ACTINOMYCES PYOGENES IN EMBRYONIC LOSS IN CATTLE.

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By

Daniel Kiwanuka Nelson Semambo B. V. M. (Makerere University, Uganda).

Departments of Surgery / Reproduction and Pathology
University of Glasgow.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DEDICATED TO MY MOTHER AND FATHER (late)
AND FAMILY FOR THEIR LOVE.

THOSE WHO SOW IN TEARS WILL REAP WITH SONGS OF JOY. HE WHO GOES OUT WEEPING, CARRYING SEEDS TO SOW, WILL RETURN WITH SONGS OF JOY, BRINGING IN THE SHEAVES.
Psalms 126:5-6.

BLESS THE LORD OH MY SOUL AND ALL THAT IS WITHIN ME PRAISE HIS HOLY NAME FOR HE HAS DONE GREAT THINGS. **Psalms104:1-2.**

REJOICE IN THE LORD ALWAYS. I SAY AGAIN REJOICE. **Philippians 4:4.**

"FOR GOD AND MY COUNTRY"

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PREFACE.

The work presented in thesis was composed by myself under the supervision of Dr D. J. Taylor and J. P. Renton and has not been used in any previous application for a degree. The pregnancy specific protein B assay was kindly carried out by Dr R. G. Sasser (University of Idaho, Moscow, U. S. A.).

SUMMARY.

Actinomyces pyogenes is one of the bacteria commonly found in the bovine reproductive tract and it has been considered not to be a primary pathogen. The bacteria has also been isolated from foetuses and pus after abortion but its role as a primary or secondary pathogen in bovine abortion has remained an area of controversy and as yet little experimental work has been carried out to determine its role. In this thesis the potential role of *A. pyogenes* in bovine embryonic loss is examined.

Eight animals were inoculated with live *A. pyogenes* (10^9 - 10^{10})between days 30 and 44 of pregnancy and the organism was introduced into the pregnant uterus by laparotomy using a laparoscope. Control groups of animals used in the study were given intrauterine sterile saline (N=2), killed *A. pyogenes* (N=2) and *A. pyogenes* toxin (N=2) or injected with cloprostenol (N=8).

Of the eight animals infected with live *A. pyogenes*, six aborted 29 - 144 hours after infection. Animals were examined for the effects of the bacterium on the embryo by use of ultrasound, rectal temperatures, rectal palpation and vaginoscopy. Ultrasound was able to determine the time of embryonic death which occurred between 11 - 96 hours from the time of infection. Other intrauterine ultrasonographic changes included the separation of the allantochorion from the endometrium and endometrial thickening, accumulation of cloudy (echogenic) fluid in the uterine lumen and opening of the cervix and expulsion of the embryo from the uterus. The corpus luteum was maintained from the time of infection to abortion.

Ultrasonographic examination after cloprostenol administration revealed embryonic death between 55 - 72 hours after the treatment. Other

ultrasonographic changes seen in this group were dark non - echogenic intrauterine fluid and regression of the corpus luteum which commenced prior to embryonic death. These changes were followed by oestrus. Regression of the corpus luteum was accompanied by development of a dominant follicle which ovulated after oestrus.

Other clinical signs in the infected group included increase in the uterine tone (score 2 - 3 on a scale of 0 - 5), presence of embryo or mucopurulent material in the vagina, pus in the perineal region and on the byre floor. *A. pyogenes* was isolated from the smears in profuse culture.

Pathogenetic studies of *A. pyogenes* in bovine abortion were carried out in two heifers which were slaughtered 18 and 24 hours after infection for post mortem, bacteriological, histological, scanning and transmission E. M. examinations. Changes after infection were most severe in Heifer 20 slaughtered 24 hours after infection. At post mortem the infected uterus was congested and enlarged and contained cloudy fluid and the allantochorion was separating from the uterine wall. The embryo from Heifer 19 was alive at slaughter but the embryo from Heifer 20 was dead. Haemolysin (toxin) was demonstrated in both allantoic and amniotic fluids but the organism was only recovered from these fluids in small numbers in the embryo of Heifer 19.

Tissue debris containing neutrophils was seen in histological sections of the infected uterine horn. Erosion of the epithelium and the presence of neutrophils in the endometrium were seen. Gram positive organisms were seen in the tissue debris and on the eroded surfaces of the endometrium in Gram stained sections. Neither scanning E. M. nor transmission E. M. revealed substantial bacterial attachment to the uterine epithelium. Some

epithelial cells were hypertrophied.

Histological sections of the embryo were characterised by degenerative changes in the kidney, liver and the lining of the gut. Gram stained sections failed to demonstrate presence of *A. pyogenes*.

Whereas abortion occurred in one of the cows infected with killed *A. pyogenes* (6.5 days after inoculation), pregnancy was maintained in the second one and in the two inoculated with *A. pyogenes* toxin. The results of the pathogenetic studies suggest that *A. pyogenes* does not invade the embryo since it does not seem to cross the allantochorion. The pathogenesis of *A. pyogenes* in early embryonic death may be indirect through damage to the maternal - foetal junction or through the toxin.

Animals that aborted after bacterial infection came into oestrus 18 - 34 days after abortion and antimicrobial treatment was given between 6 - 10 days after abortion. Ultrasonography revealed the presence of a corpus luteum, echogenic fluid in the uterine lumen, thickened endometrium and an open cervix. Using ultrasound and vaginoscopy complete evacuation of pus from the uterus was seen and was accompanied by regression of the corpus luteum and oestrus. Systemic antimicrobial treatment was not conclusively shown to be effective in this study.

To further understand the pathogenesis of *A. pyogenes* induced abortion and the period prior to the first oestrus, assays were performed on serum or plasma for (a) antibodies, (b) acute phase haptoglobin (Hp), (c) progesterone and oestradiol 17 ß and (d) pregnancy specific protein B (PSPB).

(a) Using the enzyme linked immunosorbent assay (ELISA), antibodies to *A.*

pyogenes were detected at titres between 1:100 - 1:1000 >5 days after infection in all aborting animals. The ability of the antibodies to protect the animal against *A. pyogenes* infection was difficult to determine.

(b) Hp concentrations increased from, 15 mg/100 ml on day zero of infection to ≥150 mg/100 ml in 72 hours after infection thereby indicating inflammation and damage to the uterus caused by *A. pyogenes*.

(c) Progesterone concentration was >4 ng/ml from day zero of infection to just prior to the first oestrus (18 - 34 days after abortion) suggesting that the corpus luteum then present was functional. During the same time oestradiol 17 ß concentration was low (0.8 - 2 pg/ml). Complete evacuation of pus from the uterus was followed by regression of the corpus luteum, decline of progesterone (<0.5 ng/ml) and elevation of oestradiol 17 ß (>4. 5 ng/ml) at oestrus. Progesterone levels after cloprostenol induced abortion declined from >5 ng/ml at treatment to <0.5 ng/ml within 48 hours after treatment.

(d) PSPB declined from >1.5 ng/ml at infection to <0.8 ng/ml within 7 days following abortion. Similarly PSPB concentration declined from >1.2 ng/ml at cloprostenol treatment to <0.8 ng/ml within 7 days after abortion. The potential role of PSPB in embryonic death and the relationship between PSPB and progesterone in diagnosis of embryonic loss are discussed in the study.

CHAPTER I.

REVIEW OF THE DEVELOPMENT AND MAINTENANCE OF THE CONCEPTUS IN MAMMALS WITH SPECIFIC REFERENCE TO CATTLE AND OF FACTORS INFLUENCING EMBRYONIC LOSS IN THIS SPECIES.

INTRODUCTION.

Embryonic wastage in mammals (Boyd 1965, Vandeplassche 1968) including man (Edmondson *et al.*, 1988), is a phenomenon that has been known for some time. The embryonic period is considered to be from conception to completion of differentiation.

In cattle embryonic loss has been found to comprise a major portion of reproductive failure (Fischer and Beier 1986, Sreenan and Diskin 1986). It is generally accepted that calving rates to a single insemination are about 55% (Diskin and Sreenan 1980). Fertilization rates following natural or artificial insemination in heifers and cows have been estimated as 88% and 90% respectively (Sreenan and Diskin 1986). The difference between fertilization rates and calving rates suggests a total pregnancy loss of 33-35%. From ovum and embryo recovery experiments, estimates of embryonic loss up to day 42 after insemination range from 20% (Ayalon 1978) to 42% (Diskin and Sreenan 1980) with the greatest loss occurring between days 15 and 18 (Sreenan and Diskin 1986).

The economic loss due to the loss of pregnancy caused by brucellosis in bovine in England and Wales was investigated by Hugh-Jones *et al.* (1975) while carrying out cost-benefit analysis for the control of the disease. Using a series of "value judgements", loss due to following factors was calculated:- abortion

(loss in calves and from buying in replacement animals), infertility, depression of milk yield and cost of more land (for the extra animals bought). They assumed that annually, dairy herd losses due to brucellosis prior to the intensive control scheme were £ 3, 128,199. Losses due to the disease in beef herds using parameters including calving interval, non-pregnant cows per 100 mated, live calves per 100 cows mated, reduction in calve live-weight gain per day, cost for replacement of cows and resources freed for other use, mounted to a loss of to the National herd £ 3,545,780 (Hugh-Jones *et al.*, 1975).

Although the economic impact of pregnancy loss is realised, the relative contribution of different factors is still debatable. Aetiological factors associated with embryonic mortality such as fertilization failure, genetics, nutrition, hormonal status and uterine environment have been identified (Fischer and Beier 1986, Diskin and Sreenan 1986, Wiebold 1988).

The relative contribution of specific infections (viral and bacterial) to embryonic mortality is well documented, but other bacterial infections are recorded and infections with them have scarcely been investigated and its importance has seemingly been underestimated (Bouters 1986). Hartigan (1978) reported that uterine infection was about 100% in the first three weeks post-partum and that it was reduced spontaneously to approximately 30% during the fifth week. The presence of non-specific uterine infection or endometritis around the time of service was not considered to be an important cause of infertility in otherwise clinically normal cows by Griffin *et al.*, (1974), *A. pyogenes* was the only bacterium they associated with endometrial lesions.

A. pyogenes has been considered to be a secondary organism by some authors and to be of primary importance by others. It has been isolated from cases of endometritis, pyometra, metritis, and from cases of abortion from domestic

animals (Boyd 1965, Smith and Reynolds 1971, Hartigan *et al.*, 1978, Anetzhofer 1989). While investigating the fetopathogenicity of summer mastitis in cows, Richardson *et al.* (1982) found that not only does the disease lead to foetal growth retardation but that abortion can occur. The bacteria isolated from infected animals were *A. pyogenes* and *Peptococcus indolicus*. *A. pyogenes* has also been found in genital tracts of apparently healthy animals (Wolfgang and Gunter 1988). Bouters (1986) suggested that with increasing herd size and more cows being kept under loose conditions or cubicles, the transmission of *A. pyogenes* inside the herd could become more important and thus necessitate more rigorous hygienic measures.

The mechanism of action for non-specific infection in embryonic loss is poorly understood. Theoretically, the bacteria may interfere with fertility by:-

(i) directly killing the gametes or conceptus,

(ii) changing the uterine milk,

(iii) causing endometritis,

(iv) producing chronic histological lesions (Bouters 1986).

Dafalla and Hartigan (1983) used intrauterine diffusion chambers as a model system in rabbits to study some of these mechanisms. They found that neither the presence of diffusable bacterial products nor a persistent active infection had any "carry over" effect on fertility. Infusion of glycogen (a leucocyte chemotactic agent) caused complete termination of pregnancy and significantly reduced fertility before and during implantation in rats, though later stages were not affected (Anderson and Alexander 1979). These investigations threw some light on the possible pathogenetic mechanisms but clearly work remained to be done (Bouters 1986).

Kelly (1981) observed attachment of bacteria to infertile bovine eggs using electron microscopy. Later, in the Department of Bacteriology, Glasgow Veterinary school, *A. pyogenes* was found to adhere to bovine infertile eggs. It is these findings which led to the undertaking of the study described in this thesis. It was also believed that the improved diagnostic tools in scientific investigation such as ultrasound, laparotomy and laparoscopy, electron microscopy, bacteriological and biochemical tests, would help in understanding the pathogenicity of *A. pyogenes* infection in early pregnancy in cattle as one element of non specific infection.

As the study was intended to examine the effects of *A. pyogenes* on early embryo in cattle, the literature dealing with fertilisation, implantation, and the mechanisms of maintenance of pregnancy are reviewed below. Emphasis has been placed, throughout, on the situation in cattle but where information from other species is of relevance, papers and subjects illustrating these points have been reviewed. The literature reviewed below has been divided into the following sections which were felt essential for carrying out this project. They are:-

(a) the normal development and maintenance of pregnancy to parturition,

(b) loss of the conceptus and methods of identifying this and reasons why such loss might occur, and

(c) finally, *A. pyogenes* and its role in loss of the bovine conceptus.

I EMBRYONIC DEVELOPMENT AND LOSS.

A. DEVELOPMENT.

In mammals the conceptus or embryo is formed and grows in the female reproductive tract which consists of two ovaries,two uterine tubes,two uterine horns,

the body of the uterus), the cervix and the vagina. Due to a number of factors, the conceptus so formed does not always develop to term. The literature reviewed below sets out the events leading to development, maintenance and loss of the conceptus, with most emphasis laid on the factors that may cause the loss in cattle.

Endocrinology.

Oestrous cycles occur approximately every 21 days in cattle with a range of 17-25 days, and are about one day shorter in nulliparous heifers (Thatcher and Collier 1986). The cycle has been divided into four phases, oestrus (Day 0), metoestrus (Days 1-3), dioestrus (Days 4-18), and proestrus (Day 19 to oestrus), and the morphological, functional and behavioural changes during the cycle occur in response to and result in endocrine patterns. These manifestations depend on the integrated regulation between the hypothalamus, pituitary, ovaries and uterus (Knickerbocker *et al.*, 1986a). Hansel and Convey (1983) reviewed the physiology and endocrinology of the oestrous cycle in 2 endocrine phases: (i) Pregonadotrophin surge, and (ii) Luteal. The pregonadotrophin surge is characterised by an increase in oestrogen(E_2) and accelerated growth of the preovulatory follicle after regression of the corpus luteum (CL) and decline of progesterone (P_4) (Peterson *et al.*, 1975). Sustained increases in E_2 occur only after the decline of P_4 levels. This is due to the fact that P_4 exerts a negative feed back on the hypothalamus and or pituitary leading to reduction of gonadotrophin release. Removal of this negative influence results in elevated luteinising hormone (LH) base line concentrations with a pulsatile release pattern (Rahe *et al.*, 1980). Follicular stimulating hormone (FSH) alone can stimulate follicular growth, but a combination of FSH and LH has been shown to induce maximal E_2 production.

Peak follicular production and the resulting plasma concentration of E_2 potentiate the onset of behavioural oestrus and trigger the preovulatory surge and release of both LH and FSH from the anterior pituitary. Evidence in the cow and ewe suggests that E_2 acts via increasing pulsatile gonadotrophin releasing hormone (GnRH) secretion by the hypothalamus and by enhancing pituitary responsiveness to GnRH. Both these factors and low basal P_4 concentrations are essential for the preovulatory LH and FSH surge, which occur near the onset of a 12 to 22 hour behavioural oestrus period in the cow and last for 8 to 10 hours.

Following the preovulatory surge, follicular production of E_2 and circulating levels of the hormone rapidly decline as follicular luteinization occurs. During the greater part of metoestrus, plasma concentrations of E_2, P_4 and LH are low. A second elevation in FSH concentration but of a lower magnitude and unknown function has been reported between 9 - 12 day after oestrus (Dobson 1978, Kazmer *et al.*, 1981).

Ovulation occurs on Day 1 of the oestrous cycle, approximately 24-30 hours after the preovulatory surge of LH and FSH, and this is followed by development of the CL and a rise in plasma P_4 concentration from about 1 ng/ml on Day 3 after oestrus to a plateau of 6 to 10 ng/ml from Days 7 to 18. LH secretion is altered in response to steroid hormone concentrations so that low frequency and high amplitude LH pulses occur during the P_4 - dominant phase of the cycle (Rahe *et al.*, 1980). Follicular growth and atresia occur continuously, throughout the cycle (Choudary *et al.*, 1968, Donaldson and Hansel 1968, Matton *et al.*, 1981), with moderate rises in plasma E_2 (Peters 1985). Only after Day 18 is there an increase in the probability of ovulation of the largest follicle. Thus the rapid growth of a large antral follicle, which is subsequently identified as a mature preovulatory Graafian follicle, occurs from

Days 15 to 18 in the cow (Dufour *et al.*, 1972), accompanied by high levels of plasma E_2 (Chenault *et al.*, 1975, Peterson *et al.*, 1975). E_2 produced by the developing large antral follicle, is thought to initiate the process of luteal regression during late dioestrus via induction of uterine prostaglandin F 2α (PGF 2α) production. Experimentally administered exogenous oestrogens initiate luteolysis in cattle (Thatcher and Collier 1986), but removal of ovarian follicles also results in extended luteal function (Eley *et al.*, 1979). Current data suggests that PGF 2α produced by the horn adjacent to the corpus luteum-bearing ovary is transferred from the venous drainage into the ovarian arterial supply via a counter-current transfer mechanism (Ginther 1981).

Evidence in cattle and sheep (Flint and Sheldrick 1983) has demonstrated the release of ovarian oxytocin during luteolysis, and oxytocin is thought to augment uterine PGF 2α release, thus ensuring a rapid and complete luteal regression (Flint *et al.*, 1990).

Pregnancy results when the liberated mature ovum released from the Graafian follicle is fertilised by the spermatozoon in the oviduct after service by the male at oestrus. The fertilised ovum is referred to as an embryo and the process is called conception. In mammals the fertilised egg proceeds through the cleavage stages in a relatively autonomous manner, up to formation of the blastocyst. The duration of the pre-implantation stage varies between species, ranging from about 4-6 days in mice and rabbits, through nearly 20 days in kangaroos and wallabies, to 12-45 days in sheep and cattle (Arthur *et al.*, 1989a). The pre-attachment phase may be extended for even greater periods by the suspension of blastocyst development, usually when the blastocyst grows to around 100 cells as in marsupials (Renfree 1982). The transformation from the fertilised egg (with 2-cell stage eggs placed on oviductal epithelial cell monolayer or granulosa cell layer culture systems (Gordon 1991)) to the blastocyst stage can take place *in vitro*, so growth during the pre-implantation period is not necessarily dependent on the uterus,

but the
transition from blastocyst to implantation and expansion of the embryo requires a receptive uterus and a physiological trigger to initiate the transition. During implantation and for some time thereafter, the embryonic membranes form and develop into a placenta, which makes possible efficient nutrient exchange between the dam and embryo.

All mammalian embryos need to shed the zona pellucida in order to be free to adhere to the uterine epithelium. In the Artiodactyla (cloven-hoofed ungulates which include cattle), the blastocyst elongates considerably before attachment and implantation occurs at predetermined sites known as the endometrial caruncles. The horse, pig, kangaroo and wallaby do not have such specialised areas and attachment occurs at the unspecialized endometrial surface and over the entire trophoblastic surface.

Pregnancy (the period from fertilization to parturition) in cattle extends for approximately 280 days after mating. Each oestrous cycle provides the opportunity for the establishment of pregnancy. When conception occurs after service at oestrus, development of the corpus luteum becomes essential for establishment of pregnancy. Elevated P4 concentration also leads to reduction of the uterine tone and myometrial contractility, thus allowing for expansion and attachment of the conceptus. The first 15 days of the oestrous cycle and of pregnancy are identical and presence of a viable embryo is not necessary for the changes that occur up to that time. Beyond this point the presence of a viable embryo within the uterus plays an important role in the perpetuation of an embryotrophic environment. Thus maintenance of the corpus luteum reflects the response of the uterus and ovary to the physiological conditions initiated by the embryo and its products and the whole process is referred to as "Maternal recognition of pregnancy" (Short 1969, Thatcher and Collier 1986).

Maternal recognition of pregnancy. The bovine embryo is active endocrinologically by Day 13 of gestation, producing an array of steroids, prostaglandins and proteins within the uterine lumen but alterations in maternal physiology due to this production occur from Day 16 or 17, when the cyclic events involved with corpus luteum regression are modified to accommodate the maintenance of pregnancy. There is evidence of at least 3 possible ways in which the conceptus may prevent lysis of the corpus luteum:-

(i) both progesterone and oestrogen produced by the blastocyst may act directly or indirectly respectively to suppress the uterine release of PGF 2α,

(ii) the blastocyst may secrete an anti-prostaglandin synthetase or a substance which blocks the release of uterine arachidonic acid,

(iii) chorionic gonadotrophic-like activity produced by the blastocyst could act locally to stimulate direct or indirect progesterone secretion, through increasing blastocyst steroidogenesis (Shemesh 1989).

Betteridge et al. (1980) showed that in cattle transfer of viable day-16 conceptuses into suitable recipients up to day 16 after ovulation can result in maintenance of the corpus luteum and normal pregnancy. Using a culture system developed for the maintenance of day 13-18 conceptus tissue, Hickey and Hansel (1987) found significant ($P < 0.05$) luteotrophic activity in the culture medium in 80% of the conceptuses. The active material (Mr $< 10,000$) was found to be lipid soluble, heat labile and with the ability to bind onto dextran-coated charcoal, suggesting that it is a steroid. Reynolds *et al.* (1983) found that intrauterine infusion of 150 ng oestradiol-17ß plus 250 ug PGE-2 at 6 hourly intervals from day 13 post oestrus to day 21 resulted in maintenance of corpora lutea. There is also evidence to show that early cow embryos (day 16-18)

produce a number of prostanoids, including PGE-2 and PGI-2 which are luteotrophic (Shemesh *et al.*, 1984, Hansel and Dowd 1986). The two have often been referred to as possible luteotrophic signals from the conceptus to the dam to invoke maternal recognition of pregnancy in the cow and ewe (Pratt *et al.*, 1977, Silvia *et al.*, 1984).

Thrombocytopaenia has been found to be an initial response to pregnancy in mammals and man (O'Neill 1985 a). Upon activation by embryo derived Platelet Activation Factor (PAF), platelets slowly release histamine, serotonin, arachidonic acid derivatives, growth factors, and other factors that have roles in cellular attachment and adhesion (O'Neill 1985 b, O'Neill *et al.*, 1987). Habenicht *et al.* (1985) reported that human platelet-derived growth factor increases synthesis products of the cyclo-oxygenase pathway of arachidonic acid metabolism, especially PGI-2 and PGF. Furthermore serotonin, released from platelets by PAF, has been shown to increase progesterone secretion by bovine luteal cells (Battista and Condon 1986). Hansel *et al.* (1989), carried out *in vivo* and *in vitro* experiments to find out whether platelets, or their products might be involved in early pregnancy recognition in cattle. They found that serotonin and platelet-derived growth factors appeared to be the major products of platelet activation responsible for the luteotrophic activity of the platelets.

Experimental introduction into the uterine lumen of proteins produced by Day 16-18, by the bovine embryo prolonged luteal function when administered between Days 15 and 21 in a normal cycling cow (Knickerbocker *et al.*, 1986b). These secretory proteins may reduce the capacity of the uterus to synthesize and secrete PGF2α thus providing for corpus luteum and pregnancy maintenance (Knickerbocker *et al.*, 1986c). Kindahl *et al.* (1976) have also reported that pulsatile episodes of prostaglandin secretion are depressed during early pregnancy.

In the cow, sheep and pig the signal for maternal recognition is transmitted to the mother even before embryonic tissue becomes intimately attached to the uterine epithelium and is therefore clearly distinct from implantation. Such findings lead to the concept that the blastocyst might participate in the events leading to its own implantation by signalling its presence in the uterus. In the cow, the early effect of the conceptus on plasma progesterone is evident as early as day 10 of gestation (Helmer *et al.*, 1987). In species such as the pig, the blastocyst remains free in the uterine lumen, where it may produce a substance capable of diffusing into the uterine fluids and across the uterine lumen, exerting a local effect on the uterine tissue (Shemesh 1989).

Other maternal functions influenced by the presence of the embryo include :- maternal vascular permeability, uterine blood flow, fluid movement, uterine synthetic activity, nutrient transfer and immunosupressive activity, mammary gland growth and development.

Apart from P_4 produced by the corpus luteum (6-15ng/ml) throughout gestation, the placenta contributes the bulk of the P_4 and is able to maintain pregnancy in absence of ovaries from Day 200 but adrenal glands must be present (Wendorf *et al.*, 1983). Elevated concentrations of plasma P_4 and placental oestrogens (predominantly oestrone-sulphate, 17α, oestradiol-(17αE_2)) during pregnancy, inhibit pituitary gonadotrophin production so that a preovulatory surge of LH and ovulation does not occur. Other oestrogens found in foetal plasma and fluids and maternal plasma include pooled 17-α/ß oestradiol sulphate (E_2SO_4), androstenedione (A_2), and testosterone (T) and are demonstrated throughout pregnancy (Eley *et al.*, 1981, Gaiani *et al.*, 1984), and concentrations in maternal plasma are depleted soon after parturition and expulsion of the placenta. Levels of LH and the growth hormone do not change significantly during

pregnancy but prolactin concentration increases from 80ng/ml 2 weeks before parturition to 400ng/ml just prior to calving. About 20 days prepartum, secretion of corticosteroids from the foetal adrenal cortex activates placental oestrogens which in turn stimulates endometrial PGF2α production. The PGF2α production is accompanied by a decline in plasma progesterone just prior to parturition (Peter and Ball 1987). Endocrine events during the preparturient period represent the final stages of foetalplacental maturation and provide the impetus for synchronous mammary gland function at birth, post-parturient involution and reinitiation of post-partum ovarian cyclicity.

1. Uterus - the site of embryonic growth.

The uterus is a highly specialised organ located in the pelvic cavity in heifers and sometimes extending into the abdominal cavity in cows. In cattle the uterus consists of the uterine horns, the body and the cervix. The horns are between 35-40 cm long and decrease in size towards the utero-tuberal junction with the fallopian tubes. The body is about 3-5 cm long. The wall of the uterus varies in thickness from 1 to 1.5 cm and consists of three layers, which from outside to inside are: (i) the thin serous layer or serosa, (ii) myometrium and (iii) the mucous membrane or endometrium.

The **serosa**, which is the peritoneal covering of the organ consists of a single layer of mesothelial cells supported by a thin layer of connective tissue.

The **myometrium** consists of three strata of smooth muscle fibres and connective tissue. The outermost and innermost strata are thin, with the outermost having longitudinal fibres and the innermost having oblique or circular fibres. The middle stratum is much thicker and its smooth muscle fibres are circular. Because larger blood vessels of the uterine wall are found in this layer, the layer is sometimes referred to as the stratum vasculare. Smooth muscle fibres in this layer increase in number and in length during pregnancy.

The increase in number is thought to be both due to division of the pre-existing fibres and transformation of the undifferentiated cells in the connective tissue (Sisson 1975, Blandau 1977, Leeson et al., 1985, Junqueira *et al.*, 1989).

The **endometrium** (mucous membrane) of both the horns and the body bears endometrial caruncles which are more prominent in cows than heifers. These are oval prominences scattered irregularly over the surface or arranged in rows and are about 15 mm high in the non-gravid uterus. They increase in size and become pedunculated in pregnancy. The underside has a hilus at which blood vessels enter whereas the surface appears spongy due to crypts which receive the villi of the allanto-chorion at placentation (Sisson 1975). The endometrium consists of a columnar epithelial lining and connective tissue lamina propria or endometrial stroma which is continuous with the myometrium. The stroma consists of glands which are long and branched in cattle and open through the epithelium to the uterine lumen. The glands are composed of columnar epithelium. During oestrus, under the predominant influence of oestrogen, the endometrial stroma grows in thickness and the glandular epithelial cells secrete mucus. Soon after conception in cattle, luteal progesterone (P4) stimulates the glandular epithelium of the endometrium to become secretory, providing nutrients (histotroph) for the conceptus growth and development of the conceptus prior to placentome formation (Day 45).

Immunological properties. The uterus is very responsive to changing hormonal levels, and either the trophoblast and/or the uterus must have some unusual immunological properties, for the uterus does not reject the immunologically alien conceptus, although it is capable of rejecting other foreign tissues. Certainly the uterus is a rejection site and is an organ in which transplantation antigens are readily detectable and transplacental immunity is fully expressed

(Hansen *et al.*, 1986), but the reason for the exemption of the embryo from immunological attack is a field that has extensively been investigated (Renfree 1982). Beer and Billingham (1974) have postulated that the embryo seems indifferent to the maternal immune response, the trophoblast layer of cells being continuous and unbroken, acting as a dialysis membrane and being in direct contact with the maternal uterine tissue and blood. Evidence seems to be emerging that the embryo establishes an immunological "truce" with the dam, and either jams or controls her immune system. The embryo probably achieves this in a number of ways:

(i) In species that form decidua at implantation, the decidual tissue can give the early conceptus some immunological protection. The trophoblast is a privileged tissue, because it not only has fewer histocompatibility antigens on its surface, but trophoblast cells are coated with a physiological barrier of sialomucin.

(ii) The trophoblast tissue also sheds surface antigens (Clark *et al.*, 1986) which may reduce immunological recognition, and provides a stimulus for the production of maternal protective or blocking antibodies that prevent the proliferation of antigenically reactive cells.

(iii) There is evidence that placental hormones may have immunosuppressive properties. Despite the differences in the type of placentation in different species, a common feature in all mammals is that the trophoblast epithelium produces progesterone at some stage of gestation. High tissue progesterone profiles have been shown to suppress the maternal cellular response (Hansen *et al.*, 1989), possibly preventing rejection. Progesterone has been demonstrated to prolong the life of skin allografts placed in utero in the sheep. This is thought to be due to production of uterine immunosuppressants induced by the hormone (Hansen *et al.*, 1986, Stephenson and Hansen 1987).

The presence of transplacental antigens in the sheep (Clark *et al.*, 1986) has

been shown to pose a risk for fetal rejection. The sheep uterus has also been shown to possess an immune system capable of rejecting tissue grafts (Reimers and Dziuk 1974, Hansen *et al.*, 1986) and antibodies against the conceptus have been found in maternal blood. Despite the above findings, foetal rejection does not occur. Experiments and hypotheses have shown that the sheep conceptus produce several molecules that inhibit immunorejection. These molecules include prostaglandin (PG) E-2, which is produced by the pre-implantation conceptus, cotyledons and allanto-chorion (Hyland *et al.*, 1982, Evans *et al.*, 1982, Risbridger *et al.*, 1985), and is found to be immunosuppressive at concentrations as low as 10^{-8} pg/ml. Another protein, a lactosamino-glycan containing glycoprotein (Mr 800,000-900,000) is also secreted by the pre-implantation conceptus (Masters *et al.*, 1982, Newton and Hansen 1988). Ovine trophoblastin protein-1 (oTP-1) and the alpha-interferons produced by the conceptus (Imakawa *et al.*, 1987) are also immunosuppressive (Ohman *et al.*, 1987). Futhermore the definitive placenta has been shown to release immunosuppressive factors when placed in culture (Low *et al.*, 1988). The pregnant uterus also produces molecules that alter lymphocyte responsiveness to the embryo *in vitro* (Bazer *et al.*, 1979, Sergerson and Libby 1984, Hansen *et al.*, 1987, Stephenson *et al.*, 1988)

It is however important to stress that the uterus is a basically hostile environment which must be modified by hormones and or products of the conceptus to accept the foetus (Hansen *et al.*, 1989).

2. The Placenta and its function.

Formation of foetal membranes: Foetal membranes are essentially auxiliary structures necessary for embryonic growth and are derived from three basic extraembryonic germ layers (ecto-, meso-, and endoderm) which support the

growth, nutrition, respiration and excretion of the embryo throughout pregnancy. The membranes may vary in shape and size in different species.

The single layer **trophoblast** (or trophoectoderm) is the first of the cell layers to become extra-embryonic, and it has an important role in the attachment and implantation of the embryo. It later fuses with the mesodermal cells forming the chorion, the outer envelope that encloses the embryo and the other two foetal membranes, the amnion, yolk sac and allantois (**Fig. 1**).

The **yolk sac** occurs in all embryos with an amnion and in the mammals it develops early from the blastocoele cavity and in them contains only yolk bodies or very small yolk masses with accumulated nutrients from the uterus. After fusion with the chorion, the yolk sac may be vascular (trilaminar) or avascular, and the chorio-vitelline placenta formed makes the first attachment, a site for exchange with the mother. In some mammals like man, the yolk sac becomes vestigial after about one week, but in others like rabbits, the yolk sac placenta remains an important organ of nutrient and antibody exchange throughout pregnancy.

The **amnion** develops from extra-embryonic ectoderm and avascular mesoderm to surround the embryo proper completely and provides a fluid-filled environment in which the embryo can float and develop. The amniotic fluid also provides protection from mechanical shock and desiccation. The amnion is never vascular.

The **allantois** is an outer growth of the embryonic hind gut, and is continuous with the urinary bladder and the fluid is thus rich in urea and nitrogenous waste products. The allantois is derived from endoderm and vascular mesoderm, and it expands into the extra-embryonic coelom to fuse with the chorion forming a definitive chorio-allantoic placenta. In most mammals the

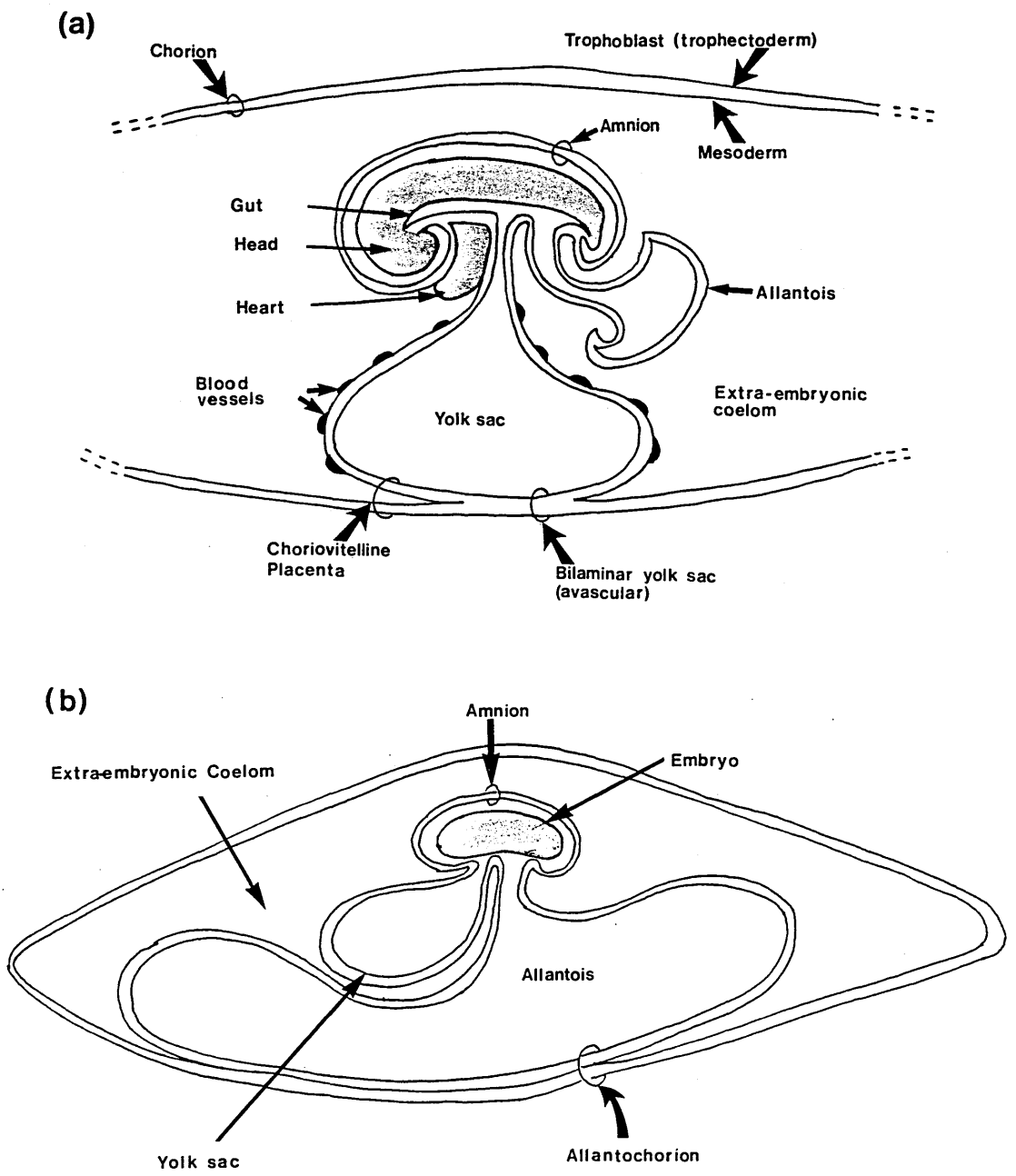


FIG. 1: Placental formation in animals. This is based on the pig showing elongation of the embryonic vesicle:- (a) shows the embryo surrounded by the amnion and allantois being formed from the embryonic hind gut, with a choriovitelline placenta and (b) the allantois and chorion fusing to form the allantochorion and the yolk sac regressing (From: Renfree 1982).

chorio-allantoic placenta takes over from the chorio-vitelline placenta as the main organ of respiratory and nutritive exchange (Perry 1981).

3.Placental types.

One aspect of the placental classification of Grosser (Renfree 1982) into 5 types, was that efficiency of the placenta is inversely proportional to the number of cellular layers separating the maternal and foetal circulations. The 5 were types (a) epitheliochorial (3 maternal and 3 foetal) eg in mare, pig, cow, sheep; (b) syndesmochorial (2 maternal and 3 foetal); (c) endotheliochorial (1 maternal and 3 foetal) eg in seal, dog, cat, ferret; (d) haemochorial (no maternal layer and 3 foetal layers) and (e) haemoendothelial (no maternal layer and 1 foetal layer). Though the classification was useful for categorisation the functional implication was disproved by Amoroso (Renfree 1982).

The other type of classification based on gross morphological appearance and sites of chorionic attachment to the endometrium includes (a) diffuse (eg. in horse, pig, camel); (b) cotyledonary (eg. ungulates - cow, sheep); (c) zonary (eg. dog, cat, and ferret) or (d) discoid (eg. in man, rat, mouse and guinea pigs). In ruminants embryos elongate before forming a placental attachment. Sheep embryos grow from a 1mm sphere to a 1m elongated sac between 12-14 days of gestation (Renfree,1982)

B. Pregnancy loss.

In theory every successful union of a male and female gamete should result in birth of a normal offspring, but the reality is that apart from the enormous loss of gametes prior to fertilization, there is a high rate of embryonic or foetal loss after fertilization and this loss can occur at any time during pregnancy.

Pregnancy loss can occur any time from fertilization to parturition and in cattle

this can be divided into 3 stages each of 3 months and designated as the first, second, and third trimesters. A number of methods have been developed for monitoring fertility and pregnancy and or its loss in cattle and these will be dealt with below.

1. Diagnosis of pregnancy, embryonic/foetal loss in cattle.

Clinical examination has three aspects: the animal, the history and the environment all of which require adequate examination to avoid errors. In fertility control for investigation of pregnancy or its loss , a complete and thorough examination of the female reproductive tract is essential for diagnosis of the physiological state of the animal. This determines whether the animal is cycling or pregnant and provides an estimate of the stage of such a pregnancy, and characterises any pathological features of reproductive tract. It also allows the clinician to predict events in the future such as oestrus, ovulation, parturition or abortion and allows for a rational approach to therapy and establishing a prognosis. Additional information gathered from the laboratory may add weight to the findings (BonDurant 1986). The examination can be carried out as follows: (A) external examination, (B) rectal palpation, (C) vaginoscopy, (D) laparoscopy, (E) ultrasound, (F) blood sampling for hormonal study, (G) pregnancy specific proteins and (H) laboratory examination for microbiological and parasitic study.

(a) External examination. This should always precede acquisition of the proper case history which should include; date, course and outcome of the previous pregnancy (if any); state of sexual cycle, including intensity and duration of oestrus; previous mating or insemination; previous treatment; stage of lactation and milk yield. The examination should then continue by analysis of

environmental factors such as feeding and management, size and shape of the abdomen, changes in the udder and perineal regions (Rosenberger 1979). It may be beneficial to repeat examinations for example when checking the oestrous cycle. Signs of muddy hoof marks over the flanks indicate that the animal in standing heat has been ridden by other cows (Williamson *et al.*, 1972, Esslemont and Bryant 1974, Foote 1975). The pelvis and the broad pelvic ligaments (ligamenta sacrotuberalia lata) which are normally tightly stretched, should be examined to determine the degree of relaxation in approaching parturition, relaxation at any other time may be pathological, as in cystic ovarian degeneration (Rosenberger 1979). Inspection of the underside of the tail and the area around the ischial tuberosities for traces of mucus or encrusted secretion, may provide evidence of a vaginal discharge as in oestrous or genital catarrh (Arthur *et al.*, 1989b). The discharge may be pathological and may consist of pus (Noakes 1986).

Freemartins and females with genital hypoplasia have relatively small vulval lips. The vulval lips are relatively large during oestrus, in ovarian cysts, just before calving and when inflamed. Vulval asymmetry may be due to neoplasia, haematoma, abscess or injury. The surface of the vulval lips is normally smooth but is folded between oestrous cycles. The colour may vary from pale pink, reddened or anaemic or to yellowish in jaundice. Closure of the vulvar cleft may be incomplete, there may be a lateral or dorsal laceration, cystic degeneration of the vestibular glands, or inversion or prolapse of the vagina. Examination of the hairs at the ventral commissure may find these dry, moist or matted with mucus, pus and or faeces.

(b) Rectal palpation. It is possible to feel parts of the reproductive tract through the rectal wall, but this is not always easy. The first structure which can

easily be identified is the cervix as the vagina is frequently flaccid and thin walled and is not prominent. It lies on the floor of the pelvis close to the pelvic ridge. In cows the cervix is firm, cylindrical, 7-10cm long and 2-7cm thick, but it is smaller and softer in heifers (Rosenberger 1979, BonDurant 1986). It increases in size with the increase in the number of calvings and in old cows may be 10cm long and 5-6cm diameter (Noakes 1986) but abscesses associated with calving or AI may cause marked distortion. Symmetrical enlargement indicates diffuse inflammation or recent abortion whereas asymmetrical enlargement indicates abscesses, injury or scar formation. Whereas the size and location of the cervix may also differ in different breeds, it is normally less mobile as pregnancy advances due to the pull of the gravid uterus. A similar effect can result from pathological conditions such as pyometra, adhesions and tumours.

Just beyond the cervix in the non pregnant uterus is the **body of the uterus** which is about 5 cm long and is demarcated from the uterine horns by the intercornual ligament, which forms a false bifurcation. The empty uterus usually contracts on palpation and is mostly found located in the pelvic cavity near the pelvic brim in heifers and young cows. In older cows the uterus sinks in the abdominal cavity when abnormal uterine contents are present (pneumo-, hydro-, myxo-, pyo- and haemometra) and also during pregnancy and soon after parturition and also some breeds as a natural process.

