To the Graduate Council:

I am submitting herewith a dissertation written by Gary Wayne Randall entitled "Prolactin-Enhanced Uteroglobin Synthesis and Secretion by Uteri of Estrogen and Progesterone Treated Juvenile Rabbits." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

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PROLACTIN-ENHANCED UTEROGLOBIN SYNTHESIS AND SECRETION BY UTERI OF ESTROGEN AND PROGESTERONE TREATED JUVENILE RABBITS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Gary W. Randall

December 1986

ACKNOWLEDGEMENTS

It has been a great pleasure to be able to work with the individuals associated with this research project. I am particularly grateful to Dr. Pat Coulson for allowing the use of her microspectrophotometer since the sample volumes involved in this study were very small. I appreciate Prof. M.L. Pan's analytical assistance as well as technical guidance involving the immunological techniques used in this study. Prof. Alex Shivers also provided ideas about immunological techniques and a work place for the completion of this project. I further express my gratitude to Dr. Beverly Chilton for her assistance in gel electrophoresis. I appreciate Dr. Jeff MacCabe and Dr. John Koontz serving as committee members. I am eternally grateful and appreciative of my mentor, Dean Joseph Daniel, Jr., without whose guidance and encouragement, this project could not have been completed. I must express my appreciation to my wife Robyn R. Large-Randall, whose love kept my heart in the right place.

Support for this project was provided as part of an overall project funded by NIH Grant Number 1 R01 HD 20271-03, Joseph C. Daniel, Jr., principal investigator. This support is greatly appreciated.

ABSTRACT

Juvenile (22 day old) and neonatal (nine day old) female rabbits were exposed to a nine day injection protocol. Each day, the animals were either sham (carrier only) injected or received a subcutaneous injection of estradiol (either 10 or 100 micrograms/kg/24 hr^{-1}) for the first two days, ovine prolactin (14 I.U./kg/24 hr^{-1}) for the following three days and progesterone (3 mg/kg/24 hr^{-1}) for the remaining four days of the nine day injection period. Eleven treatment groups existed for each age group. Juvenile animals, which received the 100 micrograms estradiol/kg/24 hr^{-1}, sham, progesterone protocol or the 100 microgram estradiol/kg/24 hr^{-1}, ovine prolactin, progesterone protocol, produced detectable amounts of uteroglobin (UG) in their uterine secretions (0.034 $\pm$ 0.030 mg UG and 0.098 $\pm$ 0.023 mg UG/mg total protein, respectively). Verification of *de novo* uterine UG synthesis was verified by ^{35}S-methionine incorporation, *in vitro*, into UG, at a significant level relative to total protein incorporation of radiolabel.

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I. INTRODUCTION

In mammals, following ovulation and subsequent fertilization of the ovum, a suitable environment must be available for normal embryonic development. Prior to implantation, this environment is provided by the reproductive tract secretions, including those of the oviduct and uterus, which make accessible to the embryo certain essential nutritional and biochemical components.

The early embryo is contained within a covering, the zona pellucida. At the blastocyst stage, the growing embryo must escape the zona, and any other embryonic coverings that might exist (such as the mucin coat in rabbits), for nidation and subsequent placentation for exchange with circulating maternal factors. Reproductive tract secretions, including oviducal and uterine proteins, change in the types and relative concentrations from the time of ovulation, until blastocyst expansion and implantation (Daniel, 1968; Johnson, 1972; Murray and Daniel, 1973; Beier, 1974; Katzenellenbogen and Greger, 1974; Daniel, 1976; Kirchner, 1976a,b and 1980; Guilbert-Blanchette and Lambert, 1978; Katzenellenbogen, et al., 1980; Manak, et al., 1980; Roberts and Bazer, 1980; Bazer, et al., 1981).

The secretory protein profile of uterine luminal fluid from the time of fertilization until implantation has been characterized in several species, primarily the domestic rabbit (Oryctolagus cuniculus). Krishnan and Daniel, (1967), and Beier, (1968), have established that a variety of proteins are synthesized and secreted into the uterine lumen during this time, of which the best characterized is uteroglobin (UG).

Rabbit Uterine Fluid Proteins

Uterine protein synthesis and secretion is necessary for the survival of preimplantation embryos of several species. In the rabbit (Krishnan, 1971) and the swine, immunological inactivation of uterine proteins results in abnormal embryonic development and decreased reproductive success (Daniel, 1972).

Estrus rabbit uterine fluid contains proteins which are similar to those found in blood serum and oviducal fluid with albumin comprising the major fraction; however, transferrin and alpha-macroglobulin are found in significant amounts as well (Beier and Beier-Hellwig, 1973). The albumin fraction of estrus uterine fluid has a different electrophoretic mobility than serum albumin which may be attributed to bonding of fatty acids (Cowan and Daniel, 1972). The presence of these proteins in the estrus uterus are attributed to transudation from plasma. During early pregnancy, pseudopregnancy or after progesterone (P) administration to ovariectomized (OVX) does, changes occur in the type and concentrations of pregnancy-specific uterine luminal proteins. Daniel, (1976), has described several rabbit uterine proteins including UG, a pre-albumin fraction which migrates ahead of albumin in polyacrylamide slab gel electrophoresis, a group of non-specific esterases (initially demonstrated by Tyndall and Daniel, 1975) comprising up to ten separate proteins by day three post coitum (p.c.), a reverse transcriptase fraction reaching maximal concentration by day four p.c. (Yang, et al., 1976 and Chilton and Daniel, 1976), a protein fraction which binds 67-Gallium, reaching maximal concentration by day three p.c. and a crystalloid inclusion body protein fraction which

reaches peak concentrations by day six p.c. (Tyndall, et al., 1976). A macroglobulin fraction exists in rabbit uterine fluid, which is composed of a mixture of at least six proteins with molecular weights greater than albumin (Dunbar and Daniel, 1979). Also from rabbit uterine fluid, an endometrial arylamidase I fraction (leucine amino-peptidase) and a broad beta-glycoprotein fraction which comprises 35 percent of the total protein by day seven p.c. (Denker, 1980; Kirchner, 1980). These uterine, pregnancy-specific proteins are characterized by temporal changes in their concentrations and have been proposed to be related to specific requirements of the preimplantation embryo (Beier, 1970; Daniel, 1971; Lutwak-Mann, 1971). Evidence has been presented for an embryotoxic component in the low molecular weight fraction (< 500 daltons) of pregnancy rabbit uterine fluid (Guilbert-Blanchette and Lambert, 1978). Of particular interest to the present discussion is UG, which comprises 50-80 percent of the total protein concentration in uterine luminal fluid by day five or six p.c. (Barfield, et al., 1976; Daniel and Booher, 1977; Dunbar and Daniel, 1979; Kirchner, 1980; Chilton and Daniel, 1984).

UG was first reported by Schwick, et al., (1965), and referred to as B_1-U-globulin. Krishnan and Daniel described it in 1967 and called it "blastokinin" because it was found to promote <u>in vitro</u> growth and development of blastocyst-stage rabbit embryos. Beier described it in 1968 and called it "uteroglobin", which was generally believed to be more descriptive of its location and structure. Immunohistological localization of UG in rabbit endometrial epithelium has been demonstrated (Kirchner, 1972, 1976 and 1979; Aumuller, et al., 1985;

Hegele-Hartung and Beier, 1985). However, it was later shown to exist in other tissues of the rabbit and in other species.

UG in Other Tissues and Organs of the Rabbit

UG-like antigens, which cross react with monospecific antisera preparations against purified UG from the rabbit uterus, have been found associated with both reproductive and nonreproductive tissues of the rabbit. Uterine and oviducal fluids and the female reproductive tract tissues, contain UG-like antigens (Torkkeli, et al., 1977). The uterine epithelium, myometrium and endometrium contain UG-like antigens (Daniel and Milazzo, 1976; Torkkeli, et al., 1977). The myometrium, vagina, and ovary have also been shown to contain cross-reacting antigens to rabbit uterine anti-UG antisera preparations (Noske and Feigelson, 1976). UG has been localized in the oviduct as well (Kirchner, 1976). Male rabbit reproductive tract components found to contain these antigens include the cauda epididymis, ductus deferens, seminal vesicle and seminal plasma (Noske and Feigelson, 1976; Noske and Gooding, 1978). Minimal UG-like antigenicity has been shown in the epididymis (Torkkeli, et al., 1977). Immunocytochemical techniques have localized UG-like antigens in the epididymal epithelium of caput and proximal corpus regions; however the ductus deferens, prostate and seminal vesicle were tested negative (El Etreby, et al., 1983). Nonreproductive tract components of the rabbit, which contain UG-like antigens include the lung, lung lavage, trachea, tracheal flushings, esophagus, esophageal flushings and jejunal flushings (Noske and Feigelson, 1976). These antigens exist in the urinary bladder and thyroid gland (Noske and Gooding, 1978). Rabbit

lung UG has been purified and shown to be indistinguishable from uterine UG (Beato and Beier, 1978).

UG in Species Other Than the Rabbit

UG-like antigens have also been demonstrated in tissues and fluids of other species. In the order Lagomorpha, the Hare (Lepus capensis) and Pika (Ochotona rufescens) have been found to contain these antigens (Nieto and Lombardero, 1982; Lopez de Haro and Nieto, 1983; Lopez de Haro, et al., 1984). A UG-like antigen exists in the Northern Fur Seal (Callorhinus ursinus) (Daniel, 1972). UG-like antigens were demonstrated in uterine flushings from 20-day pregnant mink and eight-day pregnant dogs (Daniel and Krishnan, 1969). There is significant amino acid sequence homology between the C_1 and C_2 polypeptide chains of rat prostatic steroid binding protein and rabbit uterine UG (Baker, 1983). UG immunoreactivity exists in rat ventral prostate (Aumuller, et al., 1985).

UG-like antigens have been shown in reproductive tract components and body fluids of the human. UG-like antigens have been found in the human uterus and in human seminal plasma (Daniel, 1973; Noske and Feigelson, 1976). These antigens exist in mid-secretory phase human endometrium (Shirai, et al., 1972). Cross-reacting antigens have been detected in human uterine washings by radioimmunoassay (Cowan, et al., 1986). UG-like antigenicity has been shown in tracheal aspirates from human neonates (Dhanireddy, et al., 1985).

Several other species secrete a progesterone-dependent uterine protein fraction prior to implantation. For example, the purple protein (uteroferrin), secreted by the pig uterus is known to bind iron and is

responsible for iron transport between the maternal and embryonic compartments (Bazer, et al., 1981). This protein probably has only temporal coincidence in common with UG (Daniel, 1976). Immunoreactivity to anti-UG antiserum has been demonstrated in uterine fluids of women (Johnson, et al., 1972; Shirai, et al., 1972; Daniel, 1973; Cowan, et al., 1986).

Biochemical Properties of UG

UG is a globular protein (Nieto, et al., 1977; Ponstingl, et al., 1978; Popp, et al., 1978; Menne, et al., 1982). UG formed in rabbit uterine fluid under conditions of pregnancy, pseudopregnancy (induced by cervical stimulation), exogenous P administration and hCG administration has been characterized (Garcea, et al., 1974). The authors have concluded that the same protein is produced in each case. It is composed of two identical polypeptide chains, each comprised of 70 amino acids, connected by two disulfide bridges and noncovalent interactions (McGaughey and Murray, 1972; Beato, et al., 1980). Two crystalline forms, a triclinic and an orthorhombic have been reported (Mornon, et al., 1978 and 1979). The molecular weight of UG is approximately 15,000 daltons as determined by equilibrium sedimentation, meniscus ultracentrifugation depletion and gel filtration techniques (Murray, et al., 1972; Bullock and Connell, 1973). The amino acid sequence has been established, which revealed the location of cystine residues at positions three and 69 and a single tyrosine at position 21 (McGaughey and Murray, 1972; Popp, et al., 1978; Beato, et al., 1980). UG migrates to the postalbumin region in nondenaturing polyacrylamide gels. Estimation of the isoelectric point of UG has been established to be

5.37 by electrofocusing experiments on polyacrylamide gels (McGaughey and Murray, 1972). These investigators have also shown UG to be carbohydrate poor and have an electrophoretic mobility of Rf = 0.68 in 6 percent acrylamide. The sedimentation coefficient of UG is 1.38 Svedberg units (Beier and Beier-Hellwig, 1973).

Proposed Functions of UG

Several workers have proposed possible functions of UG. These include UG acting as a sequestering agent for P in the uterus, a necessary component for the growth and development of morulae and blastocysts, a histotrophe, an immune blocking agent, and as a protease inhibitor.

