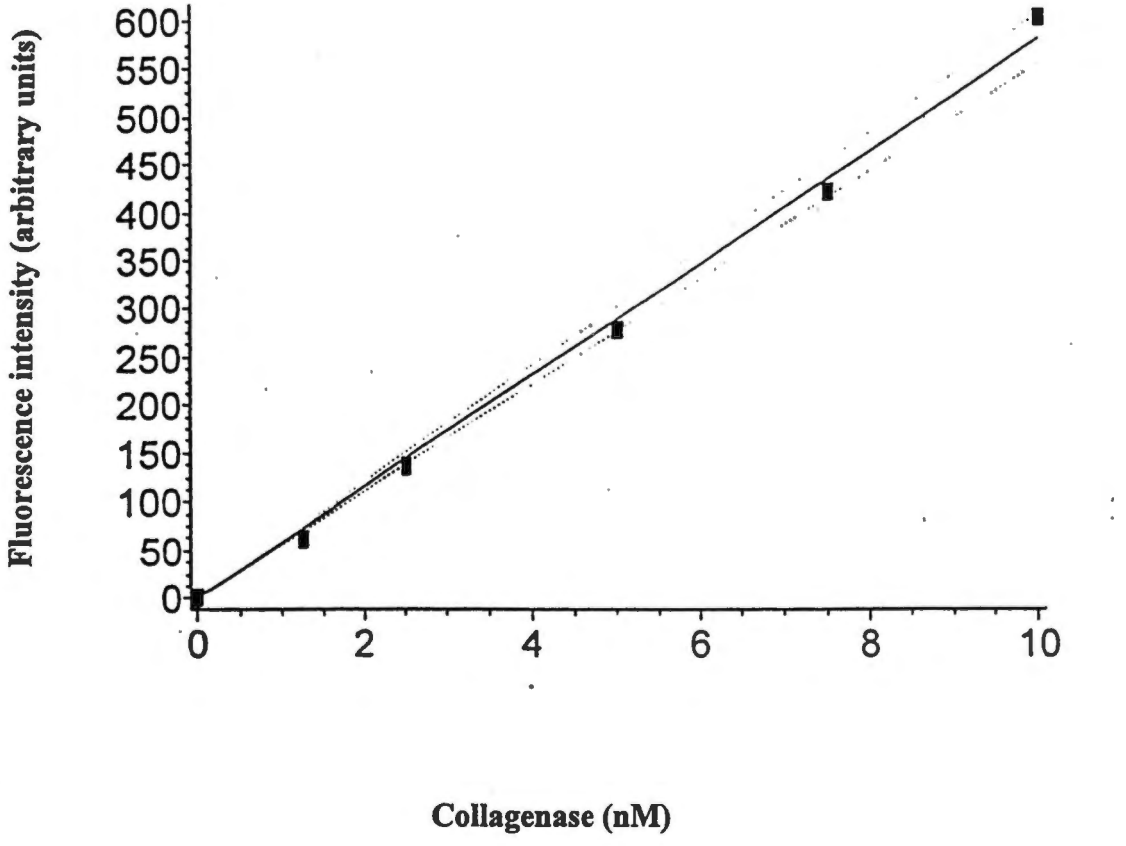



**Figure 4.1.** Standard curve for MMP assays. The X-axis represents increasing concentration of enzyme *Clostridium histolyticum* collagenase type I. Each solid box represents mean value of collagenase concentration and broken lines represent 95% confidence interval for 5 different assays performed in duplicate. The Y-axis represents arbitrary units of fluorescence intensity.

5000, Shimadzu Corporation, Kyoto, Japan) set at $\lambda_{ex}$ 328 nm, $\lambda_{em}$ 393 nm. During sample analysis, tubes were kept warm in a water bath at 40°C. The fluorescence intensity value of the blank was subtracted from values obtained for standards and samples.

*Isolated Placentome Experiments*

The objective of this experiment was to determine if 5-HT could induce MMP activity in isolated placentomes. MMP activity was assessed indirectly by determining force needed to separate the cotyledon and caruncle of the placentome, hydroxyproline (collagen breakdown product) release, and total protein release in the fetal-maternal interface.

Two different experiments were conducted. The objective of experiment 1, was to determine whether 5-HT added to perfusion blood would cause detachment of cotyledon from caruncle. In this experiment 4 different cows were used. Two placentomes were selected from each cow. One placentome was used as control (perfused with blood only), and one placentome was experimental (perfused with blood plus 50 μM 5-HT). Placentomes were perfused according to a method by Eiler and Hopkins (1992). Each placentome was placed in a plastic chamber. The most prominent umbilical vessel entering the placentome was catheterized with a 20-gauge 3.81-cm length venous catheter. This was used as a blood perfusion port. The other major umbilical vessels, if leaking, were ligated to assure a deep perfusion of the cotyledon. Blood outflow from the capillaries was drained into the chamber that contained the placentome and then dripped into a collection tube. Placentomes were perfused with fresh, oxygenated bovine blood by use of an open circulation device and a gravity driven perfusion system (blood was not recirculated). Perfusion rates varied between 2.0 cc and 5.0 cc/minute. Bovine blood used for perfusion was collected in saline (1/5 blood volume) and heparin (10 units/cc). Before use, fresh blood was oxygenated to arterial red from dark venous

color by using a rotary evaporator under flow of oxygen, then supplemented with dextrose (400 mg/dL). Placentomes were moisturized with saline and covered with a plastic sheet. The blood and placentomes were kept at 39°C during the experiment. Placentomes were perfused for 4 hours. Blood perfusion was stopped and placentomes remained in the incubation chamber for an additional 4 hours. Metabolic activity of placentomes was assessed at the beginning and at the end of the 4-hour perfusion by measuring inflow/outflow $CO_2$ and oxygen concentrations.

The objective of experiment 2, was to determine if infusion of 5-HT in Ringer solution would induce collagenase activity in metabolically active placentomes. Four different cows were used in the experiment, and 8 placentomes were selected from each cow. Four placentomes were used as control and four were experimental (injected with 5μM 5-HT). Placentomes were injected through an umbilical vessel with fluid volume equal to 10% of the placentome weight (each placentome approximately 100 g). After 5-HT infusion, placentomes were incubated in sealed plastic containers in a stationary water bath at 39°C for 4, 6, 8, and 11 hours. At the end of 11 hours, two selected control placentomes, that were not part of the experiment, were perfused with oxygenated blood. Differences in utilization of oxygen and glucose and production of $CO_2$ in inflow and outflow blood indicated metabolic activity.

After incubation of both infused and perfused placentomes, a previously described manometric technique (Eiler an Hopkins, 1992) was used to determine force needed to separate cotyledon from caruncle. Briefly, a balloon (4.5 cm in diameter) was inserted between cotyledon and caruncle. The balloon was connected to both a mercury manometer and a hand-operated air pump and was inflated until the two membranes separated. This was the point at which the pressure was recorded. The separated membranes of the placentome were placed in a container with 50 mL of distilled water and shaken (30 strokes per minute) for 10 minutes in a Dubnoff Metabolic Shaking

Incubator. After this, the surface fluids of membranes were gently "milked" out and filtered through 10 cm x 10 cm three layer gauze sponges into a beaker. Membranes were discarded and fluids were analyzed for hydroxyproline release by the method of Kivirikko et al. (1967). Total protein release was determined by the Kjel dahl method of nitrogen determination as described by Branstreet (1965).

*Experiment in Cows*

The objective of this experiment was to determine if 5-HT treatment of cows could override the retention of placenta that frequently (> 70%) results when delivery is induced with dexamethasone (20 mg) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$, 25 mg) injections. Although the mechanisms of action of dexamethasone and $PGF_2\alpha$ in causing retained placenta have not been elucidated, it has been proposed that the causative factor may be a lack of MMP activity. This is supported by the fact that injections of bacterial collagenase into the retained placenta via umbilical cord vessels has been highly effective (>80%) in causing detachment of the induced retained fetal membranes.