Examination of the **uterine horns** should include the size, symmetry, consistency, contractility (tone and motility) and presence of any content. Cyclic changes in the uterine horns can be predicted on palpation, the tone is flaccid and the horns are difficult to identify during dioestrus, but as the corpus luteum regresses and follicular growth occurs a couple of days before oestrus, the tone increases so that the horns become turgid and coiled especially on manipulation (Arthur *et al.*, 1989c). The tone declines 1-2 days after oestrus

(Noakes 1986). Uterine pathological conditions that can be diagnosed on rectal palpation include inflammation which may take the form of moderate or severe endometritis, acute metritis or pyometra; uterine or utero-ovarian adhesions which frequently result from inflammatory insults involving the serosal surface of the uterus; abscesses which occur predominantly after dystocia or the improper use of an intrauterine catheter; tumours like lymphosarcomas, leiomyomas and carcinomas which are not common but occur predominantly in older cows; and foetal remnants after foetal maceration and mummification (BonDurant 1986).

The **oviducts** (Fallopian tubes) which are about 20-25cm long are located at the anterior edge of the broad ligament and are difficult to feel in a healthy animal, but in salpingitis, they are hard, thickened and lumpy as in tuberculosis and fluctuating in hydrosalpinx.

Ovaries. Palpation of the ovaries is of particular importance in determining the stage of the female reproductive cycle. Their location depends on the age of the animal, how many times the animal has calved, the size and state of the horns (pregnant or diseased). Features that can be felt on palpation include the size, surface consistency (smooth or lumpy; protrusion or cavities) and passive motility as well as structures like vesicles (follicles or cysts), and corpora lutea (Rosenberger 1979), corpora albicantes and smooth ovaries (with no significant structure). Ovarian cysts (follicular or luteal) are fluid filled structures normally larger than 25mm in diameter. Experience has shown that rectal examination of ovaries gives false results in about 20% of cases (Dawson 1975). The main causes of error are the failure to recognize follicles and small cysts, missing a corpus luteum and confusion between corpora lutea, cysts and follicles. Some rare pathological conditions of the ovaries include abscesses and tumours (BonDurant 1986).

(c) Vaginal examination. This may yield supplemental information that may refine the tentative diagnosis made after external and rectal examinations. In some intensive dairy practices vaginal examination has almost replaced rectal examination because it has been shown to be a more sensitive method for the diagnosis of mild to moderate endometritis (BonDurant 1986). Initially the vestibule of the vagina is examined to determine the colour of the mucus membrane, the amount and consistency of any secretion adhering to the mucosa, and any pathological changes (vesicles, nodules, bruises, tears, scars, narrowing, retention cysts of the vestibular glands, neoplasms and any other findings) (Rosenberger 1979). Either a vaginal speculum or manual examination could be used.

(i) Vaginal speculum. A sterile lubricated speculum is used and the vaginal lumen is examined for location of the cervix, its shape and degree of openness, the colour and moistness of the external part of the cervix and vaginal mucosa. Vaginal length should be noted, since this is abnormally short in freemartins. Physiological secretions such as cervical mucus, may indicate accurately the stage of the oestrous cycle - large quantities are present in the follicular phase and only scanty secretion in luteal phase. Abnormal vaginal contents may include faeces (in complete perineal rupture, vagino rectal rupture or fistula), urine (urovagina), air (pneumovagina), mucus (mucovagina) or pus (pyovagina). Other features of the vagina are constriction of the lumen due to adhesions, scarring or persistent hymen (as in white heifer disease of Shorthorns) and swelling of the vaginal wall due to abscesses, haematoma, cellulitis, neoplasia, retention cysts of Gartner's ducts and prolapse of the first cervical ring (Rosenberger 1979, Noakes 1986).

(ii) Manual examination. This is only recommended in cows during

difficult calving or to feel cords or septa stretched across the vaginal lumen. It may also be recomended in perivaginal tumours (Noakes 1986).

(d) Laparoscopy. The reproductive tract can be visualised by laparoscopy and endoscopy after making a laparoscopic incision in either the right or left flank (Rosenberger 1979).

(e) Ultrasonographic Examination. Direct observation of the uterus and ovaries without surgical intervention has been made possible by ultrasound. The technique originally used was "amplitude-depth" (A-mode) ultrasound, in which the presence of fluid-filled viscera was inferred from sonar-like echoes displayed on a cathode ray tube/oscilloscope screen. This device, originally developed for swine back fat measurement and adopted for pregnancy diagnosis, proved less accurate than rectal palpation (BonDurant 1986). Real-time ultrasound in which a two-dimensional picture is generated from the echoes, has offered considerably greater accuracy and has been successfully employed for following events of the oestrous cycle in domestic animals (Rantanen *et al.*, 1982, Omran *et al.*, 1988) and more commonly in pregnancy diagnosis (Boulet, 1982, Ginther 1983, Tainturier *et al.*, 1983(a), Boyd *et al.*, 1988).

Pregnancy diagnosis in cattle is possible from 1 month onwards using a B-mode scanner with a transducer frequency of 3.5mHz transrectally (Chaffaux *et al.*, 1982, Tainturier *et al.*, 1983(b), Reeves *et al.*, 1984). Using a 3.5mHz sector transrectal transducer with a B-mode scanner, Kahn (1990) was able to document the anatomical features of 71 bovine foetuses between day 30 and 10 months of gestation. Characteristic images of the head, neck, vertebral column, thorax, stomach, liver, kidneys, urinary bladder, limbs, scrotum, teats, umbilical

cord, amnion and allantois could be discerned. A reliable positive diagnosis was possible from 45 days onwards and embryonic deaths of 23% were recorded between Days 30 and 60 of pregnancy (Chaffaux *et al.*, 1986). Using a higher frequency transducer (7.5mHz), Boyd *et al.* (1988) could diagnose pregnancy as early as 9 days after conception. Some work has been carried out using ultrasound to study embryonic loss induced by the prostaglandin analogue cloprostenol (Estrumate) (Guilbault *et al.*, 1988, Omran 1989).

In an attempt to evaluate the reliability of ultrasound (with a 7.5 mHz transducer) in 148 cows between 21-33 days after AI, Pieterse *et al.*, (1990), found a considerable difference between the reliability of scannings performed at an early stage (Day 21-25) and those performed at a later stage (Day 26-33). Sensitivity and specificity between Days 21 and 25 were only 44.8% and 82.3% respectively, but were 97.7% and 87.8% respectively between Days 26 and 33. Low accuracy or a high frequency of false negatives between Days 21-25 was attributed to difficulty in detecting the small amount of fluid present at that time. Eleven cows were incorrectly diagnosed as pregnant between Days 21-33 and this was thought to have been due to the animals being in proestrus at the time of examination, while others could have had a pathological accumulation of fluid in the uterus, or were pregnant and subsequently experienced early embryonic death. Little investigation of pathological conditions of the reproductive tract has been carried out by ultrasound in cattle except for examination for cystic ovaries (Edmondson *et al.*, 1986, Omran *et al.*, 1988), and even less has been done ultrasonically to investigate the pathology of the reproductive tract with infectious causes of bovine embryonic loss (Fissore *et al.*, 1986).

(f) Hormonal tests. The hormones most commonly (and routinely) investigated

in the diagnosis of pregnancy are oestrogen and progesterone.

Oestrogen. Oestradiol-17ß (E_2) is the major oestrogen produced by follicles in the ovary in many species. In cattle the level increases from about 2 to 15 pg/ml in the follicular phase reaching the highest levels during oestrus. After ovulation with or without fertilization of the ovum, ovarian follicular activities are drastically curtailed and the levels of E_2 falls between 2-5 pg/ml. The changes in the concentrations (in plasma or serum) are measured using radioimmunoassay (RIA; Nachreiner 1986). High levels of E_2 in an animal thought to be pregnant may point to a possible loss of the conceptus. Because the levels of E_2 are very low, the assay has to be very sensitive. Another drawback is that the time taken for the test is long (1.5 to 2 days). The few enzyme immuno assays (EIA) kits currently being developed, are even less sensitive than the RIA and are very expensive.

Oestrone sulphate (E_1S) is the other oestrogen and is fetoplacental in origin, and can be used for pregnancy diagnosis (Hamon *et al.*, 1981). In cattle E_1S can be detected in serum or milk from day 120 of pregnancy (Nachreiner 1986) and can be measured using the RIA.

Progesterone. When cattle fails to come into oestrus 21 days after service coupled with persistence of the corpus luteum, then pregnancy may be suspected (Arthur *et al.*, 1989c). The progesterone (P_4) produced mainly by the corpus luteum can be measured in blood (serum or plasma) or milk. Using the RIA, the concentration of P_4 in blood can be measured and is between 5-10ng/ml during pregnancy (Arthur *et al.*, 1989c). EIAs have been developed which are as sensitive as RIA, but with an added advantage of earlier generation of results.

Milk P_4 correlates closely with the blood concentrations (Laing and Heap 1971,

Nachreiner 1986). Initially the method used to measure milk P_4 was RIA (Heap *et al.*, 1973), but it has two drawbacks, the use of radioactivity and the need for a specialist laboratory. Many new assays have been developed and can be used with adequate sensitivity to quantitate P_4 levels in skimmed or whole milk, and are used on the farm with minimum equipment. The assay can be done on the farm with results obtainable within one hour. The best of these assays is the enzyme linked immunosorbent assay (ELISA). The accuracy of the assay in detecting pregnancy can be between 80-88% (Heap *et al.*, 1976, Hoffmann *et al.*, 1976a, Nachreiner 1986) and detection of absence of pregnancy can be 100%. Borderline cases can be seen in some cows between days 21-23. If these persist up to day 30 or more, it may indicate an impending abortion due to early embryonic death (Nachreiner 1986).

(g) Pregnancy Specific Proteins. A feature of pregnancy in many species (best documented in man) is the increase in protein levels and the appearance of new proteins in the maternal circulation for the maintenance of the maternal foetal inter-relationship. These proteins have been found to be specific or associated with pregnancy and are present at widely varying levels during gestation. The first of these so-called pregnancy specific proteins to be identified, human chorionic gonadotropin (hCG) was in man, but others have also been found in the blood (plasma and serum), ovaries, embryo/foetuses, amniotic and allantoic fluids, placenta and foetal membranes of other species. In man some of these proteins reach their maximum levels early in gestation whereas others like hCG do so at term. Thus the progress of pregnancy can be monitored and embryonic death accurately predicted. Some pregnancy specific proteins have been identified and characterized in domestic animals (especially early in pregnancy) others are yet to be characterised, and more may still be identified.

Early pregnancy factor (EPF). EPF is a pregnancy associated immunosuppressive substance in the blood of humans, cows, sheep, goats and pigs (Morton *et al.*, 1976, Noonan *et al.*, 1979, Nancarrow *et al.*, 1981). EPF has its highest activity during the first third of the gestation period; this activity then drops and at the end of pregnancy it can hardly be detected. It is suggested that the ovaries and oviducts take part in the synthesis of EPF *in vivo* as a result of the combined action of signals from the pituitary gland and the zygote (Cavanagh *et al.*, 1982, Nancarrow *et al.*, 1981). It has been demonstrated at different molecular weights and depending on the stage of pregnancy, different forms predominate (Clarke *et al.*, 1978, Clarke *et al.*, 1980). Studies have been carried out in the cow and sow using the Rosette Inhibition Test (RIT) according to Morton *et al.*, (1979). The test has been developed on the basis of the test for demonstrating the immunosuppressive action of antiluteolytic sera (ALS; Bach *et al.*, (1969) Shaw and Morton (1980)). Koch (1986) showed that the test could be used with a great degree accuracy to control the efficiency of A. I., for the diagnosis of early pregnancy and in the estimation of early embryonic mortality rate. However the test is very labour intensive and not easily reproducible, thus making it difficult to deploy as a routine procedure.

Pregnancy specific proteins and hormonal interact in early pregnancy in cattle. Maternal recognition, establishment and maintenance of pregnancy have been important areas of research but until recently progesterone has been the best indicator of positive or negative pregnancy diagnosis, at approximately 21-22 days (Pope *et al.*, 1969, Pennington *et al.*, 1985). At Days 16-21 after service, while high levels of progesterone are maintained, the foetus is believed to produce a luteotrophic factor to counteract the luteolytic effect of PGF 2α

produced by the endometrium. Parkinson (1991) reported that PGF2α is the luteolysin and that oestradiol stimulates uterine production of PGF2α (measured by PGFM - the stable metabolite of PGF2α) and therefore has a luteolytic effect in the cycling cows and ewes but not in pregnant animals. Oxytocin is luteolytic in cows as it stimulates uterine production of PGF 2α (Lafrance and Goff 1985) through oxytocin receptors whose development is induced by oestradiol 17ß. Oestradiol - induced PGF2α produced by the uterus is inhibited by the presence of the conceptus and this is evident by Day 18 (Parkinson 1991).

Likewise Lafrance and Goff (1985), suggested that the bovine conceptus inhibits uterine production of PGF 2α in response to oxytocin.

The conceptus factor has been found to be very closely related to **ovine trophoblastin (oTP-1)** (Rowson and Moor 1967) and is first detected in the gravid horn on day 16 (Roberts and Parker 1974) and has been shown to disappear by Day 24 of pregnancy (Thatcher *et al.*, 1985). Cows from which the conceptus was removed between Days 17-19 had interoestrus intervals of 25 and 26 days respectively as compared to 21 days when the conceptus was removed on Day 13 (Northey and French 1980). Furthermore, twice daily intrauterine injection of a Day 17-18 conceptus (Northey and French, 1980) or a freeze-killed Day 16 conceptus (Dalla Porta and Humblot 1983) on days 14-18 or 15-19, extended corpus luteum function by 4-8 days, but injection of a Day 12 conceptus had no effect on the life span of the corpus luteum (Bazer *et al.*, 1986). Bovine conceptuses cultured in the presence of 3H-Leucine, and also sheep conceptuses, secreted several polypeptides into the medium. The major proteins secreted between Days 16 and 27, identified by sodium dodecyle sulphate-polyacrylamide gel electrophoresis (SDS-PGE), fluorography, and gel and ion-exchange chromatography, have molecular weights (Mr) of 22,000 to

26,000 and isoelectric point (pI) of 5.6 to 6.8 (Bartol *et al.*, 1984). These proteins are thus referred to as **bovine trophoblast protein 1** (bTP-1), and antiserum to oTP-1 reacts with two parts of the bTP-1 thereby confirming their homology (Helmer *et al.*, 1987).

Martal *et al.*, (1987) suggest that bTP-1 may exert its antiluteolytic effect in one of several ways:

* by stimulating the synthesis of endometrial luteotrophic prostaglandin PGE which would behave as antiluteolysin in the ovarian system;

* by inducing the synthesis of endometrial peptides, which would be involved in numerous biochemical processes and in various vessels and tissues; or

* by inhibiting directly or indirectly the synthesis of endometrial oxytocin receptors, and thus reducing the pulsatile secretion PGF 2α.

The antiluteolytic effect, in which the dynamics of PGF are altered, is referred to as an anti-PGF action and evidence to support the concept was reviewed by Thatcher *et al.* (1986). A perfusion experiment was performed to compare the secretion rates of PGF and PGE-2 from the luminal and stromal surfaces of endometrium from cyclic and pregnant cows at day 17 after oestrus (Gross *et al.*, 1988a). Secretion rates of PGF were lower from the endometrium of pregnant than from cycling cows, whereas PGE-2 secretion was not affected by the pregnancy status. To assess the role of bovine trophoblastin protein-1 (bTP-1) in the prevention of corpus luteum regression, *in vivo* and *in vitro* experiments were carried out. Incubation of endometrial explants of day 17 cycling cows with bTP-1 (Thatcher *et al.*, 1989), induced PG-synthesis inhibitor activity and reduced PGF secretion and a slight increase of PGE-2. Likewise intrauterine infusion of bTP-1 from Days 15.5 to 21 extended the interoestrus interval from 19.5 to 26.0 days. Thus it was assumed that bovine conceptuses exert a paracrine effect through the secretion of bTP-1 to induce an endometrial

intracellular inhibitor of PGF synthesis.

Interferons are products of the normal body immunological reaction to viral infection in man and animals. Martal *et al.* (1988) have recently demonstrated homology between *interferons* and *trophoblastin* in two ways (a) antiviral activity and (b) similarity in the amino acid compositions of portions of their polypeptide chains (Imakawa *et al.*, 1987, Stewart and Stevenson 1984). Plante *et al.* (1988) tested the possibility of an antiluteolytic effect of recombinant bovine interferon class I, Type 1 (interferon-α1) using two groups of animals each of 5 cows. Twice daily intrauterine infusion of interferon-α1 from day 15.5 to 21 extended the intraoestrus interval from 22.8 to 26.8 days with an extended CL life span. *In vitro* examination revealed that the antiluteolytic effect did not affect the secretion of PGF by endometrial explants, but it increased endometrial secretion of PGE-2. Thus it was concluded that the shared role of both bTP-1 and interferon-α1 in increasing the PGE-2/PGF ratio may contribute to their common net effect of corpus luteum maintenance.

The bovine placenta also contains a **placental lactogen** (bPL) (Fellows *et al.*, 1976) which is produced by binucleated cells of trophoectoderm and, as in sheep, binucleated cells appear at about 16 day of gestation. The process of attachment of the trophoblast to the endometrium on the other hand does not begin until the third week of pregnancy in the cow (Leisser 1975) and the two events with which the appearance of placental lactogen may be associated are widely separated by an interval of about a week. Using whole conceptuses between 17-25 days of pregnancy, bPL was measured by radioreceptor assay. Levels of bPL were found to increase between Days 17-25 from 49 to 1,091 ng/mg conceptus protein. It is therefore suggested that the hormone is related

to the appearance of the binucleated cells. The bPL was partialy purified and had a molecular weight (Mr) of 45,000 by gel filtration and an isoelectric point (pI) of 5.3 (Hayden and Forsyth 1979). This was in contrast to PL isolated and purified by Bolander and Fellows (1976), which had a Mr of 22,000 by gel filtration. Higher levels were found in animals bearing twins and in dairy cows when compared with beef cows. In early stages of gestation, bPL is believed to contribute to maintenance of the corpus luteum, by acting as an antiluteolysin, but it has also been shown to possess a lactogenic potency, especially in late pregnancy (Roy *et al.*, 1977).

Chorionic gonadotrophin has been demonstrated in cattle (as in other ruminants) but it has not been refined because of its very variable rate of secretion (in very small amounts) and its study has been considerably limited because it does not crossreact with equine and human chorionic gonadotrophins (Martal *et al.*, 1988).

Pregnancy specific proteins A and B. Two pregnancy specific proteins have been identified in the cow by immunoelectrophoresis by using antisera developed to homogenates of bovine extraembryonic membranes (Butler *et al.*, 1982). One protein designated as protein A, had an estimated Mr of 65,000-70,000 and pI of 4.6-4.8, findings which resemble bovine alpha-fetoprotein in both Mr and pI (Marti *et al.*, 1976, Lai *et al.*, 1978). A reaction of identity between alpha-fetoprotein and protein A (Kithier and Poulik 1972, Marti *et al.*, 1976) by immunodifusion confirmed that these were the same protein. The physiological importance of α-fetoprotein in pregnancy is not known but it suppresses immune function in vitro (Murgita and Tomasi 1975, Auer *et al.*, 1976, Ishitani *et al.*, 1979, Yachnin and Lester 1979) and therefore may prevent

immunorejection of the embryo.

The second protein, **Pregnancy Specific Protein B** (PSP-B, Mr 47,000-53,000 and pI 4.0-4.80), has been described as an acid glycoprotein associated with the binucleated cells of the trophoblastic ectoderm which may be responsible for its synthesis (Eckbald *et al.*, 1985) but its physiological role is still obscure. A double antibody radioimmunoassay (Sasser *et al.*, 1986) was able to measure PSPB in serum and plasma of pregnant cattle 24 days after breeding and detected pregnancy earlier and more accurately than rectal palpation. Humblot *et al.*, (1988a), compared the radioimmunoassay of PSPB and progesterone assay (Pennington *et al.*, 1985) in pregnancy diagnosis, embryonic death and during the post partum period in cattle. They found that the former method is more accurate than progesterone assay as a method of pregnancy diagnosis 28 days after service (in multiparous animals bred from 70 days post partum) giving a 90% accuracy compared to the 61.0% accuracy found using progesterone assay (Pieterse *et al.*, 1990).

Over 60% of pregnancy failure occurs in the first 50 days of pregnancy due to non-fertilization or early embryonic death (Ayalon 1978, Diskin and Sreenan 1980, Humblot 1986) as demonstrated by the assay of progesterone concentration on day 24 and observation of early return to oestrus. Serum and plasma PSPB may be a more responsive and accurate marker for embryonic loss, at least from Day 30 onwards as its secretion continues to increase during normal pregnancy. However the physiological response of PSPB to embryonic death is not known, although post partum it has been established that PSPB can be detected in blood for 70-100 days (Humblot *et al.*, 1988b, Sasser *et al.*, 1989).

2. FACTORS AFFECTING PREGNANCY.

In general all pregnancy losses and birth defects result from either genetic

abnormality of the offspring or adverse environmental effects eg. sub optimal conditions for growth due to inadequate or abnormal conditions of the maternal reproductive system, inadequate nutrition or exposure of the conceptus to an environment insult eg. infection, a harmful drug or physical trauma at birth. The relative importance of these factors varies with the stage of pregnancy. These are reviewed below with particular emphasis on infection.

(a) Chromosomal abnormalities. These are best documented in man. Spontaneous abortion in man has been linked with chromosomal abnormalities in the ovum. These result in unbalanced chromosome complements in the conceptus and in the death of the conceptus which is subsequently aborted spontaneously (Jacobs 1982). Many breeds of cattle have been found to carry a translocation of chromosomes 1 and 29 with individuals thus having 58 or 59 instead of 60 chromosomes (Robertsonia translocation), and this has been assummed to lower fertility in the carriers. Reduced fertlity in carrier females has been associated with the formation of a trivalent at meiosis which at disjuction produces unbalanced gametes and zygotes leading to early embryonic death (Gustavasson 1979).

(b) Single gene defects. Reproductive losses and birth defects can result from abnormalities at the level of the single gene. Both recessive and dominant conditions governed by single genes are recorded in cattle (Leipold and Dennis 1986) but the proportion of abortions caused by genetic factors is not clear (Miller 1986).

(c) Maternal limitation. In all species synchrony between the dam and the conceptus is obligatory to establish pregnancy. The uterine environment undergoes typical changes which can be defined morphologically, enzymologically, endocrinologically and biologically (as described earlier in the chapter) to achieve the synchrony. An inadequate uterine milieu may provoke

embryonic mortality (Fischer and Beier 1986). For example, maternal hormonal imbalances may lead to a sub-optimal or detrimental uterine environment. Ayalon (1978) reported on the composition of secretions from normal and repeat-breeder cows. He found differences in concentrations of ions, energy substrates, and proteins, with remarkable differences in ion content between the cows with normal and abnormal conceptuses.

The developing embryo may also be adversely affected by maternal factors factors such as an abnormally shaped uterus, a functional abnormality eg. an incompetent cervix or faulty implantation of the blastocyst. These may act in a number of ways but principally influence foetal nutrition. Maternal nutrition and the ingestion of toxins are considered below.

(d) Environmental factors. This is a general term which includes the effects of nutrition, season, exogenous hormones and toxic compounds some of which are dealt with below.

Nutrition. Deficiency in minerals or vitamins may be important in bovine abortion. **Iodine** deficiency has been reported to caused the birth of weak or dead calves with goitre (Hiridoglou 1980). Although no correlation has been reported between the deficiency of **vitamin E** and **selenium** and abortion in cattle, lesions due to deficiency of the two are common in aborted bovine foetuses (Miller and Quinn 1975, Hiridoglou 1980). Deficiency of vitamin E and selenium has also been associated with weak or dead lambs (Hartley and Dodd 1957) though some evidence is contradictory (Blood *et al.*, 1983). **Vitamin A** deficiency is associated with defective bone growth, birth of weak calves and / or abortion (Moustgaard 1969) and a high rate of fetal membrane retention. To investigate the role of **dietary protein** on pregnancy in cattle,

Waldhalm *et al.* (1979) fed two levels of proteins (high and low) to two groups of pregnant cows. Animals on a low-protein diet had a higher rate of calf mortality and dystocia. The authors concluded that protein malnutrition may be an important factor in neonatal mortality.

Temperature. Sudden rise in environmental temperature has been reported to cause abortion in pregnant cows but it occurrs rarely (Miller 1986). Experimentally, extreme maternal hyperthermia causes fetal hypertension, hypoxia and acidosis (Morishma *et al.*, 1975). Rise in maternal temperature as in fever due to infection may be more important in abortion than environmental temperatures (Miller 1986) and is considered more fully bellow.

Toxins. Certain plants and chemicals can cause abortion in cattle and the following have been implicated:- the ingestion of needles of *Pinus ponderosa* (Stevenson *et al.*, 1972); nitrates and nitrites, though reports are equivocal (Davison *et al.*, 1964); ingestion of warfarin from food contaminated with rat bait; coumarin occurring naturally in some mouldy sweet clover (Fraser and Nelson 1959); locoism a disease produced by a variety of toxic plants in the genera *Oxytropis* and *Astragalus* (Jolly and Hartley 1977); intravenous sodium iodide, although experimental treatment failed to produce abortion (Miller and Drost 1978) and *Conium maculatum* or hemlock (Kubik *et al.*, 1980) which has been shown to cause congenital defects in calves (Keeler *et al.*, 1980).

(e) **Infections.** These have been reviewed in detail in a separate section (section II) below.

II. INFECTIONS AFFECTING PREGNANCY.

Infection is a common cause of embryonic or foetal loss. A wide variety of infectious agents can affect the embryo or foetus directly. They include viruses, bacteria, fungi and protozoa. Some affect both dam and conceptus, some the conceptus alone and others produce a direct effect on the dam and an indirect effect on the conceptus. The subject is reviewed below in general terms and specifically with reference to infectious causes of embryonic or foetal loss in cattle. The part played in such loss by *A. pyogenes* is reviewed in particular detail.

Infection can cause damage to the conceptus by:-

1. The **haematogenous route** ie. through the maternal blood stream. Infection then gains access to the conceptus via the placenta for example *Bovine viral diarrhoea* (BVD), *Infectious bovine rhinotracheitis* (IBR), *Brucella abortus*, *A. pyogenes*, leptospira, *Listeria monocytogenes*, *Salmonella typhimurium*, and fungi like *Fusarium* and *Aspergillus* species (Anon 1988). The agents impart their pathenogenic effects in one of two ways :-

(a) by being invasive eg BVD, IBR, *B. abortus*, leptospira, salmonella and fungal infection or (b) direct effect of toxin(s) *Listeria monocytogenes*, *A. pyogenes* (doubtful), and most fungal infections.

2. **Ascending infection** spreads from the maternal genital tract associated with organisms that normally inhabit the tract eg coliforms, *Proteus spp.*, *Bacteroides* spp., or vaginal flora like group B streptococci, anaerobic streptococci, or mycoplasma in man (Pierson and Anderson 1970). Organisms may

gain entry to the uterus in cattle after A. I. has been accidentally performed during pregnancy (Kolar 1963, Hinton 1972).

3. The **effect on the dam** (hyperthermia, hypoxia and nutritional problems). **Hyperthermia** (fever) can occur in many infectious diseases. During some epizootics eg. Foot and Mouth Disease (FMD), rinderpest, Rift Valley Fever, the high rate of abortion is due to indirect constitutional disturbances in the mother rather than to infection of the conceptus. The mechanism leading to foetal death and expulsion is not clear but may be fever alone especially before or during implantation. Hyperthermia is a disease complex produced by the presence of substances called **pyrogens** in the circulation. Pyrogens are produced by polymorphonuclear leucocytes, monocytes and macrophages (Atkins 1960, van Miert and Atmakusuma 1971, van Miert and van Duin 1974, Blood and Studdert 1988a). They appear to act as mediators of fever through the action on prostaglandin and calcium levels in the hypothalamus (Feldberg 1975). Endotoxins also appear to act in a similar manner in the hypothalamus. It has not been established whether pyrogens may effect death of the conceptus during infection by the same mechanisms. **Hypoxia** or diminished availabitity of oxygen to the body tissues may be due to any infection which causes pulmonary disorders, anaemia or circulatory disorders, oedema or local effects on the tissue. Foetal hypoxia mostly occurs during difficult parturitions and that may lead to birth of weak or dead foetuses (Blood and Studdert 1988b).

Nutritional disorders that affect the dam may also affect the conceptus as the first physiological response will be to cut off energy for production and sustain maintenance energy levels (Noakes personal communuication). **Teratogens** may be bacterial or viral in origin. When the embryo is exposed to teratogens these are more likely to lead to structural abnormalities, whereas during the foetal period disturbances may occur affecting developmental processes.

Immunorejection may play a part in the pathogenesis of abortion. As already described (pages 13-15) there are some peculiar immunological properties in the pregnant uterus that act to prevent rejection of the immunologically alien conceptus from the uterus. It is suggested that in some infections there is a breakdown of this immunological system resulting in the dam mounting an immunological response against the conceptus (Morgan and Wrathall 1977). The abortifacient action of rat anti-trophoblast serum absorbed with lymphocyte cells was reported by Beer *et al.* (1972). It was removed by absorption with trophoblast cells. Similar experiments were done to study mycotic abortion in sheep by Corbel (1972a). Precipitins were found against placental extracts in the animals that aborted following experimental infection but not in those that failed to abort even after the infection.

It is important to note (when considering infections in pregnancy), the number of young normally born at parturition. In polytocous animals such as the pig, the time at which embryonic death occurs and the number of surviving embryos in the litter are both important criteria for the continuation of pregnancy (Dunne *et al.*, 1974) - and determines the maintenance or termination of pregnancy depending on the time of death.

In cattle and sheep the fertilized ovum reaches the uterus at about 5 days after mating. In cattle, attachment begins at about Day 21 so that by Day 35 nutrition of the embryo becomes primarily haemotrophic via placentomes (Melton *et al.*, 1951). Placentation is established by Day 45 and not complete until day 90. It is important to be aware of this timetable when investigating embryonic loss in order to determine whether the main syndrome is placentitis leading to foetal death or whether the organism attacks the embryo in its pre-attachment stage.

The above mechanisms may act alone or together in any individual infection.

III. INFECTIONS AFFECTING PREGNANCY IN DOMESTIC ANIMALS WITH SPECIAL REFERENCE TO CATTLE.

An overview.

Infections affecting the female reproductive tract affect fertility by altering the uterine environment thereby impairing sperm transport or leading to sperm death, or if fertilization takes place causing embryonic or foetal death, stillbirth, or birth of weak neonates. Infection may be **specific** or **non-specific.** Specific infections develop without predisposing causes and involve clearly defined pathogens. Non-specific infections on the other hand require a predisposing cause and tend to affect individual animals (eg. most bacterial infections including Group C streptococci, staphylococci, *Actinomyces pyogenes*, and *E. coli* which are considered to be opportunist pathogens (Arthur et al., 1989d).

A. pyogenes which is included in the later group will be reviewed in greater detail.

Viral infections responsible for abortion:-

* Infectious bovine rhinotracheitis (Pustular vulvo vaginitis) a herpes virus.
* Bovine virus diarrhoea / mucosal disease (BVD/MD).
* Parainfluenza 3 virus.
* Various enteroviruses.

Efforts to isolate virus from aborted foetuses or embryos are often disappointing. Viruses often penetrate the placenta to reach the embryo or foetus and the outcome depends on the virulence of the virus, its specific tropism and cytopathogenic action and the timing of infection in relation to the stage of gestation (Catalano and Sever 1971, Hubbert *et al.*, 1973a, 1973b). Effects of the virus on the conceptus may thus vary from no effect, to functional deficiency, growth retardation, malformation and death.

The significance of immunity. Neonates in new born farm animals are born virtually agammaglobulinaemic, and it was thought that the conceptus was immunologically incompetent. Precolostral antibodies antibodies to *Leptospira saxkoebing* were demonstrated over 30 years ago by Fennestad and Borg Petersen (1957), since when more work has been done in sheep, cattle, pigs, and horses (Ingram and Smith 1965; Sterzl and Silverstein 1967, Curtis *et al.*, 1986, LeBlanc 1986, Taylor 1989a). Development of immunological functions varies according to the species and is related to the length of the gestation period (Morgan and Wrathall 1977). Bovine foetuses become immunologically competent to some antigens at 90 to 120 days; sheep foetuses at about 35 days and pig foetuses at 70 days. Raised levels of immunoglobulins (IgAand IgM) in the foetal cord blood in animals are frequently due to intrauterine infection. Organisms which have been isolated from aborted foetuses or where specific antibodies have been found in foetal sera include blue tongue, BVD, IBR, enteroviruses and parainfluenza and the bacteria brucella, campylobacter, leptospira and chlamydia.

Diagnosis. Diagnosis of infectious causes of abortion depend upon:-

i) demonstration of the agent in the tissue of the foetus,

ii) demonstration of the agent in foetal tissue sections, the placenta, vaginal discharge, maternal blood circulation etc,

iii) antibodies or antigens may be demonstrated in foetal blood or transudate,

iii) paired serum samples from the dam for demonstration of antibodies or and antigens.

A. SPECIFIC INFECTIONS.

Over the last 30-40 years there has been a change in the importance of different specific pathogenic organisms that influence reproductive function in cattle and other domestic animals. The use of A.I. and clean bulls at A.I.

studs has reduced the incidence of venereally transmitted diseases such as campylobacteriosis and trichomoniasis. Likewise specific eradication programmes with vaccination, blood testing and slaughter schemes have reduced the prevalence of diseases like brucellosis. Conversely other diseases like IBR/IPV, BVD/MD and leptospirosis, have assumed much greater importance possibly due to a genuine increase in prevalence or to better diagnosis, while the prevalence of other organisms has remained static (Arthur *et al.*, 1989d). It is also important to note that the trend of events varies from one part of or one country to another. The percentage of foetopathies from which a specific infectious agent has been identified in the U.K. varies from 3.4-5.5% (Anon 1988) to 23-37% (Kirkbride *et al.*, 1973, Jerrett *et al.*, 1984). Factors like the type of sample collected for laboratory investigations and possible destruction of causal organisms after embryonic or foetal death, may contribute to the relatively low diagnostic rate.

1. VIRAL INFECTIONS.

(a) TOGAVIRUSES.

These are RNA viruses with a lipoprotein envelope and a characteristic nucleocapsid symmetry. The group includes the virus of bovine virus diarrhoea / mucosal disease (BVD/MD), Border disease of sheep and swine fever. These viruses are genetically related, and cross infection leading to disease has been demonstrated between some of them. Border disease can infect cattle.

(i) Bovine virus diarrhoea / Mucosal disease (BVD/MD).

This is the most important viral cause of abortion in cattle in the U.K. (Anon 1988) with more than 60% of adult cattle having significant levels of serum neutralizing antibody (Duffell and Harkess 1985). The disease occurs in two

forms:-

(a) *A mild syndrome* with fever, depression, nasal discharge and diarrhoea with high morbidity and low mortality called **bovine viral diarrhoea.**

(b) *A severe form* with oral lesions, respiratory distress, loss of weight and death characterised by low morbidity and high mortality referred to as **mucosal disease** and sporadic in nature.

The two forms are caused by the same agent (Ramsey and Clivers 1953, Gillespie *et al.*, 1959, Thomson and Savan 1963, Roeder and Drew 1984, Duffell and Harkness 1985). The virus affects the lymphoid system including Peyer's patches and the spleen leading to loss of differentiation between the cortical and medullary areas of lymph nodes and general atrophy of lymphoid tissue. Severe leucopaenia is a feature of the disease. The disease has been reported world wide.

Transmission is by direct or indirect contact leading to viraemia and alimentary mucosal localisation. Venereal infection may occur and, for this reason, semen from infected bulls is not recommended (Wentink *et al.*, 1989). Insemination of six susceptible and six preimmunised heifers with semen from a persistently infected bull led to poor conception rates, though eventually all but one heifer conceived (Paton *et al.*, 1990).

In pregnant animals the virus crosses the placenta affecting the foetus and causing abortion, mummification and varying degrees of teratogenic damage eg cerebellar hypoplasia, ocular lesions eg cataracts, retinal degeneration and hypoplasia and neuritis of the optic nerves, mandibular brachygnathism, musculo-skeletal abnormalities and alopecia (Kahrs 1973, Scott *et al.*, 1973, Braun *et al.*, 1973, Archbald *et al.*, 1973). Roeder and Drew (1984) reported an investigation in which introduction of a persistently infected heifer into a herd of susceptible pregnant animals lead to the development of some of the signs mentioned above in the herd. In the first weeks of pregnancy the virus may cause death and resorption of the embryo. Experimental inoculation of

nine early pregnant cows (63-107 days) with a cytopathic strain of BVD, led to seroconversion in all the infected cows (Brownlie *et al.*, 1989). However though all the calves developed to term, four were stillborn, one with seroconversion but no virus recovered, and 5 were born alive with no apparent seroconversion or virus isolation. It was suspected that failure to develop foetal viraemia or immune tolerance, could be due to lack of presence of the cells in the foetus needed to support the mechanisms at this time. There is considerable evidence to demonstrate, that, before 90 days the foetus can become viraemic and immunotolerant. Between 90 and 120 days the foetus is capable of mounting a specific antibody response to the virus and this is of diagnostic importance (Hubbert *et al.*, 1973b, Harkness and Lamont 1975, Braun *et al.*, 1973, Duffell and Harkness 1985). The birth of persistently infected but apparently normal and seronegative calves has been reported. Such animals secrete for long periods (Westbury *et al.*, 1979, Roeder 1982, Leiss *et al.*, 1983, Cranwell and Drew 1984).

There is a hypothesis that persistent infection resulting from foetal infection and foetal immunotolerance within the first 125 days of pregnancy, is a prerequisite for the occurrence of mucosal disease. It has also been observed that persistently infected cattle have a high mortality rate in the first two years of their life (McClurkin *et al.*, 1984). Observations show that calves born of persistently infected cows are persistently infected themselves and are often seronegative. In one study of the disease, one third of 35 cows in the herd showed a febrile diarrhoea and abortion in 4 of the 24 pregnant cows with many weak new born calves. It was thought that the disease was due to a calf previously bought in the herd (Sol *et al.*, 1989). In other investigations, despite observation of the clinical signs of the disease, sometimes the virus or the serum antibody or both were not detected (Torgerson *et al.*, 1989, Doll and Gerbermann 1989).

In experimental infections of pregnant cows, no macroscopic changes were

apparent in the foetuses but mild microscopic lesions were seen (Braun *et al.*, 1973). Postnatal experimental infection of susceptible non-pregnant cattle leads to subclinical disease (BVD) in most of the animals. Animals develop serum neutralising antibody to the virus between 3 to 4 weeks after infection and the levels are maintained for long periods or life. In one experiment it was found that infected semen led to early embryonic loss. The animals failed to conceive until they had acquired immunity to the virus and they required 2.3 services before they conceived (Duffell and Harkness 1985).

Diagnosis. i) Clinical signs typical of BVD-MD should cause suspicion to the disease. The breeding history of the herd for at least 2 previous years including events like long inter-oestrous intervals, embryonic death and abortion still births and poor growth rates is vital.

ii) Laboratory tests include:-

* virus isolation which can be done from blood or tissues collected postpartum, but it is slow,

* serum neutralisation test for assay of antibody, but the assay takes a long time to provide results,

* An ELISA which correlates highly with the virus neutralization test ($r=0.83$) and has a high reproducibility (Durham and Hassard 1990),

* BVD virus antigens have been demonstrated in cell cultures and in paraffin-embedded tissue sections using monoclonal antibodies (Belak *et al.*, 1989),

* virus antigen has also been detected using immunofluorescence staining of unfixed frozen tissue sections so that results are available in 24 hours (Roeder and Drew 1984).

Results from laboratory tests can be summarised as shown in Table 1.

Control. This can be achieved in two ways:-

i) To prevent transplacental infection could be achieved by (a) eliminating all infected animals form the farm or (b) introducing only animals known to be immune to the farm. Immunity in a herd could be effected by vaccination or deliberate exposure of incoming heifers to persistently infected animals. Live vaccine has a drawback of either crossing the placenta or superinfection from the persistently infected animals thus precipitating the disease. Inactivated vaccines used have these as advantages over the live vaccine. Vaccines are not used in the U.K.

ii) Persistently infected cattle if kept should be isolated from the rest of the herd.

iii) In a pedigree herd the animals should be tested and should be free of the disease.

Border disease and swine fever can infect cattle and as such they will be briefly reviewed below.

(ii) Border disease (Hairy shaker disease).

In sheep the disease is caused by border disease virus which is very closely related to or identical to the BVD virus (Fielden 1986). The disease is transmissible and is characterised by an abnormal hair coat, (hairy fleece), congenital tremor (shaker lambs), abnormal body conformation, defective myelination of the CNS, poor growth and viability. Foetal death with or without abortion may be an important feature (Winkler *et al.*, 1975), infertility with a marked increase in barren ewes, foetal mummification and/or maceration.

Cell free culture extracts from affected lambs can experimentally cause disease in pregnant ewes on inoculation (Shaw *et al.*, 1967, Gardiner and

TABLE 1: Interpretation of laboratory test results of virus isolation and serology for bovine viral diarrhoea / mucosal disease.

	ANTIBODY	
VIRUS	NEGATIVE	POSITIVE
Negative	A Susceptible	B IMMUNE
Positive	C Positively infected	D Persistently infected

Barlow 1972). The virus is a small particle with an ether soluble envelope and is antigenically related to BVD/MD and to swine fever viruses (Plant *et al.*, 1973). The viruses of BVD-MD and border disease are indistinguishable on physicochemical grounds and they cross-react serologically. The difference in the strains is thought to be more important than the host from which the virus is isolated (Harkness and Vantsis 1982). Experimentally infected early pregnant ewes gave birth to infected lambs which spontaneously developed mucosal disease (Gardiner *et al.*, 1983).

Transplacental transmission from ewe to the foetus is well established, and venereal spread is possible. Though most of the infected lambs die soon after birth (unlike in BVD-MD), some survive and these secrete the virus for long periods. Infected lambs may effect transmission of the virus both vertically and horizontally (Cranwell and Drew 1984, Fielden, 1986).

Experimentally the disease can be reproduced in pregnant goats with abortion and the birth of ataxic and shaker kids. Placentitis occurs in the doe (Smith 1986). Experimental infection of pregnant sows (Shaw *et al.*, 1967) led to foetal infection and birth of piglets with striping of the hair coat and a minor degree of cerebellar hypoplasia at term.

(iii) Swine fever (or Hog cholera).

The swine fever virus (like BVD-MD) can cross the placental barrier in pregnant sows leading to abortion. Viraemia may occur in the foetus (with no antibody) and can persist to adult life (Taylor 1989). Foetuses infected after 65 to 67 days may develop the antibody, be aborted or become mummified or be born with haemorrhagic lesions. Foetal resorption may occur or piglets may be born weak with tremors. Though the virus belongs to the same group as BVD-MD and Border disease, its isolates make a distinct antigenic group from the other two (Duffell and Harkness 1985). Some swine fever isolates however share a soluble antigen with the BVD-

MD virus. Swine fever virus can replicate in sheep and cattle though no clinical signs are produced. An antibody response may be elicited when BVD virus infect pregnant sows. Border disease virus may also infect pigs (Taylor 1989b).