Progesterone Binding to UG

An interesting characteristic of UG is that the inducing steroid, progesterone, specifically binds to it (Urzua, et al., 1970; Arthur, et al., 1972; Beato and Beier, 1975; Fridlansky and Milgrom, 1976; Beato, et al., 1980). The dimeric form of UG only weakly binds P whereas the reduced form of UG has a significant affinity for P and, to a lesser degree, for estrogens (Beato, et al., 1980). A correlation between the progestational activities of certain steroids (other than P), and their potential for binding UG exists (Beato, 1976). Certain substituted diphenyl ketones inhibit P-binding to UG in a dose dependent manner (Saxena, et al., 1983). A structural characteristic of the UG molecule includes a hydrophobic pocket which is thought to accommodate the P molecule (Mornon, et al., 1980). Two UG monomers bind to each P molecule (McGaughey and Murray, 1972). Modification of the histidine at

position eight of UG with diethylpyrocarbonate, decreases the P-binding activity without significantly altering the secondary structure of the protein (Saavedra and Beato, 1980). These findings spurred interest in a P binding function for UG. Evidence has since been presented, that the P-binding form of UG is similar in structure to myosin alkali light chain (Baker, 1985).

Studies directed toward a role for UG in sequestering P in the embryo have led to the hypothesis that P enhances embryogenesis; however, UG may function to decrease P availability to the early embryo. High P concentrations such as exist during early pregnancy may inhibit cleavage (Whitten, 1957; Daniel and Levy, 1964; Pratt and Daniel, 1967; El-Banna and Daniel, 1972). During the preimplantation period of rabbit pregnancy, the presence of UG in uterine fluid significantly enhances P accumulation (Bochskanl and Kirchner, 1981). UG may function to provide P to the early embryo in a nontoxic form (Arthur and Chang, 1974).

Growth and Development of the Early Embryo

The relative amount of UG to total protein secretion during the first ten days of pregnancy is shown in Figure 1. UG increases in concentration thru day five or six p.c. in rabbit uterine fluid. A corresponding increase in P concentration also occurs in rabbit uterine fluid through day six p.c. (Cowan, et al., 1976). During this same time, the embryo develops to the blastocyst stage and a few days later to a highly expanding blastocyst before the decline of UG in the uterine luminal fluid. A relationship exists between the presence of UG and subsequent growth of morulae and blastocysts (El-Banna and Daniel, 1972). In evidence of growth was an increase in RNA synthesis as

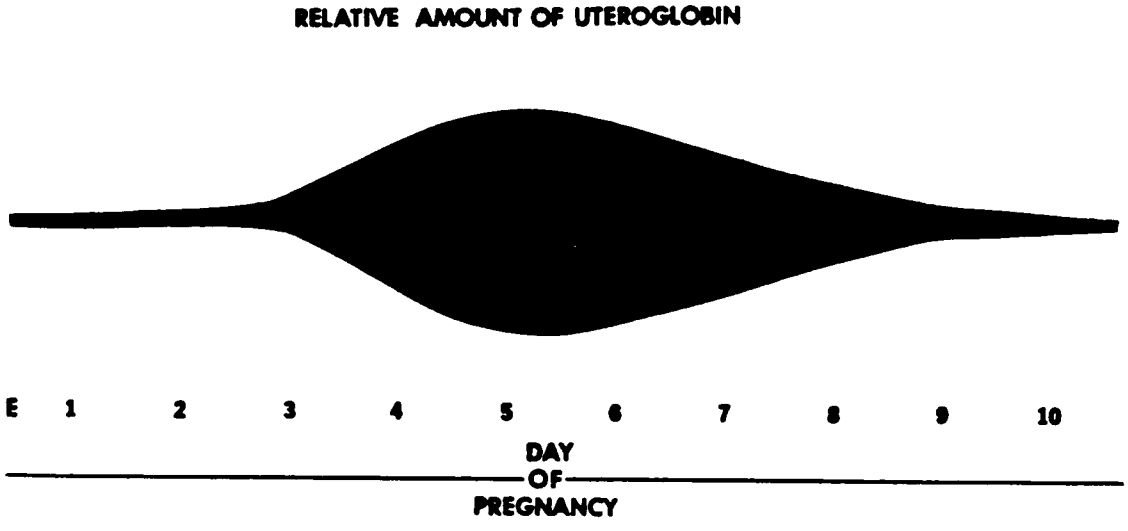


Figure 1: The relative amount of UG to total protein, secreted by pseudopregnant rabbit uteri. See the text for details.

evidenced by ^{3}H-uridine uptake followed by an increased protein synthesis characterized by increased ^{3}H amino acid uptake. The greatest rates of RNA and protein synthesis occurred when UG was in the presence of P.

UG as a Histotrophe

Eutherian mammals produce ova which have a limited supply of nutritional components (Wales and Pike, 1977). Thus embryos produced by fertilization of these ova are essentially dependent upon exogenous sources of energy substrates for continued development throughout their preimplantation stages. During this time, these substances are provided by secretions of the oviduct and uterus. Early mouse embryos synthesize and store glycogen for energy substrate (Thomson and Brinster, 1966; Stern and Biggers, 1968). Exogenous metabolic substrates are necessary for preimplantation rabbit embryos in vitro (Daniel, 1967; Biggers and Stern, 1973; Van Blerkom and Manes, 1974). This process is probably representative of mammals in general.

During the preimplantation period, rabbit uterine fluid consists primarily of secretions from the glandular and nonciliated cavum endometrial epithelium (Beier, 1974; Hegele-Hartung and Beier, 1985). In other mammals, the uterine fluid has been referred to as uterine milk (Squire, et al., 1972). Components of this fluid are thought to support certain anabolic processes of the developing embryo. Uterine-specific proteins comprise the majority of components of uterine fluid in mammals during the preimplantation period (Shirai, et al., 1972; Renfree, 1971). UG may contribute in some way to the metabolic substrates necessary for

these early embryos but no evidence as yet has been presented to support this hypothesis of a nutritional role.

UG as an Immune Blocking Agent

The transplantation of tissues from one individual to another of the same species but of different genotype constitutes an allogenic graft. These types of grafts are generally rejected by the immune system of the recipient because certain components of the graft are recognized as foreign or antigenic. At least one exception to this rule of allograft rejection exists. The reproductive tracts of female mammals are exposed to sperm and to embryos which have genetically expressed antigenic components on their membranes which reflect the paternal genome. The maternal immune system does not normally recognize these surface antigens of sperm and embryos and allows for successful pregnancy to result. UG may be involved in masking of surface antigens of sperm and embryos (Mukherjee, et al., 1980; a report from the NIH, 1981; Mukherjee, et al., 1982; Mukherjee, et al., 1983; Manjunath, et al., 1984). These workers have presented a hypothesis which states that beta-2-microglobulin, which is present on the surface of sperm and embryos, reacts with UG in the presence of transglutaminase and results in cross-linking of beta-2-microglobulin and UG in such a way that cell surface antigens are masked. This is thought to prevent maternal lymphocytic recognition of sperm and embryonic antigens, thereby allowing fertilization and normal embryonic development to ensue without immune rejection.

UG as Protease Inhibitor

Although UG is not the only uterine protein of significance regarding embryonic development in the rabbit, its importance has been clearly demonstrated (Krishnan, 1971; Johnson, 1974). Thus inactivation of UG by monospecific anti-UG antiserum *in vivo* inhibits implantation. UG specifically inhibits thrombin-mediated, blood platelet aggregation in rabbits and humans (Levin, et al., 1985). UG may exert its effects thru inhibition of phosphorylase A_2. In this context, the protease-inhibiting activity of the UG fraction of uterine fluid is especially interesting.

A necessary step in early embryogenesis involves the escape of the expanding blastocyst from its coverings prior to implantation. Changes in the structure of these coverings, in particular the zona pellucida and mucoprotein layers, must occur in order for the developing blastocyst to escape and make contact with the endometrium. It is at this time that the uterine fluid UG concentration has begun to decline.

Protease inhibitors are important biochemical regulatory substances which are necessary for controlling the rates of several physiological chain reactions including complement activation, glycoprotein hormone biosynthesis, blood coagulation, fibrinolysis and other cascade reactions involving amplification of relatively small stimuli into maximal physiological responses. Evidence for a protease inhibitory role of UG includes an observation commonly seen in the *in vitro* culture of rabbit embryos. Culture media containing bovine serum albumin as the only protein source often yields embryos which appear to grow and develop normally but herniate when attempting to escape their embryonic

coverings (Beier, et al., 1980). These embryos are characterized by a rigid mucin coat and thickened zona pellucida on day four of development whereas embryos cultured in vivo show significant thinning of the embryonic coverings. Thus UG may be involved in these processes during the preimplantation period. The declining concentration of UG in uterine fluid after day five or six p.c., may allow certain proteases to react with the macromolecular structure of embryonic coverings in such a way as to allow subsequent embryonic escape after thinning or disolution.

Control of UG Synthesis and Secretion in the Rabbit

The fact that steady state concentrations of uterine protein secretions are under the control of ovarian steroids is well established. This is evidenced by the effects of ovariectomy on preimplantation uterine secretion. The rise in uterine luminal UG content thru day five p.c. is due to the inducing action of serum P secreted by the corpus luteum. It can also result from exogenous P, or ACTH administration (Rahman, et al., 1975). Further stimulation by P during early pregnancy beyond day five or six p.c. does not maintain this rise in UG concentration since an elevated serum estrogen titer occurring at this time antagonizes the action of P (Bullock and Willen, 1974; Browning, et al., 1980; Shen, et al., 1983). A similar inhibitory effect on UG secretion results from exogenous estrogen administration during pregnancy, pseudopregnancy or other modes of P-stimulated UG secretion (Barfield, et al., 1976). Although estrogen administration following P stimulation causes a decline in UG synthesis and secretion, low dosage estrogen priming in adult animals prior to P stimulation,

enhances the UG secretory response (Urzua, et al., 1970; Heins and Beato, 1981). The mechanism of action of this estrogen priming effect is thought to be thru increased P receptor synthesis (Baulieu, et al., 1975; Chan and O'Malley, 1976; Janne, et al., 1978; Neulen, et al., 1981; Rahman, et al., 1981). Lower priming doses of estrogen potentiate P-induced UG secretion in younger animals and adults, respectively, whereas larger dosages of estrogen suppress UG secretion in response to P (Hemminki, et al., 1980; Kopu, et al., 1981). Estrogens have also been shown by these workers to potentiate the UG secretory effects of lower P dosages.

Although detectable levels of UG exist in estrus glandular endometrium under the influence of estrogens, P is primarily responsible for the increase in UG synthesis and secretion during pregnancy and pseudopregnancy (Cato and Beato, 1985). The synthesis and secretion of UG has been induced in primary cultures of endometrial cells, in endometrial explants and in isolated uteri in vitro (Whitson and Murray, 1974; Beato and Arnemann, 1975; Joshi and Ebert, 1976; Garcea, et al., 1976; Nieto and Beato, 1980; Kao and Bullock, 1982; Rajkumar, et al., 1983 a,b; Saxena and Sahib, 1984). The concentration of UG in rabbit uterine fluid increases from day three thru days five and six p.c., declining thereafter and reaching nondetectable levels by day ten (Krishnan and Daniel, 1967; Urzua, et al., 1970; Johnson, 1972; Bullock and Connell, 1973; Mayol and Longenecker, 1974; Daniel, 1976; Barfield, et al., 1976; Rahman, et al., 1982). This same profile of temporally increased secretion may be obtained thru injections of P; however, in immature and OVX animals, further stimulation by P beyond this time does

not result in corresponding increases in UG secretion (Rahman, et al., 1981; Kumar and Bullock, 1982). The lack of UG responsiveness to P in young rabbits may be a selective and inherent effect of P (Isotalo, 1983).

UG synthesis and secretion have also been induced in rabbit uteri by synthetic steroids. The synthetic steroid R2323 has been shown to be weakly progestomimetic, since the amount of UG found in uterine fluid following treatment of OVX rabbits was only a fraction of that in progesterone-treated controls or normal pregnant animals (Daniel and Booher, 1975). Rabbits were exposed to a group of synthetic steroids containing a C-19 methyl group, which enhanced the uterine-UG secretory response (Arthur and Chang, 1974). Investigation of the effects of certain synthetic progestins containing a C-19 methyl group, on UG secretion by rabbit uteri have been performed (Chilton, et al., 1977). All of the progestins investigated were effective; however, a broad range of responses was observed. The progestin responsible for the highest levels of UG secretion possessed a C-6 methyl group as well as the C-19 methyl.

A mechanism of action for P-induced UG synthesis and secretion is the same as for other steroid-induced protein synthesizing systems (Loosefelt, et al., 1981; Kumar, et al., 1982; Suske, et al., 1983). Thus P is made available to the general circulation by the corpus luteum of early pregnancy and is available to all tissues including the endometrium. P diffuses into target tissues and binds to specific cytoplasmic receptors. Estrogen is known to affect the population of cytoplasmic P receptors. The hormone receptor complex is subsequently

translocated to the nucleoplasm where interactions with DNA in the nucleus results in derepression of the UG genome. This allows transcription of the UG gene to occur and the production of UG mRNAs. The UG mRNA content of rabbit uterine endometria increases in response to exogenous P administration and to pregnancy (Atger and Milgrom, 1977). These mRNAs move to the cytoplasm and bind to polysomes of the granular endoplasmic reticulum (Nieto, 1985). Subsequent translational events yield the preuteroglobin (preUG) molecule, first described in the domestic rabbit (Bullock, et al., 1976). PreUG contains about 20 additional amino acids in the Hare (Lepus capensis) (Lopez de Haro, et al., 1984). It is later cleaved to yield UG prior to its secretion.