Fourteen cows near term pregnancy (> 8 months) were purchased from a local stockyard and induced to deliver. Twenty-four hours after dexamethasone/ $PGF_2\alpha$ treatment 7 cows were injected with 5-HT-creatinine sulphate (50 µg/kg, IM) in saline. Injections of 5-HT were given every 12 hours thereafter for a maximum of 3 days, or until delivery, whichever came first. Seven control cows received no 5-HT injections. The occurrence of retained placenta (≥ 12 hours) was evaluated clinically.

Statistical Analysis

Statistical analysis was performed using the General Linear Models procedure in SAS. Probability of significance was reported at $P \leq 0.05$. Least Square Means were computed and compared using least significant difference techniques.

## RESULTS

Cell Culture Experiment

*Effect of FBS or NBS on MMP activity*

Supplementation of culture medium with either 10% FBS or 10% NBS increased ($P \leq 0.01$) MMP activity of medium 3.1-fold and 2.5-fold, respectively, compared to medium alone (Table 4.1).

**Table 4.1.** Pooled MMP activity of 5-HT treated and non-treated cells grown in serum-free medium (DMEM/F12) or medium supplemented with 10% NBS or 10% FBS

| Medium | MMP activity (nM) |
|---|---|
| DMEM/F12 | $1.36 \pm 0.71^{a}$ |
| DMEM/F12 + NBS | $3.40 \pm 0.72^{a,b}$ |
| DMEM/F12 + FBS | $4.28 \pm 0.76^{b}$ |

$^{a,b}$Different superscript indicates significant difference ($P \leq 0.01$).

*Effect of 5-HT on MMP activity*

In serum (FBS or NBS) supplemented medium, MMP activity was decreased between 38% and 47% in medium treated with 5-HT compared to controls, however differences between control group and treated group at different incubation times were not significant ($P \geq 0.93$) (Table 4.2). However, when control groups were pooled and 5-HT groups were pooled there was significant ($P \leq 0.05$) inhibition of MMP activity in the 5-HT group. Mean ± SEM activity of pooled control and 5-HT treated groups was 3.81 nM ± 0.59 nM and 2.05 nM ± 0.60 nM, respectively. From these results, inhibitory effects of 5-HT may be statistically debatable. However, it is clear that 5-HT at 5 μM was not stimulatory of MMP activity as reported for cultured human and rat myometrial smooth muscle cells.

**Table 4.2.** Effect of 5-HT on MMP activity* of cell medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) or 10% newborn bovine serum (NBS) at different times of cultured placentome cell incubation

| Treatment | Protease activity (MMP nM/20 minutes) at hour | | | | Mean |
|---|---|---|---|---|---|
| | 0 | 12 | 24 | 48 | |
| DMEM/F12 + FBS | | | | | |
| Control | $3.3^{a,b}$ | $5.0^{a,b}$ | $9.0^{a}$ | $9.2^{a}$ | $6.6^{x}$ |
| + 5-HT | - | $1.7^{b}$ | $1.7^{b}$ | $7.0^{a,b}$ | $3.5^{x,y,z}$ |
| DMEM/F12 + NBS | | | | | |
| Control | $5.4^{a,b}$ | $3.5^{a,b}$ | $3.6^{a,b}$ | $4.1^{a,b}$ | $4.1^{x,y}$ |
| + 5-HT | - | $2.4^{b}$ | $1.6^{b}$ | $3.5^{a,b}$ | $2.5^{y,z}$ |
| DMEM/F12 (serum-free) | | | | | |
| Control | $1.4^{b}$ | $1.5^{b}$ | $1.8^{b}$ | $1.7^{b}$ | $1.6^{y,z}$ |
| + 5-HT | - | $1.0^{b}$ | $1.5^{b}$ | $1.3^{b}$ | $1.2^{z}$ |

*Type I Clostridium histolyticum collagenase equivalent (nM/20 minutes).

$^{a,b,c}$ Different superscripts indicate significant difference ($P \leq 0.05$) among time means within a row, while same superscripts indicate no difference.

$^{x,y,z}$ Different superscripts indicate significant difference ($P \leq 0.05$) between mean within mean column, while same superscript indicates no difference.

Note: when all control (6.6, 4.1, 1.6) and all 5-HT (3.5, 2.5,1.2) means were pooled, there was a significant ($P \leq 0.05$) inhibition of MMP activity by 5-HT.

Isolated Placentome Experiments

Five-HT treatment did not affect ($P \leq 0.05$) manometric pressure, hydroxyproline or total protein within and between experiment 1 and 2. Therefore, results of experiments 1 and 2 were pooled. However, results of pooled data indicated a discrete but significant increase ($P \leq 0.05$) in the amount of hydroxyproline released from the 5-HT treated placentomes compared to that of control placentomes (Table 4.3).

**Table 4.3.** Effect of 5-HT added to blood perfusion or added to Ringer infusion on manometric pressure needed to separate cotyledon from caruncle, and fetal-maternal interface hydroxyproline and total protein content in isolated placentomes

| Treatment | Manometric pressure (mm Hg) | Hydroxyproline (μg/cc) | Total protein (g/dL) |
|---|---|---|---|
| Control | $118 \pm 4.3^{a}$ | $1.57 \pm 0.47^{a}$ | $1.70 \pm 0.14^{a}$ |
| 5-HT | $116 \pm 4.2^{a}$ | $2.06 \pm 0.08^{b}$ | $1.61 \pm 0.09^{a}$ |

Numbers are mean ± SEM for pooled blood perfusion (n = 4 cows) and Ringer infusion (n = 4 cows) experiments.

[a,b] Different superscript under same column indicates significant difference ($P \leq 0.05$). Same superscript indicates no difference. Results pooled due to no difference ($P \geq 0.05$) between blood perfusion and Ringer infusion experiments and no difference at pre-set times within infusion experiment.

MMP Activity in Cows Injected with 5-HT

There was no difference in length of retention between control cows and cows injected with 5-HT. All cows injected with 5-HT retained their placenta between 2 days and 9 days (Table 4.4).

**Table 4.4.** Number of cows induced to deliver and injected with saline or 5-HT prior to delivery that retained placenta

| | Day postpartum | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Saline | 6/7 | 6/7 | 5/7 | 4/7 | 4/7 | 4/7 | 3/7 | 0/7 | 0/7 |
| 5-HT | 6/7 | 6/7 | 5/7 | 5/7 | 5/7 | 5/7 | 5/7 | 5/7 | 3/7 |

Number of cows affected / total number of cows.

## DISCUSSION

The experiments performed in this study were comprehensive, in that they accounted for the effects of 5-HT at the cellular level *in vitro*, isolated organ level, and at the level of the whole animal *in vivo*. Among all of the systems used, there was convincing evidence that 5-HT was not stimulatory of MMP activity, as was reported for cultured rat and human myometrial smooth muscle cells.

Jeffrey et al. (1991) reported that 5-HT added to cultured postpartum rat myometrial smooth muscle cells at a 5 μM concentration was maximally stimulatory of collagenase activity. Half-maximal stimulation of these myometrial cells was reported at 2 μM. In our experiment, 5 μM 5-HT was not stimulatory of MMP activity in cultured bovine placentome cells. Our results displayed more of an inhibitory tendency, since the overall mean of MMP activity of 5-HT treated cells (2.05 nM) was significantly lower than the overall mean of MMP activity of control cells (3.81 nM).