Infected sows may fail to show the clinical disease after recovery, but the virus may cross the placenta causing disease in the piglets which may excrete the virus for over 56 days after birth (Harkness 1985, Williams and Mathews 1988).

(b) HERPES VIRUSES.

In this group viral diseases IBR is the only one of great significance in cattle and as such it is the only one being reviewed in depth below.

(i) Infectious bovine rhinotracheitis (IBR).

This is characterised by a wide range of syndromes including *infectious pustular vulvovaginitis, infectious balanoposthitis, rhinotracheitis, conjunctivitis, meningoencephalitis, mastitis* and *abortion* in experimental animals. All these could have a profound effect on the reproduction in both the cow and the bull. The respiratory and genital forms of the disease are caused by a virus of the herpes group 1 (Kahrs 1986, Arthur *et al.*, 1989d). It occurs in at least one form in N. America, Europe, Australia, and New Zealand. Abortion rates in a herd may be as high as 60%.

Because the virus grows rapidly in a variety of cell cultures with distinct cytopathogenic changes are useful for virus isolation , virus titration and neutralization tests for antibody in the serum, the disease has been extensively studied. The mode of entry is primarily by direct contact or ingestion but it may also be venereal. The virus is perpetuated in individuals or populations in a latent form which on reactivation due to stress of any form leads to the shedding of the virus. Cattle are the best reservoir,

although most animals can be infected (Kahrs 1986).

Primary exposure to infection can cause clinical signs and lesions or a mild inapparent disease. In the female reproductive tract the syndrome is referred to as infectious pustular vulvovaginitis. Infection is normally followed by a viraemia accompanied by the production of antibodies by the dam. The invasion of the placenta and foetus causes foetal viraemia, foetal death and abortion 1-7 days after infection (Kerdrick and Osburn 1973). In a recent experiment (Guerin *et al.*, 1989), ten cows were intravenously inoculated with HPV 1, ovariectomized 8-10 days later and 100 oocytes collected. Eighty of the oocytes were fertilized *in vitro* and 27 of the 80 which began to develop were cultured on layers of oviductal cells. Using the HPV 1 assay (on bovine kidney cells for the cytopathogenic effect) the virus was found in embryos of 5 (of the 9 cows) 4 of which also had the virus in their ovaries, follicular fluids, granulosa cells, CL and oviductal cells. Embryos from the infected cows had a lowered rate of development. It was thus demonstrated that HPV 1 could infect oocytes and the embryos, affecting their development. Abortion normally occurs between 4-7 months and may be the only significant sign. Experimental infection to seronegative animals has shown that abortion can occur at any time of exposure during pregnancy. The aborted foetus is frequently extensively autolysed though the changes are normally secondary to impaired foetal circulation. However even in such circumstances focal necrosis and intranuclear inclusion bodies in the autolyzed foetal liver or adrenal glands can be found. It has been suggested that if the disease is so acute that the foetus dies before responding immunologically with antibody, thus accounting for the difficulty in detection antibodies in the foetal fluids.

Investigations of the mechanisms effecting infertility have shown that the virus causes embryonic death by direct invasion of the cells (Bowen *et al.*, 1985, Miller and van der Maaten 1986). There is further evidence that virus

has a direct effect on the ovary causing necrosis of the follicles and luteal tissue following intrauterine, intravenous or intramuscular inoculation (Arthur *et al.*, 1989d).

Diagnosis. Clinical history and examination of the herd in diagnosis is very important. Isolation of the virus from foetal liver, spleen, abdominal fluids, and cotyledons is confirmatory (Arthur *et al.*, 1989d).

Serological tests include :-

* Fluorescent Antibody Tests (F.A.T.) can be of great value (Kahrs 1986).

* ELISA. A number of tests using the ELISA technique have been developed (Krause *et al.*, 1989).

* The intradermal test for HPV 1 infection is a relatively new technique requiring further perfection (Wizigmann *et al.*, 1989).

Gene mapping of viral DNA genome has been used to study HPV 1, it is believed that this will improve the current knowledge of the disease (Simard *et al.*, 1990).

Control. Vaccination of the animals can be undertaken. The virus may cross the placental barrier causing abortion in the third trimester (McKercher and Wada 1964). A temperature sensitive mutant of IBR virus can be used safely even in pregnant animals or a killed vaccine which is better still. Both the intra nasal and intramuscular routes can be used (Kahrs 1986, Arthur *et al.*, 1989d).

(c) Other miscellaneous viruses.

Other viruses which may affect pregnancy include:- Blue tongue virus (a Reo virus) which is teratogenic to sheep foetuses (Young and Cordy 1964, Dennis and Leipold 1986), Parainfluenza 3 virus (paramyxovirus) in cattle and pigs

(Swift 1972, Dunne *et al.*, 1974, Osburn 1986), Japanese B encephalitis and porcine cytomegalovirus in pigs (Mengeling 1986, Cutler 1986, Taylor 1989c), and a range of enteroviruses in both cattle and pigs (Afshar *et al.*, 1964, Dunne *et al.*, 1973). The latter include SMEDI viruses (still birth, mummification, embryonic death and infertility) in pigs (Dunne *et al.*, 1965, Huang *et al.*, 1980, Taylor 1989c) and the F46e virus in cattle (Afshar *et al.*, 1964). Bovine and porcine Parvo virus have been isolated and studied (Mengeling 1986, Osburn 1986 and Taylor 1989e) Specific immunoglobulins to these viruses can be found in foetal or precolostral sera.

2. PROTOZOAL INFECTIONS.

Abortions may occur in trypanosomiasis or babesiosis outbreaks but the infections that have been thought to be most important are trichomoniasis (only in cattle) and toxoplasmosis (pathogenic to sheep, but can potentially affect other species).

(a). Trichomoniasis.

The disease is caused by *Trichomonas fetus* and may cause temporally infertility (Laing 1970) and is distributed world wide. The disease can be transmitted mechanically amongst bulls from an infected bull, the bull being a symptomless carrier (Parsonson *et al.*, 1974), and in the female population venereally at natural service or A.I.

The parasite colonises the vagina, uterus and oviducts. Fertilization is not hindered, but implantation may fail and foetal death or abortion may occur at about 4 months, and this may be accompanied by pyometra (Kimsey 1986). The uterine inflammatory response to infection which occurs one and a half to two months after infection, and may be responsible for the embryonic wastage and death (Parsonson *et al.*, 1976). Placentitis and foetal

septicaemia may occur. Abortion and pyometra may not be the main features of the disease (which may occur in 5% or less of the animals in the herd). Infertility is the main manifestation of the disease sometimes extending to 2 to 5 months in animals exposed for the first time. The disease may be acute (with embryonic death and delayed return to heat), or may be sub-acute (with foetal death and abortion or delivery of a dead calf). The female normally acquires immunity and complete recovery ensues. Then the disease becomes chronic in a herd with all the animals normal except in heifers exposed for the first time and animals newly arrived. Latent infection can be established with the disease reappearing under stress.

Diagnosis. This must include herd record examination and laboratory tests. The organisms may be isolated from preputial samples (Clark *et al.*, 1974a), vaginal mucus (5 weeks after infection), proestrus mucus, or from a pyometra (Kimsey, 1986) by culture on Diomond's medium.

Control. A.I. should be carried out with semen from clean bulls since females recover from the infection. It may also be beneficial to cull old bulls. Treatment of infected bulls can be attempted with acriflavine on the preputial mucosa, Dimetridazole (orally) or Metronidazole (intravenously or locally) (McLoughlin 1968) may eliminate infection.

(b) Trypanosomiasis.

The disease is complex in domestic animals and wild life and is caused by a number of different species of the parasite. Although the disease is of no importance in temperate regions, it is of great economic importance and causes reproductive loss in the tropics especially in Africa where in about 40-60% of the productive areas the disease is endemic. In Africa the major causes are *Trypanosoma congolense*, *T. vivax*, and *T. brucei* which are

transmitted by flies of the genus *Glossina*, the tsetse fly. *T. evansi* causes disease in the camel, but it is also transmitted non-cyclically by biting flies like *Stomoxys* or *Tabanus* causing disease especially in cattle and buffalo in the Middle East, Asia and South America. *T. vivax* is also transmitted by biting flies in Brazil, Colombia and Venezuela where it is pathogenic in cattle and buffalo (Luckins *et al.*, 1986)

Although information may not be precise as to the impact of trypanosomiasis on live stock production, it is an agreed fact that the disease can cause death after an acute or chronic infection, with death occurring from a few months (with low numbers of the parasite found in the blood) to the animal surviving for many months (Morrison *et al.*, 1981). The pathogenesis of the disease includes extensive tissue and organ damage, affecting the heart, skeletal muscles, central nervous system, endocrine organs and the reproductive tract (Ikede and Losos 1972a, Morrison *et al.*, 1981). A major feature of the disease in livestock is anaemia.

The disease has been reported to cause abortion and infertility (with maintenance of the corpus luteum) in infected animals and it can exert long lasting effects on ovarian function. In cases of abortion reported by Leeflang (1975) the organisms were rarely isolated in the foetus or new born (Woo and Limebeer 1971). In some experimental infections, organisms have been found within the uterus including a case of *T. vivax* in the blood of a new born lamb (Ikede and Losos 1972b) and in a calf (Ogwu *et al.*, 1985). *T. congolense* has also been found in mice (Griffin 1983), suggesting a transplacental infection. Ikede et al. (1988) have suggested that because most aborted foetuses do not show evidence of intrauterine infection, it is possible that abortion is related to maternal factors like hormonal imbalance due to pituitary damage, stress of infection, hyperthermia and hypoxia due to anaemia. It has also been suggested that maintenance of the CL could be facilitated by alteration of production of the uterine PGF2α, by the presence

of the trypanosomes within the microvasculature effectively preventing luteolysis (Llewelyn *et al.*, 1988).

In their experiment with cycling goats and cattle, Lleweyln *et al.*, (1987) and Llewelyn *et al.*, (1988) found that infection with *T. congolense* caused disruption of the normal cycle, leading to prolonged inter - oestrus periods accompanied by maintenance of a functional corpus luteum (Stephen 1966, Llewelyn *et al.*, 1988). Some of the infected cows became acyclic. Treatment with the trypanocidal drug Berenil (diaminazine aceturate) led to recovery and resumption of normal function in most of the experimental animals. The presence of trypanosomes in the uterine micromusculature was associated with lack of PGF2α production leading to corpus luteum maintenance, whereas disruption of the normal pattern of LH secretion was thought to be the main reason for the disturbances in the acyclic animals (Llewelyn *et al.*, 1988).

Changes leading to pituitary dysfunction occur in trypanosomiasis, especially in *T. brucei* infection (Ikede and Losos 1975) but no evidence of pituitary damage was found in cattle infected with *T. conglense* (Valli and Forsberg 1979). Hublart *et al.*, (1990) performed *in vivo* and *in vitro* experiments to study the pituitary gonadal axis in the rat (as a model) using *T. brucei* and trypanosomal preparations respectively. They found that although there was a decrease in the testosterone, coupled with an increase of FSH and LH pituitary production during the acute phase of the disease, the levels of circulating LH remained unchanged. Failure of the levels of circulating LH to increase was attributed to possible dissociation of LH sub-units or carbohydrate and/or polypeptide chain hydrolysis. It was assumed that the gonadal imbalance seen could be mediated through release of parasitic components.

3. FUNGAL INFECTIONS.

Fungi are common in nature, and are generally saprophytic but may sometimes become invasive. Mycotoxins in food contamination may cause reproductive disorders eg zearalenone and aflatoxin produced by *Fusarium*

and *Aspergillus* species respectively.

Fusarium spp. are oestrogenic plants and zearalenone (F2 toxin a metabolite of *Fusarium spp.*) has a potent oestrogenic activity. Mouldy feed fed to pregnant animals may lead to abortion, stillbirths or weak neonates. Changes in the reproductive tract depend on whether the corpus luteum is maintained causing pseudopregnancy due to progest a tional influence or the oestrogenizing effects of the toxins may be seen. The toxin is thought to have negative feed back effects on the hypothalamo-hypophyseal secretion of gonadotrophins (Dial and Britt 1986, Taylor 1989f).

Infection of the pregnant uterus leading to abortion in between 1-20% of cases has been reported mostly due to species. of Aspergillus and mucoraceous fungi, the commonest being *A. fumigatus* (Hill *et al.*, 1971). The disease leads to sporadic abortions in cattle, pigs, sheep and horses, mostly in the last trimester.

The mode of infection is most probably haematogenous, from the respiratory or alimentary system following ingestion of spores (Hill *et al.*, 1971, Cysewski and Pier 1968, Miller 1986).

The disease is characterised by the following clinical signs, placentitis, retention of granules in the foetal cotyledons, and marked thickening of the allantochorion. Foetal invasion leads to bronchopneumonia and skin lesions. The following histological features are found; thrombosis, perivascular necrosis, haemorrhages and cellular infiltration (Corbel and Eades 1973, White and Smith 1974). Antibodies to placental extracts were demonstrated after experimental infection in sheep, so immunological rejection may be involved in the pathogenesis (Corbel 1972b).

4. BACTERIAL INFECTIONS.

A wide variety of bacteria have been isolated from aborted foetuses and reproductive tracts of domestic animals and these have been assumed to be

causative agents of reproductive failure. These vary from one animal species to another in importance. Important genera associated with reproductive disease from one animal to another are listed below:-

In **cattle** the following bacteria have been isolated:- *Brucella spp., Campylobacter spp., Chlamydia spp., Leptospira spp., Salmonella spp., Listeria spp.* and *Coxiella burneti* (Hubbert *et al.*, 1973a, Kirkbride *et al.*, 1973, Arthur *et al.*, 1989d). *A. pyogenes* is increasingly being isolated from bovine aborted foetuses but its pathogenic role as a primary or secondary infection is still doubtful (Hinton 1972, Schiefer *et al.*, 1974, Biancardi *et al.*, 1982).

In **other species** additional bacteria have been incriminated in abortion. These include streptococci and *Klebsiella spp.* in horses, and *Erysipelothrix rhusiopathiae* in the pig. All bacteria infecting cattle can also infect the sheep.

The part played by many of the bacteria mentioned above is not clear or proven. The bacteria may enter the uterus at service or soon after causing inflammatory reaction and death to the fertilized egg or the prevention of attachment. Entry may also be haematogenous leading to endometritis or entry to the embryo through the placenta. Bacterial toxins especially enterotoxins produced in the intestinal tract may also cause abortion by inflammation leading to production of substances such as prostaglandin and resulting in termination of pregnancy (Dennis 1966).

(a) Brucellosis.

The disease has been described in the cattle, sheep, goat, pig, and dog and has a world wide distribution (Olitzki 1970, Morgan 1970). Their route of entry is predominantly by ingestion of contaminated feed or through the

conjunctiva and bacteraemia is followed by infection of tissues including lymph nodes and bone marrow. Presence of the bacteria in the placenta is encouraged by a carbohydrate, erythritol, produced by the fetoplacental unit of cattle, which stimulates growth of virulent bacteria after the animals become infected by ingestion of contaminated food or water. The stage of gestation when abortion occurs depends on the degree of infection, virulence of the infecting strain, the stage of pregnancy at the time of infection and the resistance of the host. In cattle the disease is mainly caused by *Brucella abortus*, and is characterised by abortion while in sheep the same organism causes sporadic abortion. *Brucella melitensis* causes disease in sheep and goats, and can also be transmitted to cattle.

The disease is important economically because of abortion occurring from the second trimester, though at the onset of the outbreak earlier abortions may take place. Abortion occurs once or rarely twice and is followed by retained placenta (especially late in gestation), after which subsequent pregnancies are carried to term but may also be followed by retention of the placenta. Retention of the placenta due to a combination of uterine inertia and placentitis is coupled with delayed involution and leads to secondary bacterial infection and metritis. Chronic endometritis may occur leading to infertility and the infection may predispose to adhesion of the ovarian bursa to the ovaries. After involution is complete the organism localises in the udder and supra-mammary lymph nodes until the next gestation. The organism reaches the pregnant uterus via the blood stream and affects the allanto-chorion and amnion, after which foetal death occurs. The earliest lesion is inflammation of the connective tissue between the uterine glands leading to ulcerative endometritis. The cotyledons become involved and large numbers of organisms appear in the endothelial cells lining the foetal blood vessels of the of chorionic villi. This is followed by tissue destruction and eventual loss of the villi. Death of the conceptus is due to interruption

of the function of the placenta and the effects of the endotoxin (Nicoletti 1986), which is followed by abortion. Failure to abort leads to mummification or maceration of the foetus (Arthur *et al.*, 1989d). Calves born to infected dams are usually free from infection, but a few (about 5%) may retain the infection for long while and remain seronegative until the first calving.

Diagnosis. This depends on the history of the disease in the herd, the clinical signs and isolation of the bacteria from contaminated material (Farrell 1974). The bacteria can be isolated from uterine fluid, milk, colostrum and non lactating udder secretions, placental tissue, and stomach contents or lungs of the foetus (Nicoletti 1980). Recently Hopper *et al.* (1989) have seen able to demonstrate *B. abortus* in mammalian tissue using biotinylated whole genomic DNA as a molecular probe.

Antibody detection plays a major role in diagnosis and detection of carriers. The **milk ring test** is done in surveys for the disease in dairy herds and is carried out monthly in the U. K.. Serological tests are done after cultural screening using the **Rose Bengal test** which is very sensitive.

Palmer and Douglas (1989) analysed brucella lipopolysaccharide (LPS) with monoclonal antibodies. They detected four unique epitopes on brucella LPS - (A, M, C and C/Y) demonstrated by blot analysis. The authors suggested that with greater knowledge of brucella LPS antigens, monoclonal antibodies may be useful in monitoring the antigenic content of brucella vaccines and for diagnosis.

Enzyme immunoassays (EIA) have been developed for the detection of *B. abortus* antibodies in cattle. It has been found that purified antigen for EIA can be correlated with the results of the Rose Bengal test and the serum agglutination test (Cecchini 1989).

Other serological tests can be deployed using standardised antigens and the

techniques include, serum agglutination tests, complement fixation tests, antiglobulin tests and plate tests using buffered antigens.

Control. In cattle this can be done by the stamping out method ie. killing all infected animals and in-contacts and all animals tested and found positive (Blood and Radostits) but up to 15% of infected cattle recover from natural infection and become seronegative.

Living Strain 19 vaccine or the Strain 45/20 vaccine - with killed cells in adjuvant can protect animals and reduce the incidence to a point where eradication may become unnecessary (Nicoletti 1986).

This should be coupled with hygiene, disposal of infected materials, separation of animals post abortion or calving, and animal quarantine.

Many European countries are free of the disease or it is present at very low levels because of a combination of testing, eradication, vaccination and movement controls.

(b) Vibriosis.

The new name for the genus is *Campylobacter* and a number of campylobacters affect the female reproductive tract. *C. fetus* causes abortion and infertility in cattle and sheep. *C. fetus intestinalis* causes abortion in sheep and sporadic abortion in cattle. *C. cryoaerophila* has been isolated from the reproductive tract, aborted foetuses and infertility but its significance is not clear. *C. fetus venerealis* causes temporary infertility in cows due to a mild endometritis and salpingitis, and leads to death of the developing embryo and return to heat at irregular intervals. *C. fetus venerealis* does not affect fertilization, though its colonisation of the uterus may cause a tissue reaction with accumulation of exudate in the lumen of the uterine glands and periglandular infiltration by lymphocytes. Such a hostile environment leads to interference with the implantation of the embryo or its

continued nutrition and embryonic loss (Dekeyser 1986, Arthur *et al.*, 1989d). Sometimes placentitis occurs with death and expulsion of the foetus at the fourth or fifth month, but abortion is not the main feature of the disease. Experimental vaginal or intrauterine inoculation with cultures of *C. fetus venerealis* have been studied (Schurig *et al.*, 1974).

The disease is transmitted venereally by natural service and by A.I. from infected bulls (which are symptomless carriers). Bulls become infected after serving infected cows or at A.I. centres, mechanically from semen collectors or by serving an infected artificial vagina. Young bulls less than 6 years of age may lose the infection after the initial disease. In cows inseminated by an infected bull, 5-30% may conceive while the rest return to heat between 21-30 days after service depending on the time of embryonic loss. So the first important reaction to be seen by the stockman will be repeat regular or irregular heats in a number of animals in a herd especially after introduction of a new bull. However fertility never regains its normal ideal state, and in some animals bilateral salpingitis leads to permanent sterility.

Diagnosis. Herd history, and clinical signs should be recorded. Demonstration of specific agglutinins in mucus, growth of the bacteria, especially in cultures from the stomach contents of aborted fetuses or from its other organs, on selective media are confirmatory (Bryner 1991). Fluorescent antibody tests on preputial samples (Dekeyser 1986), can be done. Multiple examination of the bulls for 6 weeks should be done to confirm their negative or positive status.

Control. It is important to note that most cows develop convalescent immunity between 3-6 months from service and conceive even to infected bulls and most bulls remain carriers for life; also service of infected animals with uninfected semen leads to self cure. At A.I. centres only semen from

clean bulls should be used. Infected bulls can be treated with penicillin and streptomycin (Lander 1988). As a routine, antibiotics should be incorporated while processing semen.

Vaccination of both male and female can be done using killed cells in an adjuvant annually (Bouters *et al.*, 1973). It is possible that immunity results from the production of antibodies in cervical-vaginal secretions causing immobilisation of the organisms and preventing uterine invasion. Vaccination of infected bulls leads to 70% cure and the remaining 30% are cured after the second vaccination, 6 weeks later.

(c) Leptospirosis.

The disease has been found both in man and animals. Saprophytic leptospira belong to the "biflexa" complex and the pathogenic strains to the "interrogans" complex, including a number of "serogroups" with "serovars" having different antigenic characteristics. Normally there is an animal reservoir. Most domestic animals are affected, the commonest serotypes being:-

(a). **Cattle;** *pomona*, *grippotyphosa*, *australis*, *hebdomadis* (especially *hardjo*, *sejroe* and *hebdomadis* serotypes), *icterohaemorrhagiae* and *tarassovi*.

(b). **Pig;** *pomona*, *canicola*, *icterohaemorrhagiae* and *grippotyphosa*.

Other animals like dogs, sheep, goats and to a lesser extent horses, are also affected.

The most widely spread infection in cattle is the *hebdomadis* serogroup, and the most severe disease is due to members of the *pomona*, *hardjo* and *grippotyphosa serogroups*. The major form of transmission is direct contact between animals from infected urine, post abortion uterine discharge, infected placenta, sexual contact or in utero (Ellis 1986). The clinical signs vary from being severe including high fever, icterus and haemoglobinuria, to subclinical and inapparent infections. The bacteria have the ability to

localise in the kidneys producing a shedder state of variable duration. After experimental infection *L. pomona* has been shed in urine for up to 120 days following infection (Ferguson *et al.*, 1957) while *L. hardjo* was shed for 215±26 (up to 542) days (Hellstrom and Blackmore 1980, Theirmann 1982). It has been proposed that some animals may excrete the bacteria for life. *L. pomona* has also been shown to localise in the pregnant or non-pregnant uterus for up to 141 and 92 day respectively (Thiermann 1982) and also in the fallopian tube. The bacterium persists in the uterus and causes infection of the conceptus and subsequent chronic reproductive wastage. *L. hardjo* contributes to a high rate of infertility in the infected herds (Ellis *et al.*, 1982a).

Serotype *pomona* produces a haemolysin or haemotoxin leading to massive erythrocyte destruction, icterus and haemoglobinuria. Young animals are particularly susceptible to the infection and the acute disease leads to death of calves and lambs. Infection leading to death of the foetus takes 1 -2 weeks after infection depending on the subgroup (Ellis 1986). Foetal death is followed by autolytic changes and abortion but the organisms are destroyed by the changes and thus difficult to recover by cultural examination. The foetus has been shown to produce specific antibodies (Moojen *et al.*, 1983).

Infection with serotype *hardjo* is subacute in cattle and has been shown to be endemic in many countries (Ellis *et al.*, 1981 and 1984). It causes abortion, stillbirth or birth of weak calves. Abortion can occur any time during pregnancy especially after 6 months and may occur without any other sign (Thierman 1982) and there is evidence to suggest the occurrence of embryonic death (Ellis 1983). Sometimes, however, abortions are seen 3 months previous to mastitis or agalactia caused by leptospira (Ellis and Michna 1976). Following examination of 245 aborted foetuses randomly selected and another 103 aborted foetuses from farms with abortion

problems, Ellis et al., (1982b) isolated the bacteria from 41%, 70% and 68% of the cases respectively pointing to the significance of the organism.

Acute disease occurs in pigs especially in the young ones accompanied by fever, meningitis, icterus and death. The chronic disease is very common and is characterised by abortion and dead foetuses or still births (Michna 1970, Taylor 1989a). Infection is introduced by introduction of an infected animal or direct contact through ingestion, abrasions or venereally. The fact that the disease is a zoonosis must always be borne in mind.

Diagnosis. Is best done on a herd basis and includes isolation of the organism during the septicaemic stage. Culture is very difficult because the organisms are very delicate, require precise growing conditions and are slow growing. Hamster inoculation may be more successful with isolation possible in 10 days. Because of the present difficulty of isolation of leptospira from specimens normally submitted for laboratory diagnosis, serological investigations have become indespensible for the diagnosis of leptospira.

Serological tests require antigens including the whole variety of serogroups known or suspected. The tests include:-

(i) Immunolabelling techniques for the demonstration of leptospira i.e. immunofluoresence on fresh material (Baskerville 1986, Schonberg 1986) and immunoperoxidase used on formalin fixed tissues (Ellis *et al.*, 1983)

(ii) Microscopic agglutination test - which requires live antigens. Pooled killed antigens are very useful for screening but are less specific (Schonberg 1986).

(iii) A skin test for leptospirosis reported by Schonberg (1981), has been used with success in cattle, goat, horses, pigs, and man (Matus and Mike 1978, Schonberg *et al.*, 1981) using an allergen (leptospirin), and based on

the development of erythema at the site of inoculation (Schonberg *et al.* 1986).

(iv) C.F.T. - using patoc strain of the biflexa complex or pooled antigens of the different interrogans serotypes. Sera from a wide range of groups of animals in the herd should be taken from the normal to the very sick.

(v) Monoclonal antibodies and restriction endonuclease analysis (REA) of DNA can be used to type leptospiras (Terpstra *et al.*, 1986, Tamai *et al.*, 1988, Yamaguchi *et al.*, 1988, Barriola and Saravi 1989). Though complex, the REA has been found to be more accurate and reliable than the conventional serological methods.

(vi) In one investigation Bolin *et al.* (1989) compared three techniques for detection of *L. hardjo* in bovine urine in a controlled experimental infection of 75 pregnant animals. They found that 60 of the 75 urine samples from infected cows were positive by repetitive sequence element nucleic acid probe (nucleic acid hybridization), 24 samples positive by fluorescent antibody test and 13 samples by bacteriological culture. No leptospira was detected from the unchallenged controls.

(vii) Enzyme linked immunosorbent assays (ELISA) have been used in leptospira diagnosis with great accuracy (Hartman 1986, and Bercovich *et al.*, 1990).

(viii) Another method used is the detection of Aminopeptidase activity in leptospira, which is important for the identification and differentiation of leptospira from other leptospira-like cultures using a spectrophotometric assay (Neil *et al.*, 1986).

Treatment. Treatment can be carried out using dihydrostreptomycin and tetracycline (Blood and Radostits 1989).

Control. Good management and hygiene are very important. Test and quarantine of newly arrived animals may be useful, and these should treated

with dihydrostreptomycin and vaccinated on arrival. Vaccines against *pomona*, *grippotyphosa*, *canicola*, *hardjo* and *icterohaemorrhagiae* are available and vaccination should be used in all cows or heifers that are pregnant, once every year in a closed herd or twice every year in an open herd (Ellis 1984).

(d) Salmonellosis.

In animals the disease may be acute, chronic or symptomless and may be septicaemic enteritic or intrauterine. With about 1,000 serotypes in the genus, the few important species that may cause disease in animals include *S. dublin* for cattle or sheep, *S. abortus ovis* in sheep. *S. typhimurium* is not host specific and may cause disease in many animals. In Britain about 97% of salmonella infection in cattle is caused by *S. dublin* and *S. typhimurium* (Sojka and Field 1970). The disease incidence varies from one country to another.

The bacteria can be excreted in faeces, urine and milk of sick or carrier animals which then act as a source of infection for others. The main route of entry is by ingestion.

The acute disease in cattle is characterised by fever, dullness, loss of appetite, drop in milk production, and severe diarrhoea or dysentery, sometimes with high mortality and abortion in pregnant animals after transplacental infection. Abortion is usually sporadic, but it may occur as an epidemic. Although abortion can take place at any time during gestation, it is mostly seen between 6 and 9 months. The disease may be sub-acute. In either state, recovered animals become carriers and the disease may be reactivated under stress eg. of pregnancy or fascioliasis (Schaaf 1972). *S. dublin* can be isolated from aborted foetuses even in the absence of clinical signs in the dam (Hinton, 1974, Richardson and Watson 1971). Abortion is mostly between 6-8 months of pregnancy.

The mechanism of abortion in the symptomless carriers has been thought to be as a result of the uterus being a predilection site. Hall (1973) in his studies after experimental inoculation, concluded that the initial lesions in the placenta were like those produced by endotoxins of Gram-negative bacteria in the rat placenta, characterised by slowing and stasis of the placental circulation. Extending the work to cattle, he found that after inoculation with 10^8 of the organism, 6 out of 10 animals aborted 8-10 days later and after the second febrile stage had subsided, this was accompanied by retained placenta, but with an otherwise normal subsequent reproductive performance (Hall and Jones 1976 and 1977).

Diagnosis. Confirmation is by isolation and identification of the organism from aborted material or faeces. Serological tests are not reliable but S.A.T. may be used although the agglutinins fall to low titres soon after infection. ELISA tests for salmonellosis have been described (Barrow *et al.*, 1989, and Smith *et al.*, 1989). A DNA probe has been used in the study of *S. typhimurium* (Poppe *et al.*, 1989).

Treatment. Treatment can be carried out using tetracyline.

Control. This is by provision of clean feed and water, proper disposal of the infected material, and quarantine. Vaccination can also be carried out (Smith 1965).

(e) MYCOPLASMOSIS.

Mycoplasma spp. were known to cause genital disease in cattle when *Mycoplasma bovigenitalium* was isolated from infertile cows and the semen of bulls (Blom and Erno 1967). The mycoplasmas that have been implicated are *M. bovigenitalium*, *Acholeplasma laidlawii* and *Ureaplasma* spp and these

cause two specific reproductive disorders ie granular vulvovaginitis and abortion.

The organisms most frequently isolated in disease outbreaks are *Ureaplasma* spp (Doig *et al.*, 1979) and may thus be the main causal agents. The disease can be transmitted at natural service and A.I.. *Ureaplasma* spp. and *M. bovigenitalium* have been isolated from raw and processed semen (Hodge and Holland 1980, Rae 1982) and *M. bovigenitalium* can infect the genital system of the bull (Blom and Erno 1967).

Infection is characterised by a sudden onset of a purulent vulval discharge 4-10 days after service, inflammation of the vaginal mucosa with raised granular lesions especially on the ventral surface of the posterior vagina, vulva and around the clitoris with difficult urination. Pyrexia and systematic infection are not common manifestations but pregnancy rates are reduced. Abortion has been observed with infection (O'Berry *et al.*, 1966) and with isolation of mycoplasmas from aborted foetuses or premature calves. Similarly, Langford (1975) cultured mycoplasma from 8.7% of aborted bovine foetuses.

Experimental introduction of Ureaplasma into the bovine genital tract via the vulva and into the uterus (Doig, et al., 1980 a and 1980 b), produced a granular vulvovaginitis and endometritis / salpingitis respectively. Experimental inoculation with *M. bovigenitalium* into the cervix produced similar granular lesions and depressed pregnancy rates (Saed and Al-Aubaidi 1983).

Diagnosis. Diagnosis depends on isolation of the organisms after culture of vaginal mucus or discharge.

Control. Can be achieved by both systemic and local treatment of infected animals, and use of semen from only clean bulls for A.I.. Infected bulls

should rest until recovery from infection after treatment is achieved.

(f). Listeriosis.

Listeria monocytogenes is usually associated with central nervous system disorders, causing encephalitis in cattle and sheep, but it is frequently isolated in cases of bovine abortion, and has been shown to cause abortion in sheep and goats. The organism is ubiquitous in the environment and is particularly resistant to effects of drying, sunlight, and extreme temperatures. *L. monocytogenes* gains entry by ingestion, via mucus membranes of the respiratory system, the conjunctiva or the C.N.S.. A septicaemia or bacteraemia may result to give localisation in the placenta. In the placenta, *L. monocytogenes* causes placentitis and affects the foetus. Abortion may be sporadic mainly occurring towards the end of gestation and may be proceeded by pyrexia in some cases. The aborted foetus frequently has characteristic multiple yellow or grey necrotic foci in the liver (Arthur *et al.*, 1989d).

Diagnosis. Depends on identification of the organism from the aborted foetus, membranes and discharge. Culture of the organism is not easy.

Control. Treatment with oxytetracycline and penicillin may be attempted especially for symptomless carriers which may excrete the organism in milk and faeces. Suspected infected feed should be with-held especially from pregnant cows.

(g) Q. fever (*Coxiella burnetti* infection).

This is a condition caused by infection with a rickettsia *Coxiella burnetti*. The organism affect a wide range of mammals including cattle, sheep, goats, man and wild life. It has been associated with cattle , sheep, and goats (Miller *et*

al., 1986, and Little 1988). Little work however has been done as regards its pathogenesis in bovine reproduction.

(h) Chlamydiosis.

The organism possesses both bacterial and viral characteristics. The chlamydia group includes the causal agents of psittacosis, epizootic bovine abortion (E.B.A), and enzootic abortion in ewes (E.A.E). The vector for E.B.A. causative agent in cattle is thought to be the Argasid tick *O. coriaceus* (Koch). This was proven when a similar condition was experimentally reproduced after feeding the Argasid tick on susceptible pregnant cattle (Shmidtmann *et al.*, 1976). Chlamydia are obligate intracellular parasites which undergo a complicated reproductive cycle that includes a non-infectious (initial-body) phase which divides by fusion, the daughter cells then reorganize and condense to become elementary bodies which can infect cells. Chlamydia has been previously thought to cause intrauterine infection in cattle, sheep, small rodents, rabbits and pigs (Storz 1968). In cattle however, evidence like vaccine trials (McKercher *et al.*, 1969, McKercher *et al.*, 1973), experimental reproduction of the disease (Kennedy *et al.*, 1983, Kimsey *et al.*, 1983) and maternal serological foetal assays (Wada *et al.*, 1976, Kimsey *et al.*, 1983), seem to point to another cause other than chlamydia.

In Cattle, E.B.A has been recorded in Britain, U.S.A and Europe and though experimental cross-infection of ewes is possible experimentally, no natural cross-infection has been recorded. Abortion in cattle occurs at 7-9 months or earlier and may be as high as 40% with seasonal calving. Studies have revealed that the lesions characteristic of E.B.A are seen 100 days or more after maternal tick exposure. Foetuses surgically collected between 50 and 100 days showed mild to moderate lymphoid and mononuclear cellular

hyperplasia pointing to the chronically progressive nature of the disease in the foetus (Kennedy *et al.*, 1983, Kimsey *et al.*, 1983). Calves may be born dead or weak and die soon after birth. The aborted foetus may show oedema of the skin and internal organs (if 7 months old) or may be fresher near term. The foetus shows many more pathological changes than the lamb (Kennedy *et al.*, 1960). Recently the organism which has for long been thought to the cause of E.B.A., has been implicated as a cause of abortion in U.S.A. and Southern Europe but with different pathogenesis and clinical history. So as yet no particular organism has been identified as the cause of E.B.A. (Arthur et al., 1989d). It is thought that the two disease syndromes i.e. bovine chlamydiosis abortion (BCA) and E.B.A are caused by two different entities (Kimsey 1986).

In sheep, the disease caused by *Chlamydia psittaci* leads to the abortion in the last month of pregnancy, stillbirth or delivery of weak lambs (Linklater 1979, Blewett *et al.*, 1982, Aitken 1986, Aitken et al., 1990). Abortion first occurs at 100 days, the disease thereafter becoming enzootic with between 1-5% abortions every year.

The disease is essentially a placentitis with oedematous and frequently haemorrhagic fetal membranes. Autolysis of the foetus may occur depending on the stage of gestation at infection. It has been postulated that the placentitis destroys the placental barrier causing a host versus graft reaction (Studdert 1968). *Chlamydia psittaci* has been shown to cause disease in cattle. During an experimental investigation Gupta and Galthotra (1986) reproduced chlamydiosis in crossbred calves after intratracheal inoculation of 6 ml of the laboratory-propagated yolk-suspension of the organism.

Diagnosis. The geographical location, history of the condition and post-

mortem examination of the cases are of vital importance in diagnosis. Cytoplasmic inclusion bodies in impression smears, using modified Z-N or Macchiavello's method, are pathognomonic for chlamydiosis.

Isolation and identification of the organism from infected material is made possible by infecting 6-7 day chick embryos or using tissue cultures such as McCoy cells.

Serological tests include F.A.T. and C.F.T. but these may not be very useful in cattle.

Control. Vaccination is used in sheep (Foggie 1959).

B. NON-SPECIFIC INFECTION IN CATTLE.

The female genital tract has mechanisms which can act to prevent opportunist pathogens from becoming established. These are the physical barriers of the vulval sphincter and cervix the flow of mucus and the natural defence mechanisms which include phagocytosis, antibodies and the normal vaginal flora and its effect on the pH. All these may be significantly influenced by the endocrine system. The physical barriers are lowered on two occasions ie. at oestrus and parturition, but at both these times the genital tract is at its most resistant under the influence of the endocrine system. It is now accepted that under oestrogen dominance the genital tract is most resistant to infection (ie. at oestrus and parturition), whilst under progesterone dominance it is more susceptible (ie. the rest of the oestrous cycle and during pregnancy). At both oestrus and parturition there is an increase in blood supply to the genital tract and concurrently an increase in migration of white blood cells from the circulation to the uterine lumen, thus enabling vigorous and active phagocytosis of bacteria to take place. The increase in the quantity and nature of the vaginal mucus also plays an important part by providing a protective physical barrier and flushing and

dilution of the bacterial contaminants. The presence of secretory immunoglobulins at this time is also important.

Failure in any or some of these natural barrier mechanisms will lead to establishment of infection. Failure could be brought about by any of these factors:-

(i) Damage to the vulva, leading to impairment of the sphincter-like barrier which may lead to aspiration of air and the subsequent development of vaginitis.

(ii) Relaxation of the cervix or its damage may cause heavy contamination which may lead to infection of the uterus.

(iii) Dystocia may lead to break down of the natural defence mechanisms especially in the face of secondary inertia or severe trauma to the genital tract.

(iv) Primary uterine inertia due to hypocalcaemia and placental retention may also lead to delay in uterine involution.

(v) Progesterone dominance increases susceptibility of the reproductive tract to infection.

Discussed below are disease syndromes seen as a result of non-specific infection of the genital tract and these are:-

1. puerperal metritis,
2. endometritis,
3. pyometra,
4. salpingitis
5. cervicitis/vaginitis.

1. **Puerperal metritis.**

Puerperal metritis commonly occurs after abnormalities of parturition including the first and second stages of labour. The condition is most

probably due to failure of uterine contractions and is characterised by foetal membrane retention and uterine infection. The organisms commonly implicated include *A. pyogenes*, Group C streptococci, haemolytic staphylococci, coliforms and to a lesser extent clostridia. Infection may affect the dam as a result of septicaemia, pyaemia or the action of bacterial toxin. The animal strains and there is vaginal discharge of foul smelling, reddish serous fluidy material. On rectal palpation the uterus is distended with fluid and the cotyledons are enlarged. Parts of the foetal membranes are seen to protrude from the cervix and vagina and the vulval lips are congested and swollen.

The animal is off food and body temperature is raised ($>40^{O}$ C). Other clinical signs include increased heart rate and respiratory rate and occasional diarrhoea. When infection spreads to the peritoneum, peritonitis may occur accompanied by adhesions of the uterus and ovaries. Formation of abscesses in other body tissues may occur due to haematogenous spread of the infection.

Control.

Infected material may be removed from the uterus but care is required as the reproductive tract is fragile. Broad spectrum antibiotics give the best results but oxytocin can also be used soon after parturition. Detailed local and systemic treatments are mentioned in the review of pyometra below.

2. Endometritis.

Endometritis is the inflammation of uterine endometrium. Its effects are localised in the uterus and affect fertility severely. Infection causing the inflammation reaches the uterus either haematogenously or by ascending through the cervix and the organisms implicated include specific infections

like trichomonas and campylobacter but these are of less importance. Non specific bacterial infections are of greater importance in this condition (Boyd 1965).

The effects of endometritis on fertility may be (a) short term or (b) long term.

(a) Short term.

These effects are shown by reduced fertility and long interoestrus intervals. In their investigation Studer and Morrow (1978) established that the interval between parturition and conception was directly related to the amount of vaginal mucopurulent discharge. The interval has been found to range between 10 - 20 days and service per conception to range between 1.67 - 2.42 (Tennant and Peddicord 1968, Erb *et al.*, 1981 and Bretzlaff *et al.*, 1982).

(b) Long term

The effects are characterised by infertility due to permanent changes in the uterus such as adhesions. Infertility, in severely affected herds, varies from 5% to reach as high as 20.6% (Bretzlaff *et al.*, 1982) and may lead to increased culling. Dawson (1963) reported endometritis in culled repeat breeders.

Endometritis is most frequently seen clinically after parturition and is characterized by a white or whitish yellow mucopurulent vaginal discharge, volumes varying in volume and increasing significantly at oestrus. In most cases the cow does not show signs of systemic illness. On palpation, poor involution is shown by an enlarged uterus which is doughy in texture. Studer and Morrow (1978) found a close relationship between size and texture of the uterus and cervix, the nature of the purulent exudate and the degree of endometritis determined by biopsy and the nature of bacterial isolation.

Endometritis develops as a result of colonisation of the endometrium by bacteria and some predisposing factors have been identified (Andriamanga *et al.*, 1984, Markusfeld 1984 and 1985) and these are:-

(i) **Dystocia** - commonly follows acute metritis.

(ii) **Retained placenta.**

(iii) **Season of the year** - commonest during winter.

(iv) **Nutritional status** eg. dietary concentrations of Vitamins A and D.

(v) **Twins and induction of calving** - both of result in retained placenta which predisposes to metritis.

(vi) **Delay in return of cyclical ovarian activity** - this may be due to a prolonged luteal phase and lowered resistance to infection thus predisposing to endometritis. Andriamanga *et al.* (1984), demonstrated the above when they found a 50% risk that non-cycling females 30-37 days after parturition would acquire endometritis as opposed to a 34% risk in the cycling animals.

(vii) **Metabolic diseases** like ketosis, hypocalcaemia and overfeeding during the dry period may predispose to endometritis probably due to the fatty liver syndrome (Reid *et al.*, 1979).