The control step in the synthesis of UG is at the level of transcriptional processing of the UG gene and only a single copy of the gene exists in the haploid genome of the rabbit (Murray and Whitson, 1974; Kumar, et al., 1982). Conflicting reports state that several copies of the UG gene may exist (Muller and Beato, 1980; Beato, et al., 1980). The UG gene has been isolated and sequenced (Lopez de Haro and Nieto, 1985). A structure has been proposed for the UG gene which consists of three kilobases and is composed of three exons separated by two intervening sequences (Menne, et al., 1982). The subsequently transcribed mRNA has been shown to be 465 bases in length of which 270 bases are known to code for the UG molecule. Translation of this mRNA yields a protein (preuteroglobin) which contains a signal peptide at the amino terminus of UG containing 21 amino acids (Snead, et al., 1981). Preuteroglobin mRNA levels increase in rabbit endometrial cells

following a single injection of either E_2 or P; however the P-effect is ten-fold that of E_2 alone (Heins and Beato, 1981).

It should also be noted that UG synthesis and secretion may be induced by corticosteroids in the lung, where a functionally conceivable role for UG binding to P has yet to be established (Lombardero and Nieto, 1981; Fernandez-Renau, et al., 1984). The primary structure of rabbit lung UG is identical to the uterine protein (Lopez de Haro and Nieto, 1985). Since the respiratory system is exposed to various antigenic substances, UG may function in an immune-blocking role in the lungs.

Other factors have been shown to significantly affect the uterine production of UG in rabbit uteri. Light deprivation thru day four, p.c. is related to changes in uterine protein synthesis and UG production (Daniel, 1980 a,d). Animals which were deprived of light for two to four days p.c. were characterized by the secretion of 10-17 percent more UG than controls by day five p.c. This alteration in specific uterine protein synthesis may, in part, reflect the mechanism thru which light deprivation reduces reproductive success.

Seasonal variation exists in rabbit reproductive tract responsiveness to ovarian steroids. Significant differences exist in UG production in the winter and spring by long-term ovariectomized rabbits treated with P plus prolactin (Daniel, et al., 1987). Winter animals were more responsive. Estrogen receptor affinity for ligand in uteri of rabbits was modified by seasonal effects (Stern and Vaughan, 1979, 1987). The Ka of uterine estrogen receptors was lowest in winter months, remaining at constant levels during summer and fall. These results may

in part, provide a biochemical explanation for the seasonal variation noted in rabbit reproductive physiology. The mechanism of action by which these changes in reproductive parameters are altered by seasonal factors, may involve photoperiodic modulation of the hypothalamo-pituitary-ovarian axis.

Rabbits require high protein content in their diet in order to maintain normal pregnancy. Rabbits deprived of dietary protein produce an excessively high number of abnormally-developing embryos, low levels of total protein and decreased UG and high molecular weight protein fractions, relative to controls (Daniel, 1980 b,d). Continued starvation results in depressed reproductive behavior.

Rabbits may live as long as 13 years (Altman and Dittmer, 1972). They are reproductively active for up to six years of age (Adams, 1970). The average life span of a healthy rabbit is about seven to eight years of age (Weisbroth, 1974). Reproductive success declines quickly after two years. Daniel, (1980 c,d), was unable to demonstrate significant differences between total uterine secretory protein and UG production in younger (6-18 months) as compared to older (24-42 months) infertile female rabbits. The authors suggest that the "uterine failure" associated with infertility of older animals occurs following implantation.

Prl and UG Secretion

Prolactin (Prl) is an ubiquitous hormone initially found responsible for milk secretion by the pigeon crop sac (Riddle, et al., 1933). Even though an extensive literature exists regarding the role of Prl in the reproductive processes of a variety of target tissues and

species, only recently has it been shown to exist as a separate entity from growth hormone in humans (Frantz and Kleinberg, 1970; Frantz, et al., 1972). Prl promotes the growth and differentiation of mammary gland ductal and alveolar epithelium (Guyton, 1976). This tissue-specific responsiveness of the mammary gland to Prl is characterized by increased RNA and protein synthesis with resultant intracellular accumulation of specific enzymes and milk proteins (Topper, 1970; Turkington, et al., 1973). A role for Prl in mammalian ovarian function has been proposed (Dorfman, 1972). It prolongs the life of the corpus luteum in certain rodents, presumably by effecting an increased cholesterol biosynthesis that provides substrate for P production (Armstrong, 1968; Armstrong, et al., 1969). These ovarian effects are species specific. Prolactin has been shown to affect ovarian progesterone biosynthesis in rabbits as well (Ensor, 1978; Hilliard, et al., 1969).

Other roles for Prl include the amphibian behavioral water drive response (Chadwick, 1940; Grant, 1961; Turner and Bagnara, 1976). Amphibians such as Notopthalamus and Triturus are examples of Urodeles which return to an aquatic existence following a Prl-induced metamorphosis of body structure (Goodman and Gilman, 1975). Prl affects its target tissues in these amphibians in such a way that growth of a tail fin and other aquatic adaptations result (Ensor, 1978).

Thus Prl is not a phylogenetically young hormone and may be responsible for other endocrine phenomena heretofore unexplained. Of particular interest to this investigation is the role of Prl in the P induction of rabbit uterine secretory activity.

Prl is known to affect the uteri of mammals. Specific binding of Prl to mammalian tissues has been demonstrated in a variety of species (Posner, et al., 1974). Specific as well as high nonspecific binding of ^{125}I-iodo-ovine Prl to rat uterine tissues has been shown (Williams, et al., 1978). Prl also has been shown to have indirect actions on uterine functions through the actions of ovarian steroids (Kennedy and Armstrong, 1972; Leung and Sasaki, 1973). More recent studies have revealed a role for Prl in the induction of estrogen receptors and DNA synthesis in the rabbit (Chilton and Daniel, 1984). Prl has also been proposed to induce P receptor synthesis in rabbit uterine endometrium (Chilton and Daniel, 1986). Thus, it may indirectly participate in the regulation of uterine endometrial secretory activity by preparing the endometrium for a progestational state and function in a synergistic fashion with P.

Models Designed to Investigate Rabbit Uterine Secretion

It was initially discovered that the long-term ovariectomized (LTOVX) rabbit (i.e. >12 weeks) showed little or no response to exogenous P administration whereas rabbits treated four weeks following ovariectomy were still capable of P induction of UG synthesis and secretion. An endometriotrophic factor exists in brain extracts that, when administered to LTOVX adult rabbits, results in reactivation of the uterine secretory response to P (Daniel, 1981). Exogenous oPrl, in conjunction with P administration, restores the ability to synthesize and secrete UG in the LTOVX rabbit (Daniel, et al., 1984). Since the LTOVX model requires the use of adult animals, certain endocrine parameters will inevitably vary. These animals have previously been

exposed to adult levels of ovarian steroids as well as other hormones. Although the primary source of the ovarian hormones has been eliminated in these rabbits, prior exposure to adult levels of ovarian steroids, as well as other hormones, plus those produced by the adrenal gland, may contribute to the variability in steady state levels of uterine secretory components.

Purpose of the Present Study

The present study is an attempt to elucidate the series of events involved in the interaction between the adenohypophyseal hormone, Prl, and the secretory activity of the progestational rabbit uterus. Quantitation of UG in uterine flushings, following the administration of the hormones 17B estradiol, ovine prolactin and progesterone was used to represent uterine secretory responsiveness. Previous experimentation using LTOVX rabbits, yielded evidence that Prl was an important factor for P-induced UG synthesis and secretion (Daniel, et al., 1984). Prl causes an increase in cytosol P receptor concentration in the uterine endometrium of rabbits (Chilton and Daniel, 1986). Prepubertal (26 day old and four week old) female rabbit uteri, are competent to secrete UG following P administration (Daniel and Milazzo, 1976; Loosefelt, et al., 1981). Immature female animals prior to these times, are unable to respond by synthesizing and secreting UG, following P (Daniel and Milazzo, 1976; Booher and Daniel, 1977; Loosefelt, et al., 1981). The endometria of these animals may not be mature enough to respond to the exogenous steroids, estrogen and progesterone. Assuming this was the case, the P receptor population of the glandular endometrium may not have been at an appropriate level for adequate P-binding, which is

necessary for the UG secretory effect. Immature endometria may also be deficient of other components such as estrogen receptors or components which may be involved in certain intracellular mechanisms involved in the progestational response, which must be present for UG synthesis and secretion. Animals older than 26 days may have been exposed to sufficient Prl (and other hormones or factors) prior to the P injections, whereas pituitary Prl may not have been secreted at appropriate levels for the younger animals to respond by secreting UG.

The purpose of this study was to investigate this phenomenon in younger animals. In particular, experiments were designed to test the hypothesis that the uteri of neonatal or juvenile rabbits (i.e. $\leq$ 22 days of age) were capable of synthesis and secretion of UG in response to progesterone, if oPrl is also present. It was assumed that these animals could provide a more sensitive and less variable model for further investigations of hormone action on the mammalian uterus. This model could provide a system whereby one may investigate the mechanism of action of protein and steroid hormones within the same system.

From the preliminary evidence, the polypeptide hormone, prolactin, primes the glandular endometrium for the subsequent action of a steroid hormone. This is the first demonstration of such a dependency interaction in mammalian reproductive tissues. Since the progestational effect on endometria of LTOVX adult rabbits is enhanced by prolactin priming, a prepubertal animal system which responds in this fashion would allow further study of how polypeptide and steroid hormones interact to elicit a given response within the same system.

II. MATERIALS AND METHODS

Animals and Their Maintenance

Adult female rabbits weighing 3.4-4.4 kg each, were bred to fertile bucks. Nesting boxes were added to each cage on day 28 post coitum (p.c.). Rabbit chow (Purina) and tap water were provided ad libitum. A 14 hr light, ten hr dark photoperiod was maintained. Each litter constituted an injection group.

A virgin female goat, ten months of age (27 kg), was acquired from a local farmer and kept in pasture for the duration of antiserum production and blood collection. Shelter, fresh grain (Formax) and tap water were provided ad libitum.

Rabbit Treatment Protocol

Treatments occurred during a nine day injection protocol beginning at 10:00 a.m. on the first day post-partum. This constituted day one of injection for the newborn group. A second group of animals was injected in the same fashion beginning on day 14. Each animal received a subcutaneous injection, at the nape area of the neck, of estradiol (E_2, ten micrograms/kg or 100 micrograms/kg-24 hr^{-1}) for the first two days (the 100 microgram E_2 group was designated $E_2$10X), ovine prolactin (oPrl, 14 I.U./kg-24 hr^{-1}) for the next three days and progesterone (P, three mg/kg-24 hr^{-1}), for the remaining four days of the nine day injection protocol as shown in Table 1. Similar injection protocols have been used in other studies in this laboratory involving adult animals which responded by producing UG. The hormones were purchased from Sigma Chemicals (St. Louis, MO). Corn oil or phosphate buffered

Table 1: Treatment Protocols

	9 day injection period			
Injection Group	10 micrograms E_2/kg-24 hr-1	100 micrograms E_2/kg-24 hr-1	14 I.U. oPrl/ kg-24 hr-1	3 mg P/ kg-24 hr-1
1	+	-	sham	sham
2	+	-	+	sham
3	+	-	+	+
4	-	+	sham	sham
5	-	+	+	sham
6	-	+	+	+
7	sham	-	+	sham
8	sham	-	+	+
9	sham	-	sham	+
10	-	+	sham	+
11	+	-	sham	+
Treatment Day	1,2	1,2	3,4,5	6,7,8,9

Legend: +, received described treatment
-, no treatment given
sham, received carrier alone

Note: Each animal received a 0.1 ml injection as indicated on each of nine injection days.

saline (PBS, 8.5g NaCl, 1.15 g NaH_2PO_4, 0.21g $NaH_2PO_4 \cdot H_2O$ per liter of glass distilled water [qdw], pH=7.4) comprised carrier solutions for the steroids or oPrl respectively and were used accordingly for sham injections of controls.

Collection of Uterine Flushings

Two hours after the final injection, the nine and 22 day old female rabbits were killed by cervical dislocation. A mid-ventral incision exposed the uterine horns. Each horn was ligated with 3-0 silk (Ethicon) ligatures and transected at the uterotubal junction and cervical end, distal to the ligatures, respectively. This procedure protected against loss of uterine fluid and contamination of uterine fluid with blood, oviducal and cervical secretions or tissue debris. The uterine horns were excised, rinsed with sterile PBS, transferred to a sterile tissue culture dish (Falcon #1007) and flushed with 0.2 ml of PBS. A binocular dissecting microscope (Bausch and Lomb Stereozoom Five) aided the accurate injections of PBS and recovery of flushings. A 30 gauge hypodermic needle and one ml syringe were used for collection and injection. Each flushing was transferred to a microcentrifuge tube (Eppendorf) containing one microliter of five percent phenylmethylsulfonyl fluoride in PBS and centrifuged (5000 x g) for 5 minutes to remove cellular debris. Fifty microliter aliquots of the supernatant were transferred to labelled microcentrifuge tubes, fast frozen in an acetone and dry ice slurry and stored at $-20^{\circ}C$ until assayed for UG.