Although there was no 5-HT stimulation of MMP activity, the amount of MMP activity varied according to supplementation of medium, with cells grown in medium plus FBS responding with the highest activity, and cells grown in serum-free medium responding with the lowest activity. Fetal bovine serum contains hormones, polypeptides and other substances that are involved in controlling cellular processes, such as proliferation and protein synthesis (Freshney, 1994), whereas serum-free medium cannot support these cellular processes as effectively. The addition of 5-HT to the medium may have slowed down cellular processes such as protein synthesis. We analyzed the concentration of 5-HT in the commercial FBS and NBS used via an enzyme immunoassay specific for 5-HT (not reported). We found that FBS contained approximately 16 μM 5-HT and NBS contained a negligible amount (0.0034 μM) of 5-HT. Therefore, the cells grown in medium supplemented with FBS were actually exposed to roughly 7 μM 5-HT, which did not stimulate MMP activity in our cells. Our

decision to use 5-HT at a concentration of 5 μM was based on the positive response of the placentome cells in proliferation assays when exposed to 5-HT at concentrations between 2.5 μM and 25 μM in our laboratory (Fecteau and Eiler, 1998). Our decision was supported by the work of Jeffrey et al. (1991). The discrepancy between our results and those of Jeffrey et al. (1991) could be explained, in part, by the use of different animal species and different types of cells (postpartum myometrial smooth muscle cells versus prepartum placentome cells).

Isolated placentomes are an intermediate step between cell culture and intact animal experimentation. In the isolated placentome experiments, inactivation of 5-HT by peripheral organs (lungs, liver, and placenta) was excluded. Ringer's solution (plus 5-HT) infusion and blood (plus 5-HT) perfusion techniques were used to evaluate effects of oxygenation and blood biochemical variables introduced by whole blood use. In both types of experiments, blood perfused or Ringer's solution infused placentomes retained metabolic capacity for respiration by taking up oxygen and producing $CO_2$ and using D-glucose. The viability of excised placentomes is excellent. We were able to culture cells from explants taken from 6 day old placentomes that were kept in ice. In the placentome model, a physiological indicator (loosening of cotyledon from caruncle) and two biochemical indicators hydroxyproline release (a measure of collagen hydrolysis) and total protein release (a measure of non-specific proteolytic activity) were used. Eiler and Hopkins (1992) reported that when the degree of collagen breakdown reached a physiological point to separate fetal membranes from the caruncle, the force needed to separate cotyledon from caruncle decreased significantly (about 50%). At the same time, the amount of hydroxyproline increased 50- to 90-fold when total protein increased 4- to 6-fold. Our experiment with infused saline plus 5-HT revealed that there was no decrease in manometric force needed to separate cotyledon from caruncle. However, there was a minimal but significant ($P<0.05$) increase in hydroxyproline (from 1.57

μg/mL to 2.06 μg/mL), and no change in total protein compared to control. The interpretation of results of this experiment is that there was not sufficient collagen breakdown to cause a physiological effect. We believe that a significant difference in hydroxyproline release was attained because of the unusually low dispersion (0.04 SEM, 0.08 SEM) recorded in the control and 5-HT groups, respectively. In this experiment, 5-HT concentrations were 50 μM, which is the fetal blood maximal concentration (Jeffrey et al., 1991) and 10 times more than the concentration used in cell culture experiments. The results from these experiments support the results of the cell culture experiments in that there was no stimulation of MMP activity by 5-HT.

High incidence (over 70%) of placenta retention can be caused by inducing cows to deliver with injections of dexamethasone (Thorburn et al., 1977). In our experiment, we induced delivery by using both dexamethasone and $PGF_2\alpha$ injections. This combination usually results in delivery between 60 hours and 80 hours with over 85% placenta retention at 12 hours post delivery. This retention often lasts between 2 and 10 days (Eiler and Hopkins, 1993; Eiler, 1997). The research advantage of the induced retained placenta model versus naturally occurring placenta retention is the standardization of experimental conditions, which is not possible with privately owned cows. The induced model is a logical first step in this study.

The mechanism for placenta retention has been associated with lack of MMP activity at the placentome level (Eiler and Hopkins, 1993; Eiler, 1997; Maj and Kankofer, 1997). In fact, infusion of collagenase via umbilical cord vessels is the most effective treatment to detach retained placenta in cows (Eiler and Hopkins, 1993) and mares (Haffner et al., 1998). Although the mechanism by which dexamethasone induces placental retention is not fully understood, dexamethasone has been shown to inhibit collagenase mRNA production (Reddy et al., 1998), decrease levels of MMPs in oral fibroblastic cells but not in oral epithelial cell lines (Kylmaniemi et al., 1996), and reduce

fibronectin and collagen III synthesis in amnion cells that may be associated with prematurely ruptured membranes (Guller et al., 1995).

Injections of $PGF_2\alpha$ can also cause delivery and high incidence of retained placenta (Thorburn et al., 1977). Interestingly, both glucocorticoids and $PGF_2\alpha$ are elevated during spontaneous delivery in the cow, and it was reported that dexamethasone has a stimulatory effect on $PGF_2\alpha$ secretion by the placentome (Thorburn et al., 1977). If dexamethasone is indeed an inhibitor of MMPs, it is logical to assume that overriding dexamethasone inhibition of placental MMP activity may prevent retention. Five-HT is a substance that has the ability to stimulate MMPs. This stimulation potential is supported by two different experiments: (1) Jeffrey et al. (1991) reported 5-HT markedly stimulated secretion of collagenase by myometrial smooth muscle cells; (2) Zaiem et al. (1994) reported that intramuscular (IM) injection of Sergotonine (Rhone-Merieux, Lyon, France) within 12 hours postpartum decreased retention of placenta from 38% to 10% in cows. Sergotonine is a combination of 5-HT creatinine sulfate and ergometrine (uterokinetic substance). It is suspected that the positive effect in preventing placenta retention was due to 5-HT since uterokinetic substances have been reported as ineffective in preventing primary retained placenta in cow (Stevens and Dinsmore, 1997). In our experiment with cows, we injected 5-HT every 12 hours until delivery starting 24 hours after dexamethasone-$PGF_2\alpha$ injections. The dose of 5-HT used was roughly the same as that used by Zaiem et al. (1994) to decrease incidence of retention from 38% to 10%. It is observed in the work of Zaiem et al. (1994) that 10% retention in the treated group is still high since in this country the average incidence is about 7% (in untreated cows). However, our treatment was ineffective since 6 of 7 cows treated with 5-HT had retained placentas. Five-HT did not override the mechanism responsible for placenta retention. This result indirectly suggests that 5-HT did not promote MMP activity. In conclusion, our results indicate that 5-HT did not override dexamethasone-$PGF_2\alpha$

induced retention. Based on our results we do not see the practical potential of 5-HT injections to prevent retention in situations when early delivery is indicated. The results are consistent with the cell culture experimental results and placentome experimental results. We realize that our experimental conditions are not similar to spontaneous delivery. However, the lack of MMP stimulation by 5-HT indirectly supports our hypothesis of a withdrawal of 5-HT causing activation of MMPs.

# PART V

# LOCALIZATION OF COLLAGENASE AND 5-HYDROXYTRYPTAMINE IN BOVINE PLACENTA

## INTRODUCTION

Five-HT has been localized by immunocytochemistry in endothelial cells of human umbilical vessels (Sexton et al., 1996). More recently, Huang et al. (1998) utilized both light and electron microscopy techniques to localize 5-HT and its receptor in numerous cell types of the human placenta. Also, Yavarone et al. (1993) localized 5-HT in the ectoplacental cone and in nuclear chromatin-like material of giant cells in the mouse placenta. The appearance of 5-HT in the placenta and across species suggests that the placenta may be a target organ for 5-HT. Likewise, 5-HT may play a role in placental development (Huang et al., 1998) and regulate cellular functions such as proliferation, differentiation or secretion (Yavarone et al., 1993). Furthermore, there is evidence that a 5-HT transporter exists in the human placenta supporting the potential role of 5-HT in placental function (Balkovetz et al., 1989). It is interesting to note that the source of 5-HT in the placenta is not known. It is suspected the source may be the intestine, since the majority of body 5-HT is located in the enterochromaffin cells of the gastrointestinal tract (Tyce, 1990).