(viii) **Presence of bacteria in the environment / poor hygiene.** The environment immediately surrounding the animal especially after calving has great influence on the establishment of infection. In his studies Hartigan (1980), demonstrated that when contamination of the environment is heavy, pathogenic microorganisms may colonise the genital tract of the post-

puerperal non-pregnant cow with deleterious effects on fertility. In one herd the resultant endometritis was associated with a significantly greater depression in fertility ($P<0.001$) when the cows began cycling >30 days after calving than when cycling began earlier in the post-partum period. This can possibly explain the effect of season of the year in temperate regions where cows calving in winter or indoors in spring are likely to be in a more heavily contaminated environment (Arthur *et al.*, 1989d). A wider survey carried out by Singh *et al.* (1981) on 3662 infertile cows between January 1967 and December 1973, indicated that anoestrous was the commonest form of infertility (39%), followed by conception failure (22.7%), and cervicitis (12.78%). Functional infertility was much more common (76%) than infectious causes (24%) which were accompanied by cervicitis (12.8%), endometritis (9.61%) and pyometra (1.67%).

(ix) Infection with specific agents. Damage by any of the specific causes of reproductive failure can be colonised by other bacteria to cause endometritis.

(x) Organisms involved. A number of bacteria and, to a lesser extent, viruses have been implicated as causes of endometritis. Rose (1987), investigated cases of endometritis on 65 farms in U.K. and found antibodies to bovine herpes virus I (IBR virus) present on 7 farms, in 32% of the tested 38 cows tested, Q-fever antibodies on 19 farms in 21% of the 127 cases tested, and only one cow possessing antibodies to chlamydia. *A. pyogenes* was isolated from 126 of the 297 samples, being present on 53 of the 64 farms. Apart from *A. pyogenes*, other bacteria associated with endometritis include streptococci (63%), and *E. coli* (12%) (David and Bonner 1987). These results were confirmed by Takacs *et al.* (1990). A wider range of bacteria have been isolated including anaerobic bacteria, fungal organisms, alpha-streptococci, *Bacillus spp.*, and others including, Haemophilus (*H. somnus* frequently being strictly microaerophilic), Pasteurella spp., and

diptheroids (Ruder *et al.*, 1981, Olson *et al.*, 1984; Bonnett *et al.*, 1990) but their pathogenic role is not clear.

Diagnosis. Clinical signs are important in diagnosis and these should be supplemented by histological and bacteriological examination of endometrial biopsies (Hartigan 1974). Vaginal swabs for bacterial culture and antimicrobial sensitivity tests may be routinely undertaken. The major drawbacks with the use of swabs are contamination of the samples and the difficulty of storage for anaerobic studies.

Control. This is best achieved by both antimicrobial and hormonal treatments (details given in the review for pyometra).

3. Pyometra.

The most important clinical signs in pyometra are the accumulation of pus in the uterus and the presence of functional corpus luteum. The condition commonly occurs following chronic endometritis and may be a direct result of failure of the damaged endometrium to produce PGF 2α which is responsible for luteolysis. Accumulation of pus in the uterine lumen may be due to a closed cervix. Pyometra may also occur when embryonic death is followed by bacterial invasion of the embryo (Arthur *et al.*, 1989d).

The infection is usually localised in the uterus and there are no signs due to systemic infection. The condition is characterised by vaginal discharge and an enlarged uterus. This enlarged uterus may be difficult to differentiate from a normal pregnancy, but the symmetry of the horns the lack of cotyledons and "membrane slip" in this condition may be useful for differentiation from a normal pregnancy. *T. fetus* causes pyometra but without the above signs, and the infection is best diagnosed by identification of the trichomonads.

Diagnosis. Clinical signs especially on rectal palpation and laboratory tests, identification of the causative agents could be of benefit.

Control. This is best achieved by treatment and which also applies for the control of metritis and endometritis. Treatment can be achieved in the following ways:-

(i) Lugol's iodine can be used but it can cause severe endometrial damage. It has been used widely in the treatment of mycotic infection. Its action is thought to be by an enhancement of the immune system of the reproductive tract, by spurring the halide-peroxidase killing system of phagocytic cells (Prescott and Baggot 1988). One drawback is that iodine may cause severe damage to the endometrium (Arthur *et al.*, 1989).

(ii) Antimicrobials like sulphanilamide, penicillin, oxytetracycline and streptomycin (Ayliffe and Noakes $1978_{(a)}$) and these can be introduced into the uterus, intravenously or intramuscularly (Ayliffe and Noakes $1978_{(b)}$, Masera *et al.*, 1980). A brief review (Prescott and Baggot 1988) of the use of each of these drugs follows.

Sulphonamides are broad-spectrum and can be used in cattle with good results (Seervaald 1975, Prescott and Baggot 1988). They are bacteriostatic and act by interfering bacterial biosynthesis of folic acid, competitively preventing paraaminobenzoic acid (PABA) from transformed into the folic (pteroylglutamic) acid molecule. The drug can be given per os, intravenously (IV), or intramuscularly (IM) at the rate of 10-25 mg/kg (depending on the sulphonamide used).

Penicillins are broad-spectrum (but more gram-positive than gram-negative) and have been used widely. In cattle penicillin can be administered IV,IM, or subcutaneously at the rate of about 10,000 IU/kg. Penicillin G is most widely used at 25,000 - 45,000 IU/kg. A single IM dose of 25,000 IU/kg can maintain effective concentration for at least 12 to 24

hours. Penicillin G is bactericidal and acts by prevention of the formation of bacterial cell wall material and possibly taking part in its lysis (but lysis only occurs in grown cells).

Tetracyclines are classical broad-spectrum antimicrobial drugs mainly available as hydrochlorides. They quickly attain therapeutic levels in target tissues compared with other antimicrobials and have a very high efficiency. Tetracyclines act by prevention of protein synthesis in susceptible microorganisms. Oxytetracycline is commonly used in cattle. It is recommended that the drug be introduce slowly if the IV route is used. The drug can also be administered IM. The recommended dose is 50 mg/kg or 20 mg/kg for the long acting form of oxyteracycline.

Streptomycin is an aminoglycoside and is broad-spectrum though its activity is greatest with gram-negative anaerobic bacteria. Streptomycin causes its bacteriostatic activity in the bacterial cell by binding on receptor cells and leading to misreading of the genetic code on the messenger ribonucleic acid (mRNA), preventing ribosomal protein synthesis. The recommended dose is 20 mg/kg.

Experimental investigations have been carried out by different workers using different routes and antibiotics to study their pharmacokinetics in the infected uterus in cattle. Whereas there is general agreement on the efficiency with which drugs achieve tissue therapeutic levels after parenteral administration, there are still some controversies over the efficacy of the drugs after intrauterine infusion. Intrauterine infusion has been used for many years for routine uterine treatment.

Accumulating evidence suggests that the parenteral routes may achieve adequate therapeutic levels in the endometrium more effectively than intrauterine infusion (Saggers and Lawson 1970). After IM injection of a single dose of benzyl penicillin in healthy cycling cows, Ayliffe and Noakes (1978a) demonstrated that serum peak levels of the drug were reached at 15

minutes. The peak endometrial tissue levels were reached at 60 minutes after injection and persisted for longer times and at higher levels than the serum levels. However, Ayliffe and Noakes (1978b) also demonstrated that after intrauterine infusion of the drug, drug absorption was better during oestrus as compared to the luteal phase.

The evidence for greater usefulness of the parenteral routes compared to the intrauterine route (IU) was supported by Masera *et al.* (1980). They found that with the use of oxytetracycline (OTC), the tissues of the reproductive tract and plasma had greater concentrations after administration parenteral use than after IU infusion in a normal tract. Drug absorption was even lower in cows suffering from endometritis.

In another investigation (Dohoo 1984), four groups of cows (each of different parities) were used to study the usefulness of IU infusion before breeding (mostly using tetracycline products). The findings were compared with those animals that were bred without prior antimicrobial treatment. It was shown that animals that were treated before breeding had 0.7 chances of becoming pregnant compared to the non-treated group. This indicated that treatment could be detrimental as suggested by earlier workers including Sandals *et al.* (1979), Erb *et al.* (1981). However it was also shown that the detrimental effect decreased with increasing age and it was beneficial to treat before the fifth breeding (with 5.3 chances of becoming pregnant).

During a survey in Hungary, Takacs et al. (1990) found that the prevalence of infected uteri was very high after the third parturition. They also found that the bacteria present showed broad resistance to the commercially available antimicrobials including neomycin, oxytetracycline, oleandomycin, erythromycin, streptomycin, polymyxin-B, ampicillin, lincomycin, and sumetrolim. The significance of the resistance was not studied.

(iii) Inclusion of oestrogens in the therapeutic preparations is deemed to be beneficial as oestrogen has the capacity to increase blood flow to the uterus

stimulating changes accompanying follicular growth (Roberts 1971, Vucko 1978), but this has a possible danger of inducing cystic ovaries (Laing 1979).

(iv) The best treatment of clinical endometritis is the use of prostaglandin F2α or its synthetic analogue (Gustafsson 1986) and the treatment should only be undertaken when a definite corpus luteum is present. In a recent trial Bonnett *et al.* (1990) showed the effect of prostaglandin (PGF2α) treatment regardless of the level of progesterone, when animals were treated at 26 days postpartum and examined 14 days later (day 40). The cows examined had less vaginal discharge, less inflammation and fibrosis in the endometrium and were less likely to have bacterial isolation compared to the untreated cows. The findings confirmed earlier field trials by Young *et al.* (1984) and Etherington *et al.* (1988).

(v) GnRH (cystorelin-cella) can be used with PGF2α in the treatment of uterine infection. 100 ug of GnRH is given to induce luteinizing hormone (LH) release to cause a lutrophic effect on the corpus luteum and PGF2α given > 10 days later induces lutolysis. This is followed by follicular maturation and oestrus (Richardson *et al.*, 1982). Oestrogen produced during follicular development is thought to stimulate the uterine natural defences to combat the infection.

4. Salpingitis.

The oviducts or salpinx as are uterine tubes extending from either of the horns, thus making it easy for infection to spread from the infected uterus causing pyogenic salpingitis often followed by sterility. Evaluation of a series of infected tracts from the slaughterhouse by Arthur *et al.* (1989d), showed the tubes to be healthy in most cases. This fact may account for the high proportion of pyometra cases which conceive after treatment, thus suggesting the possibility that tubular infection may not be as common as has been suggested

5. Cervicitis and vaginitis.

These take place after obstetrical trauma during relief of a difficult calving, and it may also occur after retention of foetal membranes or puerperal metritis. The organisms commonly found include *E. coli*, streptococci, staphylococci and *A. pyogenes*, and *F. necrophorum* especially in the obstetrical contusions of the vagina commonly seen in fat heifers. Parturient trauma may be followed by severe toxaemia due to invasion by other anaerobic bacteria.

Normally these conditions resolve quickly because of the naturally strong resistance to pyogenic infection, but breakdown of the vulval sphincter may lead to prolongation of the disease and result in metritis. Sporadic bovine cervicitis is rare (Arthur *et al.*, 1989d).

Control. Damage due to trauma should be repaired surgically to restore the vulval sphincter. Systemic antibiotic treatment is preferred to local treatment due to poor distribution and penetration of the drugs in this region.

C. NON-SPECIFIC INFECTION IN BOVINE ABORTION.

A number of bacteria have been isolated from cases of abortion but have been considered to be of little significance or to be secondary in nature. They include:- streptococci, staphylococci, *A. pyogenes*, *Bacillus spp.*, Enterobacteria, *E. coli*, *Proteus spp.*, *Shigella spp.*, diptheroids, *Klebsiella spp.*, *Fusobacterium necrophorum*, and other enterobacteria (Hubbert *et al.*, 1973a, Kirkbride *et al.*, 1973, Hartigan 1980). Many of the bacteria in the environment can infect the aborting animal and the products of abortion after the event. Some of these isolations may reflect contamination either before or after abortion. Some of these will be dealt with in detail below. The inclusion of *A. pyogenes* in this group of organisms will be reviewed with

the aim of re-evaluating its pathogenic role in early embryonic death in cattle.

1. Bacillus spp.

In the U.K. *Bacillus licheniformis* has only recently been identified in some cases of abortion in cattle (Counter 1985). The bacteria normally occur in the environment and common sources of infection are contaminated feed stuff, water and bedding. Infection may be haematogenous, probably after entry via the gastro intestinal tract. Sporadic cases occur in late gestation but at other times live calves may be born with some evidence of placental lesions.

The allantochorion is oedematous especially around the cotyledons, which are themselves haemorrhagic and necrotic. If the foetus is infected there is usually evidence of a fibrinous pleurisy, pericarditis, and peritonitis.

2. *Escherichia coli.*

E. coli may lead to sporadic abortions (Habiballa and el - Zubeir 1978, Moorthy 1985) and the bacteria may reach the placenta and foetus haematogenously or by ascending the reproductive tract. Their involvement is typical of the remaining enterobacteria isolated from cases of abortion. In many cases they are considered to be the cause because more detailed examinations have not been carried out.

3. *Actinomyces pyogenes.*

Actinomyces (Corynebacterium) pyogenes is a non-motile non-sporulating, Gram-positive coccobacillus or short rod (with 0.5 - 2 um length and 0.2 - 0.3 um width) and grows both aerobically and anaerobically (Lovell 1937) producing gas but not acid from a variety of carbohydrates (Reddy and Cornell 1982, Schaal 1986, Bisping and Amtsberg 1988). *A. pyogenes* has

typical cultural characteristics. It grows well on growth media containing blood and can be accurately identified by cultural identification. The colonies are small < 2mm smooth, raised, with an entire margin and transparent, surrounded by a narrow zone of B haemolysis. The colonies may also be white or grey to white in appearance. Although colonial identification can be achieved after 48 hours (at 37° C) of aerobic incubation, anaerobic conditions lead to a more profuse growth. Chocolate agar in a 10% CO_2 also leads to increased growth (Schaal 1986, Bisping and Amtsberg 1988). The bacterium has been isolated from suppurative processes in many species of animals.

(a) Cattle.

In cattle, *A. pyogenes* has been isolated from chronic pneumonia, arthritis, liver abscesses and mastitis (Lovell and Hughes 1935). *A. pyogenes* has been isolated in apparently pure cultures from aborted foetuses at different stages of gestation. Hinton (1974) reported an incidence of *A. pyogenes* abortion varying from 0.03% to 19.6%, with a mean of 5.3%. More recent data from U.K. Veterinary Investigation Centres (Anon 1988) suggests that *A. pyogenes* was isolated from 0.28% of cases of bovine abortion investigated. Isolation from the reproductive tract is usually considered to be secondary to infection elsewhere or to follow previous damage (Arthur *et al.*, 1989d).

The pathogenesis of *A.pyogenes* infection is poorly understood (Hartigan *et al.*, 1974, Hartigan 1980) although Hinton (1972 and 1974) suggests that haematogenous infection may be more important than the ascending route. Its role as a primary or secondary pathogen in bovine abortion has remained an area of considerable controversy (Hinton 1974, Ruder *et al.*, 1981, Olson *et al.*, 1984, Farin *et al.*, 1989).

Evidence that *A. pyogenes* could have a primary role in bovine abortion comes from the literature cited below. Hartigan *et al.*, (1974), found that

endometritis was almost invariably a sequel to invasion with *A. pyogenes* with histopathological lesions of endometritis seen in 97.4% of uteri infected with the organism. Jerrett *et al.* (1984) examined material from 265 bovine abortions (including the foetus, placenta and maternal serum), by histopathological, histochemical, bacteriological, mycological, endocrinological, serological and virological techniques. Causes of abortion were identified in 98 (37%) cases, and these included 27 (28%) by fungi, 17 (17%) by *Salmonella spp.*, 11 (11%) by *Campylobacter fetus* and 10 (10%) by *A. pyogenes*. Others identified less frequently included *Leptospira spp.*, protozoan encephalitis and *Brucella abortus*. Biancardi *et al.*(1982) likewise diagnosed 101 (39%) abortions due to *A. pyogenes* of the 1411 aborted foetuses examined on bacterial isolation and identification of the 259 positive cultures. Other isolates included 45 (17%) Brucella, 37 (14%) Aspergillus, 20 streptococci, 11 Escherichia and 11 I.B.R./ I.P.V. virus.

In a brucellosis survey Guilloux (1979) found *A. pyogenes* in 109 (6.8%) brucella-free placentae of the 1596 cases examined, and this was associated with highly significant titres of anti-*A. pyogenes* haemolysin antibodies. It was concluded that the bacterium was probably responsible for a significant number of sporadic abortions in cows. Bucharova (1982) subscribed to this hypothesis when he examined 94 embryos, 936 foetuses, 35 placentae, and discharge from 285 cows with endometritis and discharge from 232 healthy cows, and milk samples from cows with mastitis. *A. pyogenes* was isolated from 124 of the 2,193 samples, including 9% of foetuses, 8% of placentae, 6% of mastitis, and 6% of endometritis cases, also from 2.8% of 280 fresh and 1.7% frozen samples of bull semen. The foetal isolates were obtained from 61 foetuses, together with *Campylobacter fetus* in 18, and with other bacteria in 20. Abortion associated with *A. pyogenes* occurred sporadically in 18 of the 38 herds and the abortions were associated with endometritis and mastitis.

Other groups of workers have suggested and proceeded to demonstrate that obligate anaerobes play a part in the pathogenesis of endometritis (Ruder *et al.*, 1981, Farin *et al.*, 1989). Evidence has been produced suggesting that there is synergism between *A.pyogenes* and *Fusobacterium necrophorum*, the latter organism producing a leucocidal endotoxin which would interfere with the host's ability to eliminate *A. pyogenes* and thus facilitating its tissue invasion and *A. pyogenes* itself producing a filterable substance which stimulates the growth of *F. necrophorum* (Roberts 1967, Ruder *et al.*, 1981). In their experiment Ruder *et al.* (1981) also showed that one of the five post partum infected animals which were infected with either *A.pyogenes* or *F. necrophorum*, had not conceived by 110 days post partum and this particular case was solely infected with *A. pyogenes*, it was not postulated however that this bacteria alone could have any primary pathogenic role.

Experimental evidence of the role of *A pyogenes* as a primary pathogen in the bovine uterus is scarce. Farin *et al.* (1989), have recently confirmed that inoculation of the non-pregnant uterus with pure cultures of the organism gives rise to endometritis with persistence of the corpus luteum. Their studies with other organisms such as *F. necrophorum* and *Bacteroides melaninogenicus* showed that these were not necessary for the production of endometritis by *A. pyogenes.* Mohamed *et al.* (1987) induced abortion in cattle after the introduction of pure cultures of *A. pyogenes* and *Staphylococcus aureus* into the amniotic cavity between 180-200 days of pregnancy.

(b) Sheep and Goats. Other evidence is available from sheep and goats. *A. pyogenes* was incriminated a cause of abortion in sheep by Dennis and Bamford (1966), Rosca (1971) and the infection is said to be sporadic (Hinton 1972). Smith *et al.* (1971) undertook the experimental infection of

ewes between 70-118 days pregnant both intravenously and by the intra - amniotic route. The animals were sacrificed 6 to 21 days after inoculation to allow the demonstration of progressive stages of the foetal placental disease. Some foetuses were alive at the time of slaughter, others were dead but retained in the uterus and other foetuses were aborted. All the dead and aborted foetuses had autolytic changes. Placental changes varied. In membranes from the live and dead foetuses, oedematous placentomes were present with the membranes still attached, but in ewes where the foetuses were aborted, foetal membranes were also expelled. The lesions of the ewes or foetuses seen were not considered to be of diagnostic significance and foetal death was thought to be due to hypoxia produced by severe placentitis. The work did not incorporate adequate controls.

Similar experiments were performed by Addo and Dennis (1979) who studied the pathological changes in the foetus and the ewe, following intravenous inoculation of the bacteria to pregnant ewes during the last trimester. Abortion occurred 12-25 days post inoculation, and although the placental and foetal changes were not thought to be diagnostic, the gross placental appearance was considered suggestive of abortion caused by *A. pyogenes*.

Abortion in the goat due to *A. pyogenes* has only rarely been reported. Rahman *et al.* (1983), reported the first case of abortion due to *A. pyogenes*, after a thorough laboratory examination of the aborted kid and its membranes. Confirmation was based upon colonial morphology and biochemical tests.

D. IMMUNE RESPONSE TO *A. PYOGENES* INFECTION AND VACCINES TRIALS.

Further evidence for the involvement of *A. pyogenes* as a primary pathogen in abortion comes from studies of the immune response to infection and

from attempts to vaccinate.

1. Immune response to infection.

For some time the only definitive proof for *A. pyogenes* infection was the isolation of the causal agent, but biochemical and immunological methods have been developed and these are considered to be of practical value in the diagnosis of bacterial infection.

Lovell (1937) described a filterable haemolysin toxin which was produced in cultures of the bacterium. He later (Lovell 1939) showed that *the A. pyogenes* anti-toxin content of sera could be measured by its power to inhibit the haemolysis of rabbit cells by the toxin and this could be demonstrated in cattle, sheep, goat, pigs, horse and rabbits. This discovery led to development of antihaemolysin titration techniques which have been used in serological diagnosis of *A. pyogenes* infection (Weitz 1949, Roberts 1968, Sorensen 1972a).

Infected pigs were found to possesses an inhibitory antibody against a protease of *A.pyogenes* (Fossum 1971), and while using the immunodiffusion test a protease antibody could be detected. In a field trial while using the test on 443 sera from pigs collected at random, 35% positive cases were demonstrated and the test was deemed beneficial for diagnosis of *A. pyogenes* infection in pigs (Takeuchi *et al.*, 1977 and 1979).

Immunofluorescent tests were also successfully used to detect antibodies against *A. pyogenes* from sera and vaginal mucus of cattle with endometritis and vaginitis and it was proposed that localised antibody formation occurred in the genital tract of cattle (Schultz *et al.*, 1978).

More recently, Schwan (1980) demonstrated IgG titres against *A. pyogenes*, in natural and experimental mastitis by the direct immunofluorescent antibody technique. He found that measurable IgG titres could be found in healthy heifers. Other authors had previously described titres in normal heifers using agglutination tests and haemolysin neutralization tests

(Sorensen 1972b), pointing to the care that needs to be taken when results from immunological tests are interpreted.

2. Animal immunization.

Because of the high frequency of isolation of *A. pyogenes* especially in cases or outbreaks of mastitis and its increasing importance in endometritis and abortion, many attempts have been made to immunise animals against the infection and conflicting results have been obtained.

Weitz (1947) was able to elicit the production of an antitoxin in sheep to *A. pyogenes*, alum precipitated toxoid (A.P.T.) which lasted for 160 days. Four groups of sheep were sensitized to *A. pyogenes* with (a) A.P.T., (b) vaccine (commercial), (c) a combination of a+b and (d) was used as control ie not sensitized. This was followed by challenge, inoculating the two udders with high and low doses of the bacteria respectively. In the udders with high inocula, infection was 100%, but in the low inocula infection was 50% in (d), 35% in (b) and 8.3% in (c) (1 out of 12); and they concluded that A.P.T. could offer protection against *A. pyogenes* mastitis (Weitz and Langbridge 1947). Field trials to protect cattle against summer mastitis by the bacterium by inoculation of the toxoid, vaccine and toxoid plus vaccine however failed to protect animals against the disease despite the evidence of antibody formation (Weitz 1949). Similarly discouraging results were reported after infection in two groups of cows, one treated with the toxoid and the other untreated. There was minimal difference in the percentages of the animals infected (1.9% and 2.3% respectively; Lovell *et al.*, 1950). The large doses of toxoid required and the rather impractical schedules were the major draw backs in the adoption of vaccination in the field.

Immunization trials have also been carried out in mice, but protection against infection by homologous strains was insufficient (Derbyshire and Matthews 1963).

In contrast, a high quality inactivated *A. pyogenes* vaccine with toxin values of 160-320 haemolytic units/ml and 0.4% packed cells was produced by Cameron *et al.* (1976). When 3 doses were administered subcutaneously at 10 day intervals, 2ml for rabbits, 5ml for sheep, and 10ml for cattle, a satisfactory antibody response was elicited. The response was at its best 1-2 months after immunization and dropped to a plateau 4 months later. Mice were also successfully immunized against the challenge with $1.5x10^8$ organisms by treatment with 3 doses of the vaccine at 3 days intervals. There are no accounts of the value of vaccination in the prevention of bovine abortion and no correlation between antibody levels and genital tract infections have been made.

E. ACUTE PHASE PROTEINS.

Acute phase proteins are a group of proteins which increase in concentration during acute phase response to inflammation or infection. The response differs from one animal to another. Much work has been done in man, and less in cattle and dog. An investigation has been done on their physiological and clinical importance (Eckersall and Conner 1988). The proteins in the group include haptoglobin, fibrinogen, α_1-proteinase inhibitor, ceruloplasmin, seromucoid and c - reactive protein.

During the reaction the body mounts a multifactorial response to remove and replace damaged tissue and one of these mechanisms is the production and secretion by the liver of acute phase proteins. As these proteins increase others like albumin decrease as the liver switches to production of the acute phase proteins required for tissue regenerating (Koj 1974 and Peeters 1986). Bovine and canine serum profiles show raised levels of α and ß globulins, in

conditions similar to that of man (Barsanti *et al.*, 1977 and Liberg 1977). These proteins are described in this study with particular reference to haptoglobin.

HAPTOGLOBIN (Hp).

Bovine plasma Hp was first recognised by Laing (1957). It is a component of the α2 - globulin of plasma and has the ability to bind firmly to haemoglobin (Neuhaus and Soigoian 1961). In cattle, Hp has been shown to have a polymeric form which is formed by association of 40 and 16 KDa sub units with albumin (Eckersall and Conner 1990). Unlike in human, Hp has been found to be in very low concentrations in normal cattle (Bremner 1964, Minocherri 1965, Spooner and Miller 1974). In a number of pathological conditions however, Hp levels have been found to increase especially in acute inflammation of bacterial origin. Some of the conditions are mastitis (Spooner and Miller 1971, Conner *et al.*, 1986), liver abscesses and abscesses infected by *A. pyogenes* (Spooner and Miller 1971), pyometra, traumatic reticulitis, traumatic pericarditis and abomasal displacement (Panndorf *et al.*, 1976, Makimura and Suzuki 1982).

Richter (1975) induced the acute phase response in cattle using small doses of turpentine. Conner *et al.* (1988) managed to stimulate the reaction in cattle with the infection of *Pasturella haemolytica* and endotoxin injection, and Hp concentration was measured. The level of response was related to the severity of inflammation and it was found that increasing amounts of turpentine caused an increased peak of Hp (Conner et al., 1988).

Although Hp can be used in disease diagnosis, it is believed that in an event of a simultaneous haemolytic crisis, presence of free haemoglobin (Hb) in circulation will lead to binding with produced Hp and the resulting complex

will be removed by the liver. The Hp produced will then be difficult to detect (Eckersall and Conner 1988). Hp was found to be absent in haemolytic crisis of babesiosis (Bremner 1964). The physiological role in tissue regeneration has not been investigated.

IV. AIMS OF THE STUDY.

This study arose from observations by Kelly (1981), that bacteria were present on infertile bovine eggs (after superovulation and A. I.) and their tentative identification as *A. pyogenes*. It was demonstrated that the organism could adhere to mammalian eggs (Taylor unpublished). The following chapters describe the production of abortion following the intrauterine inoculation of pure cultures of *A. pyogenes* in early pregnancy in the cow, describing the associated ultrasonographic and hormonal changes, the pathogenesis of infection and the changes in the uterus following infection. In view of the evidence for the association of *A. pyogenes* with reproductive disorders it was considered that *A. pyogenes* might be a primary pathogen for the bovine embryo.

CHAPTER II.

MATERIALS AND METHODS.

Procedures described in this section are those employed for the greater part of the study, more specialised materials and procedures will be dealt with in the specific chapters.

I. ANIMALS.

Source. The cows and heifers used in this study were mostly Friesian and Friesian / Hereford cross and were obtained from a dealer. They were cows from a pool that were being kept awaiting slaughter. As such no known breeding records accompanied them, but on clinical examination most of the animals had calved at least once.

Management. The cows were housed in a standing byre, restrained by the neck by a sliding chain fixed to the side wall, so as to allow free access to feed and water. Animals were fed on hay twice a day and clean water was provided *ad libitum*. The byre was kept clean.

Monitoring for oestrus. Teasing was done to determined the stage of the oestrous cycle. The animals were allowed out in the teasing yard for about 20 minutes once or twice daily. Rectal palpation and the use of ultrasound (to be described later) were also used to determine the state of the ovary and particularly the presence or absence of a corpus luteum and follicles.

Artificial insemination. A few animals came into heat normally without any

hormonal intervention, but most of them were brought into heat (after confirmation of the presence of a corpus luteum) by injecting 500ug of a prostaglandin analogue, cloprostenol (Estrumate, Coopers, U.K.) to effect luteolysis. All animals were served using artificial insemination (A.I.) with semen from a local Artificial Insemination centre, (SMMB Ayr, Scotland). Semen from two Friesian bulls was used (Brynhyfryd Officer 14233 and Landale Pearl Lad 13833). The animals were inseminated when in standing heat and were served twice with a 12 hourly interval.

II. BACTERIOLOGICAL METHODS / EXAMINATION.

The methods used to examine swabs or discharge from the vagina were as follows:-

1. Direct smear. This was made on a glass slide soon after collection, and the slide was allowed to dry and fixed with gentle heat. The fixed slide was stained by the following version of Gram's method, 0.5% crystal violet was used for about 30 seconds and washed with running tap water. After this, Gram's iodine was added on to the slide for about 30 seconds. The iodine was washed off with running water and acetone was applied for about 5 seconds for the purpose of decolourization of the smear. After washing off the acetone with water, dilute carbol fuchsin was put on the slide for about 1 minute and then washed off under running tap water. The slide was dried, examined under oil immersion and the findings were recorded.

2. Cultural examination. The media used for cultural examination:-

(a) Horse blood agar. Blood agar base No. 2 (Oxoid), was used with 7% of added sterile horse blood. Freshly poured plates were left to cool and set at room temperature before storage at $4^{o}C$.

Sheep blood agar. Blood agar base No. 2 (Oxoid CM 271) was used and 7% of sheep blood (Becton Dickinson) was added. The agar was stored at $4^{o}C$.

Chocolate agar. Blood agar base No. 2 (Oxoid CM 271) was employed. The procedures used to make up the horse blood agar were carried out but instead of the 7% of horse blood, 10% of defibrinated horse blood (Oxoid SR 50) was added and the mixture was heated to $80^{o}C$ and maintained at that temperature for 5-10 minutes, while agitating frequently until the chocolate colour was formed. The mixture was cooled to $50^{o}C$ and poured into petri dishes and later stored at $4^{o}C$.

MacConkey agar. MacConkey agar powder (Oxoid CM7) was prepared according to the manufactures' instructions and stored at $4^{o}C$. Before inoculation it was important to make sure that the surface of the gel was dry.

3. Conditions for cultivation.

(a) Aerobic. Used for horse blood, sheep blood and MacConkey agars. After inoculation, the plates were placed into an incubator at atmospheric pressure at $37^{o}C$, then examined for any growth at 24 and 48 hours after incubation and the findings were recorded.

(b) Microaerophilic. Chocolate agar cultures were incubated under microaerophilic conditions which were achieved by placing the plates in the microaerophilic jar (Don Whitley Ltd. U.K.). The jar was closed and sealed, and using a vacuum pump (Edwards High Vacuum Ltd. U.K.) air was pumped

out to create a negative pressure of 500 mm of mercury. The jar was then filled with hydrogen and CO_2 mixture ie. 95% H_2 and 5% CO_2 (BOC Special Gases, Deerhurst Rd. London) incubated at 37°C. Examination for bacterial growth was carried out after 24 and 48 hours incubation and the findings were recorded.

(c) Anaerobic. Inoculated Horse blood agar plates were incubated anaerobically with a cold catalyst. The plates were placed in an anaerobic jar (DonWhitley Ltd. U.K.), and air was pumped out using the vacuum pump to a negative pressure of 650 mm of mercury. The jar was then filled with the hydrogen and carbon dioxide mixture. Incubation was carried out at 37°C and examination for growth of microorganism(s) was carried out at 24 and 48 hours.

(d) Methods of identification of bacterial isolates.

After incubation, the plates were examined for bacterial growth. Colonies were identified presumptively by their colonial appearance. Their identity were confirmed by the examination of Gram stained smears and biochemical tests where relevant.

Confirmation of the identity of *Actinomyces pyogenes* was carried out using the biochemical tests described by Carter (1979) (Appendix).

(e) Maintenance of cultures.

Cultures were stored freeze dried. Glass tubes 8 cm long and 1.5 cm wide were prepared at least a day before the procedure by typing the name of the bacterium on thin strips of paper and placing these in the tube at the closed end. The open end was then loosely plugged with a piece of cotton wool and the tubes were autoclaved using the slow exhaust autoclave - Model 8000-DSE

(National Application Company - a Heinicken Company, Oregon, U. S. A.) at 121^{o}C for 4 hours.

After culture of the bacterium on horse blood agar for 48 hours at 37^{o}C, a freeze drying solution (peptone water and serum) was used to wash the bacteria off the agar plates. About 3 ml of the freeze drying solution was used and 0.2 ml of the bacterial suspension was put into each tube, and tubes were frozen at -20^{o}C for about 1.5 hours.

The tubes were then put onto the lyophilising machine (Edwards High Vacuum Ltd. Crawley, U.K.), and freeze dried for at least 12 hours.

The tubes were then removed from the machine and the top third of each was heated under a Bunsen burner flame to melting point and elongated leaving only a very small opening (1.5 mm), and left to cool. The tubes were put back on to the machine to continue with the drying for another 6 hours under vacuum. While still on the machine the tubes were sealed off with a small but very hot gas flame thus making glass ampoules, which were then detached from the machine. The ampoules were stored at 4^{o}C until required for use.

III. THE PREPARATION OF INOCULA.

Actinomyces pyogenes used in the experimental infections was isolated from a case of bovine endometritis in the Microbiology Laboratory, University of Glasgow, cloned and lyophilised in glass ampoules within two passages of isolation. Following this, its identity as *A. pyogenes* was confirmed by the methods described above.

Live *A. pyogenes*. The freeze dried cultures were reconstituted in 2 ml of sterile physiological saline. A drop of the suspension was placed on horse blood agar plates (in duplicate), and using a sterile wire loop (MW 193, Medical Wire and Equipment, Bath, U.K.) the suspension was spread over the agar and the plates

were incubated aerobically at 37°C for 48 hours. A typical colony was then selected and used to inoculate horse blood agar which was incubated aerobically for 48 hours at 37°C; after which bacterial colonies were washed off the plates using sterile physiological saline (0.85%), centrifuged at 15,000 r.p.m. for 10 seconds, washed again in sterile physiological saline and centrifuged and finally resuspended in sterile physiological saline for experimental animal inoculation.

1. Bacterial count.

The number of bacteria present in the inoculum was determined in two ways (a) by carrying out a viable cell count on horse blood agar plates (Miles et al., 1938) and (b) using a Coulter counter (Coulter Electronics Ltd. Bedfordshire, U.K.)

(a) Viable cell count. Tenfold serial dilutions of were made by diluting 0.1 ml of the bacterial suspension (inoculum) in 0.9 ml of sterile physiological saline and continuing with the dilutions up to 10^{-8}. Using a micropipette 20 ul of each of the 8 diluted samples was sub-cultured on a horse blood agar plate, starting with the highest dilution (10^{-8}). The plates were left out on the bench to dry for about 2 hours and then incubated aerobically at 37°C for 48 hours. Plates were examined and the colonies in each drop were counted. The number of bacteria /ml in the inoculum was calculated as follows:-

Eg. If there were 4 colonies in 20 ul at 10^{-7}

$\therefore$ c.f.u. /ml = $4 \times 50 \times 10^{7}$ = **2×10^{9}/ml.**

(b) Coulter counter - Model ZF. The equipment is more commonly used for haematology and cytology so the procedure is described below:-

The live bacteria to be counted were suspended in a counting fluid (ISOTON II) in a beaker (or disposable vial such as an Accuvatte) on the beaker platform. The aperture formed in the disc of the fused aluminium oxide and set into the side of the orifice tube, was immersed in the fluid. The two electrodes were placed in contact with the fluid, the earth inside the orifice tube, and the live electrode in the sample beaker.

A mercury manometer connected to the aperture tube by the control tap was fitted into the rear compartment and controlled the volume of the sample fluid passed through the aperture and the pressure difference across the aperture. A vacuum pump connected to the bottle provided the pressure differential.

There were three ancillaries to the system including:-

* An optical viewing system with a lamp and a monitoring screen to examine the aperture for blockages.

** A side tap connected from a beaker of fluid to a tube suspended inside the orifice tube which could be used for flushing or emptying it.

*** A pressure regulator to limit the suction developed by the vacuum pump.

The threshold control was adjusted to allow the minimum particle size of the bacteria to be counted.

The strength of the aperture current and the degree of the amplification were adjusted to produce pulses of satisfactory size to operate the counting circuit. The particles could then be viewed on the sizing oscilloscope and output from the counting circuit was displayed on a digital read-out.

A built in coincidence correction circuit could be switched on to compensate for coincidence loss, i.e. when two particles passed through the aperture simultaneously producing only one pulse. Results were expressed as organisms per ml. The equipment was disinfected with alcohol after use.

IV. EXPERIMENTAL INOCULATION.

1. Laparotomy, laparoscopy and anaesthesia.

Intrauterine inoculation was carried out by first carrying out laparotomy under local anaesthesia. Local anaesthesia was achieved by local infiltration of the right and left flanks using 40-60 ml of lignocaine (Lignol, U.K.). A 5 cm laparotomy incision (about 5 cm) was made through the skin wall of the right flank region and the trocar and cannula was forced through the obliquus externus abdominus, obliquus internus abdominus muscles and peritoneum to the abdominal cavity. The trocar was removed and a laparoscope was introduced through the cannula and via the abdominal cavity into the pelvic cavity. The reproductive tract was located after insufflation. A 60 cm 16 gauge needle was then introduced via the side tube to the laparoscope, through the uterine wall and into the uterine lumen. Before introduction of the inoculum into the uterus, an empty sterile syringe was attached to the needle and aspiration carried out to make sure that the sharp end of the needle was just between the uterine wall and the allanto-chorion, as the intention was to deposit the inoculum into the uterine lumen and not into any of the embryonic cavities. Six ml of the inoculum was then injected.

2. Inocula.

Four different inocula were used in the study, and a 6 ml volume was used in all cases. These included:-

(a) Live *A. pyogenes*.

(b) Sterile physiological saline.

(c) Killed cultures of *A. pyogenes*.

(d) *A. pyogenes* toxin.

(a) Live *A. pyogenes* was prepared as described on section III.

(b) Sterile physiological saline. The sterile physiological saline was 0.85M and 6 ml. were inoculated.

(c) Killed cultures of *A. pyogenes*. *A. pyogenes* was grown on horse blood agar and prepared as described in (i). Before the heat treatment bacterial counts were done as described above. 2 ml of the bacterial inoculum were put in Bijou bottles, and the bottles were put in a water bath at $70^{O}C$ and heated for 30 minutes. After heat treatment, subculture was attempted on horse blood and chocolate agars and incubated at $37^{O}C$ aerobically and microaerophilically respectively. The plates were examined at 24 and 48 hours to check for growth of *A. pyogenes* or any other bacteria.

(d) *A. pyogenes* **toxin.** The *A. pyogenes* toxin used was the haemolysin described by Lovell, (1937) and was prepared by the methods described by him and later modified by Lovell (1944) and Roberts (1968). The media used included Robertson's cooked meat medium and skimmed milk.

* **Cooked meat medium.** Cooked meat tablets (Oxoid CM 82) were used to prepare the cooked meat medium according to the manufacturer's instructions. It was prepared in 250 ml volumes. 12.5 gm of cooked meat medium was added to 100 ml of distilled water and the tablets were allowed to soak for about 15 minutes and latter sterilized in an autoclave at $121^{O}C$ for 15 minutes. The mixture was allowed to cool at room temperature.

* **Milk.** Pasteurised skimmed milk (from a shopping centre) was heated

to boiling point and was left to cool at room temperature before use.

The two media were mixed in equal volumes of 150 ml in a 500 ml conical flask. The **milk meat medium** thus produced was inoculated with pure colonies of *A. pyogenes* (from a horse agar blood 48 hour culture) using a sterile bacteriological loop and the flask was covered with aluminium foil to avoid contamination. It was incubated aerobically at 37^{o}C for 72 hours. During incubation the flask was periodically shaken gently to give a uniform bacterial growth.

Separation of the crude toxin. The fluid portion of the culture was poured off into universal bottles which were centrifuged, at 3,000 r.p.m. for 30 minutes (on a Centaur 2 centrifuge, MSE, U.K.). After being centrifuged the supernatant was filtered through 0.2 um ultra filters (Minisart NML, Sartorius, Germany). The filtrate containing the crude toxin was then ready for experimental use.

Haemolysin test. This was done to assess the haemolysin content of the toxin or the products of experimental inoculation, and was conducted as described by Lovell (1937). 0.5 ml of the toxin was diluted 1:2 in 1 ml volumes and the samples serially diluted to 1:2048 in glass test tubes, using sterile physiological saline. Horse and sheep defibrinated red blood cells were available and were washed 2 or 3 times with sterile physiological saline. 0.1 ml of a 10% suspension of horse and or sheep erythrocytes were was added to the diluted filtrate. The mixture in the test tubes was shaken well before incubation in a preheated water bath at 37^{o}C for 1 hour. The titre was expressed as a reciprocal of the highest dilution to produce haemolysis.

Another treatment given to control animals was **prostaglandin F2α analogue - cloprostenol** (Estrumate, Coopers, U.K.). A dose of 500 ug was given intramuscularly for the following reasons:- (i) to bring animals into oestrus or (ii) to induce abortion by lysis of the corpus luteum.

V. EXAMINATION OF ANIMALS BEFORE AND AFTER EXPERIMENTAL TREATMENT.

1. **Clinical examination.** After artificial insemination the animals were observed daily for any abnormal behaviour by recording the demeanour, appetite and the general bodily condition up to the time of experimental treatment. While under experiment the animals were under constant observation until abortion occurred or until 50 days after A.I. in those animals that failed to abort as a result of experimental intervention. The demeanour and general appearance of the animals were recorded. The presence or absence of signs of heat were noted and particular attention paid to the vulva for signs of abortion or vaginal discharge.

Rectal palpation was carried out twice daily but did not involve attempts to slip the foetal membranes. The tone of the uterus was assessed subjectively and scored on the scale of 0-5. **Rectal temperatures** were taken just before or at the time of experimental treatment and then twice daily until abortion was recorded then daily observations were made thereafter.

Vaginoscopy. The vagina and cervix were examined using a vaginoscope and a note was taken of the appearance of the mucous membranes, presence of any discharge and openness of the cervix. Embryos and their membranes or discharge were collected from the cranial part of the vagina through the vaginoscope into clean containers. Embryos were also recovered from the byre

floor on some occasions. Samples of vaginal discharge and the embryos and their membranes were examined for the presence of *A. pyogenes* and other bacteria by the methods described above.

Bacterial flora of the genital tract. Before experimentation, vaginal swabs were taken routinely for bacteriological examination by the methods described below. To avoid possible contamination of the uterus it was not thought advisable to take swabs beyond the anterior vagina.