Purification of UG

Six mature female rabbits (3.4-4.4 kg each) were given intravenous injections of 100 I.U. hCG in 0.1 ml PBS to induce pseudopregnancy. On day five after injection, (the UG content of uterine fluid is highest on the fifth to sixth day following injection) the rabbits were killed by cervical dislocation and their reproductive tracts were removed thru mid-ventral incisions. Each uterine horn was ligated at the uterotubal junction and cervical ends respectively, and flushed with five ml of PBS from the uterotubal toward the cervical end. Flushings were collected at the cervical end of each horn. Injections and collections of flushings were accomplished by using 18 guage needles and ten ml syringes. The uterine flushings were pooled and dialyzed against gdw overnight in Spectrapor #3 dialysis tubing (molecular weight cutoff 3500 daltons). The pooled uterine flushings were concentrated to a two ml total volume by covering the dialysis tubing with Aquacide. The concentrate was transferred to an equilibrated 2.5 x 38 cm column of Sephadex G-200. One hundred fractions, 40 drops each, of the eluate were collected. The eluent was 0.02 M PBS, pH 7.4, containing 0.01 percent sodium azide to prevent bacterial growth. Duplicate, 50 microliter aliquots of each fraction were assayed for protein content by the method of Lowry, et al., (1951), and the absorbance was measured at 750 nanometers (Hitachi Model 100-10 spectrophotometer) and plotted against fraction number. Results from a representative run thru Sephadex G-200 is shown in Figure 2. Fractions 54 thru 68 were pooled, dialyzed against gdw and concentrated to a final volume of two ml as described. The concentrate was then loaded onto a 2.5 x 38 cm column of

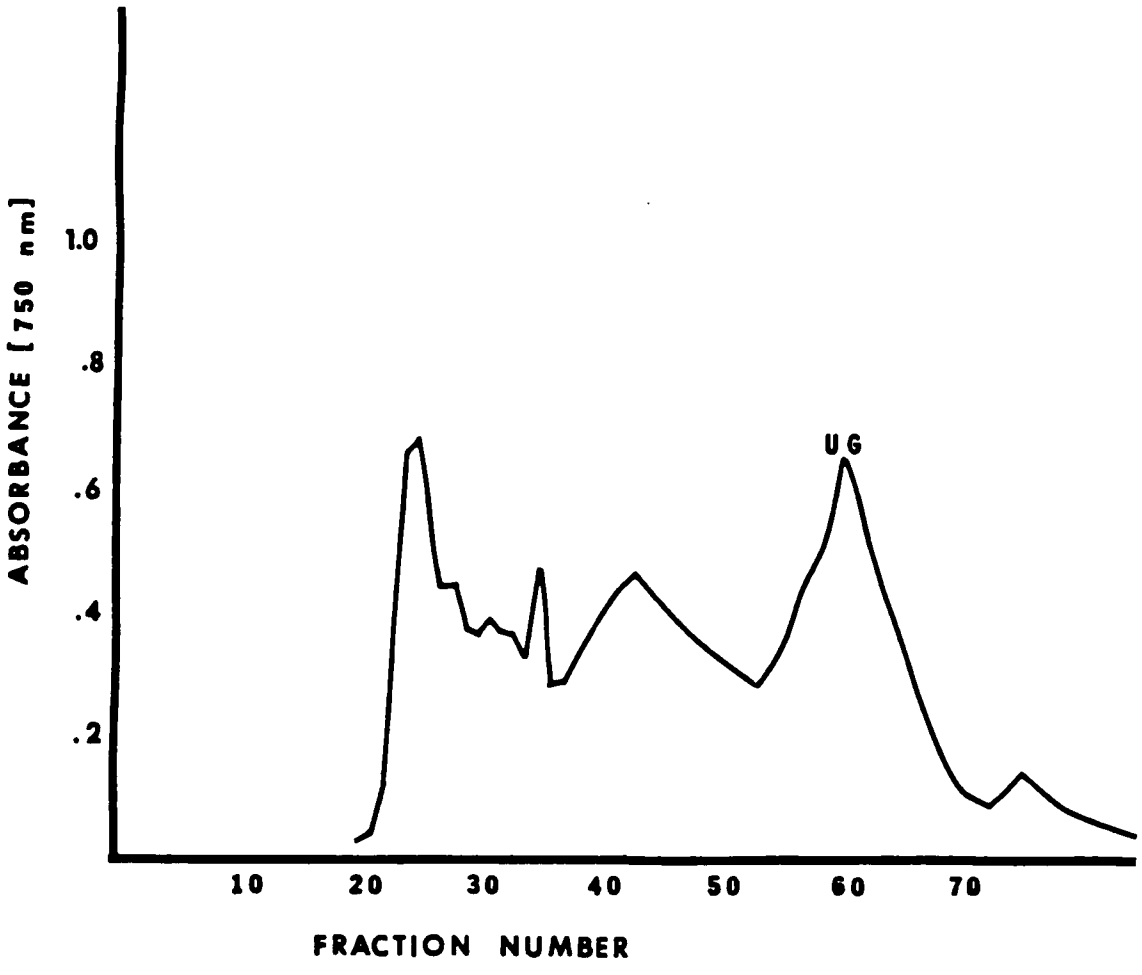


Figure 2: A characteristic Sephadex G-200 gel filtration profile resulting from separation of five-day pseudopregnancy rabbit uterine fluids. See the text for details.

Sephadex G-50. Forty-drop fractions were collected and aliquots were analyzed for protein content (Lowry, et al., 1951). Verification of the UG composition from fractions collected by these chromatographic methods was performed by running aliquots on polyacrylamide slab gels and by specific immunological reactivity against immune sera prepared against purified UG. A representative plot of the absorbance vs fraction number is shown in Figure 3. Fractions seven thru 12 were pooled and dialyzed against gdw as described for the removal of ionic components. Lyophilization of the dialyzed product yielded 5.5 mg of UG standard. All procedures were carried out at four degrees Celsius.

Production and Characterization of Goat Anti-UG Antiserum

An antiserum to the above purified rabbit uterine UG was prepared in a ten month old female goat. The immunization procedure was similar to that described by Johnson, et al., (1972). Four immunization sites were prepared by using an electric hair clipper (Oster model A2) to expose areas of skin along each suprascapular region of the goat. Blood collection sites were similarly exposed along each forelimb anterior subcutaneous vein. Preimmune blood was collected from one of these veins for production of serum. Each suprascapular injection site was swabbed with 91% ethanol and injected intradermally (i.d.) with 0.5 ml UG in Freund's Complete Adjuvant (Difco Labs). This injection solution was prepared as follows:

a. Two mg of lyophilized UG was solubilized in 0.50 ml PBS and filter sterilized (Nalgene, 0.45 micron).

b. 1.50 ml of homogenized Freund's Complete Adjuvant and this UG solution were repeatedly aspirated and expelled from one, three

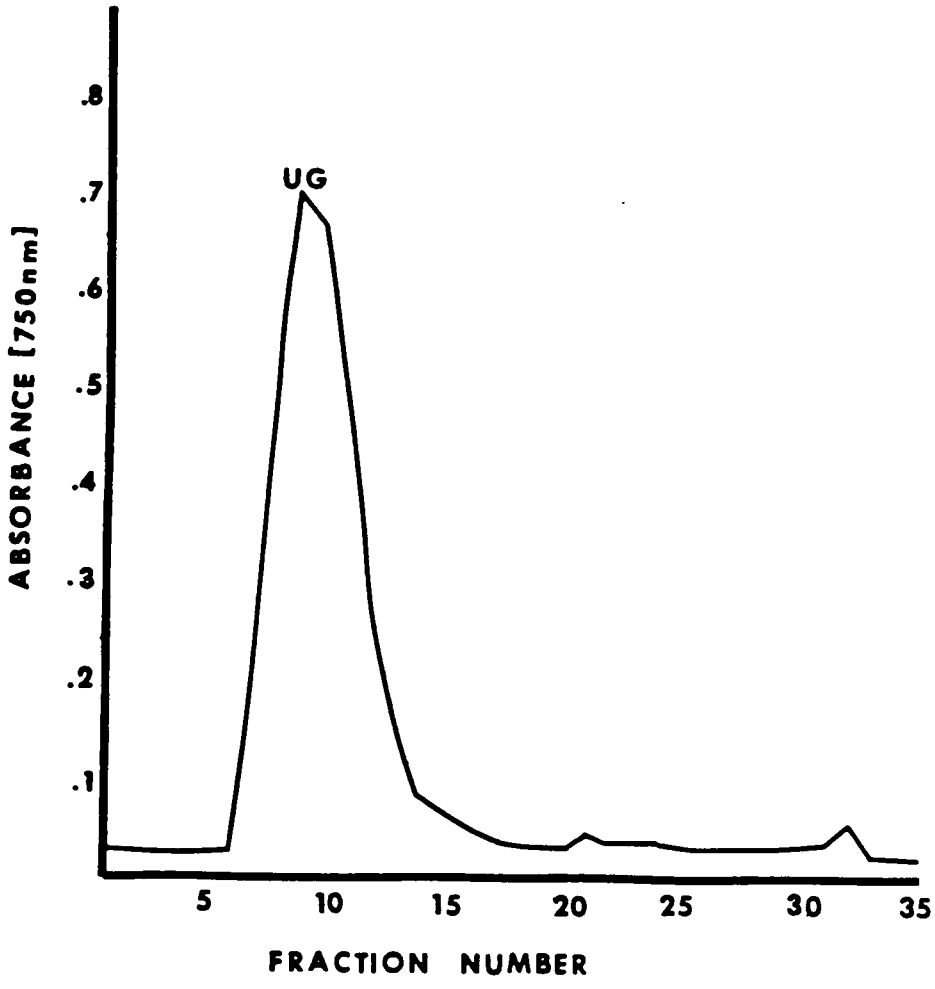


Figure 3: A typical Sephadex G-50 gel filtration of fractions from G-200 Sephadex as described in the text.

cc syringe, into another in order to facilitate extraction of UG from the aqueous into the oil phase. This solution constituted the injection-inoculation solution.

Injections were administered every two weeks for eight weeks. Incomplete adjuvant was used for preparation of all but the initial injection solution. The injection series was followed by blood collection and serum preparation for nine weeks in order to determine the optimal time of immune responsiveness. Serum collected three weeks after the final injection yielded the highest satisfactory titer of goat anti-UG antiserum (GAUG) as determined by Ouchterlony immunodiffusion analysis by the method described by Crowle, (1973) and shown in Figure 4. Immunodiffusion gels one mm thick were composed of one percent agarose in PBS. Sample wells four mm in diameter were produced in a hexagonal array.

The goat remained untreated for three months following immunization against UG, at which time a booster injection of two mg UG was administered as described. One hundred ml of blood was collected three weeks following this booster injection. Serum from this blood was used in all subsequent immunological analyses; however, further characterization of this antiserum was necessary for verification of its specificity to UG.

Pooled uterine flushings from 22 day old female rabbits and UG standard were run in tandem in a two-dimensional immunoelectrophoretic system as described by Crowle, (1973). A one percent agarose gel was prepared containing ten percent GAUG in barbital buffer, (BB, 21.35 g Veronal/L qdw, pH=8.6). Samples were loaded in sample wells and the gel