In addition to localization of 5-HT in the placenta, 5-HT in fetal bovine serum has been reported at concentrations of 30 μM to 50 μM (Jeffrey et al., 1991; Fecteau and Eiler, 1997), while newborn bovine serum 5-HT concentrations range from ≤ 1 μM (Jeffrey et al., 1991) to 24 μM (Fecteau and Eiler, 1997). It was found that a 5-HT concentration of 5 μM maximally induced collagenase in certain cultured cells, whereas higher concentrations caused no further increase in collagenase production (Jeffrey et al., 1991). We hypothesize that the high 5-HT concentrations may originate from the bovine fetal intestine and act as a "proliferation factor" to support placental cell growth during pregnancy. Partial withdrawal (by decreased secretion and/or increased inactivation) of fetal blood 5-HT may be a cause for both: (1) arrest of placental cell proliferation, and (2) activation of matrix metalloproteinases (MMPs). Activation of MMPs and arrest of

cell proliferation would lead to postpartum placenta detachment. It is important to investigate the location of 5-HT in placental tissues to further elucidate mechanisms of action of 5-HT. The objectives of the present study were to investigate the presence of 5-HT and collagenase in bovine placental tissues using light microscopy and electron microscopy techniques.

## MATERIALS AND METHODS

### Collection of Placentomes

Placentomes were obtained from Brown Packing Company, Gaffney, SC. Cotyledon and caruncle samples were taken from placentomes of cows during mid gestation (180 days to 240 days) and late gestation (≥ 270 days). Gestation age of fetus was approximated using a fetus development chart (Roberts, 1971). Samples were collected within 30 minutes and processed immediately upon collection for light microscopy study. Postpartum cotyledons (fetal membranes) were collected from cows after natural delivery at The University of Tennessee Dairy Farm. Cotyledons were obtained anywhere from 30 minutes to 4 hours after delivery of calf while membranes were still hanging from the cow. Hanging membranes undergoing detachment were cut with scissors, placed in a plastic bag and put on ice until return to the laboratory. In the laboratory, debris was removed from cotyledons by rinsing the membranes with physiological saline. The samples were then processed for light and electron microscopy study.

### Collection of Intestine and Uterus

Tissues from the uterus and intestine were obtained from female Sprague Dawley rats 2 days after they had given birth to a litter of pups. Intestine was used as a positive control for 5-HT localization and uterus (myometrium) was used as a positive control for collagenase localization. Rats were euthanized in a $CO_2$ chamber and intestine and

uterus were removed from each rat. Tissues were rinsed with physiological saline and processed for light and electron microscopy.

Preparation of Tissue Sections

*Light Microscopy*

Immediately upon collection, placentome tissues were separated and pieces (approximately 0.5 $cm^2$) of cotyledon and caruncle were cut using a single-edged razor blade. Intestine and uterus were cut using a single-edged razor blade. Pieces of cotyledon villi, caruncle (crypt region), intestine and uterus were placed in separate 50 mL conical tubes containing either Bouin fixative or 10% buffered formalin. Tissue pieces were fixed for 4 hours in Bouin fixative and for 24 hours in formalin then placed in 70% ethyl alcohol. Postpartum cotyledons were processed the same way as prepartum tissues. Alcohol of Bouin fixed tissues was changed twice a day in order to remove yellow pigment. Tissues were then embedded in paraffin (low melting point Paraplast, Fisher Scientific) using an automated processor (TissueTek VIP, Ames Division, Miles Laboratories, Inc.) (Appendix).

Tissue sections (5 µm) were cut on a microtome, placed in 20% alcohol to help reduce wrinkling of section then floated on warm water (40°C) and placed on a charged slide (Superfrost/Plus, Fisher Scientific). Slides were dried using a slide warmer.

Tissue sections were then processed to localize collagenase or 5-HT by the modified method of Lui and Godkin (1992) (Appendix).

Pieces of cotyledon and caruncle tissues and rat uterus were frozen for enzyme (collagenase) immunolocalization based on a method by Dr. Antonin Bukovsky (Department of Obstetrics and Gynecology, The University of Tennessee Medical Center; personal communication) (Appendix).

Tissue sections (5 µm) were cut on a cryostat (Reichert-Jung 2800 Frigocut) and placed directly on a charged microscope slide. Slides were stored at -70°C until used.

Tissue sections were then processed to localize collagenase (Appendix).

*Electron Microscopy*

Postpartum cotyledon villi were fixed in 2.5% glutaraldehyde/cacodylate buffer solution for 4 hours then placed in cacodylate buffer with 7.5% sucrose until further processing. Tissues were embedded in plastic (Appendix).

Technical services were utilized from the Electron Microscopy Laboratory, The University of Tennessee, Knoxville for cutting the plastic blocks and placing the cut tissues on nickel grids. Once grids were prepared, tissues were stained with saturated uranyl acetate and lead citrate (Appendix), then immunolabeled for 5-HT or collagenase (Appendix).

Specimens were examined on a Philips 201 transmission electron microscope and micrographs were made of cotyledon villi connective tissue. Negatives were exposed and 8 x 10 prints on polycontrast paper (Eastman Kodak Company, Rochester, NY) were developed. To develop the micrographs, the prints were placed in Dektol developer and stop bath (Eastman Kodak Company) for approximately 1.5 minutes and 30 seconds, respectively. Prints were then placed in Rapid Fixer (Eastman Kodak Company) for 2-3 minutes, rinsed in running water for 3 minutes and placed on a rack to dry.

## RESULTS

Light Microscopy

Attempts (frozen tissues and paraffin-embedded tissues) to localize collagenase in bovine placenta and rat uterus were unsuccessful.

Results of the immunohistochemical staining of 5-HT are shown in Figure 5.1. Results reveal 5-HT positive areas as brown and 5-HT negative areas as blue or pink. The negative controls showed negative reactions.

**Figure 5.1.** Immunohistochemical localization of 5-HT in bovine placenta. (A) Mid-term caruncle negative control. (A1) Mid-term caruncle showed diffuse 5-HT immunoreactivity. (B) Full-term cotyledon negative control. (B1) Full-term cotyledon showed 5-HT immunoreactivity on surface membrane of cells. Nuclei and cytoplasm immunonegative for 5-HT. (C) Negative control. Villi of full-term cotyledon. (C1) Tip of cotyledonary villus. (D) Villi of full-term cotyledon showed discrete immunoreactivity in connective tissue and (inset) on surface membrane of epithelial cells. (E) Blood vessel in full-term cotyledon negative control. (F) Endothelium of blood vessel showed 5-HT immunoreactivity. Bar = 200 μm (A, A1, C); 5 μm (B, B1, C1); 102 μm (D); 61 μm (D1); 25 μm (E and F); 49 μm (inset: F).