2. Blood sampling.

Plasma. Blood samples were taken from the jugular vein or the coccygeal vein before experimental treatment and then every 6-12 hours after the treatment until abortion, followed by 1 or 2 times every 48 hours to resumption of normal cyclicity. Blood was collected using heparinised Monovets, and was centrifuged soon after collection at 2,500 r.p.m. centrifuge, for at least 10 minutes. The plasma was poured into tubes, sealed and stored frozen at -20^{o}C until required. Plasma was collected to assay for progesterone, oestradiol 17_{B}, pregnancy specific protein B, and Haptoglobin, an acute phase protein.

Serum. Clotted blood for serum was collected just before experimentation and then once or twice every 48 hours thereafter. The blood was not heparinized and was first kept at 4^{o}C for at least 24 hours, centrifuged at 2,500 r.p.m. for 10 minutes and the serum was drawn off and stored at -20^{o}C until required. Serum was mainly collected for the purpose of serological studies.

VI. ULTRASONOGRAPHY.

1. Definition and principles.

As ultrasonography was an important part of the study and it is a new method of

diagnostic investigation, its definition and mode of action are briefly reviewed (Ligtuoet *et al.*, 1989).

(a) Definition. Diagnostic ultrasonography has been developed for some years now and combines more than one diagnostic parameter including **structural tomography** which allows reconstruction of the cross sectional anatomy of an organ, **structure motion** which allows observation of objects such as the foetus, and **tissue characterisation.** Ultrasound investigation is particularly useful in the study of the geometry and motions of soft tissues (Ligtuoet et al., 1989).

(b) Principles. The sound beams rapidly penetrate through the organs of the body allowing observation of moving structures on the echo principle, using an ultrasonic transducer, reflection of sound, display techniques and a two-dimensional real time principle to present the results.

The echo principle. Sound waves represent a pressure disturbance that is propagated at the velocity of sound through a given medium such as blood or tissue. For example the velocity of sound in blood or water is approximately 1,500 meters per second. The principle is best demonstrated by measuring water beneath a ship (**Appendix Fig. 1**). When the sound velocity is known, the time between transmission of a sound pulse and reception of the echo can be converted to a distance or depth measurement, the time between emission of the signal and its reception being conversely proportional to the depth.

Ultrasonographic transducer. The transducer is the apparatus by which sound waves are generated and detected. Sound waves used in clinical ultrasound have a frequency between 1 and 10 Mhz (1 to 10 million cycles per second) and can be generated using piezo electric (meaning squeeze-electric) material. A

thin piezo electric crystal is covered on both sides with electrodes. Application of an appropriate frequency i.e. an alternating polarity of the signal leads to the crystal becoming thinner and thicker at the same frequency i.e there is contraction and expansion of the crystal thus generating sound waves on the surface of the crystal (**Appendix Fig. 2**) and these are then propagated in two directions. One of the waves is suppressed with a damping material whereas the other wave penetrates the tissue in front of the crystal. When the reflected sound waves reach the transducer the piezo electric crystal is compressed and expanded producing an electrical signal which is measured over the electrodes. The operator directs the sound waves through the tissue by moving the angle of the transducer as he desires. Thus the three components of the transducer are the piezo electrical crystal, the electrode and the damping material.

Reflections. The heterogeneous nature of the tissue results in tissue interfaces where tissues of different density are in contact. Tissue may be acoustically defined by its "acoustic impedance" which is the product of the sound velocity and the density of the tissue, which is also the measure of the resistance to the propagation of sound waves. The reflectivity of the medium depends on its acoustic impedance. So surfaces like the interface between epicardium and lung, air bubbles in the colon and calcified structures much as bone are highly reflecting boundaries.

Appendix Fig. 3 diagrammatically demonstrates reflection at boundaries between I, II, and III. From the pulses transmitted in II and III there are reflections (echoes E_1 and E_2 respectively) but only parts of the echoes are reflected. The magnitude of the differences in the acoustic impedance between the tissues on each side of the interface determines how much of the waves will be reflected. Usually a small amount is reflected and the rest is available to

interact with other interfaces deeper in the tissue mass. The echo will be displayed on the screen in a manner corresponding with the depth of the tissue boundary.

Display technique. Echoes may be displayed in the A-mode or the amplitude mode (**AppendixFig. 3**), where the reflectivity at the boundary II and III is shown to be causing echo E_2 to be larger than E_1. Another display technique is the B-mode, or the brightness mode, in which the echo amplitude is converted to brightness of the echo dot, which brightness is proportional to the amplitude of the returning echoes. B-mode is the basis of all two-dimensional images and is very commonly used in the investigation of genital organs in large animals. The principle behind the B-mode is doing cross-sectional scanning and producing a cross-sectional ultrasonic image frame of the organ or tissue (**Appendix Fig. 4**). Repeated repositioning of the transducer causes the ultrasound beam to penetrate the organ along many lines in a well defined cross-sectional plane and echoes are created particularly at border lines between for example blood or fluid and tissues, or fat and tissues. Corresponding to each transducer position the echo dots can be stored in the scan converter memory on a line corresponding with the transducer beam at the moment the echoes are received. Within the scan-converter all the integrated echo information will form a cross sectional of the organ, which image will present a still frame of the structure being studied. The contents of the scan-converter memory may subsequently be displayed on the television monitor.

Efforts have been made to develop a system whereby the ultrasound beam can be readily swept through the entire cross-section, using an electric as well as a mechanical system to enable a high frame rate and thus the real time system ("Live" in which the echos are continuously being recorded). Characteristics of

the real-time system include:-

(i) The possibility of studying structures in motion, that is, the production of images that move as the structure moves which are so is important for embryonic and foetal studies.

(ii) Selection of the scan plane during observation since the echogram appears instantaneously on the display.

When the frame repetition rate is higher than 15 frame /second the eye will observe the frame sequence as a continuum. So the ideal system will have a high frame rate, a good resolution and a good dynamic content. But whereas the higher the number of lines the better the image quality, more time will be required for the formation of the image thus lowering the frame rate, so there has to be a compromise between these. For example for 30 images/sec. and a minimum depth of 20 cm, a total of 125 lines will give a good ultrasound image.

Two-dimensional real-time system. A number of two-dimensional real-time systems are currently available and may have mechanical or electric beams with a sector or rectangular format. The principle for all these systems is that an acoustic beam scans the cross-sectional plane at a high rate yielding instantaneous information about two-dimensional structures. The four main two-dimensional real-time systems include :- the Linear array scanner; the Phased array scanner; the Pivoting mechanical scanner and the Spinning wheel mechanical scanner. The linear array scanner is commonly used in bovine genital investigations. In the scanner a number of piezo electric crystals are positioned in a row. A sub group of crystals is used to form a sound beam. Adjacent and overlapping sub groups produce parallel sound beams and rapid scanning results in a high frame rate. The characteristics of this system are a rectangular longitudinal image produced with no mechanical motion of the

transducer head, but requiring a relatively a large contact area with the patient.

Resolution. Resolution refers to the ability of an ultrasound pulse to distinguish between two closely spaced reflectors. In axial resolution, the reflectors are located along the direction of the beam and a high frequency transducer gives better axial resolution due to the relatively shorter pulses, and, because of the reduced likelyhoodof overlap between two reflecting surfaces, these can be easily distinguished. For lateral resolution, the reflectors are located at right angles to the direction of the sound pulses, and lateral resolution can be either the width or thickness of the beam **(Appendix Fig. 5).**

2. Ultrasonographic equipment used in the experiment.

Ultrasound equipment. A portable real-time B-mode scanner with a 7.5 Mhz linear array transducer (Concept one, Dynamic Imaging, Livingston, Scotland) was used. The transducer was always introduced into the rectum after rectal palpation. It had an axial and lateral resolution of 0.5 and 1 mm respectively and was both water proof and electrically insulated. The ultrasound machine possessed a freeze mode and internal callipers allowing for direct on screen measurements, a magnification or zoom mode and image storage memory with recall. Using the scanner, images of organs observed on the screen were studied **(FIG. 2).**

Hard copy. From the ultrasound scanner (or console) images were stored on video tape using a VHS video cassette recorder (Ferguson BV 42, Japan) **(FIG. 2).** Detailed study of the findings was performed later by playing back the tape on a monochrome video viewer (Panasonic, Japan) through to the scanner.

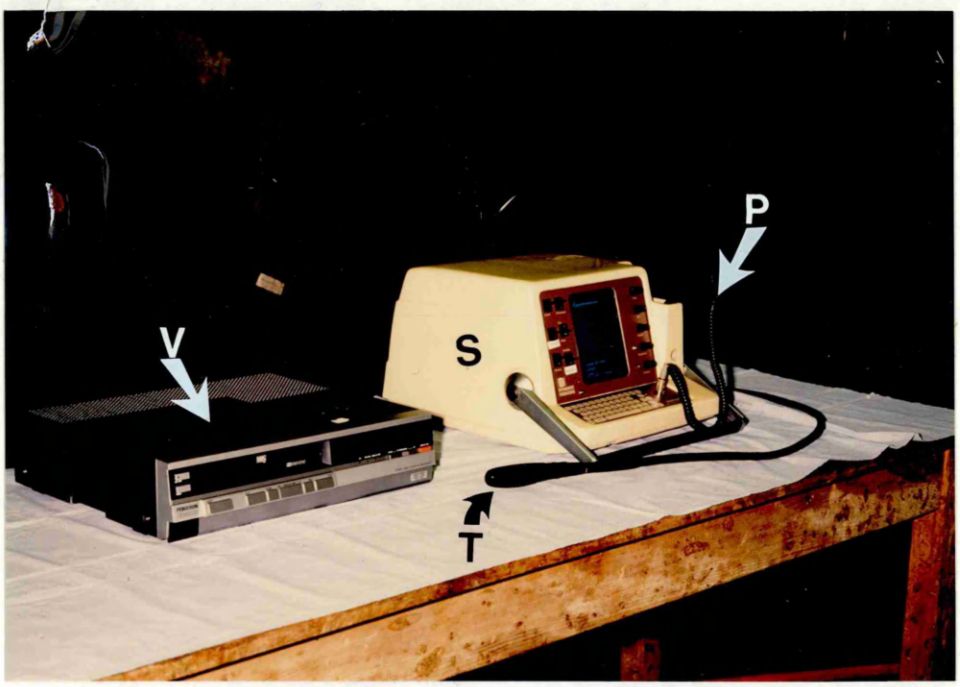


FIG. 2: The ultrasound equipment used in the study. It includes the B - Mode Scanner (S), a 7.5 Mhz transducer (T), an internal calliper or light pen (P) and the video - recorder (V) for taping the observations.

Photographs were taken for a readable printed copy using a Polaroid camera (Equisonics 210z) with No. 611 film, the camera being held directly on to the scanner TV screen. High quality photographs and slides for projection were available when photographs of the Polaroid prints were taken using a 35 mm camera system.

A Sony Video Graphic thermo printer - UP-850 was also sometimes used to produce quality hard copies, transferring images from the ultrasound scanner to the screen by screen dump using video connections and the images were printed on thermally sensitized paper using a 280 x 234 dot matrix.

3. Ultrasound scanning and method of examination.

Ultrasound scanning for confirmation of pregnancy was performed at least 3-4 times prior to experimental inoculation. Scanning first took place 3-4 hours after inoculation and continued every 3 hours for the first 12 hours and then followed at 6-12 hourly intervals until embryonic death and abortion. After abortion, scanning was continued once daily or every other day until recovery from infection or the end of the study.

Method of examination. The examiner's hand was gloved and lubricated to facilitate easy entry through the anal sphincter and the rectum was evacuated of faeces before introduction of the transducer. The transducer was also lubricated to establish a good contact with the mucosa of the rectum. The scanner and cassette recorder were placed on a mobile trolley (or a table) behind the cow so that the operator could monitor progress to be examined and at the same time providing a comfortable position for the operator and or

FIG. 3: An ultrasound examination in progress. The ultrasound examination is preformed with the operator directly behind the animal but in a position comfortable to both him and the animal. A lubricated hand and the transducer (arrows) are introduced into the rectum via the anus. Note, the ultrasound equipment must be at a safe distance to avoid damage resulting from any sudden movement either by the operator or the animal.

the assistant (**FIG. 3**). Different parts of the reproductive tract were imaged in the following order: right and left ovaries, right and left horns, body of the uterus, the cervix and the vagina some of which are demonstrated in **FIG. 4.** The position in which the operator carries out ultrasonography alone is shown in **FIG. 5** but some times an assistant is required to manage the controls on the scanner.

4. Interpretation of ultrasound images.

(a) Generally all tissues and organs are echogenic (i.e. they reflect back sound waves) and thus appear as grey to white images, whereas fluids are non-echogenic or anechoic (failing to reflect sound waves) and thus appear dark/black. Different tissues are seen in different shades of grey depending upon their echogenicity or ability to reflect sound waves. Measurements for the different structures can be made at the time (real - time) of examination (**FIG. 6**). **Specular reflection** may result when a wave strikes on an interface that is smooth, wider than the wave and parallel to the transducer. The recorded echo is normally very bright (highly echogenic). **Non-specular reflection** could occur when a wave strikes a rough interface or one that is narrower than the wave and scatter of the echoes occurs (**Appendix Fig. 6**).

Ovaries. The ovaries were examined for the presence of the corpus luteum (which was always found on the side of the pregnant horn). Corpora lutea appear as round to oval grey echogenic images. Follicles can also be distinguished as round black non-echogenic images.

Uterine horns. From day 19 after service the vesicle is approximately 3 mm in size and non-echogenic and contains the echogenic embryo which is about

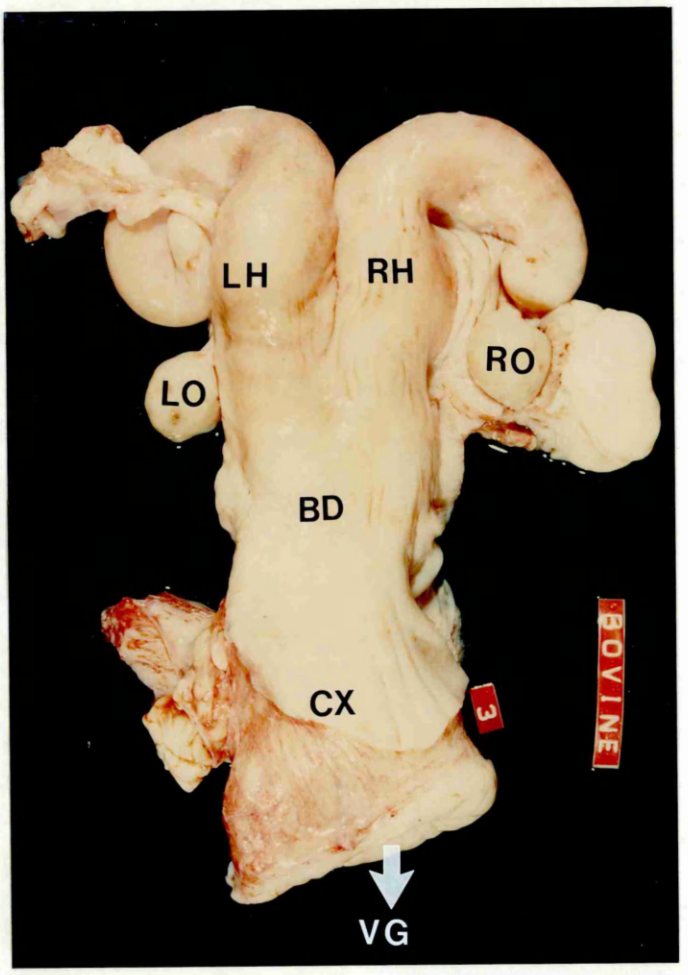


FIG. 4: The bovine non - pregnant female reproductive tract viewed from above. The following may be seen: ovaries, left (LO) and right (RO), the uterine horns (LH and RH), the body of the uterus (BD), the cervix (CX) and the vagina (VG).

FIG. 5: The operator making measurements of different sections of the uterus using the light pen with internal callipers.

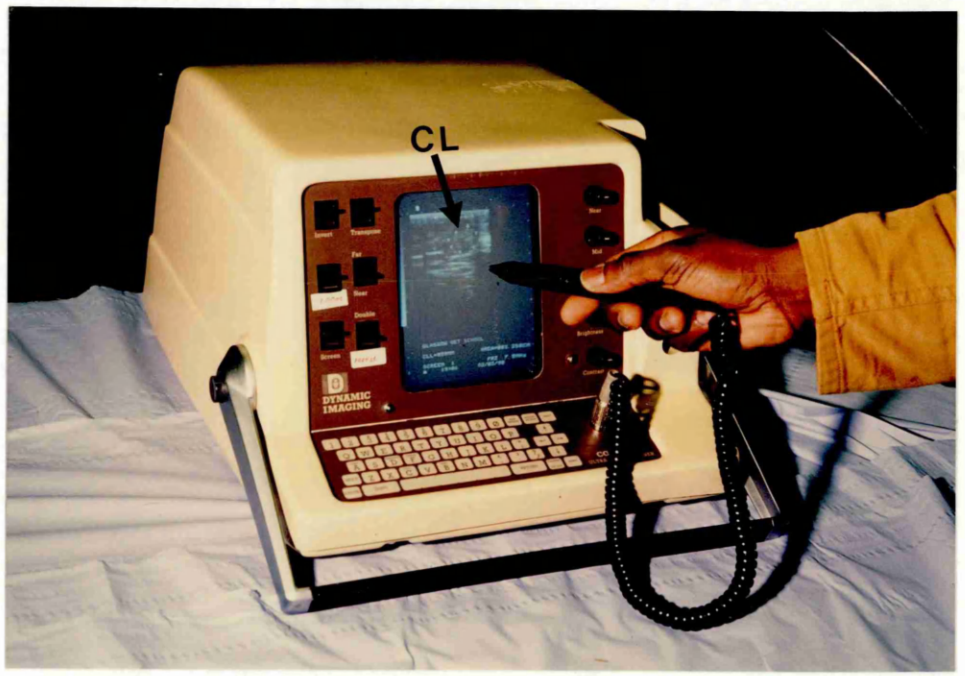


FIG. 6: Measurement of structures using the light pen. A photograph demonstrating a cross section of the corpus luteum (CL) of an ovary being measured with the use of a light pen. The real measurements of a structure is made in real - time.

0.5 mm in size. The embryonic heart beat can be identified. The amniotic cavity can be clearly differentiated from the allantoic cavity by day 25. The endometrium and myometrium give grey to white echogenic images and be identified ultrasonographically.

Uterine body, cervix and vagina. Walls of the body, cervix and vagina appear as white to grey (echogenic) images and the presence of mucus or fluid in their cavities can be seen as dark/black (non-echogenic) areas. The urinary bladder lies ventral to the vagina and is the first organ to be seen on introduction of the transducer appearing as black non-echogenic structure when filled with urine. The pubis lies ventral and posterior to the urinary bladder and is highly echogenic. The cervix frequently lies directly above to the pubis and can be distinguished with difficulty as a white (echogenic) structure.

(b) Gross pathological changes.

After inoculation the size and tone of the walls of the reproductive tract were monitored by rectal palpation and the relationship between the embryonic membranes (specifically the allanto-chorion) and the endometrium were recorded by ultrasonography. The viability of the embryo was monitored by examination for the embryonic heart beat and the presence and echogenicity of the fluid(s) throughout the reproductive tract and these were recorded.

VII. PATHOGENICITY STUDIES.

1. Post mortem examination. Animals were killed by stunning and exanguination. The internal organs were then examined including the lungs, spleen, liver, kidney and the peritoneal surfaces and any gross changes were recorded. The reproductive tract was examined and the appearance of the

ovaries, the uterine horns, the body of the uterus, the cervix and vagina recorded. This external examination was followed by opening up the tract for internal examination of the uterine luminal content, the uterine wall and the embryo.

Sections of the reproductive tract were taken and fixed for study by histology and electron microscopy (scanning and transmission).

2. Histology.

Procedure. Tissue from the reproductive tract or the embryo was taken and fixed soon after collection in Bouins or 10% Buffered Normal Formalin (B.N.F). Tissues were left for about 24 hours in Bouins fluid then changed to ethanol but they were left for 2-10 days in B.N.F.. The tissue was then trimmed and post fixed in mercuric chloride formal (formal sublimate), then dehydrated through a series of graded alcohols and paraffin wax sections of 3 um were prepared. All tissues were stained with H & E and Gram's method.

Interpretation of results. The slides were studied using a light microscope (SM - LUX, Leitz, W.Germany) at x10, x40 and under oil immersion at x100 and the results were recorded. The sections studied were photographed using a light microscope (Reichert) with an attached 35 mm camera colour - Format 2 (Cambridge Instrument Inc., New York, U. S. A.).

3. Ultrastructure. Both scanning and transmission electron microscopy (E.M) was employed. The fixing of the tissues and taking of the photographs were done in the Department of Veterinary Anatomy, Glasgow Veterinary School.

(a) Scanning electron microscopy (S.E.M.).

Procedure for tissue preparation. 1 cm blocks of tissue were washed gently in

B.N.F. to remove surface blood and mucus and then placed in Karnovsky's fixative for a minimum of 24 hours. The tissue was trimmed to expose the tissue to be scanned and washed in B.N.F. for about 4 hours. Different concentrations of acetone were used as follows:- the section was put in 70% acetone for 4 hours, then transferred to 90% acetone for 2 hours and 100% acetone for another 2 hours. The tissue was left in acetone (100%) overnight. On removal from acetone, the tissue was left to dry and later attached to aluminium stubs using silver paint, this was left to dry for 20 minutes at 37^{o}c. The tissue surface was coated with gold for 4 minutes and the tissue was ready for examination.

Scanning and data interpretation.

The tissue was examined by scanning electron microscopy using a 501B Scanning Electron Microscope (S.E.M.) (Philips, Holland). The tissues were studied at magnifications from x400 to x15,000. Photographs were taken using 120 (125 ASA) film.

(b) Transmission electron microscopy (T.E.M.).

Procedure for tissue preparation. The tissue section was minced in Karnovsky's fixative and allowed to fix for about 3 hours, the fixative was changed and left overnight. The fixative was drained and replaced with 0.1M cacodylate buffer for 1 hour. Stock osmium tetroxide was diluted 1:1 using 0.2M Na cacodylate in a fume cupboard and the minced tissue was fixed with osmium tetroxide for 1 hour. After this it was washed 3 times with distilled water.

Three different concentrations of acetone were used first 70% for 10 minutes, then 90% for 10 minutes and 100% used for 15 minutes twice. Propylene oxide was added to the tissue for 20 minutes and this was done twice. Propylene oxide and emix resin 1:1 were then used on the tissue for 1 hour. Emix resin was then

added alone for 4 hours. The tissue was embedded and put in an oven at 60^{o}C overnight. Thick sections of 1 u in thickness were cut to confirm the suitability of the area for examination and then sections 100 um in thickness were cut for staining and examination.

Transmission and data interpretation.

The transmission electron microscopy of the embedded tissue was carried out using the JEM - 100x II electron microscope (JOEL Ltd. Tokyo, Japan). Electron micrographs were taken from selected fields.

VIII. HORMONAL ASSAYS.

1. PROGESTERONE ASSAY.

Enzyme linked immunosorbent assay - EIA kits (Ovucheck, Veterinary Services, Cambridge, U.K.) were used to analyse plasma samples for progesterone concentration. The kits were provided with 4 standards containing 0.5, 1, 5, and 10 ng/ml of progesterone and each kit contained sufficient reagents for 92 tests making a total of 96 wells, held in a microwell strip carrier.

The reagents used in the assay included:-

(i) conjugate containing progesterone-alkaline phosphatase, 25 ml.

(ii) enzyme substrate tablets with p-nitrophenyl phosphatase 3x40 mg.

(iii) substrate buffer containing diethanolamine 1M and magnesium chloride (0.5 mM) at pH 9.8, 25 ml. Substrate tablets were added to the substrate buffer and dissolved by vigorous shaking. Excess substrate was stored at -20^{o}C in aliquots of 2 ml.

(iv) stopping solution containing di-potassium hydrogen orthophosphate 0.5 M and EDTA (5 mM) at pH 10.0, 20 ml.

Principles of the assay / test procedure. The assay was based on the competitive binding of unlabelled progesterone present in the standard or sample and a fixed quantity of progesterone conjugated to the enzyme alkaline phosphatase (AP), for binding sites on limited amounts of progesterone antibody. The wells were pre-coated with the antibody, providing a solid phase assay which permits convenient separation of bound progesterone from the free progesterone in the sample.

Samples and controls. Plasma in heparin was stored at -20 ^{o}C. Two controls with known amounts of progesterone (high and low) were used in the assay. The known control samples in the assay were used to find out the inter-assay and intra-assay coefficients of variation and thus test the reliability and reproducibility of the assay. Acceptable results for the low and high progesterone control samples in each run were 3.3 $\pm$ 0.7 ng/ml and 6.9 + 0.67 ng/ml respectively.

Procedure. The four standards and the thawed plasma samples were shaken well just before use, then the number of wells to be used was exposed by cutting away the foil. The contents of the exposed wells were discarded and the wells tap dried on absorbent paper.

A 10 ul aliquot of the four standards was put in the first four wells (starting with the lowest to the highest). Ten ul aliquots of the samples to be tested were added to the rest of the wells, including at least 4 or 6 replicates of the two control samples.

Two hundred ul of the conjugate was added to each well and the wells were covered with a clean sheet of paper and incubated at room temperature for at least 30 minutes. The wells were then emptied and washed with cold tap water twice then dried by tapping on an absorbent paper. The substrate reagent was

added to all the emptied wells which were covered again with a clean sheet of paper and incubated for another 30 minutes at room temperature. After this, 100 ul of the stopping solution was added to all the wells.

Reading results. After the first period of incubation, all components other than those bound to the plate wells were washed away. The amount of the bound AP-labelled progesterone remaining on the wells was inversely proportional to the concentration of the unlabelled progesterone in the sample. The bound progesterone was then measured by reacting the AP with its substrate during the second incubation and the reaction was shown by colour formation.

The colour produced was measured spectrophotometrically, the spectrophotometer in the plate reader being set to read absorbance at 405 nm. A standard curve was drawn by plotting standard absorbance values (using a computer and printer) and the concentration of progesterone in the samples was determined from the standard curve.

2. OESTRADIOL ASSAY.

A radioimmunoassay (RIA) was used to determine oestradiol 17_B (E_2B) concentrations. The assay used in the study was developed by Dr I. Jeffcoate, University of Glasgow Veterinary School. The reagents used in the assay included:- standard plasma samples with known levels of E_2B (i.e. 1.99, 3.88, 7.76,15.53, 31.5, 63, and 125 pg/ml); diethylether; working buffer; dry ice; ^{125}I - labelled E_2B antigen; E_2B antibody raised in sheep against 6-carboxymethyloxime-E_2B-BSA (provided by Dr B. A Morris, AFRC Antibody Production Unit, University of Surrey, U.K.) and a precipitating antibody - donkey anti-sheep/goat (AS) gammaglobulin, used at a dilution of 1:20 (from Scottish Antibody Production Unit, Strathclyde, U.K.). Normal goat serum

(NG) was used to aid with the precipitation.

Principle of the assay. The assay depends on competitive binding of the unlabelled E_2B in the standards / samples and radio labelled E_2B with a limited amount of E_2B antibody.

Procedure. At the start of the experiment glass test tubes (10 ml) were serially numbered from 1 onwards to accommodate each sample and standard tested in duplicate. Standards and plasma samples were then taken from the freezer at -20^{o}C and allowed to thaw at room temperature. The following were put in the first 20 test tubes:- 1-2 only the labelled antigen for total count (TC); 3-4 the labelled antigen, buffer and the precipitating antibody for non-specific binding (NSB); 5-6 labelled antigen, antibody and buffer; and from 7-20, 400 ul of the standard samples were pipetted in duplicate i.e. 1.9, 3.88, 7.76, 15.53, 31.5, 63, and 125 pg/ml. From test tube 21 onward plasma samples (400 ul) were pipetted in duplicate.

Extraction of oestrogen was carried out as described below. All tubes, apart from 1-2, received diethylether (3ml) and were vortex mixed in the fumehood for at least 5 minutes. The tubes were then placed individually in a bath of methanol and dry ice fragments (at -80^{o}C), which froze the aqueous plasma layer while permitting the diethylether layer containing extracted E_2B to be poured into clean glass tubes. The tubes were put into a rack fitted with a manifold connected to an air pump and evaporated to dryness. This left the dried oestrogen attached to the walls of the test tubes.

0.1 ml of labelled E_2 antigen (7,000 to 13,000 counts) and 0.2 ml of the oestrogen antibody (1:20,000) were added to all the test tubes except in tubes 1-

4, and all the tubes were incubated at room temperature for about 2 hours. After this 1.0 ml precipitating antibody (AS) was added to the tubes. Normal goat (NG) serum 0.1 was also added to the tubes. **AS** was added for precipitation because of its ability to bind sheep gammaglobulin and the sheep E_2B antibody. Addition of **NG** provided sufficient excess gammaglobulin to form a matrix which augments precipitation by trapping the bound oestrogen.

After addition of the precipitating antibodies the samples were incubated at 4^oC for about 18 hours. The tubes were centrifuged at 3,000 r.p.m. (centrifuge) for about 10 minutes, and the supernatant containing free E_2B was aspirated using a water tap vacuum pump to leave a precipitate containing the labelled and unlabelled bound E_2B.

Reading. The precipitate was counted after centrifugation using a Gamma-counter (Canberra Packard Ltd. U.K.). The amount of labelled E_2B in the precipitate was inversely proportional to the concentration of the unlabelled oestrogen in the sample. A standard curve was drawn, plotting percentage binding values against the Log of the known concentration of E_2B in the standards, and E_2B concentration in the samples was caltulated from the standard curve.

IX. ASSAY FOR ANTIBODIES AGAINST *A. PYOGENES* INFECTION.

An Enzyme Linked Immunosorbent Assay (ELISA) was developed to detect antibodies in serum which are directed against *A. pyogenes*. Serum samples were obtained from infected cows and from control animals. Immunolon 1 plates were coated with *A. pyogenes* antigen and only 60 of the 96 wells (ie. wells in the middle of the plate) were used, to avoid back ground interference which

is seen in borderline wells.

Reagents. The reagents used in the assay included:-

(i) *A. pyogenes*, the bacterium was sonicated and used as an antigen,

(ii) coupling buffer (10 mM $NaHCO_3$ + 1 mM EGTA at PH 9.5) , EGTA = Ethylen-Glycol - bis - (B amino ethyl ether) NNN'N' tetra acetic Acid.

(iii) Tris phosphate buffer (0.15 M NaCl + 50 mM Tris, PH 7.6) (TSB),

(iv) Dried skimmed milk powder 2% (Marvel, Cadbury),

(v) Tween 20 (T 0.5%),

(vi) Antibovine conjugate (alkaline phosphatase SIGMA),

(vii) Enzyme substrate tablets with P - nitrophenol phosphate (3 mg each),

(viii) Horseradish Peroxidase (HPR),

(ix) Stopping solution - sodium hydroxide (0.4mM NAOH).

Principle of the assay. The wells were coated with the bacterial antigen to provide a solid phase for the binding. The assay was based on binding of bovine antibody in serum to a fixed amount of *A. pyogenes* antigen. Anti bovine immunoglobulin conjugated alkaline phosphatase then bound to the bovine antibody. The ELISA was developed by use of a chromogenic, P-nitrophenol phosphate substrate. The intensity of the colour formation was proportional to the amount of the antibody present in the serum.

Procedure. The isolate of *A. pyogenes* used in the infection experiments was used to prepare the antigen. A pure culture of the bacterium (from freeze dried samples) was cultured on horse blood agar, incubated anaerobically and aerobically for 24 and 48 hours respectively. The culture was washed off the

plates using sterile normal saline, and placed in 5 ml bottles (in ice), and then sonicated for about 2 minutes using an MSE 100 watt Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd. London, England). The sonicated antigen was diluted 1 in 10 with coupling (coating) buffer and 100 ul put into each well. The plates were left overnight at room temperature with gentle shaking in a humid environment.

Plates were then washed twice with TBS (200 ul) each time, blocked with 2% dried skimmed milk in TBS (200 ul) and incubated for one hour. The plates were washed twice using TBS (200 ul) and various dilutions of sera in TBS containing 2% skimmed milk powder and 5% Tween 20 (TMT) were added to the wells in volumes of 100 ul and incubated for 1 hour at room temperature.

The plates were then washed with TBS four times and antibovine conjugate in TMT, and 100 ul was added to each well and the plates left for 1 hour. The free conjugate was then washed off the plates with TBS by washing each plate six times and this was followed by developing of the ELISA.

Development of the assay commenced by addition of 50 ul of the substrate (P - nitro - phenol phosphate in AP buffer) to each well. The plates were then left at room temperature for at least 30 minutes until adequate colour formation was observed. Colour development was stopped by the addition of 50 ul of 0.4 M NaOH to each well.

Reading of the results. The amount of bound alkaline phosphatase bovine conjugate was proportional to the concentration of the amount of the antibody to *A. pyogenes* in the sera. The amount of the bound conjugate was shown by reaction of the alkaline phosphatase with the substrate, which reaction was shown by colour formation. The colour changes were measured spectrophotometrically using the ELISA plate reader (Multiskan

Spectrophotometer). The spectrophotometer in the plate reader was set to read at 405 nm and the readings (absorbency) were proportional to the amount of antibody to *A. pyogenes* in the sera.

X. HAPTOGLOBIN.

Being one of the serum or plasma proteins that increase in concentration during the acute phase response to inflammation, haptoglobin was tested for to assess the nature of response of the animals to experimental infection with *A. pyogenes*.

Haptoglobin assay.

The method was used as modification of that of Makimura and Suzuki (1982). The assay depends on the binding of haemoglobin (Hb) to the haptoglobin in the sample, preventing the inactivation of the peroxidase activity of haemoglobin which occurs at acid pH's. The assay was standardised by using a constant concentration of haemoglobin, if all haemoglobin was protected by the binding then the haptoglobin concentration was at least 150 mg haemoglobin binding capacity per 100 ml (mgHbBC/100ml) of plasma.

Reagents for 48 duplicate samples and standards included:-

* Haemoglobin solution:- made up of stock at 3g/ml and working solution diluted by saline (0.9% w/v) to get 30 mg/ml.
* Maximum standard:- a plasma sample with >150mgHbBC/100ml.
* Zero standard:- a plasma sample with negligible haptoglobin.
* Standards:- dilution of maximum standard made with the zero standard.
* Controls:- pools of samples with known high and low haptoglobin levels.
* Chromogen:- stock solution of tetra methyl benzidine (TMB) in DMSO at 6

mg/ml, kept in the dark. The working solution of 0.06 mg/ml was made by dilution of 250 ul to 25 ml in chromogen buffer.

* Chromogen buffer:- 0.5 gm di-NaEDTA, 15.6 gm $NaH_2PO_4.2H_2O$, was dissolved in distilled water and brought to a pH of 3.8 with orthophosphoric acid and made up to one litre with distilled water.

* Substrate:- 12 ul of H_2O_2 in 10 ml of distilled water.

Procedure. A mixture of 20 ul of standard, control and sample was made with 100 ul of haemoglobin solution in glass test tubes and mixed using dilutrend and left for 10 minutes. Five ml of saline was added and mixed using the dilutrend and left for another 10 minutes. Twenty ul of chromogen solution was placed in microtitre wells and to this was added 200 ul of the diluted samples. The wells were incubated for 60 minutes before 50 ul of the substrate was added. When the colour developed (5-10 minutes) 50 ul of 2M H_2SO_4 was added.

Computation of results.

The results were worked out using the ELISA reader Flow at 450 nm. A standard curve was drawn by the computer linked to the ELISA reader and the amounts of Hp in the test sample was automatically worked out from the curve.

XI PREGNANCY SPECIFIC PROTEIN.

1. PREGNANCY SPECIFIC PROTEIN (PSPB) ASSAY.

Analysis for PSP-B was kindly done by Professor R. G. Sasser (Moscow, U. S. A). Samples were lyophilised for inter-laboratory transfer and reconstituted on arrival in U.S.A.. The assay was also validated by him and others (Sasser et al., 1986). The results from this study are described in Chapter VII.

CHAPTER III.

EXPERIMENTAL REPRODUCTION OF ABORTION IN CATTLE BY INTRAUTERINE INOCULATION WITH *ACTINOMYCES PYOGENES*.

INTRODUCTION.

The studies described in this chapter represent a single controlled experiment in which 8 pregnant cows were inoculated with pure cultures of *A. pyogenes* into the pregnant uterus to determine its effects. Two cows formed a saline control group and 6 cows were given cloprostenol, a prostaglandin F2α analogue as a further control. This experiment forms the basis of the thesis and provides material for clinical ultrasound and bacteriological studies which are presented in this chapter. Hormonal and Pregnancy Specific Protein B (PSPB) results are presented in later Chapters (VI and VII).

MATERIALS AND METHODS.

The cows used were described in Chapter II as were the conditions under which they were maintained. A total of 16 animals were used in this study. All were served by Artificial Insemination (A.I.) and pregnancy was confirmed clinically by rectal palpation and ultrasound prior to inoculation. Animals were divided into three groups and treated as follows (**Table 2**) on the days of pregnancy shown. Inoculation was carried out as described in Chapter II.

Gross morphological changes in the pregnant uterus were monitored by rectal palpation and use of a real time B - mode two dimensional scanner

TABLE 2. Summary of the treatment groups of animals used in the experiment.

Number of cattle	Days of pregnancy	Inoculum or treatment
Group A		
8	35 - 44	*Actinomyces pyogenes*
Group B		
2	27 - 34	Sterile saline
Group C		
6	40 - 50	Cloprostenol

with a 7.5 mHz transrectal linear transducer (Chapter II) at 4-12 hourly intervals from the time of experimental treatment and the findings were recorded.

Blood samples were taken for plasma hormonal and PSPB assay and clotted blood samples for the detection of serum antibodies at the intervals shown in Chapters IV and VI using the methods described in Chapter II. They were stored at -20^{o}C for analysis. Findings of the antibody production to infection and the acute phase protein - haptoglobin, are given in Chapter IV. Results from the hormonal studies are described in Chapter VI while those on PSPB are described in Chapter VII.

Vaginal swabs were taken and the products of abortion were examined for the presence of *A. pyogenes* and other bacteria by the methods described in Chapter II. Biochemical tests for the confirmation of the presence of *A. pyogenes* on isolates were carried out according to the methods of Carter et al. (1979).

Observations were carried out for up to 36 days after abortion, but only those made up in the first 8 days following abortion are reported here. The remaining observations are reported in Chapters V and VI.

RESULTS.

EXPERIMENT I.

1. CONTROLLED *A. PYOGENES* INFECTION.

a) Live bacterial infection.

Abortion occurred in 6 out of the 8 inoculated animals after intrauterine inoculation of living *A. pyogenes* (**Table 3**). Two cows failed to abort after

TABLE 3. Results of the controlled study of intrauterine inoculation with *Actinomyces pyogenes* suspensions.

COW NO	Stages of pregnancy at infection or treatment (days)	Inoculation or treatment	Time after inoculation or treatment (hours)	
			Embryonic death	Abortion
GROUP A: *Actinomyces pyogenes.*				
1	35	$2.65x10^{10}$	22	29
2	30	$1.15x10^{10}$	96	103
3	34	$2.1x10^{10}$	11	103
4	41	$7.5x10^{9}$	96	144
5 (Heifer)	33	$1.6x10^{10}$	72	86
6	44	$2.1x10^{9}$	48	96
21	40	$9.2x10^{9}$	None	
22	26	$4 x10^{9}$	None	
GROUP B: Sterile saline.				
7	27	6ml Sterile saline	None	
8	34	6ml Sterile saline	None	
GROUP C: Cloprostenol (500ug)				
9	40	cloprostenol	51	66
10	45	cloprostenol	72	78
11	50	cloprostenol	57	71
12	50	cloprostenol	72	76
13	50	cloprostenol	57	68
14	50	cloprostenol	60	72

bacterial inoculation. The time from bacterial infection to collection of the embryo or embryonic material ranged between 29 to 144 hours and is shown in **Table 3.** One of the 6 animals that aborted after bacterial infection was observed only until the time of abortion, but the other 5 animals were observed for at least 8 days after abortion. Embryos were collected with their membranes from 3 of the infected animals whereas in the other 3, pus and parts of the macerated embryo were collected. The three embryos collected with intact membranes from infected cows had cloudy membranes and were covered with pus and had shrunken blood vessels. It was difficult to distinguish the different parts of the embryo, because of haemorrhage over much of the embryo especially around the thoracic and abdominal regions (**Fig. 7 & 8**). Abortion was followed by the appearance of a creamy white mucopurulent vulval discharge (**Fig 9**). The discharge was not always evident and varied in amount from traces to 5 - 10 ml. The discharge was seen on the vulval lips, on the tail or on the floor behind the animal and its production continued for at least the next 8 days.

The animals remained alert and ate and drunk normally. No straining was noted during the period of observation and no other clinical abnormalities were seen. Rectal temperatures were generally raised after inoculation (**Table 4**). They declined after abortion to about 38.5^{o}C but in one of the 6 aborting animals (Heifer 5), the rectal temperature increased from 38.5^{o}C before infection to 40.3^{o}C during embryonic death and fell to 38.5^{o}C down after abortion.

On rectal palpation, the corpus luteum was felt in all cases and remained the same size (about 2.5 cm^{2}) throughout the period of observation. No signs of heat were seen for at least 8 days after abortion.

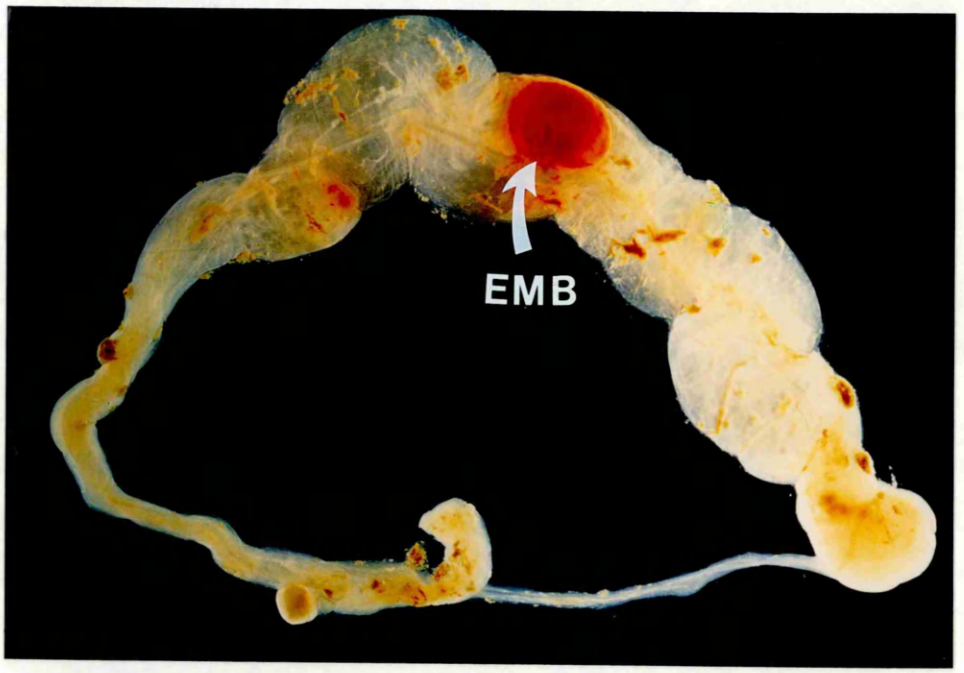


FIG. 7: Decomposing embryo (EMB) of Cow 2 after abortion due to *A. pyogenes* infection. Note haemorrhage of the organs of the embryo.