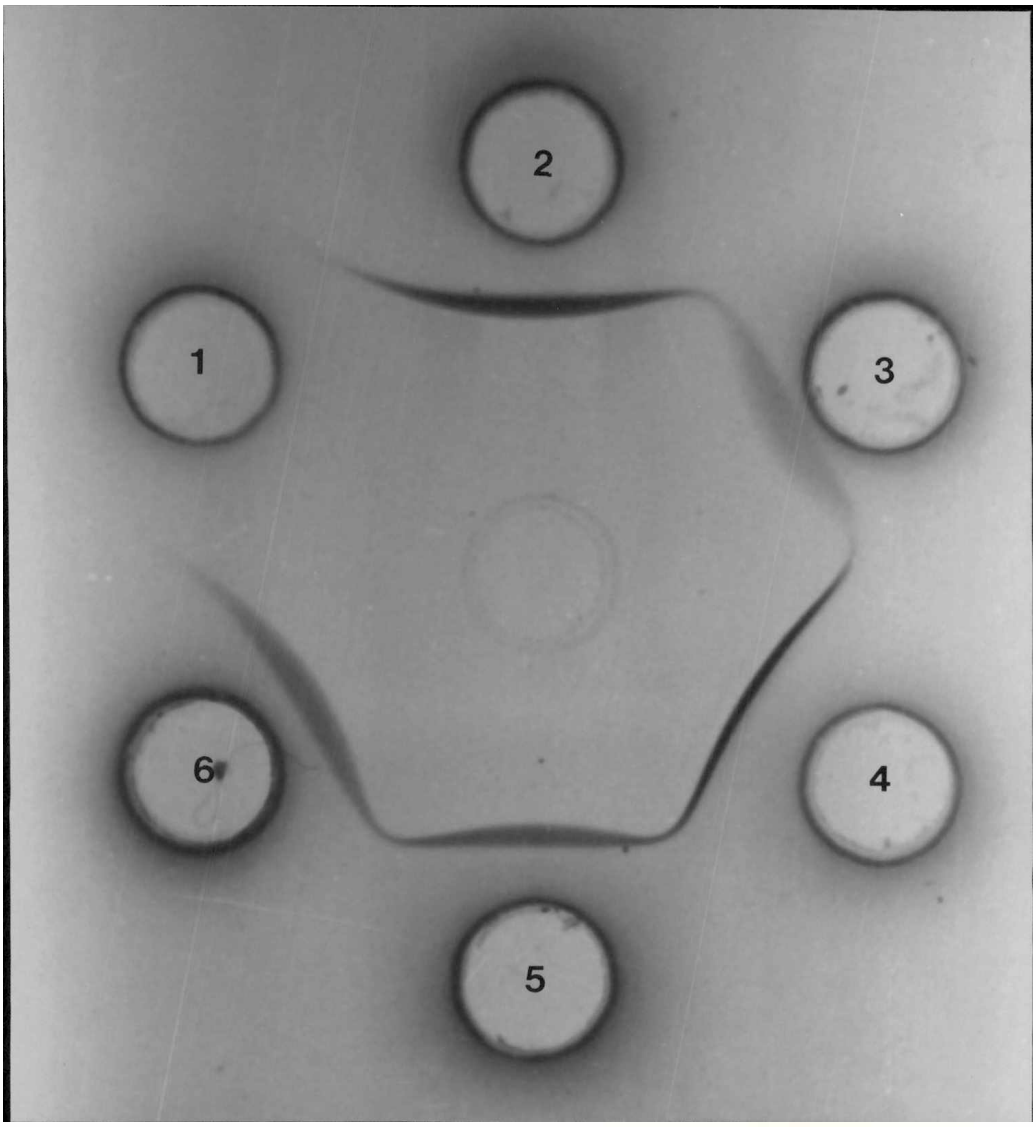


Figure 4: One dimensional immunodiffusion analysis of antisera collected at different times (peripheral wells), reacted against UG standard (center well). Sample well 1 contained preimmune sera, whereas sample wells 2,3,4,5 and 6 contained sera collected 1,2,3,6 and 9 weeks following the final injection.

was exposed to 100 V for 75 minutes in the first (horizontal) dimension and 75 V for 4.5 hours in the second dimension. Two precipitation peaks were produced upon staining with a Coomassie brilliant blue solution (0.5 w/v CBB-stain in ten percent v/v glacial acetic acid and 45 percent v/v ETOH) for 15 minutes. The plates were destained for about five minutes to remove background staining by using the same solvent system, minus the stain. The plates were then dried by placing them into contact with filter paper for 24 hours at room temperature.

Identification of cross reacting antigens with the GAUG antiserum in the two-dimensional gel shown in Figure 5, was accomplished by running aliquots of the same uterine flushing in one dimensional immunoelectrophoretic gels as shown in Figure 6. Three microliters of UG standard, 1.5 microliters of UG standard plus 1.5 microliters of uterine flushing and uterine flushing alone, were run in the one dimensional immunoelectrophoretic system as described by Crowle, (1973). UG standard and the mixture of UG with uterine fluid was reacted against GAUG, whereas the mixture of UG with uterine fluid and the uterine fluid sample alone, were reacted against goat anti-rabbit albumin antiserum (Miles Laboratories). A 1:20 dilution of each antiserum with PBS was used in this system. The gel was exposed to 100V for one hour and allowed to diffuse at four degrees Celsius overnight in a humid chamber. The gel was removed from the humid chamber and washed in BB in order to remove unprecipitated proteins. The gel was dried by placing in contact with filter paper overnight at four degrees Celsius, stained with CBB-stain and destained to remove nonspecific background staining.

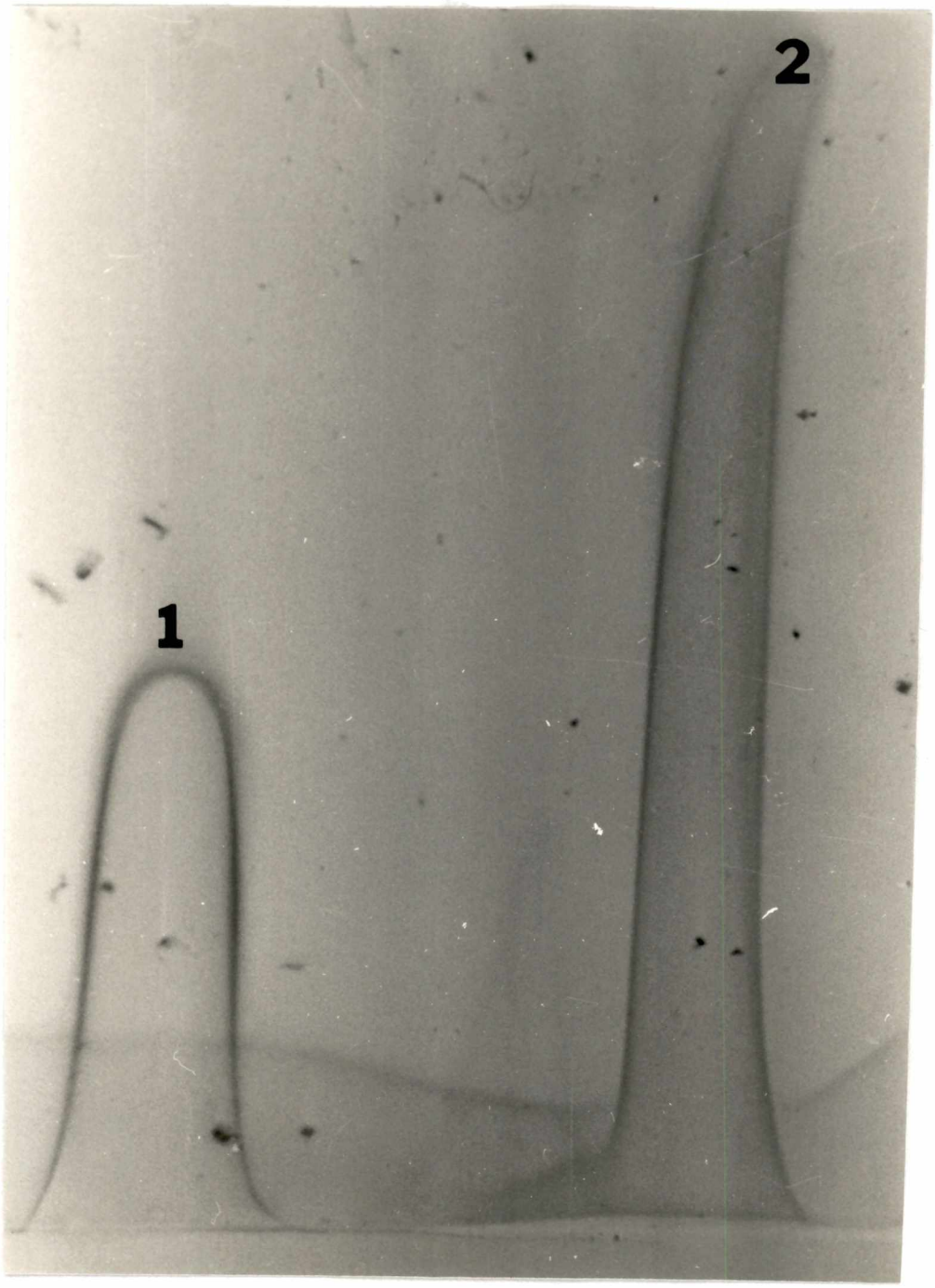


Figure 5: Two dimensional immunoelectrophoretic analysis of UG standard and a uterine flushing collected from a neonatal (nine day old) rabbit treated with the $E_2$10X,oPrl,P protocol. Samples were run in tandem. Precipitation reaction #1 represents UG reaction with GAUG, whereas precipitation reaction #2 represents GAUG reacting with some other antigen contained in the neonatal uterine flushing.

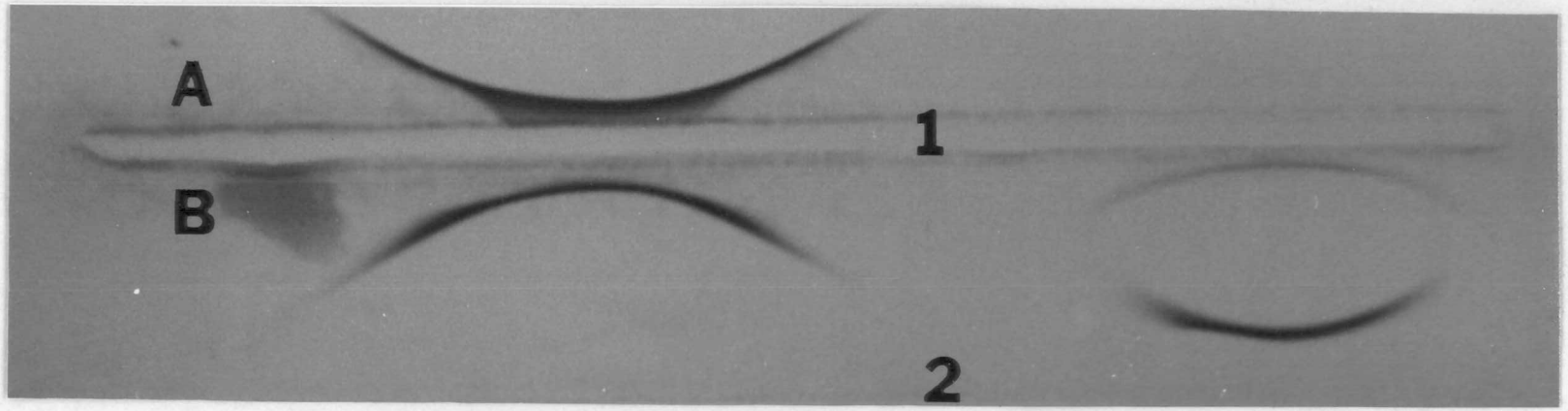


Figure 6: Single dimension immunoelectrophoretic analysis of UG standard (sample well A) and a mixture of UG standard with a neonatal (9 day old) uterine flushing from a rabbit treated with $E_2$10X,oPrl,P (sample well B). These samples were reacted against unabsorbed GAUG (trough #1). Trough #2 contained goat anti-rabbit albumin. See the text for details.

Absorption of GAUG was necessary in order to enhance the specificity to UG. Four ml of pooled whole serum from estrus rabbits was used to absorb 50 ml of GAUG. All subsequent analyses of UG were performed by using this absorbed antisera, shown to be relatively monospecific to UG by the two-dimensional immunoelectrophoetic system, shown in Figure 7, as described by Crowle, (1973).

Detection and Quantitation of UG

The presence and relative amount of UG in uterine flushings from young rabbits was determined by the one-dimensional immunodiffusion procedure performed in Oudin tubes as described by Crowle, (1973). Pyrex glass tubes, of three mm internal diameter were washed, cut into five cm lengths and sealed at one end with an orthodontic torch. Each tube was internally coated with a 0.5 percent agarose solution in gdw. Excess agarose was removed by centrifugal force and the agarose was allowed to dry. The tubes were filled about two thirds full with a final 0.5 percent agarose solution in three parts PBS to two parts GAUG. The Oudin tubes were inoculated with 20 microliter aliquots of uterine flushings and another set with a serial dilution of UG standard.