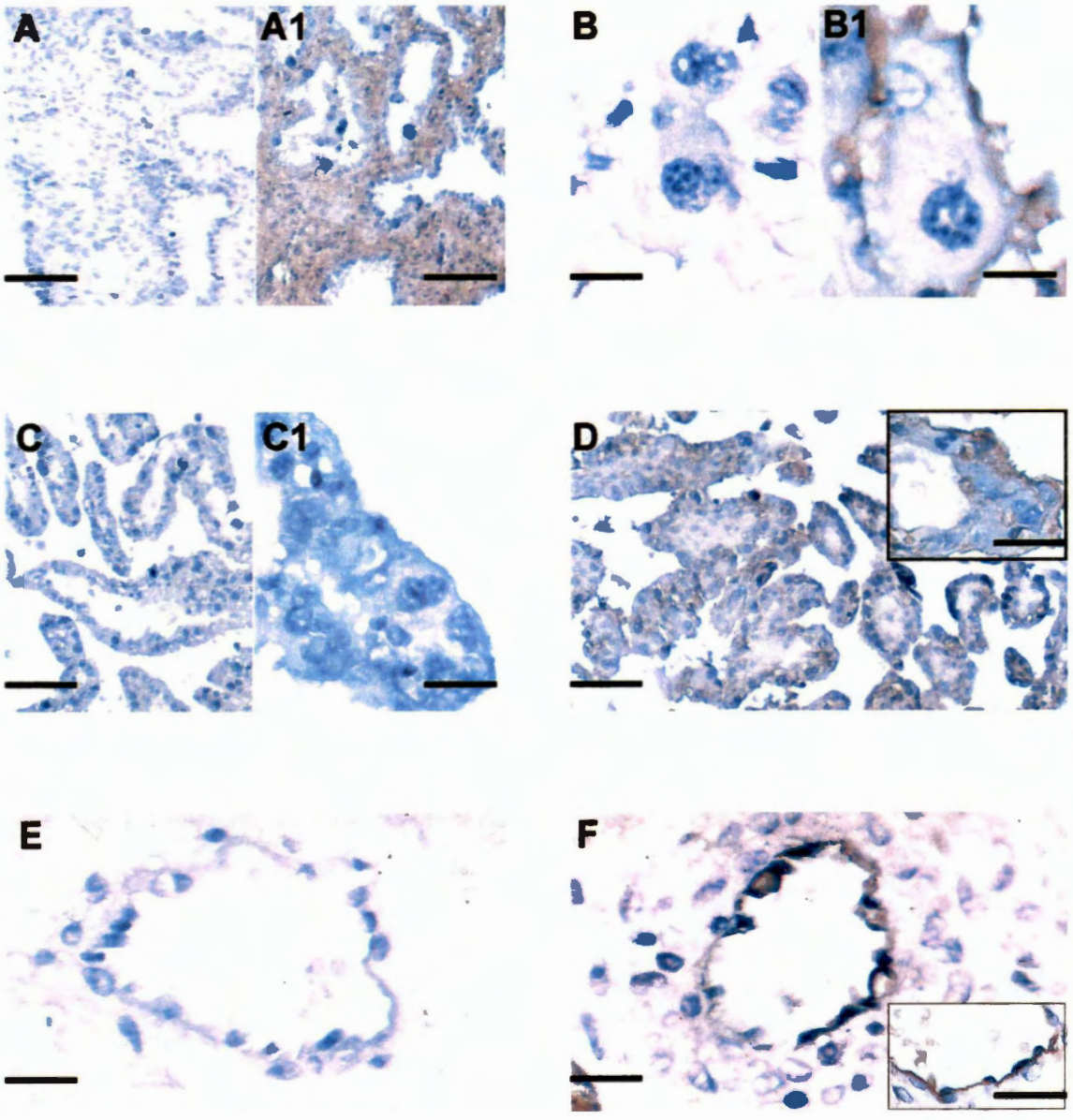

A
A1
B
B1
C
C1
D
E
F

**Figure 5.1 (continued).** (G) Postpartum cotyledon negative control; (inset) connective tissue. (H) Postpartum cotyledon showed 5-HT immunoreactivity on surface epithelial cells and cytoplasm. Diffuse 5-HT immunoreactivity in connective tissue (inset), however, isolated cells in connective tissue appear 5-HT immunonegative. (I) Rat intestine negative control. (J) Isolated cells in rat intestine immunoreactive to 5-HT. Bar = 100 μm (G and H); 25 μm (I and J); 42 μm (inset: G and H).

G
I
H
J

Surface membranes of epithelial cells of mid and full gestation cotyledon and caruncle were immunoreactive to 5-HT. Connective tissue of prepartum and postpartum placenta exhibited diffuse 5-HT immunoreactivity, however cells within connective tissue were immunonegative. Five-HT immunoreactivity was also seen in endothelium lining blood vessels of mid and full gestation and postpartum placenta. The cytoplasm of most lining epithelial cells of villi in the postpartum placenta were immunoreactive to 5-HT, however, cytoplasm of underlying cells was immunonegative. Isolated cells in the lamina propria-submucosa in rat intestine positive control tissue were immunoreactive to 5-HT.

Electron Microscopy

Attempts to localize collagenase were unsuccessful. Results of electron microscopy localization of 5-HT are shown in Figure 5.2. Five-HT positive areas are indicated by the round black (colloidal gold) particles, which are in close proximity to collagen fibers. Five-HT negative areas show no gold particles (Figure 5.3).

## DISCUSSION

Attempts to localize collagenase in the bovine placenta were unsuccessful. The collagenase antibody was species specific for bovine and possibly rat tissues and was specific for the 57- and 53-kDa forms of the proenzyme and 47- and 43-kDa form of the active enzyme. A reasonable explanation for lack of collagenase immunoreactivity in bovine and rat tissues may be that these tissues do not have the kDa forms of collagenase that cross-react with the antibody. This is supported by negative results (not shown) from Western blots performed in our laboratory in which lysates from placental cells were tested for cross-reactivity with the collagenase antibody.

Attempts to localize 5-HT in both the fetal (cotyledon) and maternal (caruncle) tissues of the bovine placenta and in the postpartum fetal tissue were successful.