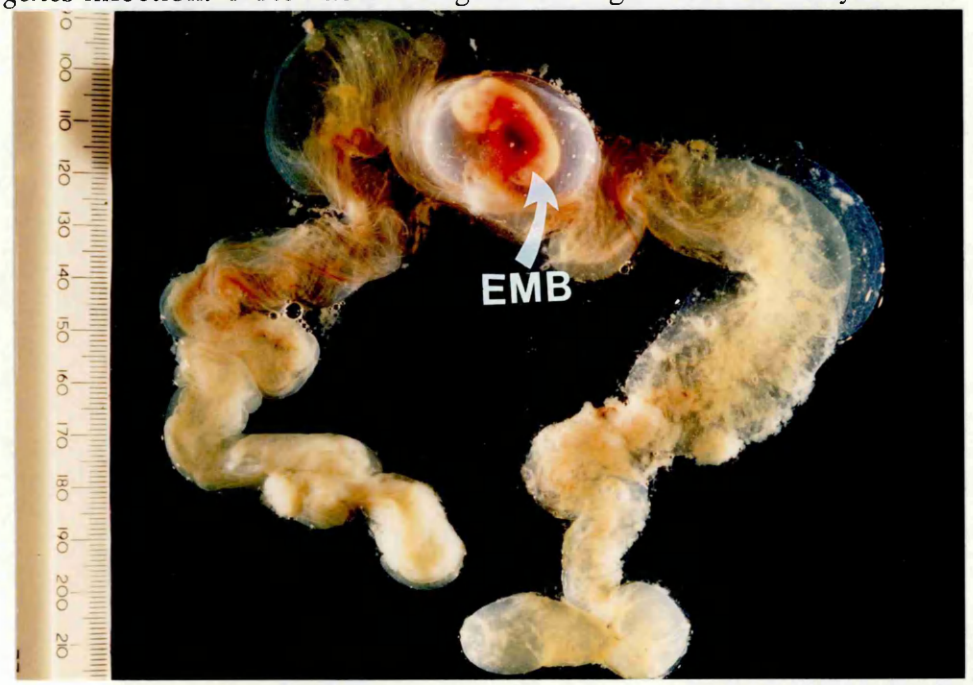


FIG. 8: Decomposing embryo (EMB) of Heifer 5 after abortion resulting from *A. pyogenes* infection. Note the cloudy membranes covered with pus.

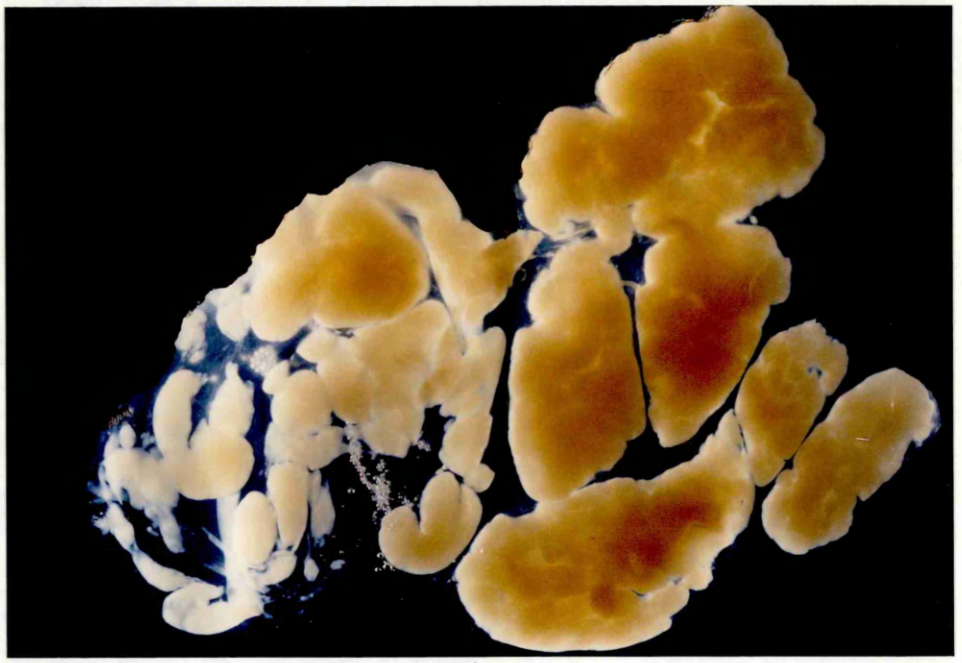
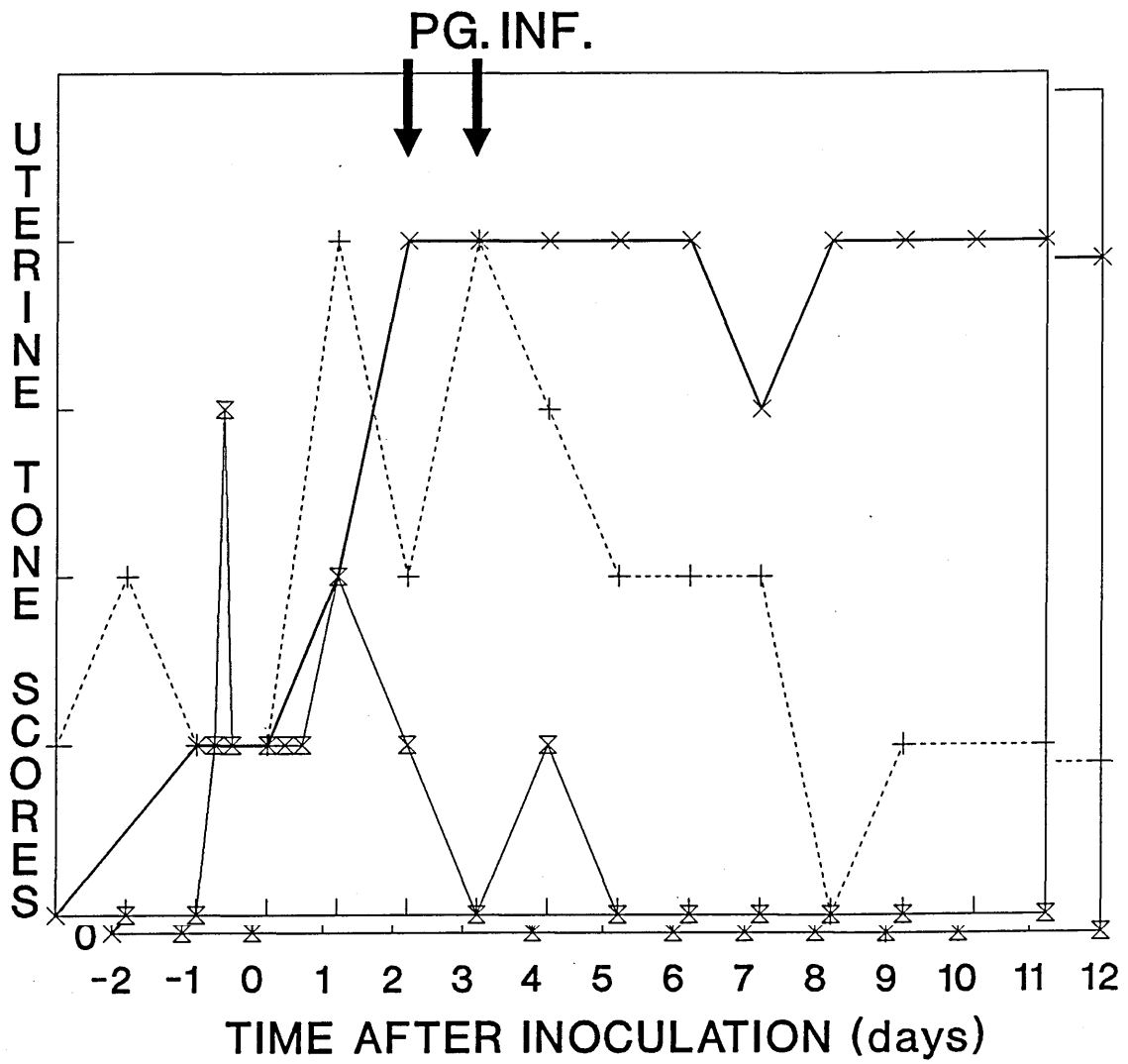


FIG. 9: Mucopurulent discharge after experimental infection. The discharge is creamy in appearance and there is no blood.

TABLE 4.
Rectal temperatures recorded from the animals in the study.

TREATMENT GROUP	TEMPERATURE RANGE (^{O}C)	
	Treatment to abortion	Abortion to 8 days later
A.Live bacteria	38.5-39.4	38.5-39.0
B.Sterile saline	38.4-38.8	Not available
C.Cloprostenol	38.4-38.7	38.4-38.8

FIG. 10: Specimen record of uterine tone for animals inoculated with bacteria, sterile saline and cloprostenol treatment. Oestrus occurred 24 hours after abortion in cloprostenol induced abortion (Cow 12) whereas in infection (Cow 3) the animal had not come to oestrus 8 days after infection.



Before infection the tone of the uterus was uniformly flaccid (score 0) but soon after infection (4-8 hours) it became moderate (score 2) and by 24 hours it was scored as being moderate/tonic (score 3). Increased uterine tone was observed in all 6 animals between infection and abortion. Maximum uterine tone (score 4) was reached 72 hours after infection and remained high for the period of observation (**Fig. 10**). Increased tone appeared to be uniform over the whole uterine horns and body. The uterine horn which had contained the embryo remained the larger of the two horns by 1 - 2 cm during the period described in this chapter.

No change in the cervical size or tone was detected throughout the period of investigation. Vaginoscopy revealed changes in the texture and appearance of the external os of the cervix and the vagina. The cervix was partially open when first observed by the vaginoscope between infection and abortion. It was fully open and remained open after abortion. Mild inflammation of the cervix was seen prior to abortion and this became more prominent during abortion. It remained inflamed following abortion. A small amount of pus was seen in the external os between infection and abortion in all the 6 cows and more pus was seen at the time of abortion and subsequent to it. The cranial part of the vagina contained small amounts of pus prior to abortion.

The vaginal wall was congested and appeared pink after the opening of the cervix and expulsion of the embryo. Pus and mucus could be seen on the luminal floor of the vagina in all 6 animals and varied in amounts from very small quantities a few hours after infection to large amounts of up to 15 - 20 ml at the first observation after abortion followed by periodic discharges of quantities of 5 - 10 ml during the 8 days after abortion. The frequency of events observed with vaginoscopy of the cervix and the vagina after infection are described for one representative animal (cow 3) in **Table 5**.

TABLE 5: Specimen record of observations in the external os of the cervix and vagina with the use of the vaginoscope in Cow 3.

Time after infection (hours)	12	48	96	120	144	168
Events in:-						
1. Cervix						
Openness	ND	++	+++	++	ND	++
Inflammation (congestion)	ND	+	++	++	ND	++
Presence of pus	ND	+	++	++	ND	++
2. Vagina						
Inflammation (congestion)	ND	+	++	+	ND	++
Presence of pus	ND	+	++	+	ND	++

b) Sterile saline inoculation.

In the two animals that were inoculated with sterile saline, pregnancy did not seem to be affected by the treatment as pregnancy was maintained up to 50 days after service (**Table 2**). Rectal temperatures varied between 38.4°C and 38.8°C from the time of inoculation to the end of the experiment and there was no consistent increase in the uterine tone although a transient rise was noted in the pregnant horn (**Fig. 10**). No clinical abnormalities were detected and pregnancy was terminated by the injection of cloprostenol 16 - 23 days after inoculation at 50 days of gestation.

c) Cloprostenol treatment.

Treatment with cloprostenol was followed 24-48 hours later by regression of the corpus luteum and an increase of the uterine tone to score 4 (**Fig. 10**). There was rapid growth of follicles with one being most prominent in either the left or right ovary. Abortion occurred 66 - 78 hours later (**Table3**) and embryos were collected from the vagina along with clear or slightly cloudy mucus using the vaginoscope. All embryos aborted following cloprostenol treatment appeared normal and well defined with clear membranes (**Figs 11 and 12**) and were accompanied by clear or cloudy mucus. The blood vessels in the membranes were prominent. Animals came back into heat 14$\pm$S.D. 6.5 hours after abortion. The cervix was seen to be open 6-12 hours before abortion using the vaginoscope and allantoic membranes could be seen protruding through the open cervix. The vaginal lumen contained mucus both before and after abortion and the vaginal mucus always had some degree of hyperaemia or congestion. The uterine tone decreased following the first oestrus (12-24 hours after abortion).

Maternal temperatures ranged between 38.4°C and 38.8°C from the time of treatment to abortion and remained within the same range between abortion

FIG. 11: Aborted embryo (EMB) of Cow 15 after cloprostenol treatment. The embryo appears normal with no internal haemorrhage as in the embryo after bacterial induced abortion.

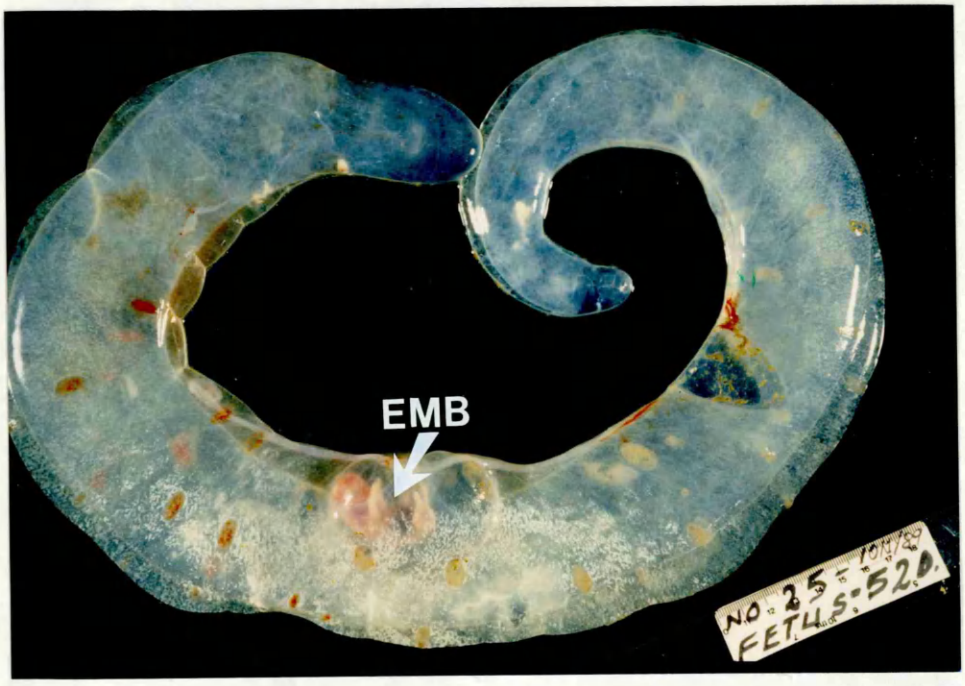


FIG. 12: Aborted embryo (EMB) of Cow 12 after cloprostenol treatment. The allantochorion is clear and there is no pus on its surface

and 8 days later (**Table 4**).

BACTERIOLOGICAL IDENTIFICATION.

A pyogenes was isolated from only one of the 8 animals before experimental bacterial inoculation (Heifer 5). The organism was isolated on blood agar as 0.5 - 1 mm grey-white colonies with B-haemolysis (**FIGS 13 and 14**) and was identified on chocolate agar from the six animals which aborted after the infection. One infected animal yielded streptococcus, staphylococcus and *E. coli* before injection but these could not be detected afterwards.

Streptococci, staphylococci and *E. coli* were isolated before inoculation from the animals given sterile saline. No other specimen were taken from the animals during the experiment since sterile saline did not seem to effect pregnancy but samples taken at the end of the study (50 days) were still positive for all the organisms and negative for *A. pyogenes*. Streptococci, staphylococci and *E. coli* were also identified before and or after cloprostenol treatment and abortion, but the numbers isolated were small and insignificant. The results are given in **Table III. 6.**

A. pyogenes was isolated in profuse culture after embryonic death and abortion from the aborted material of group A animals. Gram-positive rods and cocci resembling it were seen in large numbers with polymorphs (**FIGS 15 and 16**) in smears from the vagina and from aborted embryonic material. The gram positive rods isolated from Heifer 5 before bacterial inoculation were confirmed to be *A. pyogenes* using the biochemical criteria of Carter (1979) (Chapter II). Similar tests on the gram positive bacteria identified from the 6 animals that aborted after bacterial inoculation confirmed them as *A. pyogenes*.

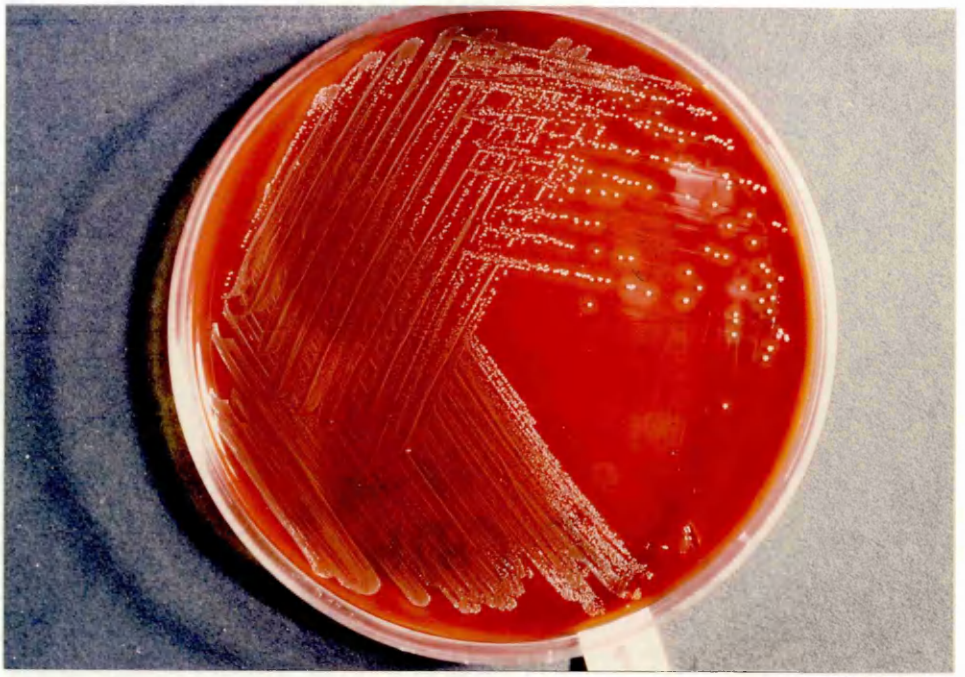


FIG. 13: Profuse culture of *A. pyogenes* on 7% horse blood agar from vaginal discharge following infection and abortion demonstrating gray - white colonies.

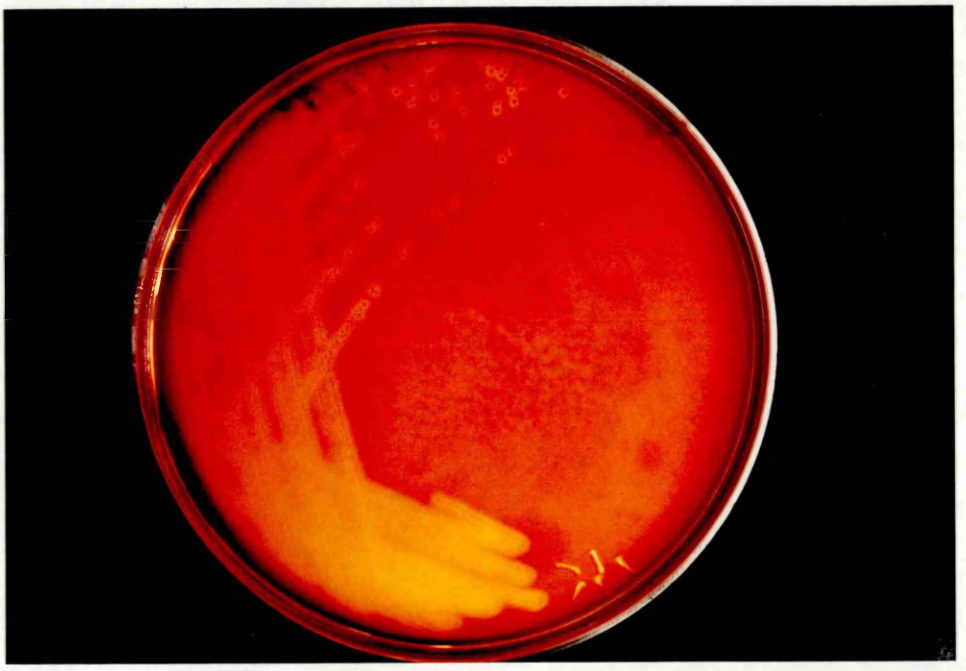


FIG. 14: Profuse culture of *A. pyogenes* on 7% horse blood agar from discharge after *A. pyogenes* induced abortion demonstrating ß - haemolytic colonies.

TABLE 6. Bacterial isolates obtained from vaginal samples during the study (i) before and (ii) after inoculation.

COW NO	*A.pyogenes* (i)(ii)	Streptococci (i)(ii)	Staphylococci (i)(ii)	*E.coli* (i)(ii)
Group A. Live bacteria				
1	-/++	+/-	-/-	+/-
2	-/++	-/-	-/-	-/-
3	-/++	-/-	-/-	-/-
4	-/++	-/-	-/-	-/-
5	+/++	+/-	-/-	+/-
6	-/++	+/-	+/-	+/-
Group B. Sterile saline				
7	-/-	+/+	-/-	+/+
8	-/-	+/+	+/+	+/+
Group C) Cloprostenol analogue				
9	ND	ND	ND	ND
10	ND	ND	ND	ND
11	-/-	+/+	-/+	+/+
12	-/-	+/+	+/+	+/-
13	-/-	-/+	-/+	-/-
14	-/-	-/+	-/+	-/-

(+) = presence of bacteria

(-) = absence of bacteria

ND = examination not done.

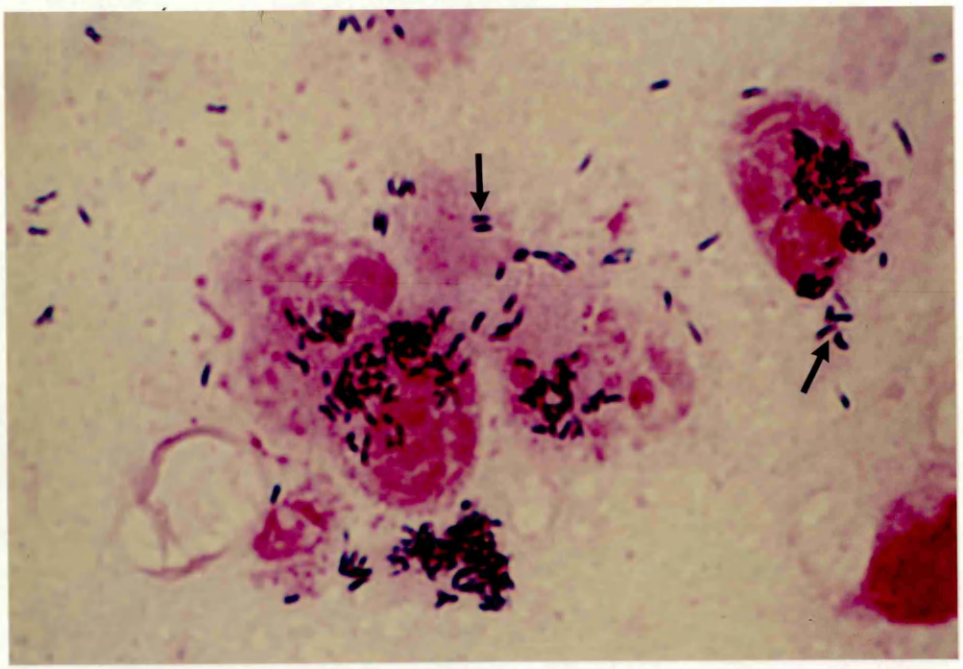


FIG. 15: Direct smears from products of abortion showing Gram positive rods and cocci (arrows) which are characteristic of *A. pyogenes* x1000.

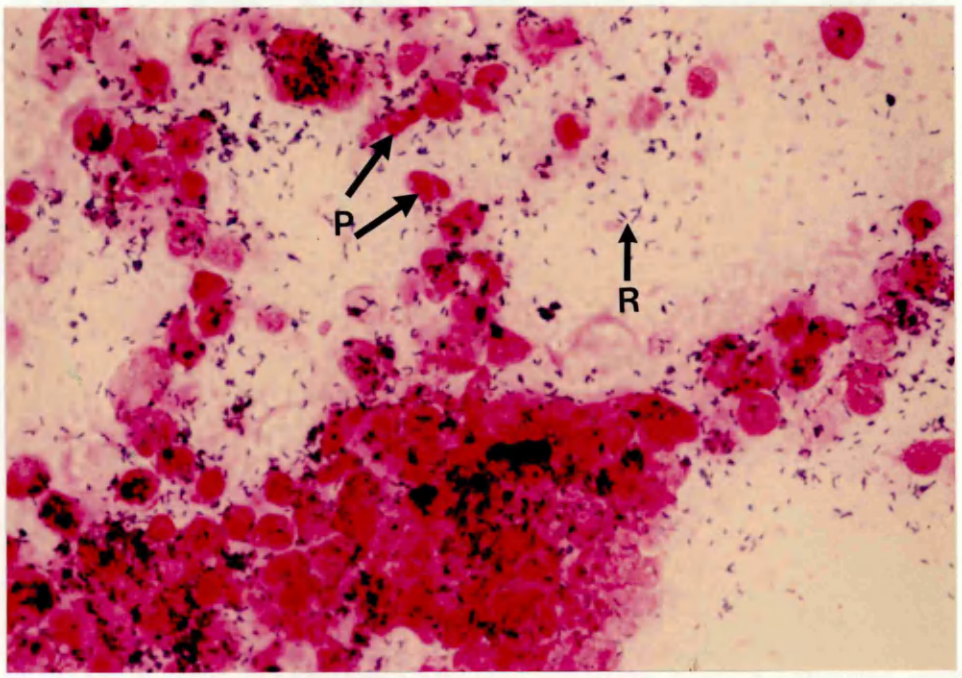


FIG. 16: Direct smears from products of abortion showing polymorphs (P) indicating uterine inflammation and Gram positive rods (R) x400.

Fusobacteria and other anaerobes and *Haemophilus somnus* were not isolated from any cow in the study.

2. ULTRASOUND.

Events leading to embryonic death and abortion in the six animals after live bacterial introduction and in the controls were recorded and are described bellow.

Prior to inoculation all the animals were confirmed to be pregnant at least 3 or 4 times by rectal palpation and by the use of ultrasound. The examples of ultrasonic observations presented here are from one animal (Cow No. 2) representing all 6 infected animals. The morphological changes described below were evident as early as 4 hours after infection and the following observations were made consistently. The *corpus luteum* was maintained between 2.1cm^2 and 2.9cm^2 from before infection (**FIG. 17 A1**), through infection, embryonic death (**FIG 18 B1 and 19 C1**) and abortion (**FIG. 20 D1**). In the *pregnant horn* thickening and separation of the allantochorion from the endometrium was seen initially in the cranial part of the horn (**FIG. 18 B2**) and membranes could be seen floating in the uterine lumen. The uterine fluid which was clear (dark and non-echogenic; **FIG. 18 B2**) started to turn cloudy (gray-moderately echogenic; **FIG. 19 C2**) from the time of infection onwards. Endometrial thickening was evident soon after infection but more so after 24 hours (**FIG. 20 D2**). This was maintained through the time of embryonic death and abortion (**FIG. 21**). The embryo was considered to be alive as long as the heart beat and other embryonic movements were still visible. Both of these stopped with embryonic death. Pending abortion was indicated by complete detachment of the membranes along most parts of the reproductive tract (**FIG. 18 B2**). Similar changes

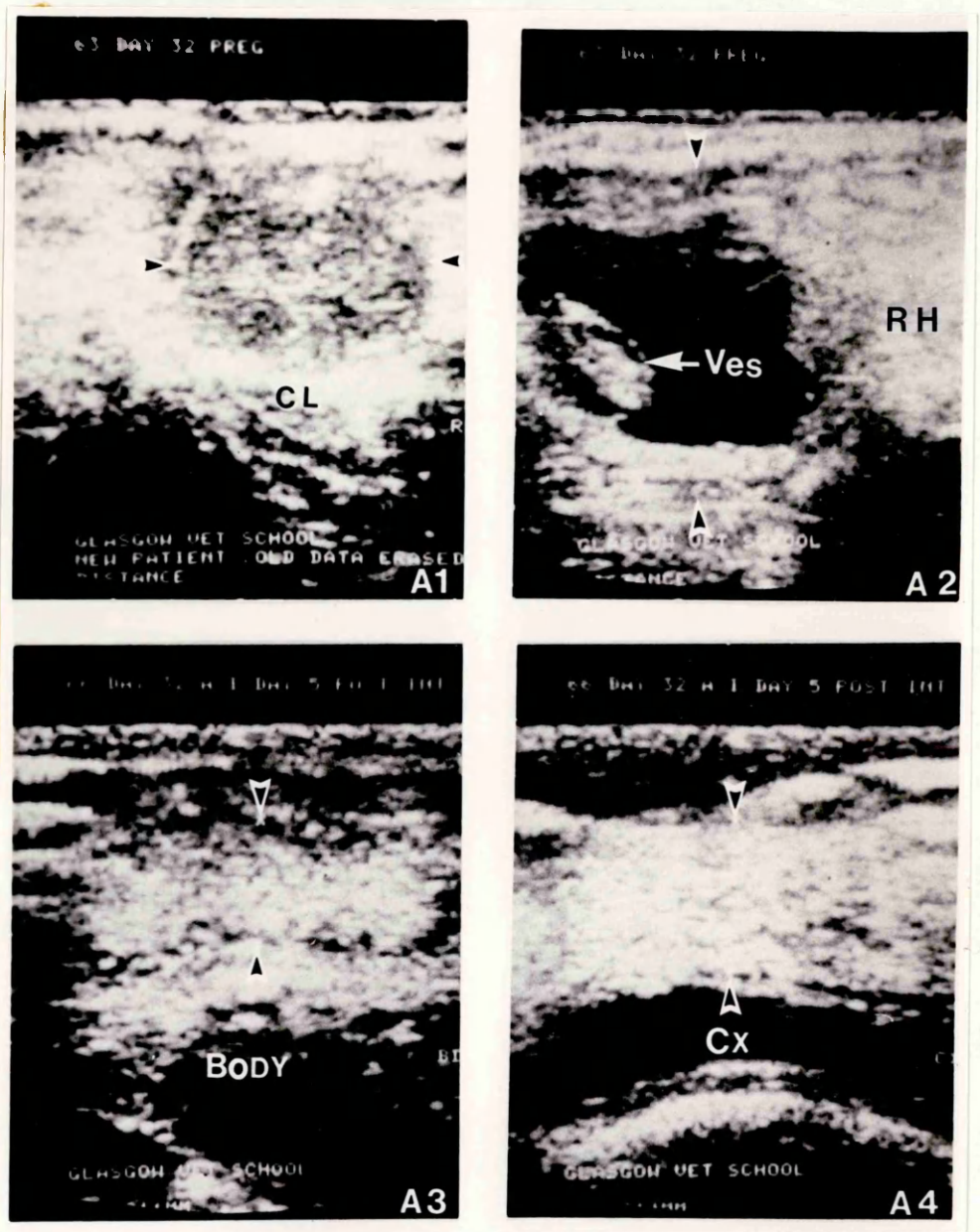


FIG. 17 (A1 - A5): Ultrasonographic appearance of the reproductive tract of Cow 1 on the 32nd day of a normal 32 day pregnancy. **A1** showing the corpus luteum (CL) = 2.3 cm^2 (arrows); **A2** pregnant horn (RH) (arrows) with embryonic vesicle (ves); **A3** body of the uterus (arrows); **A4** cervix (CX) (arrows) which is closed and;

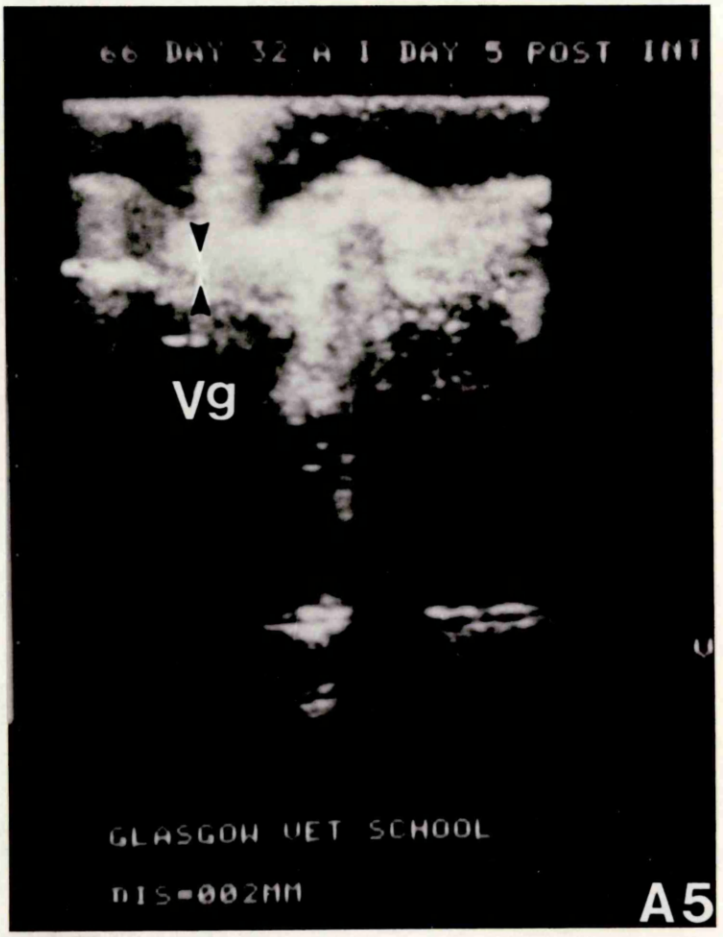


FIG. 17 A5 vagina (Vg) which is relaxed and the vaginal walls closely apposed (arrows).

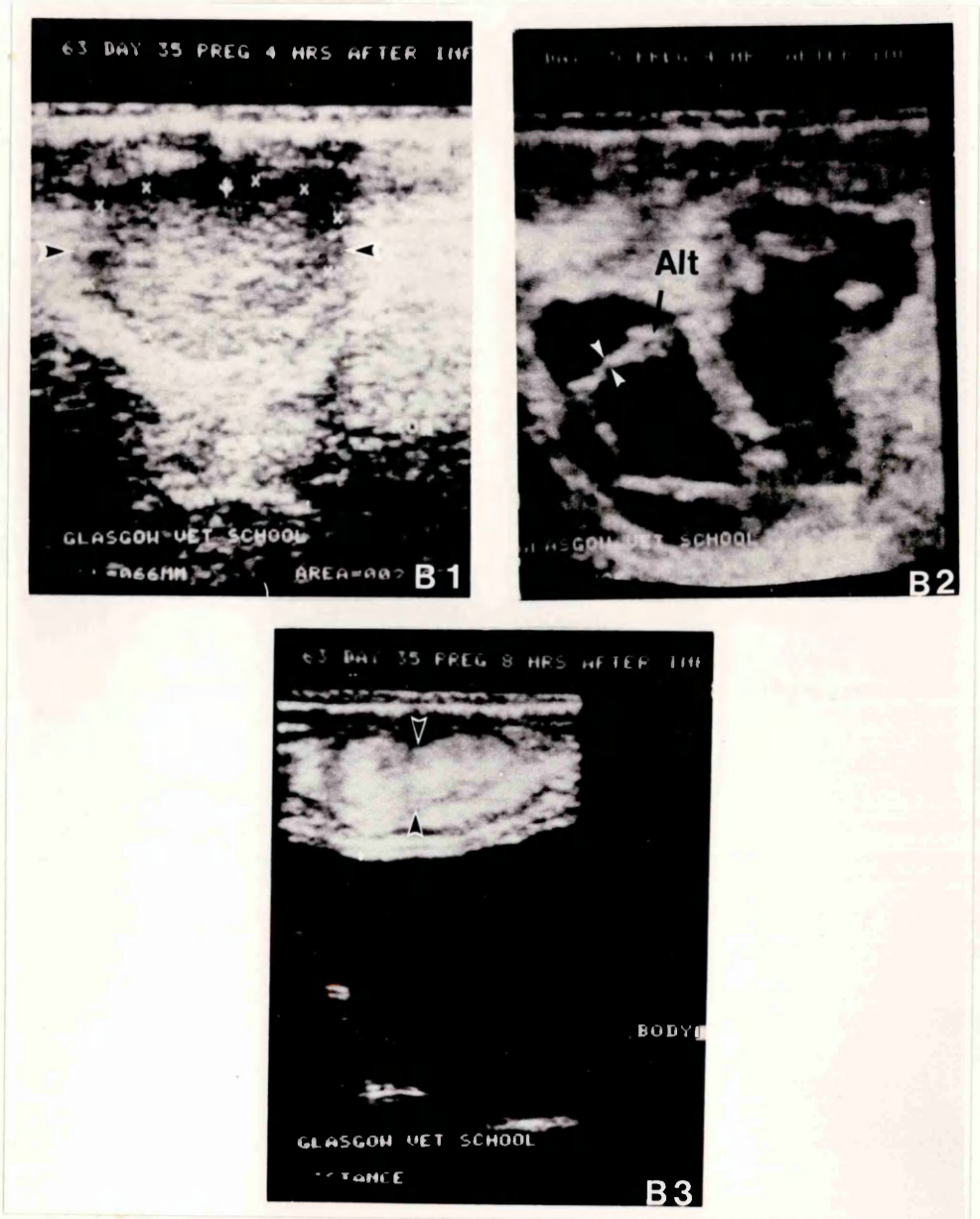


FIG. 18 (B1 - B3): The 35 day pregnant horn of Cow 1, 4 - 8 hours after infection with *A. pyogenes*. **B1** CL maintained at 2.9 cm^2 (arrows); **B2** shows thickening (arrows) and separation of allantochorion (Alt) from the endometrium, uterine fluid clear and **B3** body (arrows) shows no change from the normal state.

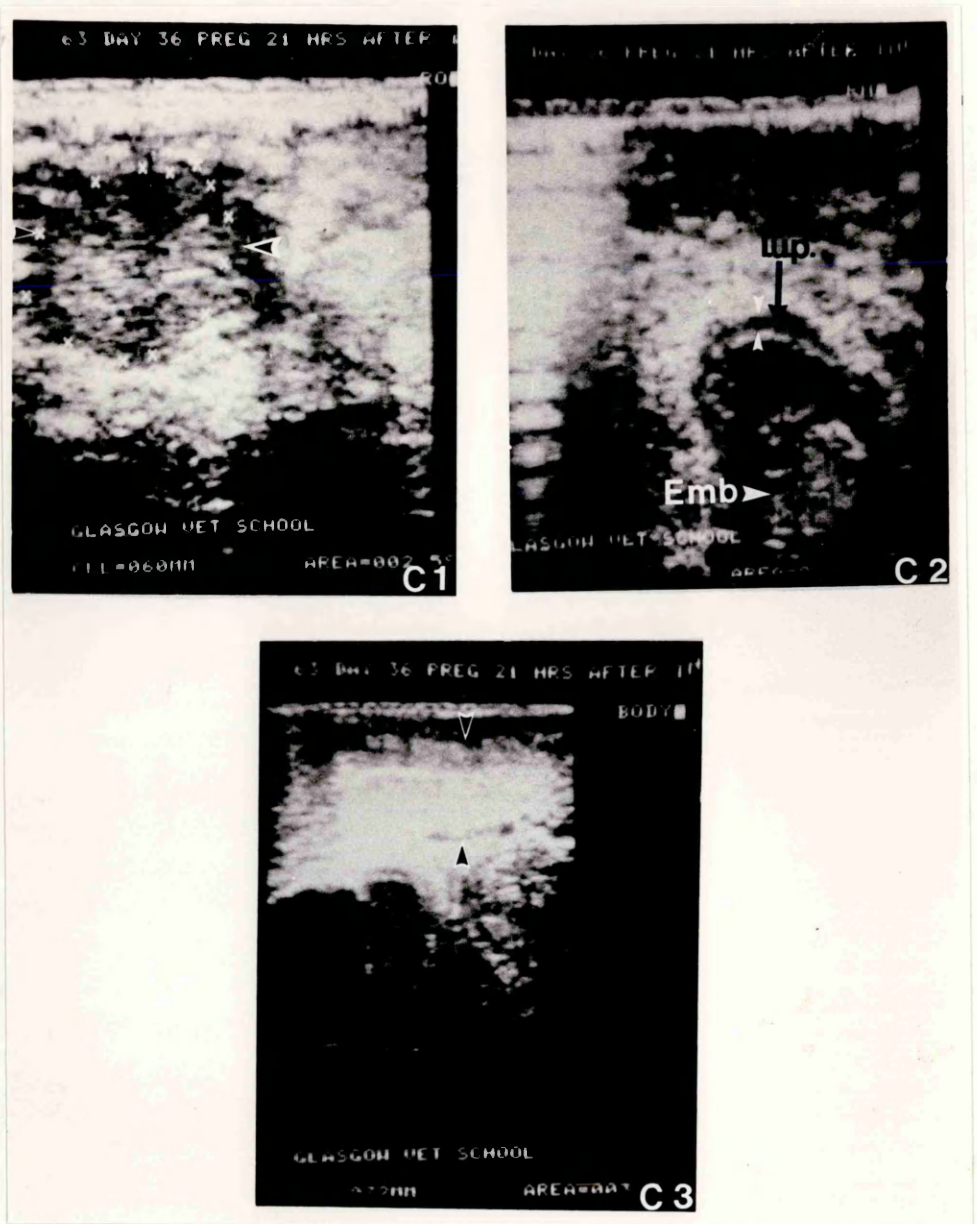


FIG. 19 (C1 - C3): Ultrasonographic findings in Cow 1 on the 36th day of pregnancy, 21 hours after infection. **C1** CL = 2.5 cm^2 (arrows); **C2** RH intra-uterine pus (i. u. p) beginning to form between allantochorion and the endometrium (arrows), embryo (Emb) still viable; **C3** body (arrows) and rest of the tract normal.