In this immunodiffusion system, a precipitin front moves thru the gel until antigen or antibody exhaustion. A value h, measured to the nearest 0.1 mm from the sample-agarose interface to the precipitin front, was recorded. Linear relationships described h values with respect to time in minutes. The slope of each line was described by the equation $k = h/\sqrt{t}$. K values remain constant for any one antibody concentration; therefore, k values for a serial dilution of UG standard were plotted with respect to their known concentrations. The resultant

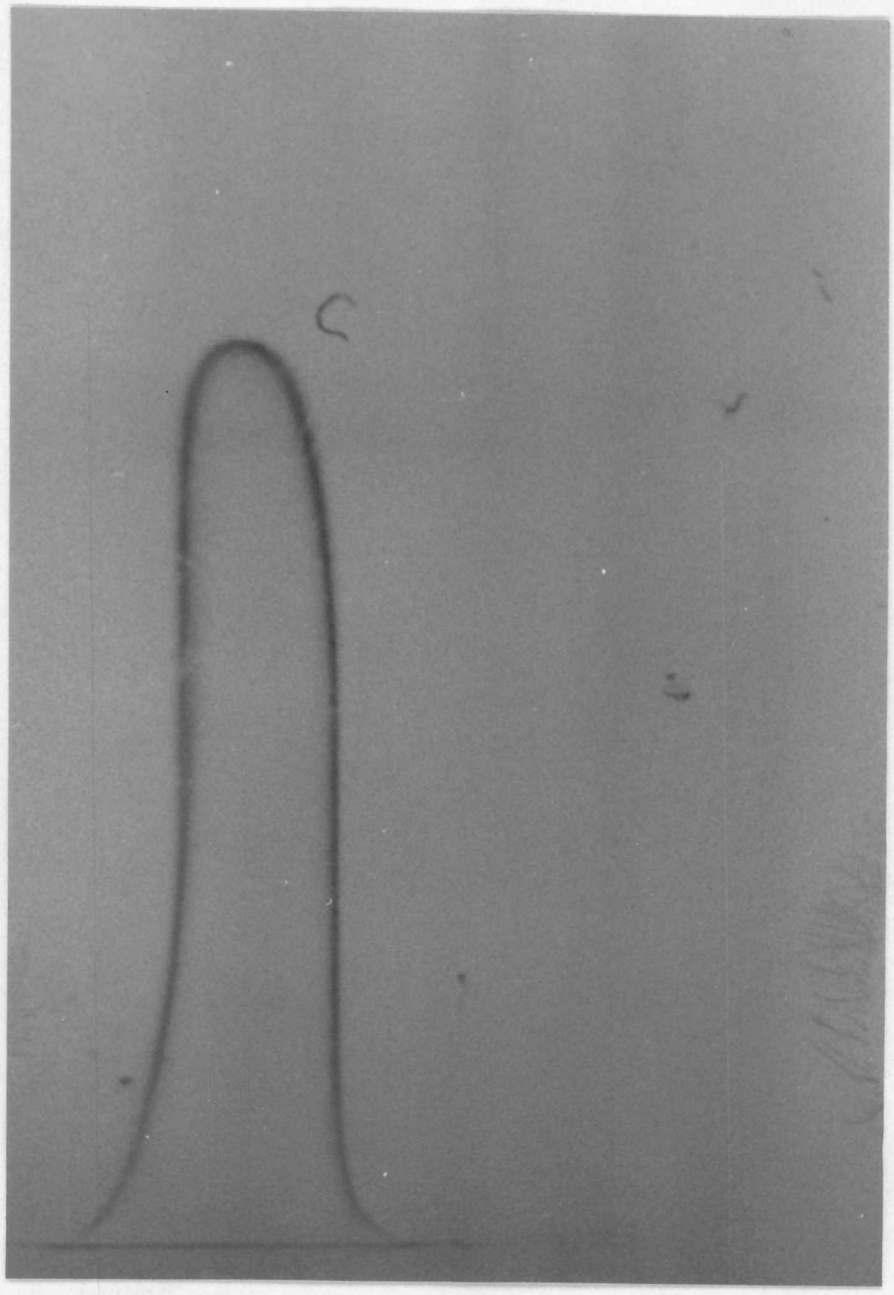


Figure 7: Two dimensional immunoelectrophoretic analysis as described in Figure 2, following absorption of GAUG with pooled whole adult rabbit sera. The single precipitation reaction represents UG standard reacting with GAUG, demonstrating relative monospecificity of the antiserum to UG.

standard relation as shown in Figure 8, was described by a straight line. From this standard relation, k values, determined for aliquots of uterine flushings, yielded UG concentrations by extrapolation.

Total protein concentration in uterine flushings was determined (Lowry, et al., 1951). A standard relation is shown in Figure 9, from which, absorbance readings yielded total protein concentrations by extrapolation. Duplicate 20 microliter aliquots of neonatal and juvenile rabbit uterine flushings were analyzed by this method. Results were expressed as percent UG of total protein.

Demonstration of De Novo UG Synthesis

Verification of the uterus as being the source of UG synthesis and secretion was necessary since the oviduct of rabbits is also known to produce this protein. De novo synthesis and secretion was demonstrated in vitro by showing significant incorporation of radiolabelled amino acid precursors into the UG molecule.

Three, 22 day old female rabbits previously treated as described with $E_2$10X (100 micrograms/kg-24 hr^{-1}), oPrl and P were sacrificed by cervical dislocation two hrs after the final injection. Their left uterine horns were ligated and removed as described in the uterine flushing procedure. Each uterine horn was externally rinsed and flushed with PBS. The uterine horns were individually placed in separate sterile tissue culture dishes (Falcon #1007) containing eight ml of Hams F-ten tissue culture medium (Gibco Labs), pH=7.4 at 37 degrees Celsius. Each uterine horn was filled with a 0.3 ml solution of culture medium containing ^{35}S-methionine (Amersham). Immediately following this, a 35 microliter aliquot of uterine luminal fluid was removed which

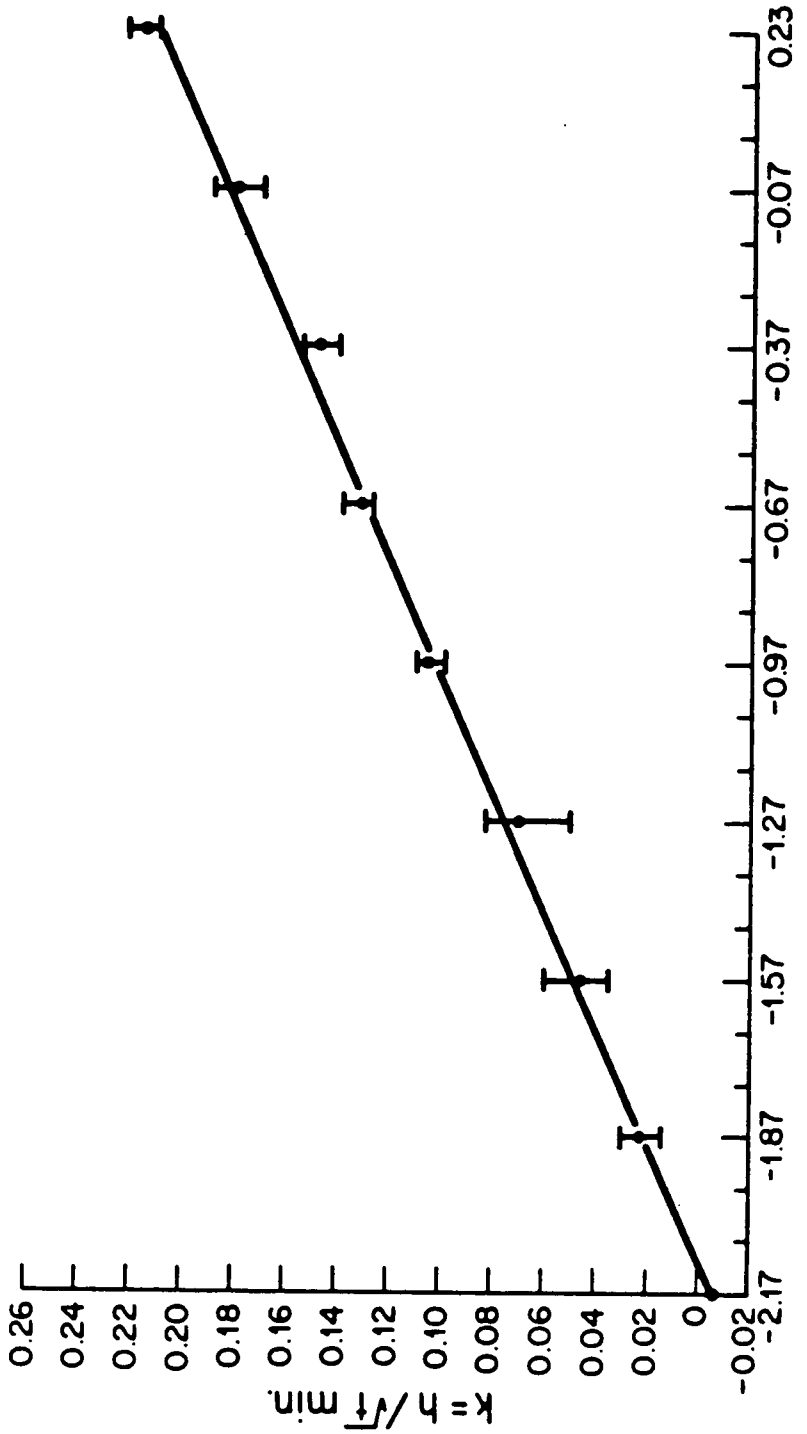


Figure 8: Oudin quantitation of UG. Standard relation which yielded concentrations of UG from K values of uterine fluids by the procedure described by Crowle, 1973.

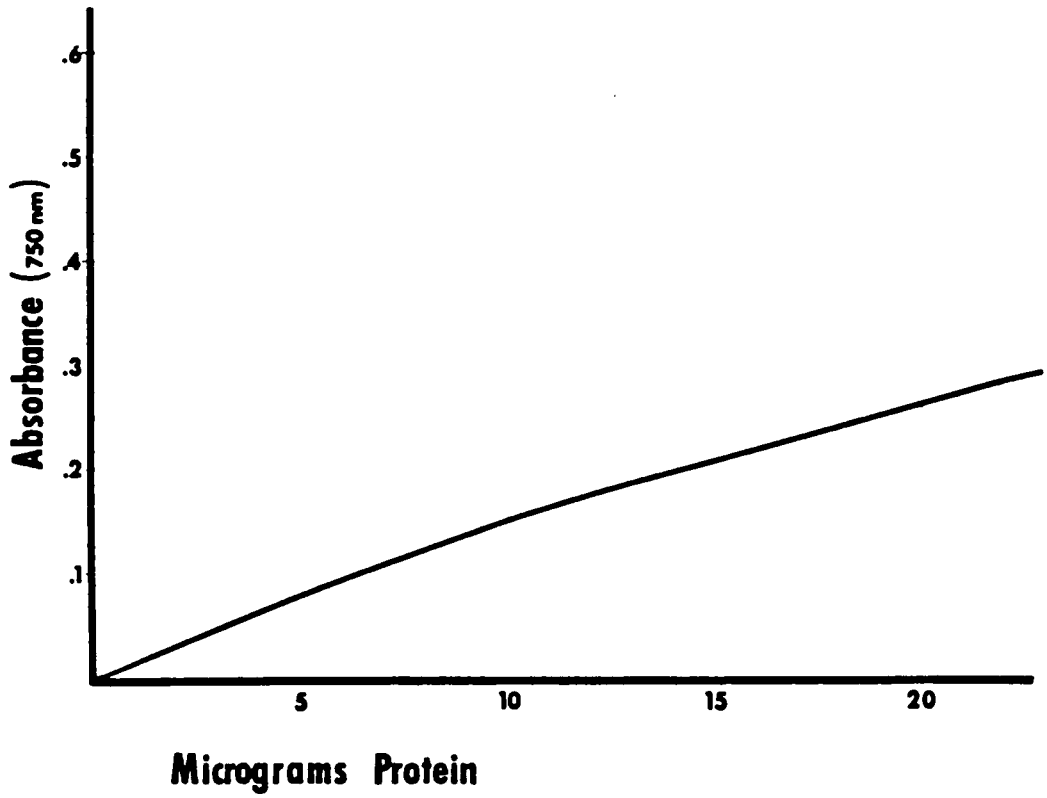


Figure 9: Lowry protein determination. Standard relation for determining unknown protein concentrations of uterine flushings from absorbance measurements.

represented the zero time point for a subsequent four hour incubation in vitro at 37 degrees Celsius (Napco, model 4100 incubator). Similarly, 35 microliter aliquots of luminal fluid were removed every 30 minutes during the incubation period. Each 35 microliter aliquot was equally divided and analyzed for total protein incorporation of radiolabel and UG incorporation of radiolabel, respectively.

Total protein incorporation of radiolabel was determined (Mans and Novelli, 1961). Each 17.5 microliter aliquot was pipetted onto a two cm diameter ashless filter paper disc (Whatman) and allowed to dry. Each filter disc was washed for 30 minutes in 100 ml of ten percent trichloroacetic acid (TCA) in PBS, five percent TCA in PBS, 95 percent ethanol, a solution of 75 percent ethanol-25 percent diethyl ether and 100 percent diethyl ether. All procedures were carried out at four degrees Celsius. Each filter disc was then air dried and placed in a scintillation vial containing eight ml of scintillation cocktail (Scintiverse).

Incorporation of radiolabel into UG was quantitated by precipitating the UG from each 17.5 microliter aliquot of uterine luminal aspirate with an equal volume of GAUG. However, previous experimentation had determined that the amount of UG present in uterine luminal fluid was insufficient to maximize UG precipitation. In order to create optimal antigen-antibody interaction and thus maximal UG precipitation, 8.6 micrograms of UG standard in 2.5 microliters PBS was added to each 17.5 microliter temporal aliquot. Each aliquot was gently vortexed and 20 microliters of GAUG was added to each tube. Following vortexing and incubation for one hour at room temperature, each sample

was incubated overnight. All subsequent reactions were conducted at four degrees Celsius. The samples were centrifuged (5000 x g) for five minutes and the pellet was washed three times with 1.5 ml of PBS. The pellet was solubilized with 15 microliters of 0.25 N acetic acid in PBS and transferred to filter discs, which were allowed to dry. Each filter disc was placed in a scintillation cocktail as described for total protein incorporation of radiolabel.