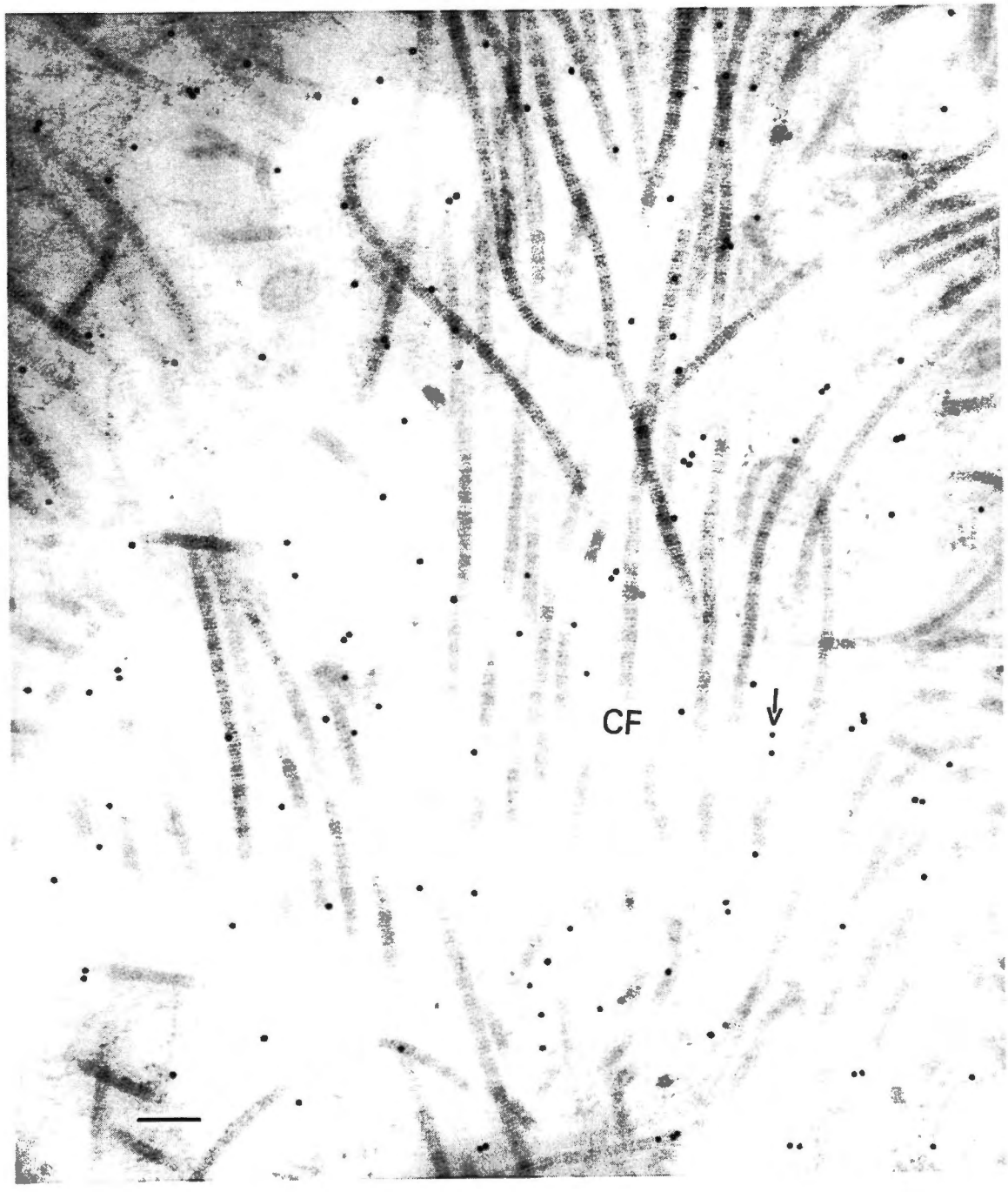



**Figure 5.2.** Postpartum cotyledon tissue. Colloidal gold particles (arrow) indicating presence of 5-HT are located on or near collagen fibers (CF). Bar = 0.158 μm.

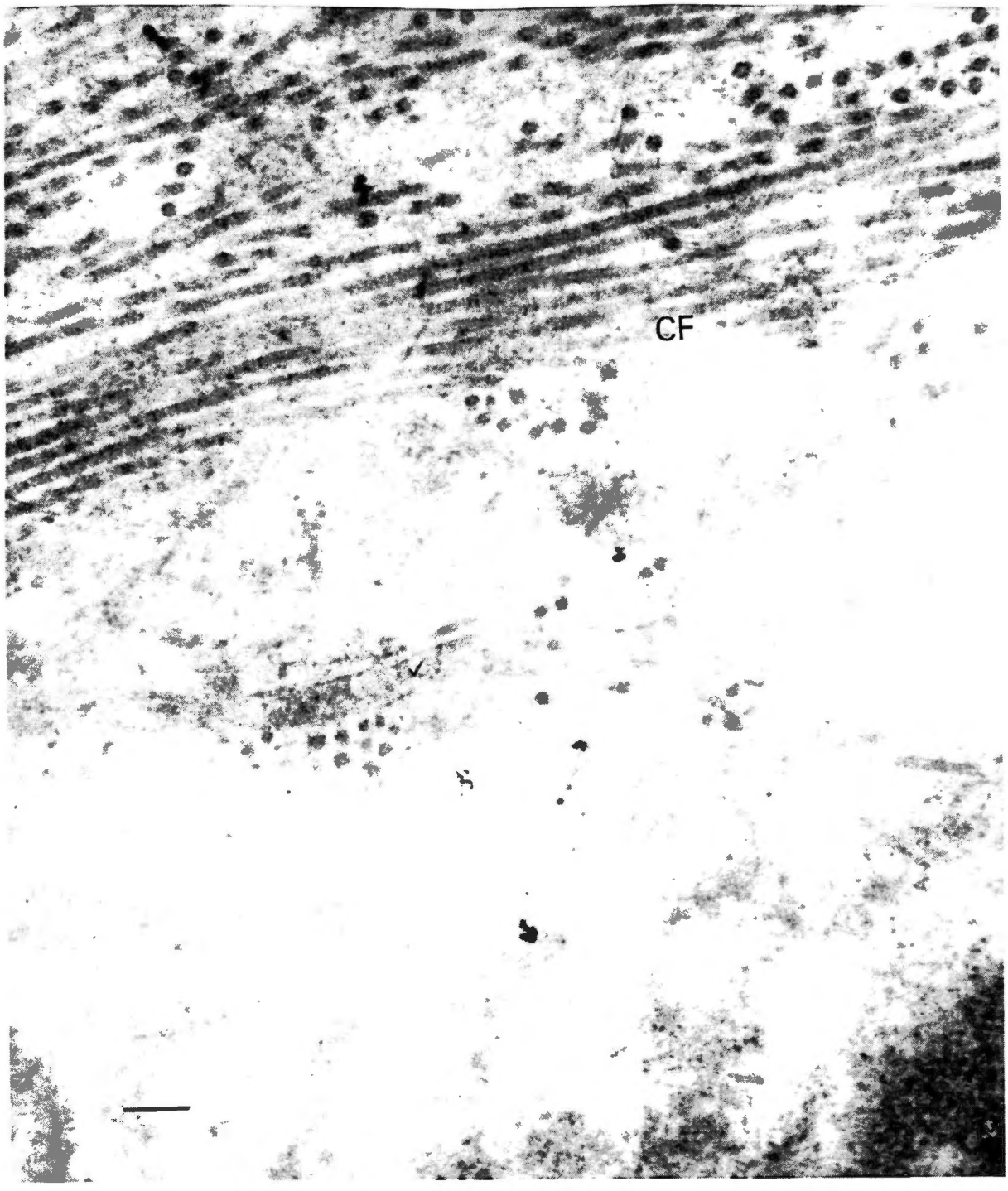



**Figure 5.3.** Postpartum cotyledon tissue. Negative for 5-HT, collagen fibers (CF) visible. Bar = 0.158 μm.

Positive staining for 5-HT was seen on the surface membranes of epithelial cells, in connective tissue and in blood vessels. Immunoreactivity of 5-HT in connective tissue was identified in postpartum cotyledon by both light microscopic methods and electron microscopic methods. For the latter, we used colloidal gold to localize 5-HT that was aligned either next to or on collagen fibers. The purpose of this alignment is not known. Kishi and Hayakawa (1989) reported localization of immunoreactive collagenase inhibitor protein along collagen fibers of bovine aortic medial tissue, suggesting that collagenase and its inhibitor could participate in control of collagen catabolism in bovine aorta. It is suspected that 5-HT is also involved in collagen catabolism. Passaretti et al. (1996) reported that 5-HT decreased the gene expression for collagen type I and III and fibronectin in rat uterine smooth muscle cells. In the placenta, it is expected that 5-HT would initiate its effect on collagen and/or collagenase at cell surface not directly on the collagen fiber. It is hypothesized that once collagen fibers are degraded, 5-HT may be released from the fiber to regulate collagen synthesis. This assumes that 5-HT is bound to collagen fibers, which is not known. At this time, there is no logical explanation for the alignment of 5-HT on the collagen fibers.

There appeared to be no change in 5-HT immunoreactivity from placentas during pregnancy to postpartum cotyledon, except surface epithelial cells on villi appeared to have 5-HT in their cytoplasm. It is interesting that cells underlying these epithelial cells did not appear to have 5-HT in their cytoplasm. One should keep in mind that technical artifact could have been introduced in the sampling of postpartum cotyledons, since the normal expelling of membranes often takes between 1 to 4 hours after calf's delivery. When possible, we tried to minimize time by cutting membranes close to the vulva instead of waiting for membranes to be expelled. Although viability of postmortem and excised placentomes is excellent, cell deterioration is expected.