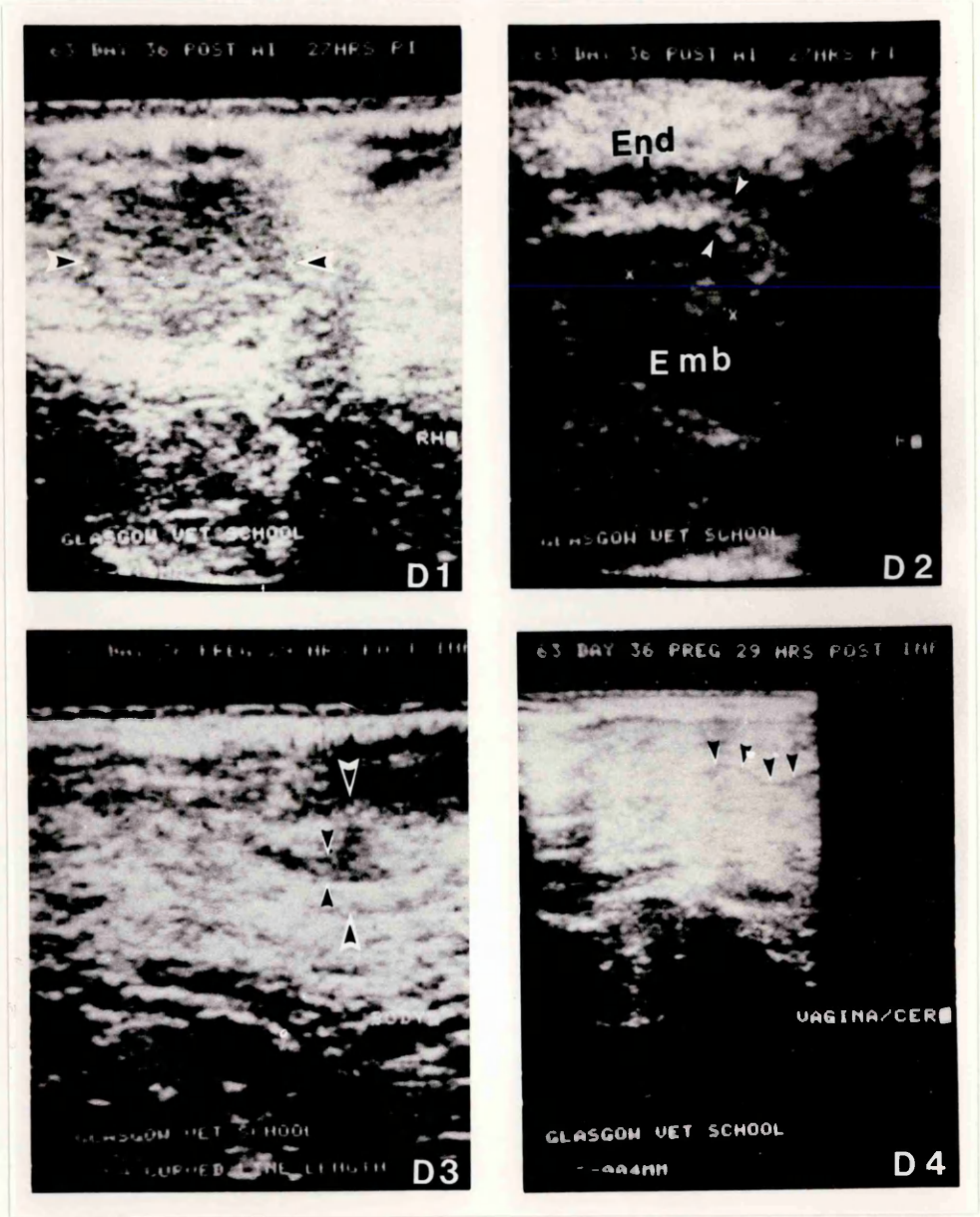


FIG. 20 (D1 - D5): Ultrasonographic findings in Cow 1 on the 36th day of pregnancy, 27 - 29 hours after infection. **D1** CL = 2.1 cm^2 (arrows); **D2** RH with marked thickening and oedema (arrows) of the endometrium (End) and a dead embryo (Emb); **D3** uterine body (arrows) starting to fill with cloudy fluid (possibly pus and slightly echogenic); **D4** cervix starting to open (arrows) = 1.5 mm;

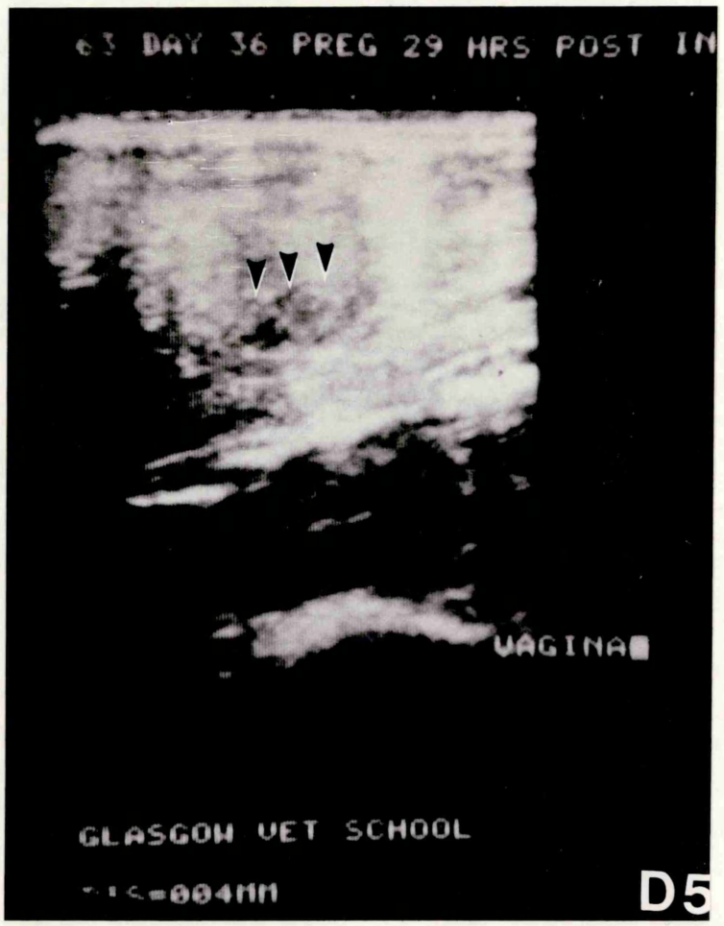
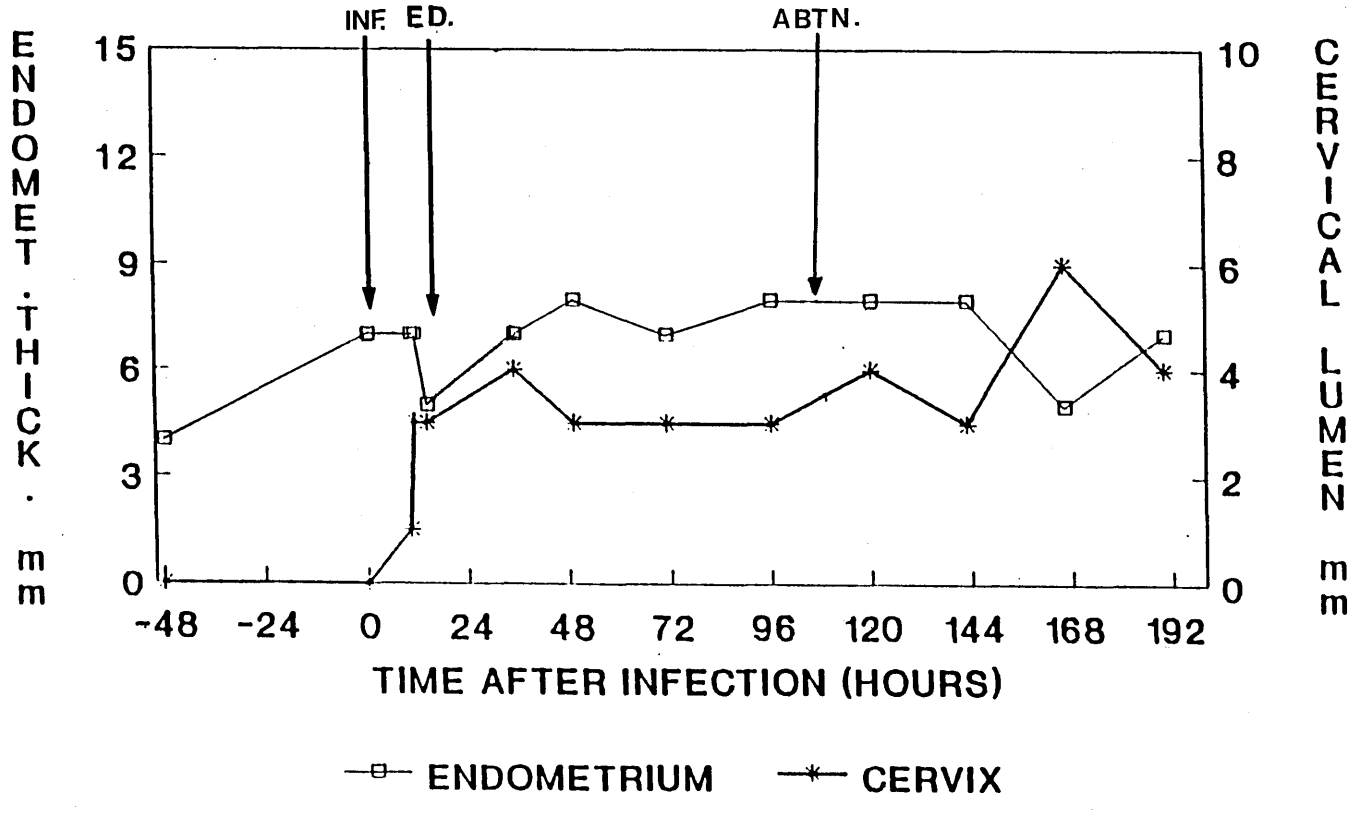


FIG. 20 D5 vagina beginning to open (arrows) = 2 mm. Abortion occurred 29 hours after bacterial inoculation.

FIG. 21 Graph to show ultrasonic changes of the endometrial thickness (mm) and cervical lumen (mm) 48 hours before infection throughout infection, embryonic death and abortion and for 8 days after abortion in Cow 3. INF = infection, ED = Embryonic death and ABTN = Abortion.



were observed in the contralateral non-pregnant horn but these occurred some 24-48 hours later.

The *body* of the uterus appeared empty and the walls were closely apposed before infection (**FIG. 17 A3**), but it began to fill with cloudy echogenic fluid 24 - 48 hours after infection, and was accompanied by moderate thickening of the endometrium (**FIG. 20 D3**). In the *cervix*, the folds of the closed cervix could be seen before infection (**FIG. 17 A4**) and up to 24 - 48 hours after infection, when it gradually begun to open and fill with cloudy echogenic fluid (**FIG. 20 D4**). The *vagina* remained empty and relaxed (**FIG. 17 A5**) as long as the cervix was closed. When the cervix opened the vagina begun to fill with cloudy fluid which increased in amount 16 hours after abortion (**FIG. 22 E5**).

Embryonic death occurred 22 hours after infection and abortion followed 7 hours later. Sixteen hours after abortion the corpus luteum (2.9 cm^2) was still present and cloudy fluid could be seen throughout the uterine horns, body of the uterus, cervix and the vagina (**FIGS 22 E1 - 5**). The gross pathological changes were still evident 6 days after abortion (**Figs 23 F1 - 5**). The changes observed through the reproductive tract were maintained for at least 8 days after abortion as shown in **FIG. 21**.

One day before inoculation with sterile saline a normal 26 day pregnancy (**FIG. 24 G1 - 4**) was seen with a distinct corpus luteum and a live embryo. Sterile saline did not affect pregnancy (**FIG. 25 H1 - 5**) and embryonic growth was seen until 40 days after service (**FIG. 26 I1 - 4**) and continued until 50 days after service when cloprostenol was given to cause luteolysis and abortion.

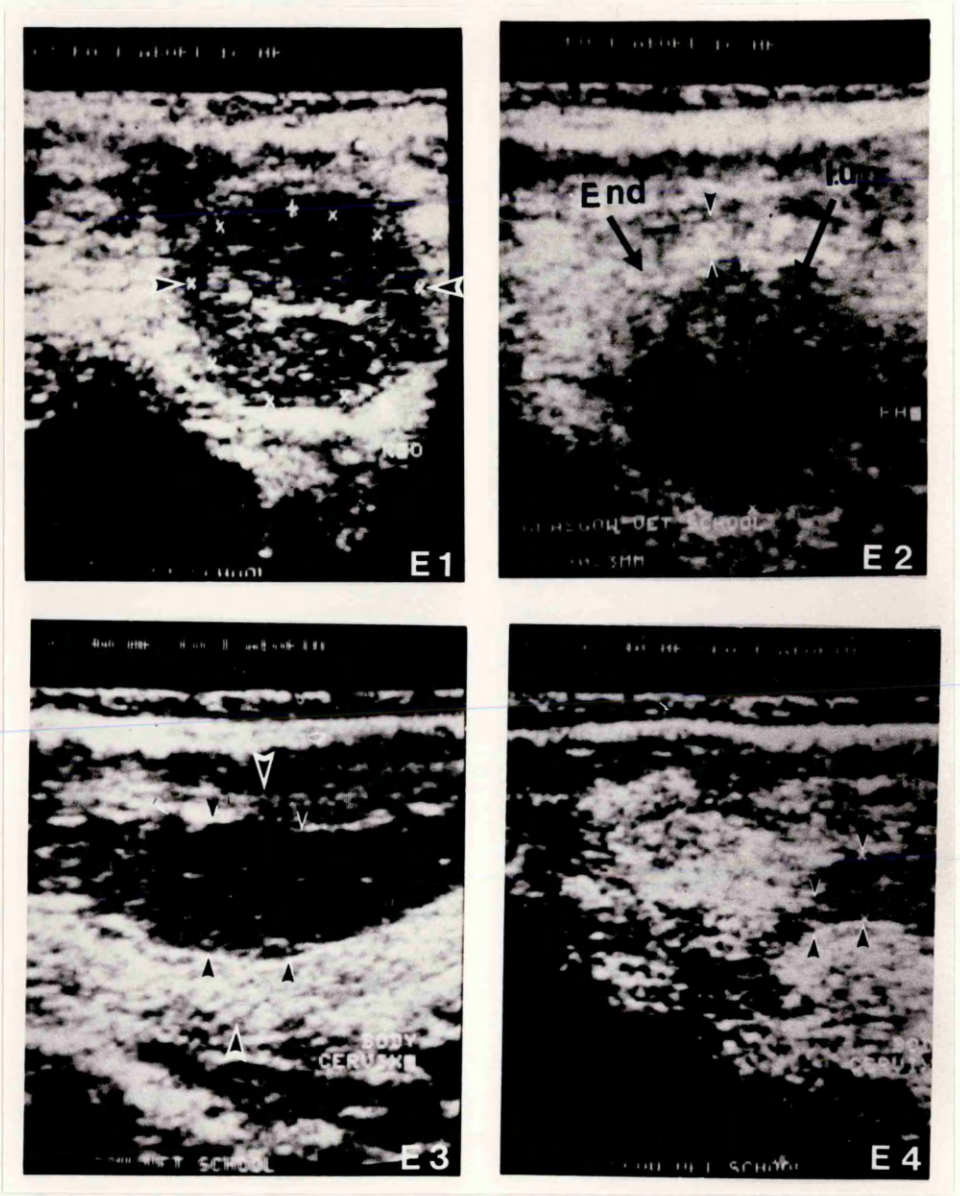


FIG. 22 (E1 - E5): Reproductive tract of Cow 1, 16 - 40 hours after abortion. **E1** CL = 2.9 cm^2 (arrows); **E2** RH lumen filled with pus (i. u. p) and endometrium (End) (arrows) thickened and oedematous; **E3** body of the uterus markedly filled with cloudy fluid; **E4** cervix open = 6 mm (arrows) and filled with pus;

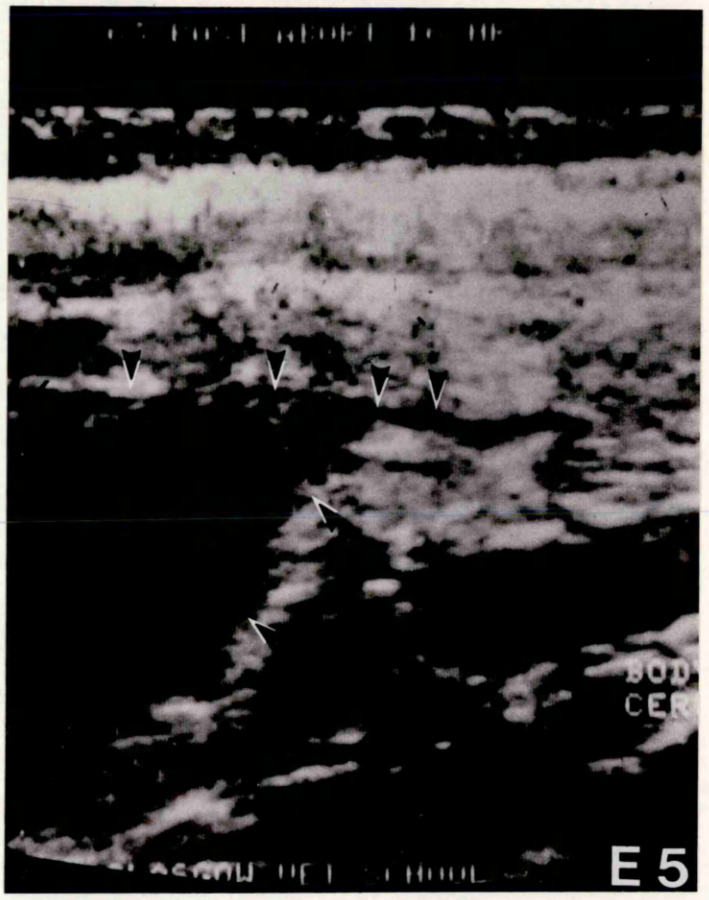


FIG. 22 E5 vagina filled with muco - pus (arrows).

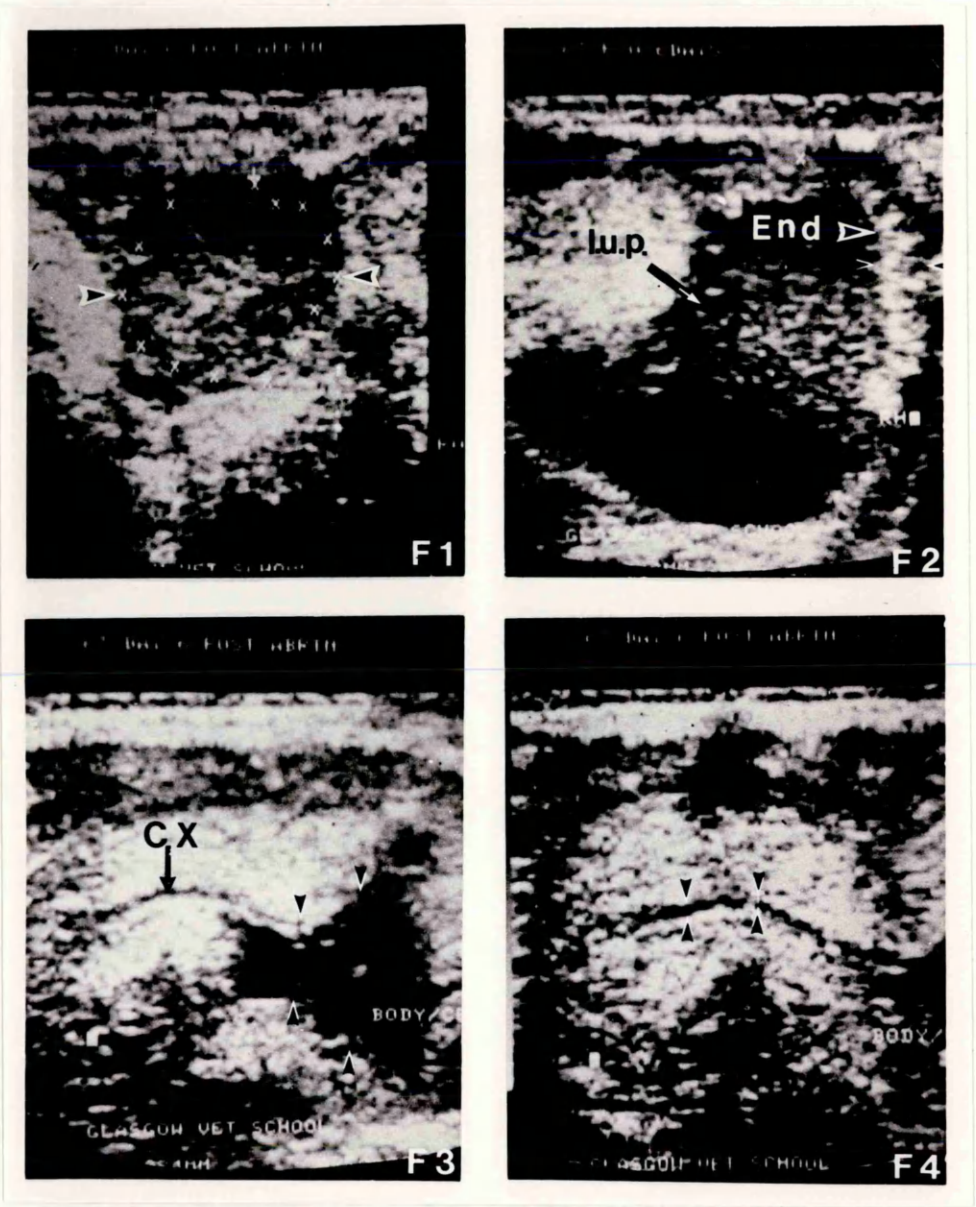
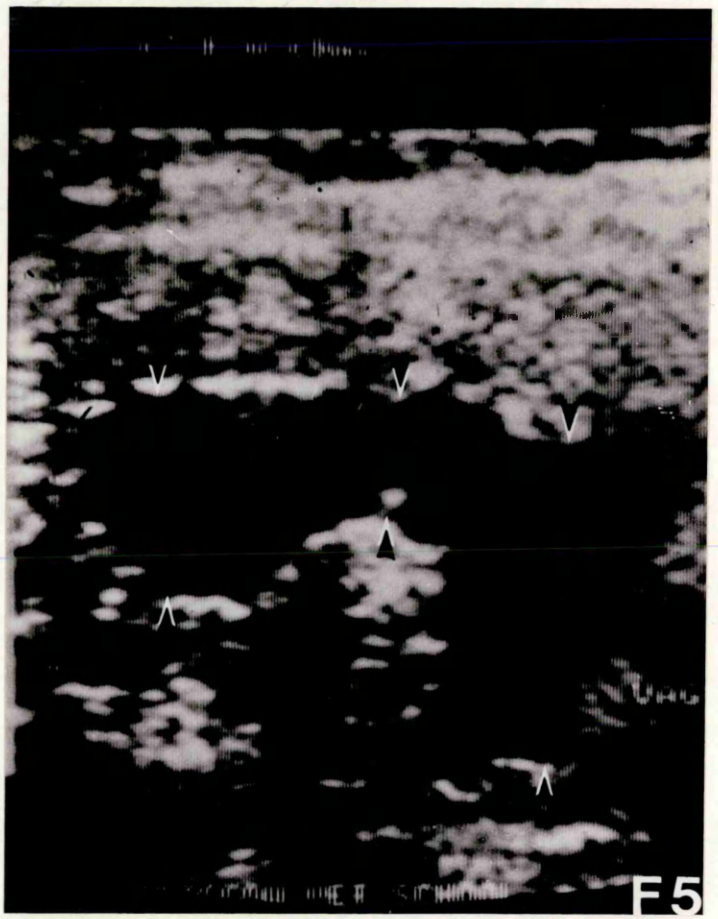


FIG. 23 (F1 - F5): Reproductive tract of Cow 1, 6 days after abortion. **F1** CL = 2.7 cm^2 (arrows); **F2** RH with a thick endometrium (End) (arrows) and pus (i. u. p), **F3** body of the uterus and **F4** cervix (CX) filled with cloudy - echogenic fluid (arrows);



and **FIG. 23 F5** vagina filled with echogenic fluid (arrows).

In the group treated with cloprostenol (**FIG. 27 J1 - J6**), embryonic death and abortion occurred with minimal thickening and separation of the allantochorion from the endometrium and the presence of clear (non-echogenic) fluid in the uterine horns. These uterine changes were accompanied by regression of the corpus luteum from 2.3 cm^2 at treatment to 0.8 cm^2 48 hours after treatment. The cervix was open (2 mm) and the vagina was filled with clear non-echogenic fluid (**FIG. 28 K1 - 7**). Abortion occurred 72 hours after the treatment. Twenty four hour after abortion there was further regression of the corpus luteum to 0.4 cm^2, marked thickening of the endometrium of the uterine horns (14 mm) and body of the uterus (7 mm) with non echogenic fluid seen in the cervix and vagina (**FIG 29 L1 - L5**)

DISCUSSION.

The clinical signs and bacteriological observations suggested that abortion was caused by the live *A. pyogenes*. The recovery of the organism in pure cultures from the products of abortion and the normality of the controls inoculated with sterile saline alone, reinforce this view. The response of the animals treated with cloprostenol was exactly as expected and the group formed a very useful control in these studies. Six of the eight animals infected with the live bacteria aborted. Failure of the infection to establish in the two cows could have been due to a number of factors. It is possible that as failures occurred towards the beginning of the series of experiments, poor technique in the inoculation of the bacteria into the uterine lumen could have led to the failure, with the inocula possibly deposited outside the uterus. It is also possible that the animals had been previously infected with *A. pyogenes* rendering them immune to the experimental infection. This possibility will be discussed further in Chapter VII. Inoculation with sterile

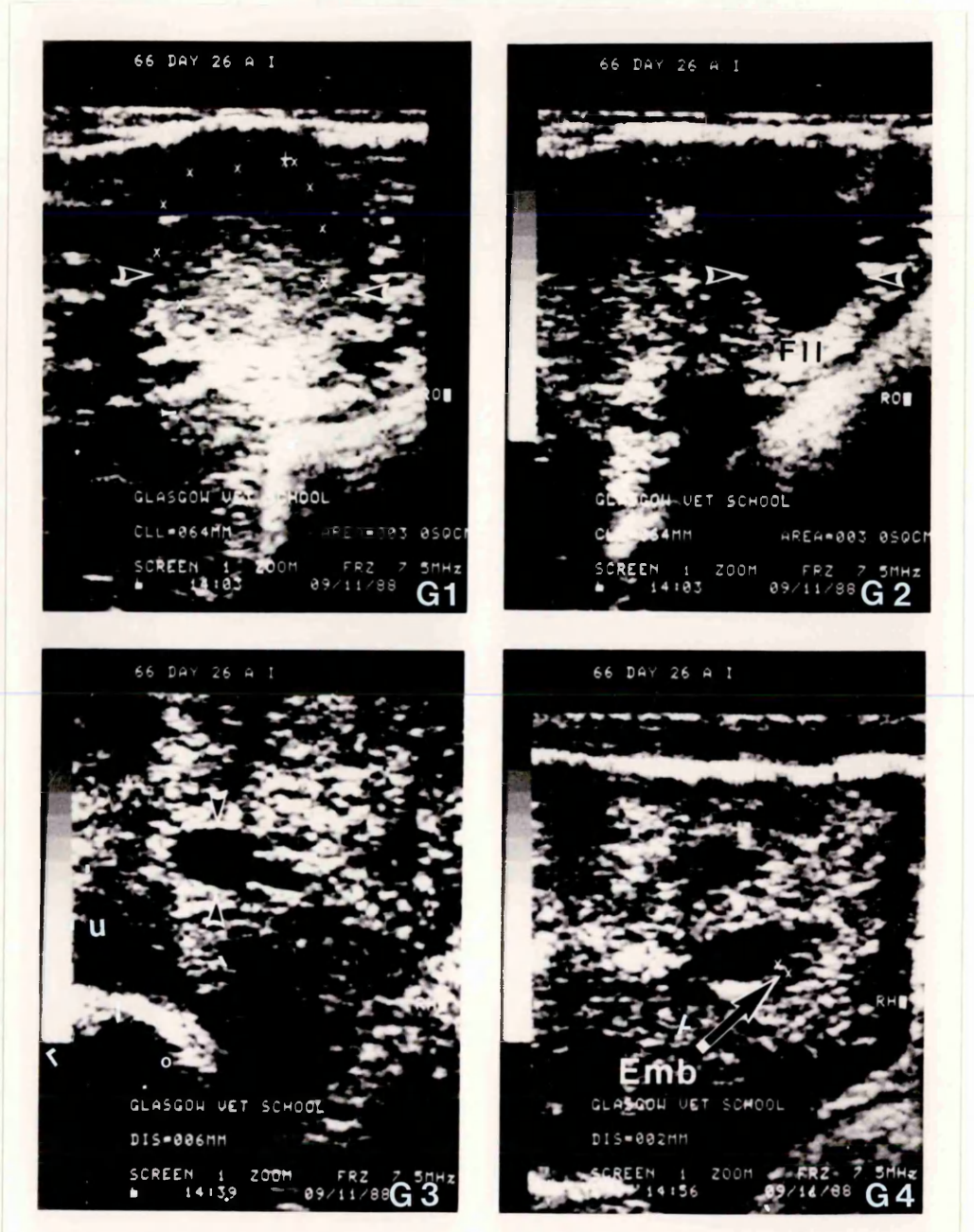


FIG. 24 (G1 - G4): Ultrasonography of a day 26 normal pregnancy, one day before inoculation with sterile saline. **G1** CL present at 3.0 cm^2 (arrows); **G2** follicle (Fll) = 10 mm (arrows); **G3** pregnant horn luminal diameter = 6 mm (arrows) and **G4** with the embryo (Emb) = 2 mm in diameter.

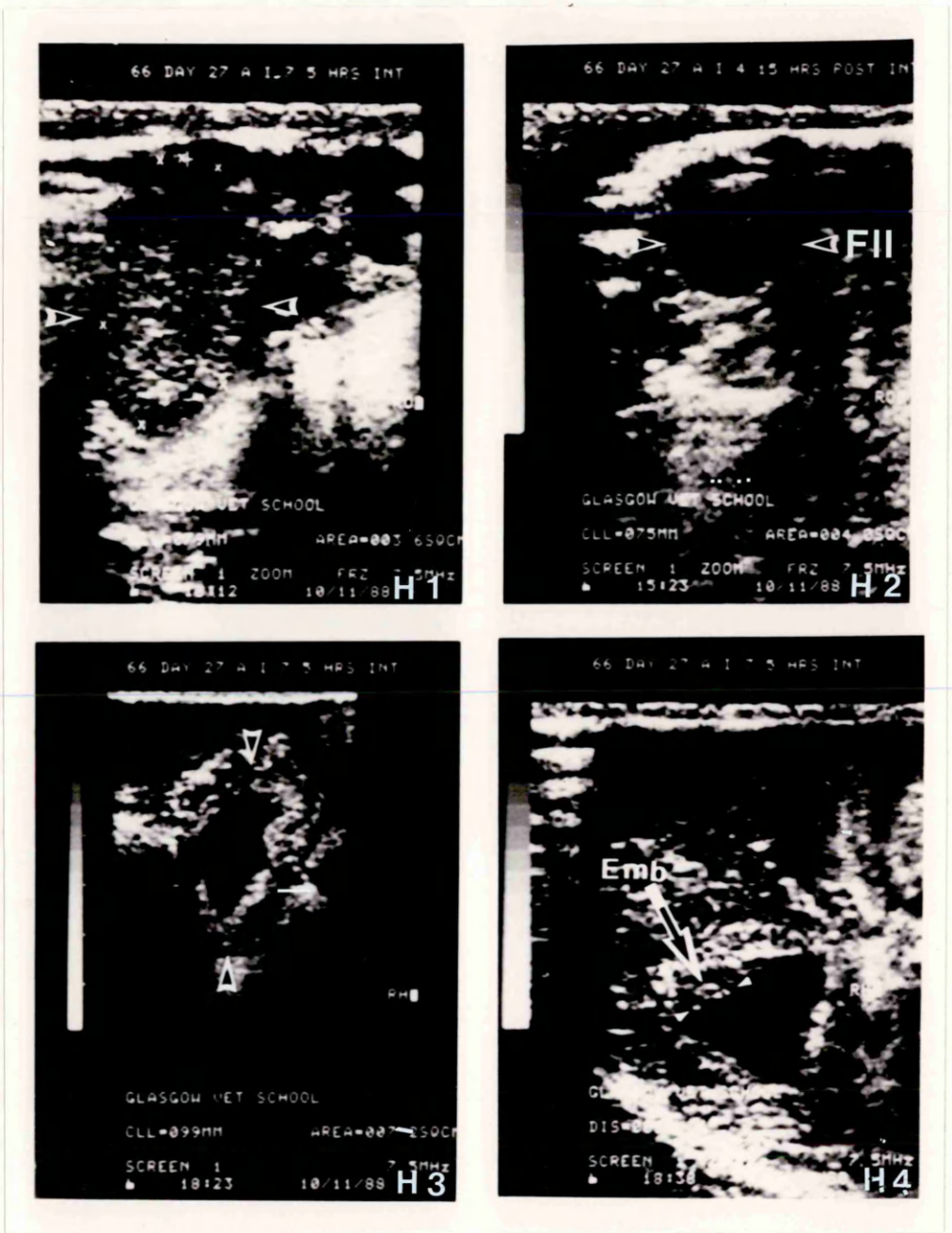


FIG. 25 (H1 - H4): Ultrasonography of the pregnant uterus 7 - 15 hours after intervention with sterile saline. **H1** CL = 3.6 cm^2 (arrows); **H2** follicle (Fll) = 9 mm (arrows); **H3** with dark non - echogenic allantoic fluid (arrows); **H4** the embryo (Emb) = 6 mm in length and 3 mm in diameter.

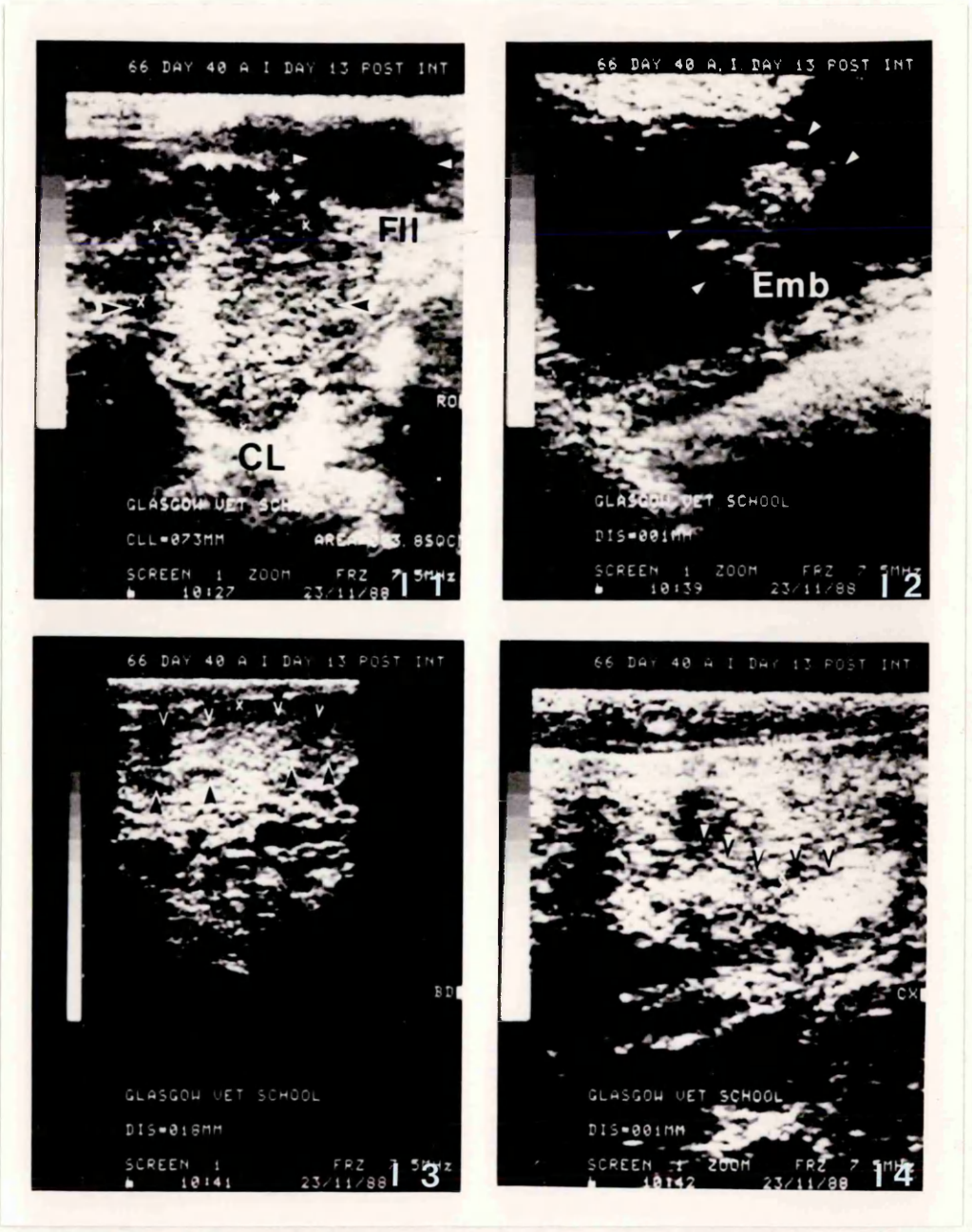


FIG. 26 (I1 - I5): A 40 day old pregnancy, 13 days after inoculation of sterile saline. **I1** CL = 3.8 cm^2 (arrows) and follicle (FII) = 8.4 mm; **I2** embryo (Emb) 15 mm long and 7.2 mm diameter (arrows); **I3** the body of the uterus with fluid and has a cross-sectional diameter of 18 mm (arrows); **I4** the cervix (CX) closed (arrows);

FIG. 26 I5 vagina (Vg) with walls closely apposed (arrows).

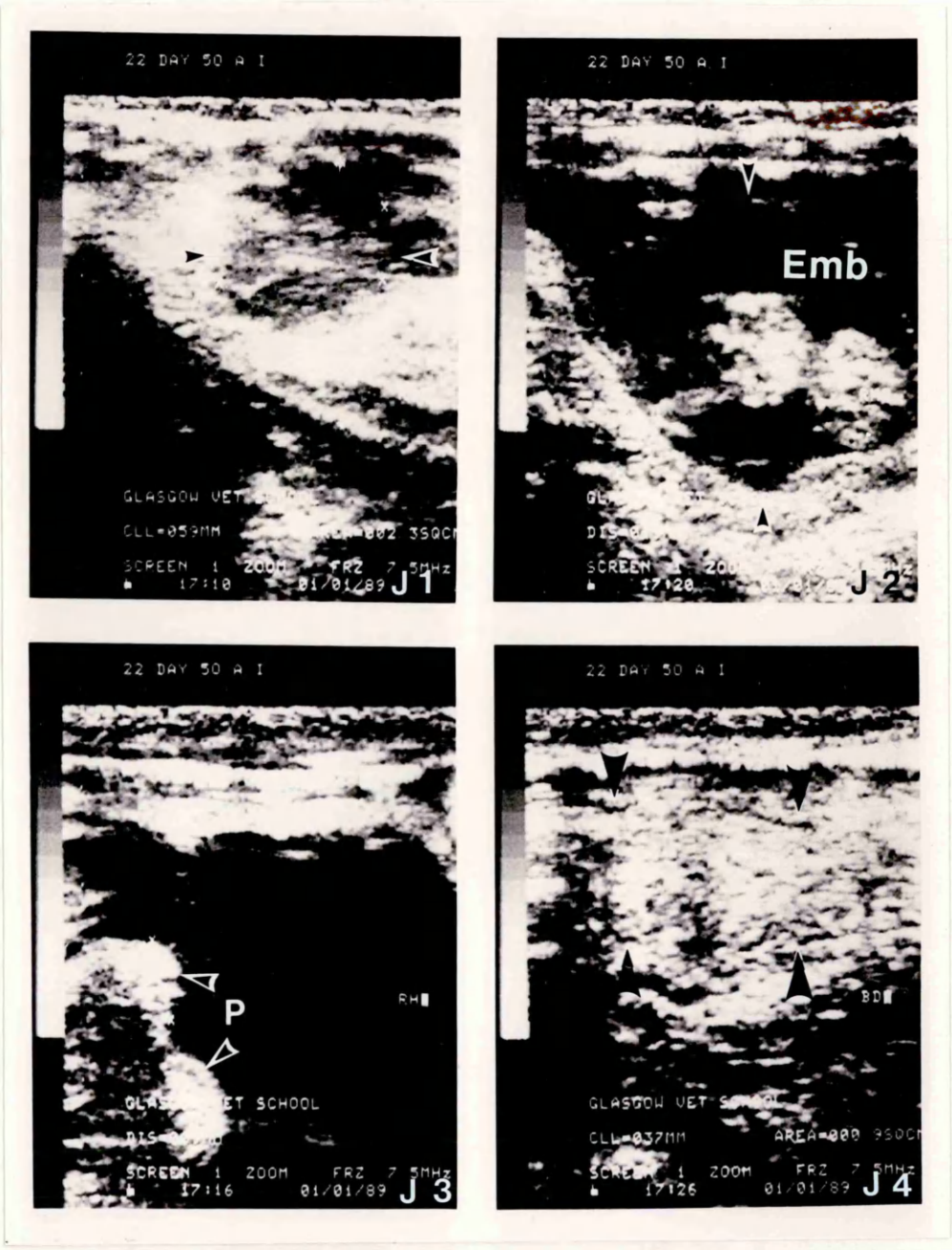
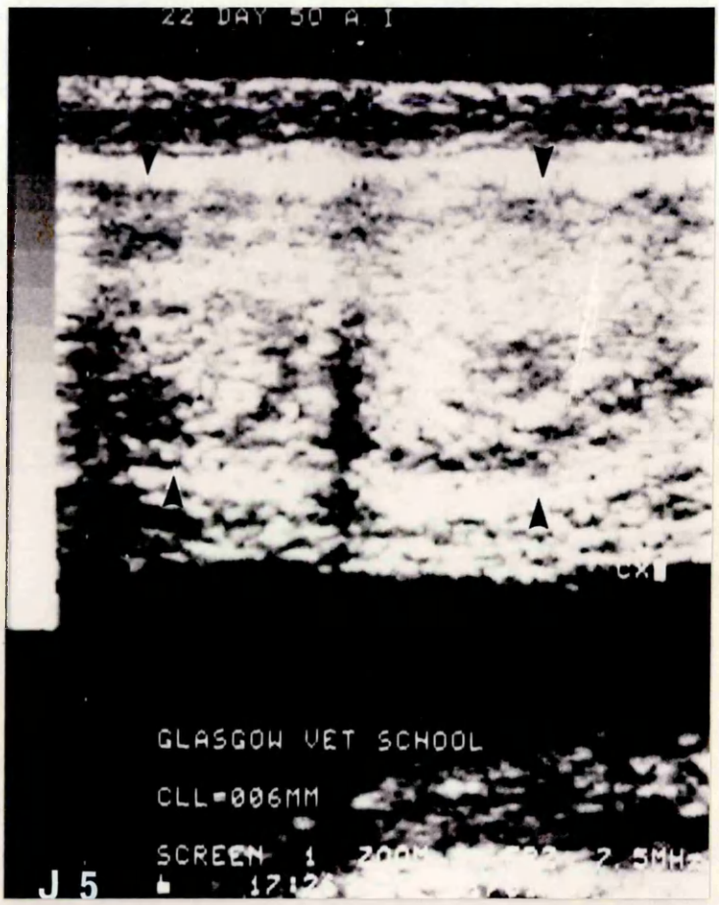


FIG. 27 (J1 - J6): Normal 50 day pregnancy in Cow 14 prior to treatment with cloprostenol. **J1** CL = 2.3 cm^2 (arrows), **J2** embryo (Emb) 18 mm long and 8 mm cross sectional diameter; **J3** pregnant horn with placentomes (P); **J4** body of the uterus with no fluid (arrows);



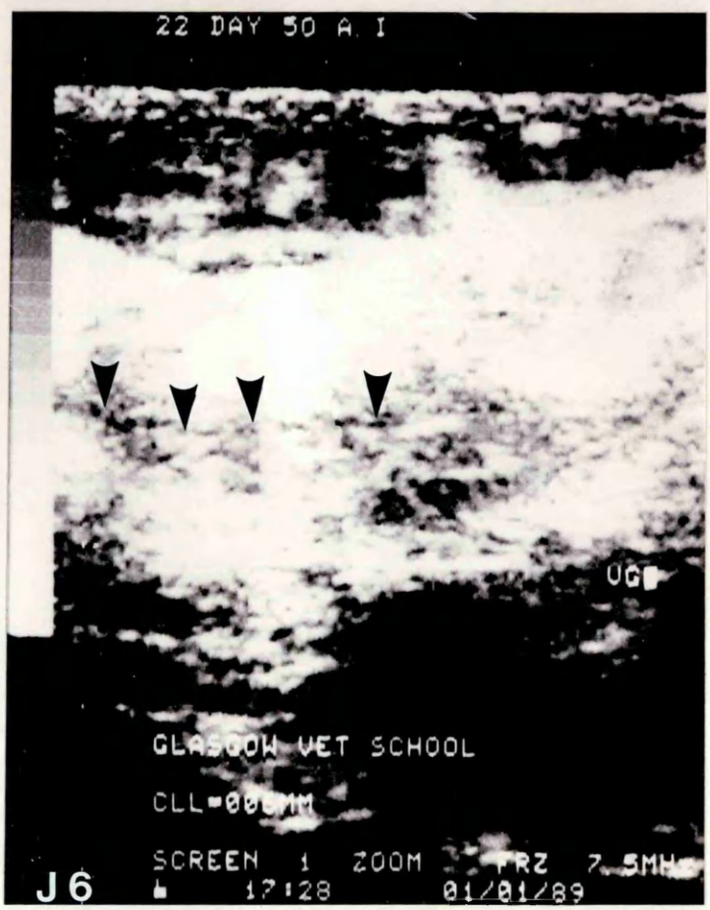


FIG. 27 J5 Cervix closed (arrows); **J6** vagina closed (arrows).