All cocktails were counted (Beckman 8100 LSC) for radioactivity by standard methods. Counts were expressed as CPM and corrected for nonspecific radiolabeled binding. Percent of total protein incorporation which is UG was plotted against time of incubation in Figure 10. Table 2 shows the initial counts and those corrected for nonspecific binding at each sample time. Nonspecific counts were those which were bound to filter disk components following washing procedures. Nonspecific counts were determined from aliquots taken immediately following injection of radiolabel into each uterine lumen (zero time samples).

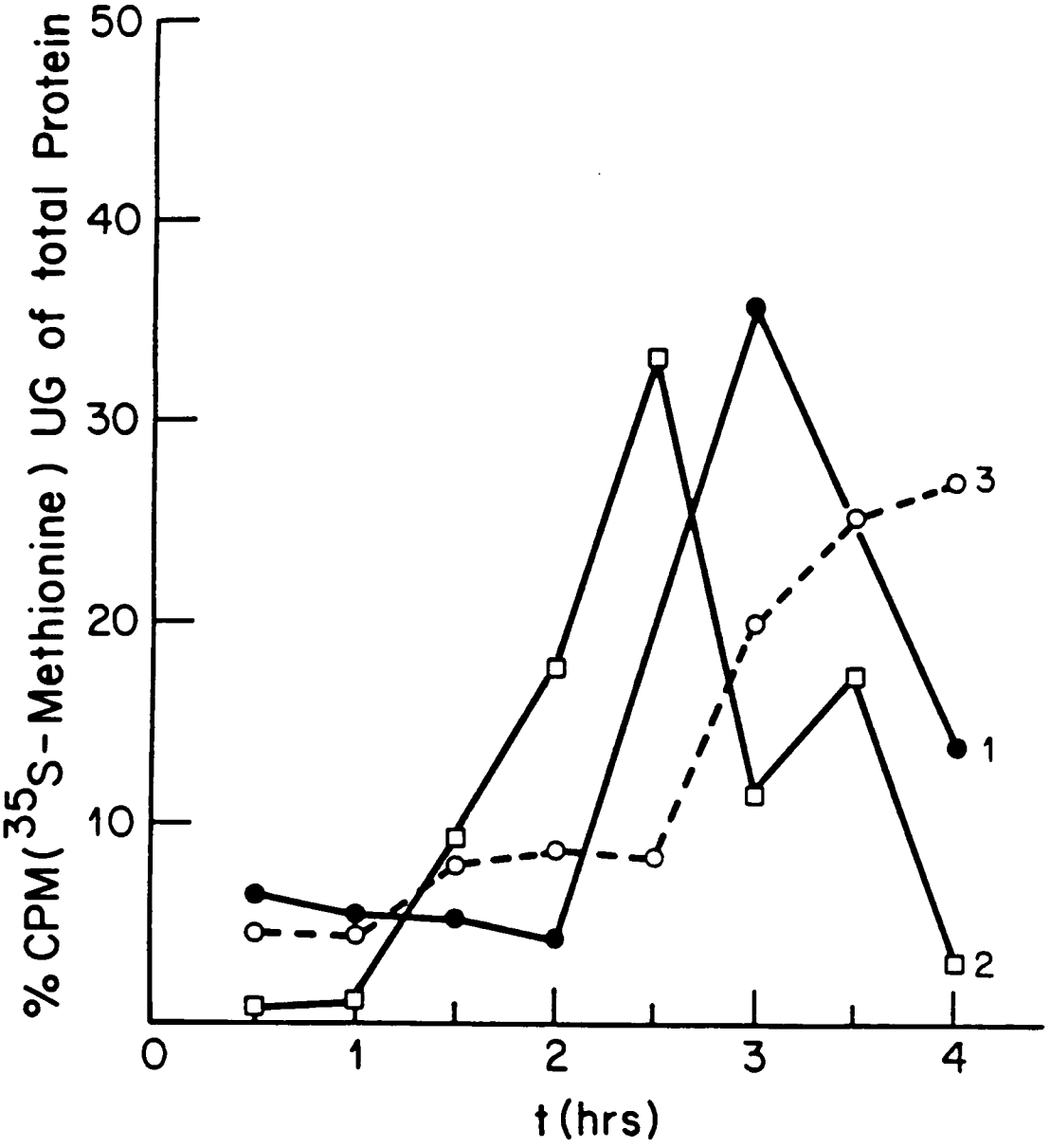


Figure 10: ^{35}S-methionine incorporation into UG. The results are expressed as the percentage of total protein incorporation of radiolabel that is labelled UG.

Table 2: ^{35}S-Methionine Incorporation Into Uteroglobin

Animal #	Incubation time (hours)	Total Protein CPM Corrected for *Nonspecific Binding	UG CPM Corrected for Nonspecific Binding	% CPM UG of Total Protein
1	0.5	285,874	18,544	6.49
	1.0	214,206	11,727	5.47
	1.5	235,741	12,636	5.36
	2.0	196,619	7,943	4.04
	3.0	170,530	60,758	35.63
	4.0	68,419	9,360	13.68
2	0.5	76,680	612	0.80
	1.0	145,053	1,649	1.14
	1.5	80,759	7,449	9.22
	2.0	123,081	21,918	17.81
	2.5	88,905	29,430	33.10
	3.0	93,986	10,411	11.08
	3.5	134,745	23,324	17.31
	4.0	95,635	2,914	3.05
3	0.5	74,332	3,514	4.73
	1.0	111,163	5,041	4.53
	1.5	86,327	5,855	6.78
	2.0	117,920	9,829	8.34
	2.5	121,223	9,853	8.13
	3.0	195,273	38,875	19.91
	3.5	101,223	25,395	25.09
	4.0	117,273	31,567	26.92

* Nonspecific binding refers to CPM associated with total protein or UG at the zero time point of incubation. See the text for details.

III. RESULTS

Characterization of GAUG Antiserum

Production of GAUG antisera yielded 50 ml of serum. As indicated in Figure 4, serum collected three weeks after the final booster injection resulted in the sharpest and most dense precipitation reaction in the Ouchterlony immunodiffusion analysis. This batch of antisera was further characterized to verify its monospecificity and was standardized against known concentrations of UG from previous studies.

Two dimensional immunoelectrophoresis of UG standard and a uterine flushing from a nine day old rabbit treated with the $E_2$10X,oPrl,P protocol, run in tandem, produced two precipitation reactions as shown in Figure 5. UG standard was pipetted into sample well A, whereas the uterine flushing was transferred to sample well B. Following absorption of GAUG with pooled estrous rabbit serum, the non-uteroglobin reactivity was removed as demonstrated in Figure 7. All GAUG antisera preparations were absorbed in this fashion and were used for detection and quantitation of UG in uterine flushings.

Figure 6 represents one dimensional immunoelectrophoresis of UG standard (sample well A) and a 50:50 mixture of UG standard with a uterine flushing from a neonatal animal treated with the $E_2$10X,oPrl,P protocol (sample well B). The antiserum of trough one, between these samples contained unabsorbed GAUG. Sample A, yielded only one precipitation reaction, which was against anti-UG. Sample B shows two precipitation arcs, one against anti-UG and the other against anti-albumin (arrows) antibodies which were in the unabsorbed GAUG

preparation. This is verified by the precipitation reaction of sample well B (arrows) against goat anti-rabbit albumin pipetted in trough two.

Quantitation of UG

Figure 8 shows the standard relation of K vs increasing concentrations of UG standard as acquired from the single dimension immunodiffusion technique in Oudin tubes as described by Crowle, (1973). From this standard relation, K values from samples of uterine fluids of neonatal and juvenile rabbits treated with the injection protocols shown in Tables 3 and 4, yielded UG concentrations. Final UG values were expressed as mg UG/mg total protein $\pm$ SEM.

Tables 3 and 4 show the treatment protocols as related to uterine weights, animal weights, the ratio of uterine to animal weight and UG production. As shown in these tables, only juvenile rabbits treated with the $E_2$10X,oPrl,P (0.098 $\pm$ 0.023 mg UG/mg total protein) and $E_2$10X,Sham,P (0.034 $\pm$ 0.030 mg UG/mg total protein) protocols produced detectable amounts of UG in their uterine flushings. Although statistical significance was not noted between the effects of these treatments, the $E_2$10X,oPrl,P group produced a mean value approximately three-fold greater than the $E_2$10X,Sham,P group.

Verification of De Novo Uterine Synthesis of UG

In order to verify that de novo synthesis of UG occurred in uteri of 22 day old animals, an ^{35}S-Methionine incorporation study was conducted. Figure 10 shows the temporal response of incorporation of radiolabel into UG as compared to total protein incorporation of radiolabel. As indicated in the figure, results were expressed as

Table 3: Treatment Categories of Neonatal Rabbits

Treatment	9 Day Old Rabbits				
	n	Mean Left Uterine Horn Wt. (g) ± SEM	Mean Animal Wt. (g) ± SEM	Ratio of Uterine Wt. / Animal Wt. ± SEM	mg UG/ mg Total Protein ± SEM
Sham Injected	7	0.015±0.016	132.4±13.5	$1.45\times10^{-4} \pm 7.80\times10^{-5}$	**ND
E_2,Sham,Sham	5	0.028±0.019	138.6±16.0	$1.80\times10^{-4} \pm 3.37\times10^{-5}$	ND
E_2,oPrl,Sham	5	0.303±0.019	204.6±16.0	$1.48\times10^{-4} \pm 1.92\times10^{-5}$	ND
E_2,Sham,P	7	0.012±0.016	200.0±13.5	$5.83\times10^{-5} \pm 2.86\times10^{-5}$	ND
E_2,oPrl,P	4	0.027±0.021	191.3±17.8	$1.43\times10^{-4} \pm 2.63\times10^{-5}$	ND
Sham,oPrl,Sham	6	0.059±0.018	165.7±14.6	$3.65\times10^{-4} \pm 2.59\times10^{-4}$	ND
Sham,oPrl,P	5	0.020±0.019	145.6±16.0	$1.42\times10^{-4} \pm 1.64\times10^{-5}$	ND
Sham,Sham,P	10	0.099±0.014	147.3±11.3	$6.84\times10^{-4} \pm 3.42\times10^{-4}$	ND
*$E_2$10X,oPrl,P	11	0.029±0.013	144.5±10.8	$2.34\times10^{-4} \pm 1.07\times10^{-4}$	ND
$E_2$10X,Sham,P	5	0.023±0.019	145.2±16.0	$1.68\times10^{-4} \pm 4.81\times10^{-5}$	ND
$E_2$10X,oPrl,Sham	3	0.019±0.025	129.3±20.6	$1.50\times10^{-4} \pm 1.00\times10^{-5}$	ND

*$E_2$10X refers to the 100 micrograms/kg-24 hr^{-1} dosage of E_2.

**Not detectable.

Table 4: Treatment Categories of Juvenile Rabbits

Treatment	22 Day Old Rabbits				
	n	Mean Left Uterine Horn Wt. (g) ± SEM	Mean Animal Wt. (g) ± SEM	Ratio of Uterine Wt. / Animal Wt. ± SEM	mg UG/ mg Total Protein ± SEM
Sham Injected	3	0.057±0.025	274.7±20.6	$2.10\times10^{-4} \pm 3.00\times10^{-5}$	**ND
E_2,Sham,Sham	3	0.317±0.025	261.7±20.6	$1.21\times10^{-3} \pm 9.17\times10^{-5}$	ND
E_2,oPrl,Sham	3	0.404±0.025	287.3±20.6	$1.41\times10^{-3} \pm 1.65\times10^{-4}$	ND
E_2,Sham,P	4	0.593±0.021	426.5±17.8	$1.41\times10^{-3} \pm 1.47\times10^{-4}$	ND
E_2,oPrl,P	5	0.320±0.019	400.0±16.0	$8.58\times10^{-4} \pm 2.28\times10^{-4}$	ND
Sham,oPrl,Sham	4	0.040±0.021	298.3±17.8	$1.30\times10^{-4} \pm 6.22\times10^{-5}$	ND
Sham,oPrl,P	5	0.032±0.019	361.2±16.0	$9.00\times10^{-5} \pm 2.35\times10^{-5}$	ND
Sham,Sham,P	5	0.042±0.019	276.2±16.0	$1.60\times10^{-4} \pm 7.31\times10^{-5}$	ND
*$E_2$10X,oPrl,P	6	0.536±0.018	379.0±14.6	$4.97\times10^{-3} \pm 8.99\times10^{-3}$	0.098±0.023
$E_2$10X,Sham,P	4	0.640±0.021	312.5±17.8	$2.05\times10^{-3} \pm 6.16\times10^{-5}$	0.034±0.030
$E_2$10X,oPrl,Sham	4	0.038±0.021	306.0±17.8	$1.09\times10^{-3} \pm 1.83\times10^{-4}$	ND

*$E_2$10X refers to the 100 micrograms/kg-24 hr^{-1} dosage of E_2.