Based on our hypothesis, it was thought that there could be weaker staining of 5-HT

in the postpartum cotyledon than in placenta during pregnancy. It appeared that this was not the case, however results are based on what can be seen, not quantitated. Based on other experiments that we performed using enzyme immunoassays for 5-HT, concentrations of 5-HT in fetal serum and tissues were significantly higher during pregnancy than during delivery or in neonatal calves (Fecteau and Eiler, 1998). In addition, MAO activity was reported to increase in human placenta at the time of delivery (Kirkel et al., 1992). Whether or not MAO activity increases in bovine placenta at delivery time is not known.

Sexton et al. (1996) reported that endothelial cells of human umbilical vessels are capable of synthesizing 5-HT. Immunoreactivity of 5-HT was observed in umbilical artery and vein from late pregnancy but not early pregnancy (Sexton et al., 1996), however, it is not known if endothelium of placental vessels synthesize 5-HT. Since 5-HT immunoreactivity was observed in blood vessel endothelium of mid- and full-gestation placentae and in postpartum cotyledon, it may be that 5-HT travels through the blood to the placentome and is not actually synthesized in the placentome.

To our knowledge, this is the first work in which 5-HT has been localized in the bovine placenta. It is unknown if 5-HT is synthesized by placental cells. We did not identify 5-HT in the cytoplasm of cells, which may suggest that 5-HT is not synthesized or stored in the cells. Furthermore, there was positive staining for 5-HT in the endothelium of blood vessels which may indicate that 5-HT has been transferred through the blood if it is indeed not synthesized in the endothelial cells.

# APPENDIX

**Procedure for Embedding Tissue with Paraffin**

80% ETOH for 2 hours

95% ETOH for 1.5 hours

95% ETOH for 1.5 hours

100% ETOH for 1 hour

100% ETOH for 1.5 hours

100% ETOH for 1.5 hours

50% ETOH/50% xylene for 1.5 hours

50% ETOH/50% xylene for 1 hour

100% xylene for 1 hour

100% xylene for 1 hour

Paraffin for 2 hours at 50°C

Paraffin for 2 hours at 50°C

**Procedure to Localize Collagenase or 5-HT**

1. Deparaffinize and rehydrate by series of xylene and graded alcohol changes.
2. Place in Phosphate buffered saline (PBS) for 5 minutes.
3. Quench endogenous peroxidase activity by addition of 3% hydrogen peroxide in 100% methanol and incubate for 20 minutes at room temperature (all incubations were in a humidified chamber).
4. Wash in PBS for 5 minutes.
5. Add 1% ammonium chloride ($NH_4Cl$) in PBS to slides and incubate for 20 minutes at room temperature.
6. Wash in PBS for 5 minutes.
7. Block nonspecific proteins with Power Block (BioGenex, San Ramon, CA) and incubate for 10 minutes at room temperature.

8. Rinse with PBS.
9. Add primary antibody rabbit anti-serotonin (Sigma, St. Louis, MO), mouse anti-rabbit collagenase (Developmental Studies Hybridoma Bank, University of Iowa) or negative control serum (rabbit serum or mouse serum) and incubate overnight at 4°C.
10. Wash slides 5 times for 5 minutes each with PBS.
11. Add secondary antibody (immunoglobulins for rabbit or mouse produced in goat) and incubate for 20 minutes at room temperature.
12. Wash as in step 10.
13. Add peroxidase-conjugated streptavidin and incubate for 20 minutes at room temperature.
14. Wash as in step 10.
15. Add chromogen diaminobenzidine/$H_2O_2$ (DAB) - prepare by adding 0.5 mL buffer to 4.5 mL distilled water, then add 4 drops of chromogen and mix well, then add 2 drops of hydrogen peroxide substrate and mix well.
16. Incubate at room temperature until color change seen (brown color) then rinse with PBS and tap water.
17. Add hematoxylin for 2 minutes at room temperature then rinse with tap water.
18. Dehydrate sections by processing through graded alcohol and xylene changes in reverse order of rehydrating procedure.
19. Mount coverslips with permount.
20. View and take pictures of slides using an Olympus microscope (Southern Micro Instruments, Inc., Atlanta, GA) and attached Olympus camera.

**Freezing Tissues for Immunolocalization of Collagenase**

1. Place enough tissue-freezing medium into plastic mold to cover bottom of mold.

2. Place tissue-piece in mold and cover tissue with freezing medium so that medium slightly exceeds mold.
3. Fill a glass Petri dish with liquid nitrogen ($LN_2$) and wait for boiling of $LN_2$ to stop.
4. Float mold with embedded tissue on the $LN_2$ in Petri dish. Do not let $LN_2$ make contact with medium in mold.
5. Freezing medium in mold becomes "milky" in color and the round "eye" closes toward the center.
6. When "eye" almost closed remove mold from Petri dish.
7. Place molds in freezer bags and store at -70°C.

**Immunolocalization of Collagenase in Frozen Tissue**

1. Fix tissue in acetone for 5 minutes.
2. Wash in PBS for 5 minutes.
3. Quench endogenous peroxidase activity by addition of 3% hydrogen peroxide in 100% methanol and incubate for 20 minutes at room temperature (all incubations were in a humidified chamber).
4. Wash in PBS for 5 minutes.
5. Add primary antibody mouse anti-rabbit collagenase undiluted or diluted 1:100 or normal mouse serum for control for 20 minutes.
6. Wash in PBS 5 times for 5 minutes.
7. Add secondary antibody (immunoglobulins for mouse produced in goat) and incubate for 20 minutes at room temperature.
8. Wash as in step 6.
9. Add peroxidase-conjugated streptavidin and incubate for 20 minutes at room temperature.
10. Wash as in step 6.

11. Add chromogen DAB - prepare by adding 0.5 mL buffer to 4.5 mL distilled water, then add 4 drops of chromogen and mix well, then add 2 drops of hydrogen peroxide substrate and mix well.
12. Incubate at room temperature until color change seen (brown color) then rinse with PBS and tap water.
13. Add hematoxylin for 2 minutes at room temperature then rinse with tap water.
14. Dehydrate sections by processing through graded alcohol and xylene changes
15. Mount coverslips with permount.

**Embedding Tissue in Plastic for Electron Microscopy Study**

1. Wash 3 times for 10 minutes each in distilled water.
2. Post-fix in 1% osmium tetroxide for 1-1.5 hours.
1. Wash as in step 1.
2. Place in 70% ETOH for 10 minutes.
3. Place in 80% ETOH for 10 minutes.
4. Place in 90% ETOH for 10 minutes.
5. Place in 100% ETOH for 10 minutes with 2 changes.
6. Place in propylene oxide for 10 minutes with 2 changes.
7. Place in 1 part plastic and 2 parts propylene oxide for 60 minutes.
8. Place in 2 parts plastic and 1 part propylene oxide overnight.
9. Remove, drain, and dry for 20 minutes.
10. Add plastic for 6-8 hours.
11. Embed tissues in molds and incubate for 48 hours at 45°C.
12. Remove from incubator and store for later study.

**Procedure for Staining Grids with Uranyl Acetate and Lead Citrate**

1. Place grids shiny side up on water droplets in a Petri dish for 5 minutes.

2. Blot shiny side on filter paper and transfer grids to drops of saturated uranyl acetate for 15 minutes.
3. Blot and rinse on a water drop.
4. Repeat step 2.
5. Put grids shiny side up on drops of lead citrate in Petri dish containing NaOH chips for 5 minutes. Put cover on Petri dish. Note: wear mask, otherwise $CO_2$ will form lead carbonate.
6. Repeat steps 3 and 4.
7. Blot dry and store in gelatin capsule.