**Not detectable.

percent of total protein CPM, represented by UG. This avoids problems associated with pool size differences for endogenous components related to the process of protein synthesis, between animals. Of the three animals studied, all of them show significant incorporation of radiolabel into UG. Animal #1 showed a maximal (35.6 percent) UG incorporation of radiolabel at three hours of incubation relative to total protein incorporation of ^{35}S-methionine. Animal #2 had a maximal (33.1 percent) UG incorporation of radiolabel at 2.5 hours, whereas animal #3 showed a continual increase in radiolabel incorporation into UG through four hours of incubation (26.9 percent).

IV. DISCUSSION

The purpose of this study was to investigate the effects of oPrl on uterine secretory activity in the rabbit. Because of prior exposure to reproductive hormones in mature animals, neonatal and juvenile rabbits were used in the hope that they would provide a better study system. Because of their age, these animals have had minimal exposure to these hormones. As indicated in Table 3, neonatal (nine day old) rabbits were unable to respond to any of the hormonal treatments by secreting detectable amounts of UG; however Table 4 shows that juvenile (22 day old) rabbits responded to the $E_2$10X,Sham,P and $E_2$10X,oPrl,P treatments by secreting detectable amounts of UG. From this study, it appears that a high concentration (100 micrograms/kg-24 hr^{-1}) of E_2 was necessary prior to P, in order to stimulate uterine secretory function. Explanations have been proposed by researchers studying the behavior of uterine estrogen receptors as to why increased dosages of E_2 are necessary to stimulate uterine function in the juvenile mammal. Elevated estrogen treatment is necessary in order to stimulate uterine function in the early postnatal development of the rat (Somjen, et al., 1973; Katzenellenbogen and Greger, 1974). A similar situation may exist in the rabbit. Rabbit uterine estrogen receptor activation may be compromised in immature uteri as characterized by an attenuated response to estrogen stimulation, since these immature animals require higher circulating levels of E_2 in order to support uterine secretion. The estrogen receptor undergoes a conformational change upon activation (Sakai and Gorski, 1984). An allosteric mechanism has been proposed for estrogen receptor activation which involves dimerization of the receptor

(Notides, et al., 1985). These authors propose that the estrogen receptor is composed of discrete subunits. Decreased subunit interaction and dimerization of estrogen receptors in uteri of juvenile rabbits may exist, so that functional receptors are in low concentration. Increased levels of E_2 would be required in order to elicit detectable effects, since the functional receptor population would be decreased.

The activated form of the estrogen receptor is capable of binding to DNA and thereby influences cell function in the endometrium. The concentration of cytosol estrogen receptor in juvenile (14 day old) rabbit uteri is significantly less than that of the adult rabbit uterus (Chilton, et al., 1986). Also noted were decreased circulating values of serum E_2 and a reduced affinity of the estrogen receptor for E_2 ($K_A=10^7$) in juvenile vs adult rabbit uteri. Further studies by these workers have shown that the K_A of juvenile rabbits was reduced, primarily by an increase in dissociation of ligand from receptor (off-rate increase) and this was correlated with a decrease in receptor activation.

Each of the studies discussed, with relevance to the estrogen receptor, agree with the response observed in the present study which required elevated levels (above what is necessary for mature rabbits) of E_2 to prepare the uterus for its secretory function.

The production and characterization of GAUG antiserum allowed quantitation of UG in uterine flushings of immature rabbits. As noted, antiserum collected three weeks following the final immune booster injection was characterized by Ouchterlony immunodiffusion analysis as shown in Figure 4. The precipitation reaction between UG standard

(center well) and the three week immune serum (sample well 4) produced the sharpest and most dense precipitation band relative to other serum collections. Therefore, the three week immune serum was used in subsequent analyses for the presence and concentration of UG in uterine fluid samples from young rabbits.

Since the Ouchterlony method does not adequately separate components of the immune serum, further characterization of the GAUG antiserum was necessary in order to verify the relative monospecificity of the antiserum to UG standard. This was accomplished by two-dimensional immunoelectrophoresis of UG standard and a nine day old rabbit uterine flushing from an animal treated with $E_2$10X,oPrl,P run in tandem. These samples were loaded into sample wells at the bottom of the electrophoresis plate and exposed to electrophoresis in the first dimension, followed by electrophoresis into the second dimension gel which contained GAUG antiserum. As demonstrated in Figure 5, GAUG antiserum reacted with UG standard (precipitation reaction A), as well as a component of the nine day old rabbit uterine flushing (precipitation reaction B). This additional component was determined, by one dimensional immunoelectrophoresis, to be albumin, as shown in Figure 6. Therefore, the GAUG antisera preparations were absorbed with pooled whole rabbit serum to remove albumin antigenicity. Rabbit serum does not contain detectable amounts of UG (Daniel and Milazzo, 1976). Figure 7 shows a two-dimensional immunoelectrophoretic, run using absorbed GAUG in the second dimension gel. This figure is characterized by only one precipitation reaction which is in the location of the UG standard. The antisera preparation absorbed in this fashion was used in

all subsequent immunological analyses for the presence and concentration of UG. The single precipitation reaction against UG verified relative monospecificity. This was necessary for identification of precipitation bands in quantitative analyses of UG by the Oudin method.

Quantitation of UG in uterine fluids was used to demonstrate uterine functional responses to exogenous hormones. As Table 4 shows, only the 22 day old, juvenile rabbits had detectable amounts of UG in their uterine secretions. Previous experiments have demonstrated detectable amounts of UG in uterine fluids as early as 26 days post partum (Booher and Daniel, 1977). In the present study, only those animals which received the higher priming dosage of E_2 (100 micrograms/kg-24 hr^{-1}) in combination with either P alone, or with oPrl prior to P, responded by secreting detectable amounts of uterine UG. UG synthesis and secretion in response to P alone, but not in endometrial homogenates from 26 day old animals has been demonstrated (Booher and Daniel, 1977). Because of this, these investigators proposed that the oviduct or some other extrauterine site of synthesis may have been responsible for the production of UG in the 26 day old animals. Since the present study demonstrated de novo synthesis and secretion of UG by 22 day old rabbit uteri in vitro, one may conclude that the UG detected in uterine flushings from animals treated with $E_2$10X,oPrl,P or $E_2$10X,Sham,P, represents uterine UG. It also appears that the priming effects of E_2 on uterine secretory structural development are required for UG production. Animals injected with the lower dosages of E_2 were incapable of responding by secreting detectable amounts of UG. This is further characterized in Table 4, when one observes the uterine weight

responses to the various injections. As shown in the table, those animals receiving E_2 followed by oPrl and/or P, have mean left uterine horn weights which are greater than other treatment groups. It is known that E_2 increases protein synthesis and enhances the glandular epithelial development of several reproductive structures including the mammary glands and endometrium. Younger animals, such as used here, are therefore unable to respond to lower dosages of E_2 in a way that allows adequate development of glandular structures, whereas higher levels of E_2 in conjunction with either P alone, or oPrl followed by P, results in uterine hypertrophy and competency of secretory function.

In regard to this research project, the major question asked, was whether or not Prl had a significant effect on the uterine function of immature rabbits. Nine day old rabbit uterine flushings did not contain detectable amounts of UG as the result of any of the treatments indicated in Table 3. Table 4 shows that 22 day old rabbits responded to the $E_2$10X,oPrl,P protocol with three-fold higher (although not statistically significant) levels of UG secretion (0.098 $\pm$ 0.023 mg UG/mg total protein) than the $E_2$10X,Sham,P group (0.034 $\pm$ 0.030 mg UG/mg total protein). This is evidence for a role for oPrl in enhancing the UG secretory responsiveness of these young rabbit uteri. One mechanism by which oPrl may have had this action, is thru the synthesis of cytoplasmic P receptors. This response was demonstrated in LTOVX adult rabbits (Chilton and Daniel, 1984). This would enhance the ability of P to induce UG production. However, since Prl has such varied functions throughout the animal kingdom and upon so many different tissue types, other explanations may exist for its effects on uterine function. For

example, the uterine estrogen receptor may be influenced by certain pituitary factors such as the adenohypophyseal hormones, prolactin and growth hormone (Pasqualini, et al., 1983; Chilton, et al., 1986). Prolactin, but not growth hormone, has a direct effect on the endometrium of LTOVX rabbits by promoting endometrial hypertrophy and glandular differentiation (Chilton, et al., 1986).

Since the oviduct is known to be a site of UG synthesis and secretion, experiments were conducted using isolated uteri from 22 day old rabbits treated with the $E_2$10X,oPrl,P protocol, in order to verify that de novo synthesis of uterine UG occurs. Figure 10 clearly demonstrates that ^{35}S-methionine is incorporated into uterine UG as well as other uterine proteins. This adequately substantiates the contention that UG synthesis, as quantitated in uterine flushings from 22 day old animals, actually is the result of uterine processes.

The uteri of young adult rabbits ($\geq$ six months old) are capable of responding to ovarian steroid induction of specific progestational uterine protein synthesis and secretion. Endogenous progesterone of the adult rabbit is supplied to the general circulation, primarily by the corpus luteum of early pregnancy and estrogens are provided to the system by the ovarian theca cells. Prepubertal animals are incapable of ovarian stimulation of uterine responsiveness because they lack the cyclic gonadotrophin secretion at levels necessary for the effect. One could speculate that prepubertal rabbits do not have a level of central nervous system development which allows for control of pituitary function.

Exogenous administration of progesterone to immature (eight week old) rabbits or progesterone in combination with estrogen, results in uterine UG production (Rahman, et al., 1975). The absolute quantity of UG produced by these two methods was not significantly different; however, this constituted only 12 percent of the adult UG response. It is therefore apparent, that by eight weeks of age, rabbit uteri are competent to respond to progesterone therapy secreting UG.

In the present study, it was shown that animals 22 days old, if treated with progesterone alone, were unable to secrete UG; whereas if estrogen "priming", plus progesterone were administered, the animals produced detectable amounts of uterine UG. Thus, the progestational response of UG secretion in these animals requires some "priming" effect of estrogens. This probably reflects the degree of maturation of the uterus at this age. When prolactin was added to this injection protocol, these animals responded with levels of UG which were three-fold of that without prolactin. Prolactin may affect the uterus directly, through estrogen or progesterone receptors.

When neonatal animals were treated in the various ways shown in Table 3, no response by UG secretion was detected. Perhaps other alterations in the injection protocol are necessary for uterine induction of UG synthesis and secretion by these animals. In any event, this further reflects the immaturity of these animals' uteri. Although UG was not secreted by this age, the various treatments had noticeable effects on the uterine weight parameter as seen in Table 3. An observation noted during hysterectomy of these animals, was that individuals treated with progesterone were characterized by increased

vascularity. Both estrogen and progesterone had noticeable structural effects on female rabbits nine days of age, even though functionally these uteri did not produce detectable amounts of UG.

Histological examination of uterine tissues from neonatal, juvenile (unpublished observations) and older immature rabbits up to eight weeks of age (Rahman, et al., 1975), shows that untreated rabbits have relatively atrophic uteri with particular regard to the endometrium. OVX adult rabbits respond to progesterone treatment by producing an endometrium which resembles that of the five day pseudopregnant animal (Chilton and Daniel, 1984). Prolactin has been shown by these investigators to enhance the progestational uterine response by increasing the population of progesterone receptors. This may be why prolactin enhanced the UG secretory response of 22 day old animals in the present study. Further histological and ultrastructural studies may lend support to this contention.

Although the 22 day old rabbits of this study required elevated levels of estrogen priming prior to progesterone in order to elicit uterine UG secretion, castrated adult animals respond to progesterone therapy alone (Urzua, et al., 1970; Rahman, et al., 1975; Daniel, et al., 1984; Chilton and Daniel, 1984). Investigators have been successful in demonstrating uterine responsiveness to progesterone stimulation in rabbits as early as 26 days of age; however, they have been unable to demonstrate the presence of UG in endometrial homogenates (Booher and Daniel, 1977). The source of uterine fluid UG in their study was not concluded to be the uterus. The oviducal secretions,

which are also known to produce UG, could be the source of the UG detected in their study.

Sham-injected immature rabbits are incompetent to secrete uterine pregnancy-specific proteins such as UG. Other treatment groups, when exposed to specific endocrine protocols, including elevated levels of estrogen priming followed by either progesterone alone or in combination with oPrl, were able to synthesize and secrete uterine UG. These results indicate that even though animals by 22 days of age are normally incapable of secreting UG, that these same animals have uterine tissues which are competent to develop progestational secretory ability if appropriately stimulated. The development, maturation and responsiveness of certain endocrine glands, including the adenohypophysis and ovaries, in these prepubertal animals are not competent to create an adequate circulating endocrine environment, necessary for appropriate induction of UG synthesis.

V. CONCLUSIONS

There were at least three conclusions resulting from the experiments presented here:

a. UG synthesis and secretion can be induced as early as 22 days post partum in the uteri of prepubertal rabbits treated with progesterone following priming doses of estradiol.

b. oPrl enhances the UG secretory response of 22 day old rabbits when added to the injection protocol with estrogen and progesterone.

c. De novo synthesis of UG occurred in uteri of 22 day old rabbits treated with the $E_2$10X,oPrl,P protocol as characterized by radiolabel incorporation studies.

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VITA

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