**Immunolabeling of Grids**

Day 1

1. Place grids in filtered water drops on parafilm for 5 minutes.
2. Remove with inverted forceps and blot shiny side on filter paper.
3. Place grids for 20 minutes on drops of saturated sodium meta-periodate.
4. Transfer grids to water drop for 5 minutes.
5. Place grids into 96-well plate with dilution/washing buffer* for 10 minutes with 2 changes.
6. Add antibody to 96-well plate. Serotonin and collagenase antibodies diluted 1:50 with dilution/washing buffer. Cover plate with parafilm and place in humidified tray on shaker.

*Combine PBS (15 mL), NaCl (438 mg) and gelatin (15 mg) then dissolve and add bovine serum albumen (75 mg) and 1 drop of tween-20.

Day 2

1. Rinse grids twice for 10 minutes with buffer.
2. Add protein A labeled gold (Auroprobe EM protein AG10, Amersham Corporation, Arlington Heights, IL) diluted 1:50 with buffer. Incubate as in step 6.

Day 3

1. Rinse grids twice for 10 minutes in buffer.
2. Rinse twice for 10 minutes in PBS.
3. Rinse twice for 10 minutes in distilled water.
4. Fix in 2.5% glutaraldehyde/cacodylate buffer for 5 minutes.
5. Rinse twice in buffer for 10 minutes.
6. Rinse twice for 10 minutes in distilled water.
7. Blot dry on filter paper and place in capsule.

# PART VI

# GENERAL DISCUSSION AND CONCLUSIONS

## SUMMARY

The end of pregnancy is characterized by placenta detachment. The mechanism of placenta detachment is not clear, but appears to involve the activation of MMPs such as collagenase (Eiler and Hopkins, 1992; Maj and Kankofer, 1997). A substance that has the distinct ability to induce collagenase in postpartum cultured rat and human myometrial smooth muscle cells is 5-HT. The concentration of 5-HT in fetal bovine serum (FBS) was reported to be 30- to 50-fold higher than in newborn bovine serum (NBS) and cow serum (Jeffrey et al., 1991), however the purpose of such high fetal blood concentrations is not known. It has been suggested that 5-HT may be involved in fetal and/or placental growth (Balkovetz et al., 1989; Yavarone et al., 1993; Huang et al., 1998). Growth stimulatory effects of 5-HT were reported for various cell types (Nemecek et al., 1986; Seuwen and Pouyssegur, 1990), however its effect on placental cells is not known. Huang et al. (1998) localized 5-HT and its receptor in the human placenta but the source of 5-HT in the placenta was not clear. Yavarone et al. (1993) reported transplacental transfer of 5-HT from the mother to the fetus in the mouse. However, Prasad et al. (1996) stated that maternal 5-HT does not reach the fetal circulation in the later stages of pregnancy. The gastrointestinal tract may be the source for placental 5-HT, since approximately 95% of 5-HT found in the body is located in the enterochromaffin cells of the tract (Tyce, 1990), and these cells develop in the fetus as early as 8 weeks of gestation for the human (Facer et al., 1989) and 12 weeks in the bovine (Totzauer, 1991). Moreover, blood concentration of 5-HT decreases after intestinal resection. Therefore, we hypothesize that 5-HT from the bovine fetal intestine may act as a "proliferation factor" to support placental cell growth during pregnancy. Partial withdrawal (by decreased secretion and/or increased inactivation) of fetal blood 5-HT may be a cause for both (1) arrest of placental cell proliferation, and (2) activation of MMPs. Activation of MMPs may contribute to uterine regression and placenta

detachment at partum.

The general objective of this study was to test selected aspects of our hypothesis. The specific objectives of this study were to:

1) Determine the concentration profile of 5-HT in the intestine, placenta, muscle and blood of bovine fetuses at mid and full gestation and at delivery, and in newborn calves up to 72 hours old;
2) Assess growth stimulatory effect of 5-HT on bovine placental cells by direct cell counts, incorporation of $^{3}$H-thymidine into DNA, and a tetrazolium-based colorimetric proliferation assay;
3) Determine effect of 5-HT on MMP activity in bovine placental cells in culture, in isolated placentomes, and in the whole animal;
4) Localize 5-HT and collagenase in bovine placenta.

The concentration profile of 5-HT in blood showed high concentrations in the fetus during gestation with a dramatic decrease in concentration during delivery and staying low in the neonate. Tissue concentrations of 5-HT followed a pattern similar to blood with the following exceptions: intestinal 5-HT was high in the 72 hour old fetus, and muscle 5-HT was low in the mid-gestation fetus. These results indicate that there is a pattern of 5-HT concentration change in the fetus and newborn calf.

Results of the proliferation assays diverged. When cells were treated with 5-HT, there was an increase in incorporation of $^{3}$H-thymidine into DNA of the cells. However, there was no effect on direct cell count, and there was an inhibitory effect when tested in the tetrazolium-based proliferation assay. Conversion of tetrazolium to a colored formazan product is presumed accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Since addition of 5-HT decreased the amount of colored product formed in a dose-dependent manner, then it is suggested that 5-HT may be inhibitory of certain cellular metabolic pathways. The possibility of

metabolic inhibition by 5-HT is supported by the results of the MMP activity experiments.

There was no observed stimulation of MMPs in isolated placentomes when either infused or perfused with 5-HT, and there was no stimulation of MMPs in cows with induced retained placenta when injected with 5-HT. Perhaps 5-HT down-regulates genes that control MMP production or re-directs enzymes or other substances needed for the production of these proteases. However, Passaretti et al. (1996) reported that 5-HT up-regulates the interstitial collagenase gene and down-regulates the gene for type I collagen and other extracellular matrix proteins in rat uterine smooth muscle cells. Since the cells and species used in our experiments and those of Passaretti et al. (1996) are different, it is a possibility that they may respond differently to 5-HT.

There is no doubt that 5-HT exists in the placenta. Concentrations of 5-HT in the placenta were determined in humans over 30 years ago (Garattini and Valzelli, 1965). Recently, 5-HT was localized in human placenta (Huang et al., 1998), and now has been localized in bovine placenta (this work). There appear to be no marked changes in location of 5-HT in early or late gestation cotyledon and caruncle or in postpartum cotyledon. However, apparently there was 5-HT cytoplasmic staining insurface epithelial cells of postpartum cotyledon. It is possible that the cytoplasmic staining, in part, may be an artifact of tissue collection due to the time that it takes the cow to expel the placenta. In human placenta, Huang et al. (1998) located 5-HT in the cytoplasm of cells, however this was accomplished in early and late gestation placenta not postpartum placenta. The immunoreactivity of 5-HT in vessel endothelium of all of the stages of bovine placenta studied supports, along with no cytoplasmic immunoreactivity, that 5-HT is probably not produced in the placenta but instead is produced elsewhere and travels through the blood to the placenta. Lack of collagenase immunolocalization in the placenta was probably the result of inadequate antibody for the MMPs in the tissue.

The conclusions of this research are: (1) there is a pattern in 5-HT concentration in fetal and neonatal blood and tissues; (2) 5-HT may be a proliferation factor for bovine placental cells; (3) 5-HT does not stimulate MMP activity in bovine placental tissues; and (4) 5-HT is visually present in the bovine placenta during pregnancy and postpartum. These conclusions support our general hypothesis. However, many aspects of the hypothesis have yet to be worked out. The physiological effect of 5-HT during pregnancy and postpartum has yet to be determined, but the results of this work combined with the supporting literature suggest that 5-HT has a definite role(s) during pregnancy and postpartum. It is hoped that there will be continued as well as new interest in this subject.

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