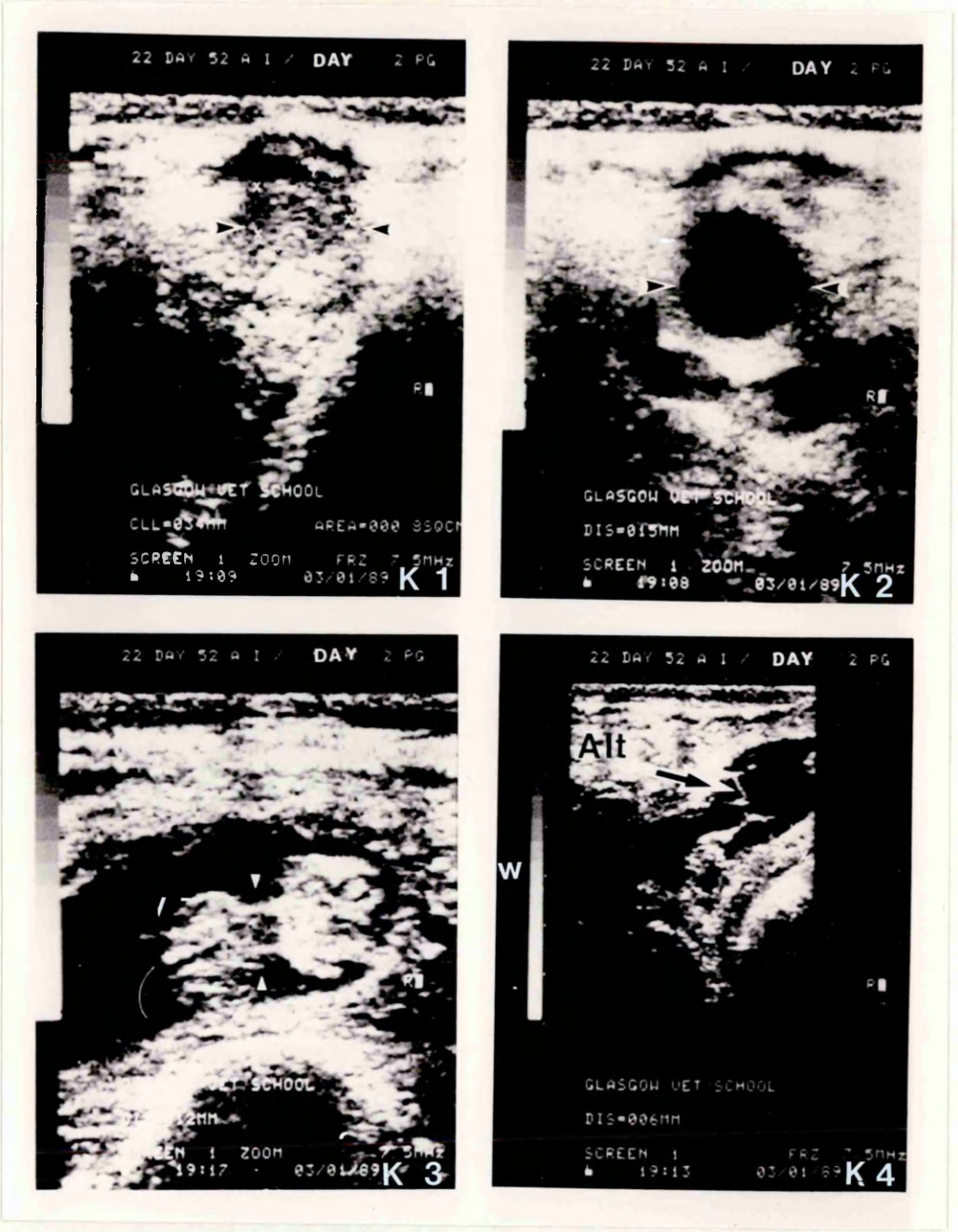


FIG. 28 (K1 - K7): Reproductive tract of Cow 14 two days after treatment with cloprostenol. **K1** CL regressed to 0.8 cm^2 (arrows); **K2** growing follicle = 15 mm (arrows); **K3** embryo = 21 mm long with the heart beat present (arrows); **K4** detachment of the allantochorion (Alt) from the endometrium;

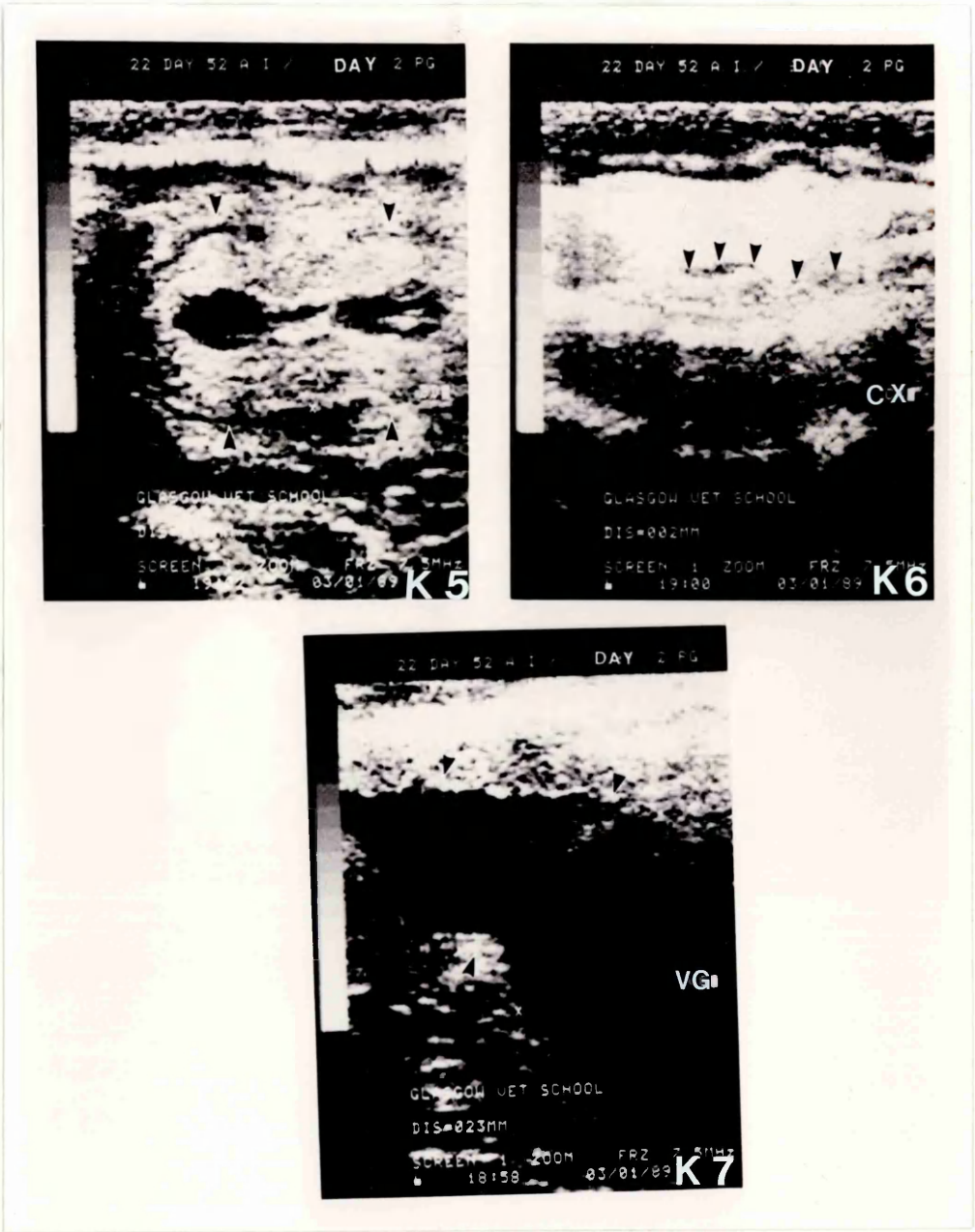


FIG. 28 K5 dark non echogenic fluid in the body of the uterus (arrows); **K6** cervix (CX) beginning to open = 2 mm (arrows); **K7** vagina (Vg) filled with non echogenic fluid (arrows). Abortion occurred 24 hours later.

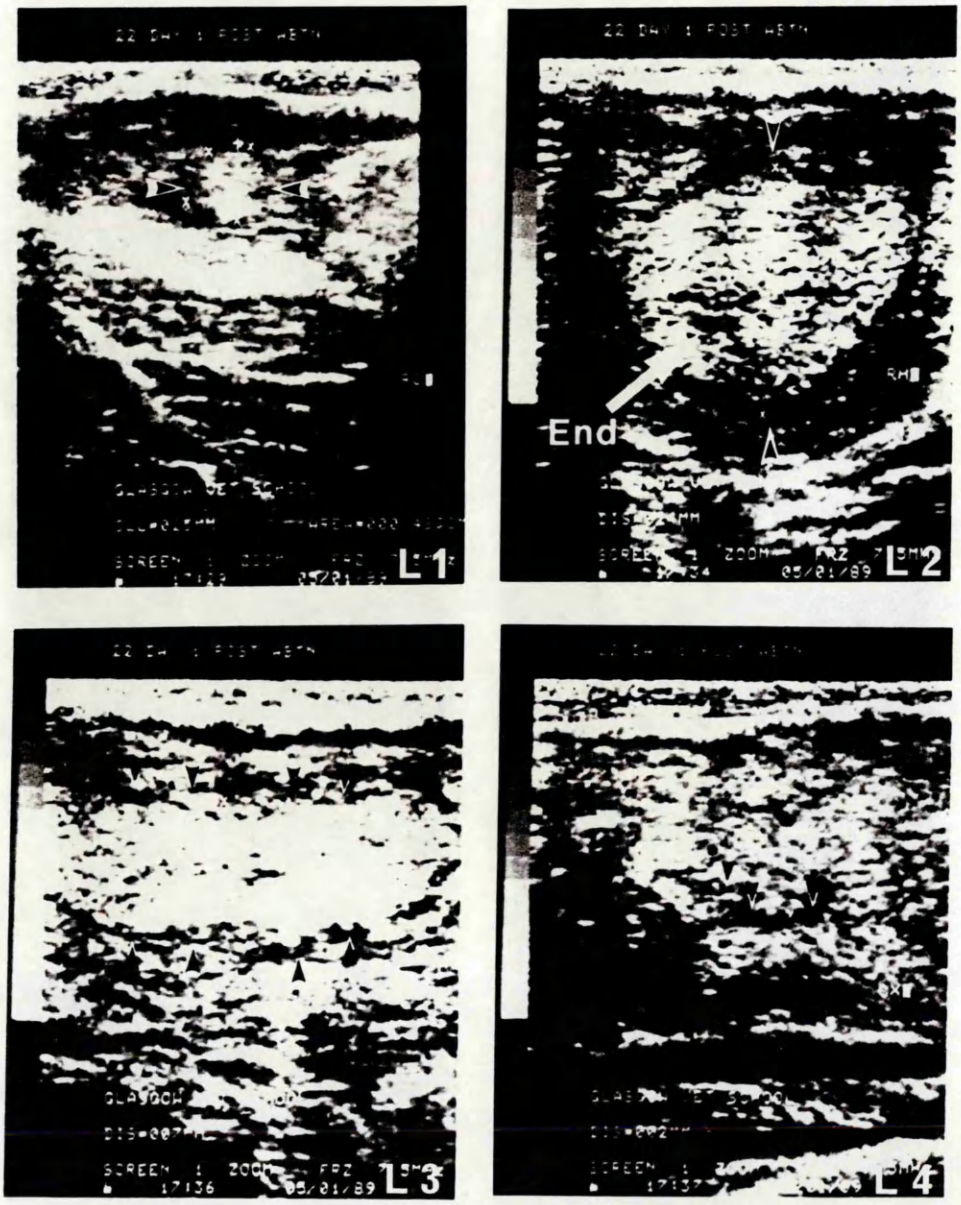


FIG. 29 (L1 - L5): Reproductive tract of Cow 14 one day following abortion (96 hours after cloprostenol treatment). **L1** CL regressed to 0.4 cm^2 (arrows); **L2** formerly pregnant uterine horn empty with a thick endometrium (14 mm) (arrows); **L3** body of the uterus empty with thick endometrium (arrows); **L4** cervix open = 2 mm (arrows);and

FIG. 29 L5 vagina (Vg) filled with non echogenic fluid (arrows).

saline did not affect the embryo or cause abortion showing that neither the saline used for preparation of the inoculum nor the needle injection through the uterine wall had any measurable effect on pregnancy and that the changes seen may have been directly related to the presence of the viable bacteria.

A. pyogenes was isolated from only one of the eight animals used in the study prior to bacterial infection (Heifer 5). Biochemical tests carried out on the bacterium isolated before experimental infection and from the aborted material showed that both isolates were of *A. pyogenes*. No tests were carried out to determine whether the two bacteria were the same or two different strains. No *A. pyogenes* was isolated from animals that were inoculated with sterile saline or treated with cloprostenol.

The appearance of *A. pyogenes* in the vagina after bacterial inoculation and the profusion of the cultures isolated following abortion from the products of abortion reinforce the conclusion that *A. pyogenes* was the cause of the abortions produced. The biochemical tests carried out on some of the isolations of *A. pyogenes* following abortion confirmed their identity as *A pyogenes* (Appendix TABLE A).

The point at which embryonic death occurred was identified by ultrasound. This specific knowledge allows the clinical signs to be compared with the ultrasound findings.

Rectal temperatures taken during the experimental period only showed a slight rise in 5 of the 6 animals that aborted after infection. The rise in temperature recorded in these animals was still within the normal range of body temperature, and could not have been used to detect embryonic death.

Likewise, signs such as demeanour and appetite were normal and would therefore not be very good guides to intrauterine events. The rise in temperature in one animal (Heifer 5) to 40.3°C after bacterial infection could have played a part in causing embryonic death and abortion. The presence of mucopurulent material around the perineum and the tail or on the byre floor appeared to be an indicator of pending loss of pregnancy or to indicate that abortion had already occurred. The increased uterine tone recorded was most obvious and sustained after abortion following *A. pyogenes* infection (**FIG. 10**). The degree of tone recorded was not seen in the saline controls and an increase of this nature in early pregnancy could therefore be a useful parameter in investigating embryonic loss. A similar level of tone was recorded in the cloprostenol treated animals (**Fig. 10**) but was not maintained. As this increase in tone is like that of normal oestrus, the duration of increased tone in infection could be a differentiating character. Whether increased tone is sufficiently specific to be used in the diagnosis of embryonic death needs to be investigated further.

Examination of the external-os and the vagina for the presence of pus and any signs of inflammation may be useful in routine examination of suspected cases. Monitoring of the clinical signs associated with embryonic loss suggested that it would be difficult to identify embryonic death or impending abortion by clinical means alone. The fact that an infective abortion had occurred might be indicated by the copious vaginal discharge and/or the finding of pus - covered embryos or foetuses of the type found in this study.

Embryos collected from animals following *A. pyogenes* induced abortion were clearly more decomposed and were accompanied by pus unlike those collected following cloprostenol treatment (**Figs 7, 8, 11, and 12**). The finding of three intact embryos (i.e. with their membranes) in the vagina,

indicates that the embryo and its membranes may be completely lost during abortion. This phenomenon would make diagnosis of pregnancy loss difficult. In the other three aborting animals this supposition was confirmed because no entire embryos could be collected and only pus with foetal parts were found. Here maceration of the embryo may have occurred following embryonic death. This possibility emphasises the importance of thorough examination of any discharge collected from the vagina in animals suspected of having aborted in order to identify any trace of an aborted embryo or foetus. The time between inoculation and recovery of products of abortion was longer in 5 of the 6 cows infected with *A. pyogenes* than in those treated with cloprostenol (>85 hours compared with 60-70 hours after cloprostenol induced abortion, **Table 3**). The interval between inoculation and expulsion appeared to be related to the number of organisms in the inoculum (comparing cows 1 and 4) (**Table 3**) but the results are equivocal and any relationship must be examined further.

Controlled induction of abortion was followed by regression of the corpus luteum and return to heat 66-87 hours after treatment as in the studies of Omran (1989). In contrast, the infected animals in this study had not come back to heat 8 days after abortion and the corpus luteum could be palpated throughout this period. This retention of the corpus luteum would make it difficult to confirm the loss of pregnancy by rectal palpation. Persistence of the corpus luteum was recorded in similar circumstances by Farin *et al.* (1989) who produced endometritis by inoculation of the non - pregnant bovine uterus with pure cultures of *A. pyogenes*. The two types of persistent corpora lutea would be difficult to distinguish and the other characters described above might be of value. The significance of the retained corpus luteum is discussed further in Chapters VI and VIII.

ULTRASOUND.

Normal pregnancy was confirmed before interference with any of the experimental treatments. The following observation were characteristic of a normal pregnancy. (a) The corpus luteum was always seen (2.5 cm^2). (b) Presence of clear non-echogenic fluid (black) in one of the two horns, containing an embryonic vesicle. The embryo was seen to increase in size with subsequent examinations and was confirmed to be alive by presence of a heart beat. (c) There were no significant changes in the body of the uterus (which is an expected finding in early pregnancy before the allantochorion extends in the non-pregnant horn). (d) The cervix was always closed. (e) The vaginal lumen was collapsed (**FIGS 17, 24, 27**).

Infection in the *pregnant horn* was primarily characterised by detachment of the allantochorion from the endometrium and thickening of the membranes. The thickness of the membranes was greater than that of the membranes of early embryonic loss induced by treatment with cloprostenol and this is in agreement with the work done by Omran (1989). The thickening of the membranes may be an inflammatory response to the presence of the bacteria or bacterial attachment. The possibility is investigated in Chapter IV. The gradual change of uterine fluid from a clear (dark none - echogenic) (**FIG. 18**) to grey - white (echogenic) image (**FIG. 22**), was most probably due to the host inflammatory reaction leading to production of phagocytes and formation of pus. The identification of pus at the external - os of the cervix and the presence of *A. pyogenes* and large number of neutrophils and monocytes in this discharge supports this view. Fissore et al. (1986) observed endometrial thickening in cases of endometritis and the findings presented above confirm this observation. Thickening of the endometrium in these cases (**FIG. 20 D2**) could be attributed to the inflammatory reaction leading to an increase in the blood flow to the uterus

and oedema, but the increase in endometrial thickness found (to 5 - 7 mm) was not the same as that seen during the follicular phase (especially around oestrus) which is between 8 - 14 mm (Omran 1989) or that seen after cloprostenol treatment (**FIG. 29 L2**) which is of a similar order. Histopathological and electron microscopic studies which resolve this question are reported in Chapter V.

Abortion was always preceded by membrane detachment from the endometrium of the reproductive tract and cessation of the embryonic heart beat, which is in agreement with Guilbault *et al.* (1988) and Omran (1989). The production of pus between the foetal membranes and the endometrium (**FIGS 18 B2 and 19 C2**) and the possible increase in endometrial secretion due to the inflammatory reaction, may be some of the mechanical factors responsible for the separation of the membranes from the endometrium. It is therefore possible that hypoxia and anorexia developed in the embryo and these may be contributory factors to embryonic death.

Gross morphological changes in the uterine body and cervix which occur some time later also point to a slow spread of infection and inflammatory reaction, the cervix being most dilated around the time of abortion. Changes in the wall of the vagina do not seem to be prominent. However changes in size of lumen and its content are significant. In normal pregnancy the luminal diameter is about 1 - 2 mm but in this study the diameter increased from less than 2 mm (at the time of infection), to more than 14 mm throughout the time of expulsion of the embryo, and even up to 6 days after abortion (**FIGS 22 E5 and 23 F5**).

The fact that no changes were seen ultrasonically following controlled inoculation with sterile saline and that pregnancy continued confirmed

further that all the changes seen after live bacterial inoculation were due to the organism alone.

Whereas pregnancy loss was induced after cloprostenol treatment, accompanying events were different. Important events only seen after the treatment with cloprostenol were, the rapid regression of the corpus luteum (24 to 96 hours after treatment), lack of thickening of the foetal membranes, presence of clear (non-echogenic) fluid in the uterus during abortion and a marked endometrial thickening (**FIGS 28 and 29**). These events represent differences between the pathogenesis of bacterial abortion and cloprostenol induced abortion.

The experiment described above suggest that *A. pyogenes* was able to cause embryonic death and abortion in cattle. This study suggests that in low grade non-specific infection *A. pyogenes* may be a primary pathogen leading to embryonic loss and infertility. Earlier work seeming to point to synergism of the bacteria with some anaerobes is not borne out by the results presented here.

In this study not only has it been shown that pure cultures of *A. pyogenes* cause death and loss of embryo in cattle but that the accompanying pathology leads to gross morphological changes which can be followed by the use of ultrasound scanning. Ultrasound has proven to be a useful and non-invasive method available to complement endocrinological and bacteriological methods for investigating the effects of low grade uterine bacterial infection leading to infertility, since gross changes and effects on the embryo and the reproductive tract can be observed directly and *in vivo* in real time.

CHAPTER IV.

STUDY OF THE PATHOGENESIS OF *A. PYOGENES* IN EARLY EMBRYONIC DEATH AND ABORTION.

INTRODUCTION.

It was demonstrated in Chapter III that *A. pyogenes* caused bovine embryonic death and abortion. The work described in this chapter was carried out in order to investigate some of the mechanisms involved in the death of the embryo. Three groups of animals were used in an attempt to determine the mechanisms, and studies of antibody and haptoglobin levels were also recorded. The first group of animals was inoculated with live bacteria and monitored ultrasonically. The animals were killed just before or at the time of embryonic death and the embryos and the reproductive tracts were fixed for E. M., histological and bacteriological studies. The animals were killed in order to avoid the post-mortem changes that take place after embryonic death. It was also proposed to study the pathological changes in the uterus caused by the bacteria at or around embryonic death.

To further understand the pathogenesis of *A. pyogenes* infection in early embryonic death, killed bacterial suspension and the bacterial toxin were used in groups two and three respectively. The killed bacteria were used to investigate the possible effect of presence of non-proliferating organisms on pregnancy in group 2. The toxin studies were considered necessary because *A. pyogenes* is known to produce a toxin that causes haemolysis of the red blood cells and death to mice after intravenous inoculation (Lovell, 1944, Roberts, 1968). Its effects on the embryo were investigated in group 3.

An ELISA was set up to evaluate the immunological response in the animals infected with *A. pyogenes* in this study as it was considered that the presence of antibody might have been responsible for the failure for cows 21 and 22 to abort after infection with the live bacteria.

Use of the assay for haptoglobin was done to measure and evaluate the extent of tissue damage caused by the *A. pyogenes* infection. Haptoglobin is one of the acute phase proteins in cattle produced by the host in an inflammatory reaction (Eckersall and Conner 1988). It was thus felt that its measurement in this study would give another dimension of the host's response to experimentally induced infection in the pregnant uterus.

MATERIALS AND METHODS.

EXPERIMENT II. Two heifers (19 and 20) in early pregnancy (35-36 days) were inoculated with cultures containing *A. pyogenes*. The methods used for inoculation were given in Chapter II. The two animals were inoculated with. $5.5x10^9$ and $3.5x10^9$ c.f.u of bacterial inoculum respectively. Rectal temperatures and other clinical parameters were monitored between infection and slaughter. Ultrasound was used to monitor the embryos before the time of slaughter. The animals were killed at 18 (Heifer 19) and 24 (Heifer 20) hours after live bacterial inoculation. The post-mortem examination of the reproductive tract was carried out by the methods described in Chapter II, and the tissue sections were fixed within 10-20 minutes after killing for electron microscopy, scanning and transmission E.M. and histology. A more thorough post-mortem examination of the carcase was later carried out including the lungs, liver, spleen and kidney and the results were recorded. Bacteriological examination was also carried out on the organs. Fluids from the allantoic and amniotic cavity were

collected for culture and tested for haemolysin by the method described in Chapter II. Swabs were taken from the fallopian tubes, uterine horns, the body of the uterus, cervix and vagina and direct smears were prepared from them and they were cultured for bacteria. The results were recorded.

In order to provide a control for the above two cases, an early pregnant uterus was collected from the abattoir and some of the examinations were repeated. Two aspects were studied in particular (a) the allantoic and amniotic fluids were used as controls for the haemolysin assays and (b) the tract was examined bacteriologically by the methods used in the two infected heifers.

EXPERIMENT III. Two early pregnant Cows (15 and 16) were used in this study. Both were inoculated with killed *A. pyogenes* culture into the uterine lumen. The methods used are given in Chapter II. Cows 15 and 16 received 2.55×10^{10} and 1.2×10^{11} c. f. u. at 27 and 36 days of pregnancy respectively.

EXPERIMENT IV. *A. pyogenes* haemolysin (toxin) was introduced into the uteri of heifers 17 and 18. Methods for preparation of the toxin were described in Chapter II, and an inoculum of 6 ml was used. Clinical examination was done by rectal palpation, ultrasound and monitoring of the temperatures in both experiments 3 and 4.

EXPERIMENT V. Antibody against *A. pyogenes* was determined by the ELISA test as described in chapter II and repeated once. In the first assay 10 serum samples were examined from one infected cow (4) to evaluate the sensitivity of the assay in the detection of antibody. Serial dilutions of 1/10, 1/30, 1/100, 1/300, 1/1,000, 1/3,000, 1/10,000 were made using tris, Marvel and Tween (TMT) and the 1/100 dilution was found to be the lowest

dilution with least background interference. Thus in the second assay 1/100 was used as the lowest dilution for all the animals that were examined. Animals were examined for values of antibody before or on the day of experimentation (Day 0) and then every 5 days for the next 20 to 25 days. Serum samples (collected as described in Chapter II) from 6 animals infected with the live bacterium (1, 2, 3, 4, 5, and22), 2 inoculated with the toxin (17 and 18) and 2 uninfected animals (one 3 month old heifer and one bull calf) were tested in the second assay. The samples were diluted using TMT to dilutions of 1/100, 1/1,000, 1/10,000. The cut-off point was calculated using the following formula, C.O.= x + 3SD (SD = standard deviation), and it was found to be 0.565 (at 1/100) and (0.273 (at 1/1,000) absorbance in blank sera (for a heifer). Serum was considered to be negative or positive if the reading was below or above the cut - off point respectively. The results obtained were compared with the results of infection.

EXPERIMENT VI. Acute phase Hp was tested for using the haemoglobin binding assay described in Chapter II. Cows 1 and 4 (Chapter III) were examined before infection, throughout infection and abortion and until the first oestrus. Samples were assayed in a single assay and the inter assay coefficients of variation were 5.3% and 5% at 20.8 mg/100 ml and 115.6 mg/ml of Hp respectively and these were calculated as for progesterone and oestradiol 17 ß.

RESULTS.

EXPERIMENT II. There were a number of similarities between the findings in both cows but the differences observed between the two animals were sufficient to present the results individually. Observation before

slaughter showed no significant changes in the clinical signs and the animals had a normal appetite. After slaughter the following post-mortem changes were seen.

Heifer I (slaughtered 18 hours after infection).

The gross appearance of the lungs, liver, kidney and the spleen was normal. There was some exudate and froth on the peritoneal surface of the pelvic cavity. Examination of the uterus revealed the presence of the corpus luteum on the right ovary (R. O.) and the pregnant right horn (R. H) was hyperaemic and about 4.5 cm in diameter (**FIG. 30**) and 9 cm long. The left ovary (L. O.) had three follicles >5 mm and 2 smaller ones <3 mm in diameter. The fallopian tubes were normal. The serosal surface of the body of the uterus, cervix and vagina were normal in colour and appearance (**FIG. 30**).

Internal findings. On opening the R. H., foetal membranes containing the embryo were exposed. The embryo was alive (as the heart beat was present) and the amniotic cavity was clearly seen. The allanto-chorion was detached from the endometrium for some distance around the area of inoculation. The much larger area of the allanto-chorion however was closely apposed to the endometrium. There was some degree of cloudiness in some parts of the membranes and they extended into the cranial end of the body of the uterus. Some cloudy fluid was seen in the uterine lumen. The endometrium was 8 mm in thickness and was slightly thickened compared with the normal endometrium which is 3 - 4 mm at this stage. The surface was congested especially at the point of injection and the caruncular areas were slightly elevated (**FIG. 31**).

The endometrium of the L. H. was 6 mm in thickness with caruncles less raised than in the R. H.. No congestion or mucosal lesions were seen but there 5 ml of cloudy fluid was present (**FIG. 31**). The fallopian tubes were

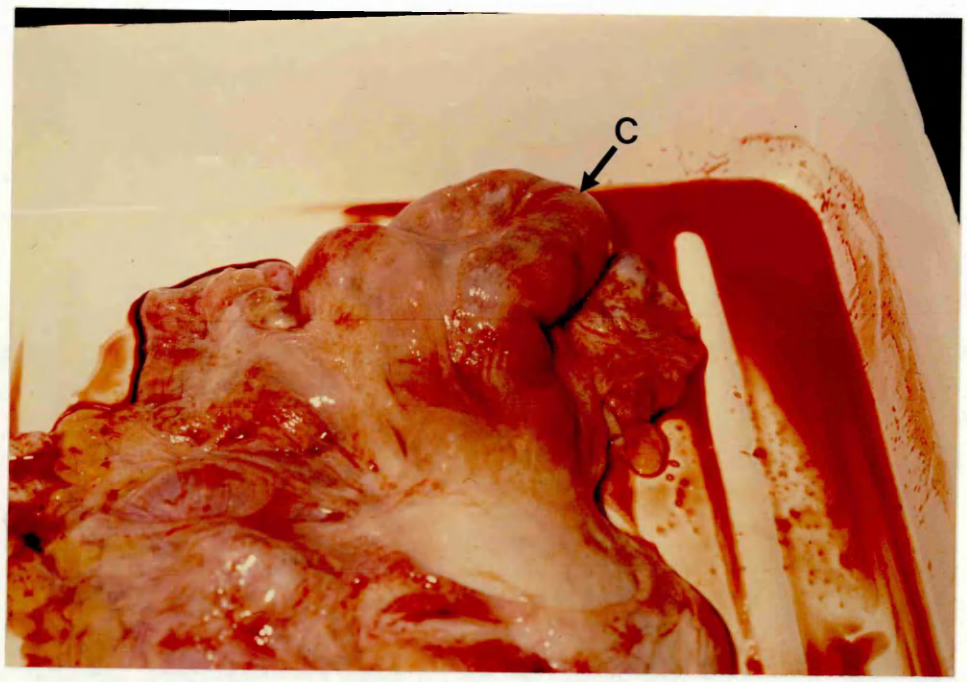


FIG. 30: Gross appearance of the uterus from Heifer 19 slaughtered 18 hours after infection. Congestion (C) in the right horn especially around the point of inoculation.

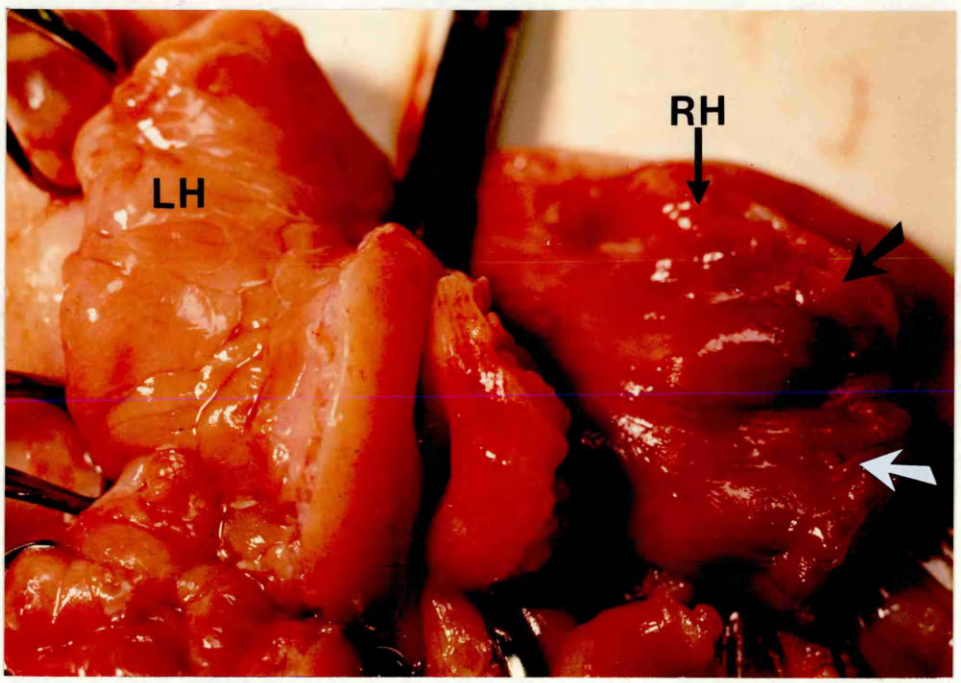


FIG. 31: Internal appearance of the uterus of Heifer 19. Congestion especially around the point of inoculation (arrows) and more congestion in the right horn (RH) compared to the left horn (LH) and the rest of the reproductive tract.

neither expanded nor congested. The body of the uterus contained similar amounts of cloudy fluid. The cervix was approximately 2 mm in diameter and a mucus plug which was loose in some parts was present. The mucosa of the vagina was normal with a pink colouration and some cloudy tenacious mucus.

Bacteriological identification. Direct smears from the vagina showed some Gram positive cocci and rods, but no bacteria were seen in the other parts of the uterus. The Gram stained bacteria were not typical of *A. pyogenes*.

Culture results from the vagina, cervix, the body of the uterus, the horns and the fallopian tubes did not reveal any significant bacterial growth. There were, however, two B-haemolytic colonies from the allantoic fluid which on staining were tentatively identified as *A. pyogenes*. There was no significant bacterial growth from culture of the amniotic fluid.

Smears made from the liver, spleen and lungs revealed presence of some inflammatory cells, but no specific findings were made from the smears of the kidney. Cultural studies revealed no bacterial growth from any of the organs examined.

Haemolysin test. The test was not carried out on uterine fluid because it was in insufficient amount. The test was carried out on both the allantoic and amniotic fluids and haemolysis was seen with both fluids up to a titre of 1:4.

Histology. **(a)** No histological abnormalities were detected in the uterus of this heifer.

(b) The embryo. There were no major changes seen due to presence of

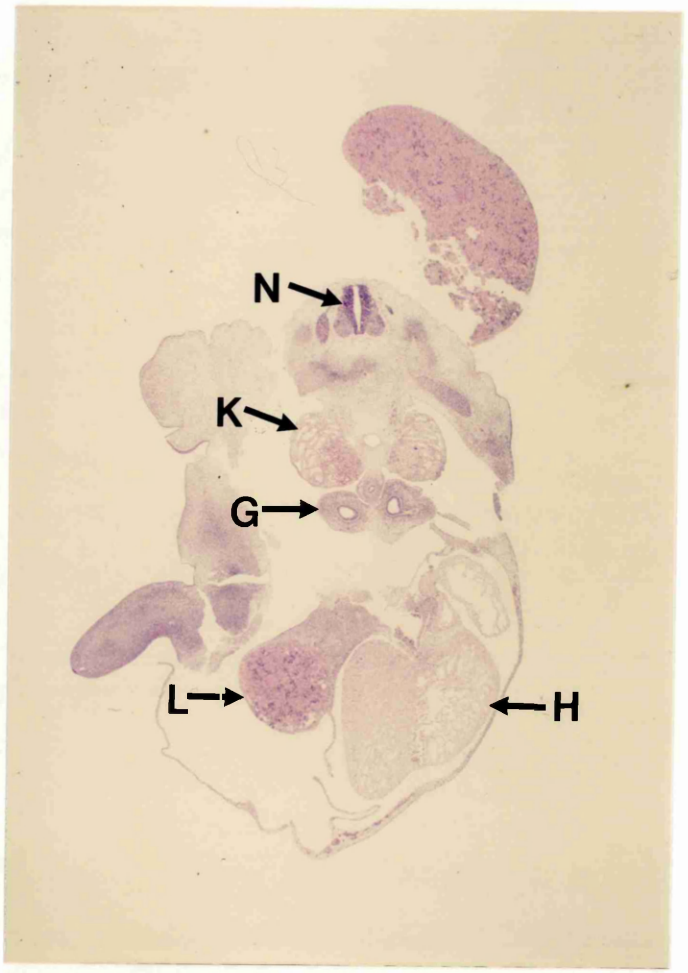


FIG. 32: A longitudinal section of the embryo from Heifer 19. The embryo appears normal with the neural tube (N), Kidney (K), gut (G), liver (L) and heart (H) showing no signs of degeneration.
H & E, x40.

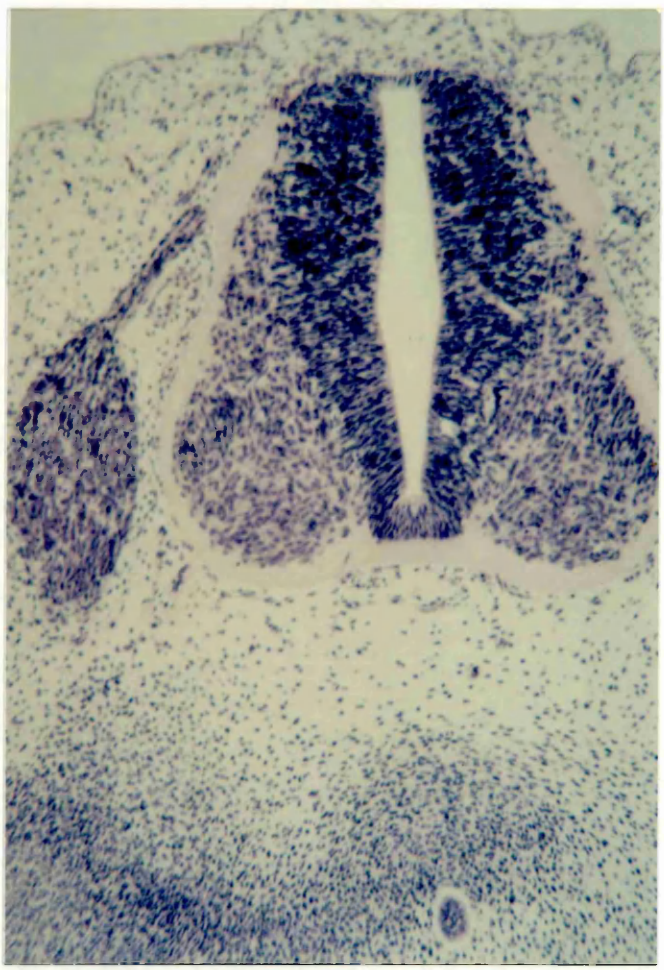


FIG. 33: Appearance of a normal neural tube of the embryo from Heifer 19 with no signs of degeneration.
H & E x100.

A. pyogenes. The findings are reported here to compare them with the more significant changes seen in the embryo from Heifer 20.

In general the findings suggested the presence of a normal embryo for 36 days of pregnancy with minimal post-mortem changes. No polymorphs were seen in any organ or tissue in H. and E. stained sections and there were no signs of tissue degeneration (**FIG 32**). The neural tube appeared normal (**FIG. 33**). The kidney had normal mesonephric tissue (**FIG. 34**). The liver was normal with intact blood vessels which were filled with blood and there was no parenchymal breakdown (**FIG. 35**). The heart showed no blood stagnation or signs of degeneration (**FIG. 32**).

No tissue breakdown was seen in H & E stained sections and there were no Gram positive rods or any other bacteria were seen in Gram stained sections.

Heifer 20 (slaughtered 24 hours after infection).

General post-mortem examination did not reveal lesions due to *A. pyogenes* infection in the lungs and spleen. There was infection of the liver with liver flukes but the parenchymal tissue was relatively normal. The kidney was mottled. Oedema was present on the peritoneal surface over the psoas muscle and over the associated lymph nodes and at the pelvic inlet.

A corpus luteum with an ovulation papilla was present in the R. O. but no follicles were recorded. The L. O. contained small follicles <3 mm in diameter. The fallopian tubes were normal and the R. H. was swollen, 4 cm in diameter, 7 cm in length and had a local area of inflammation at the site of injection. There was some degree of inflammation seen in the wall of L. H. and the body of the uterus (**FIG. 36**).

Internal findings. Five to 10 ml of fluids containing floccules of pus were seen when the right horn was opened. The endometrial lining was inflamed

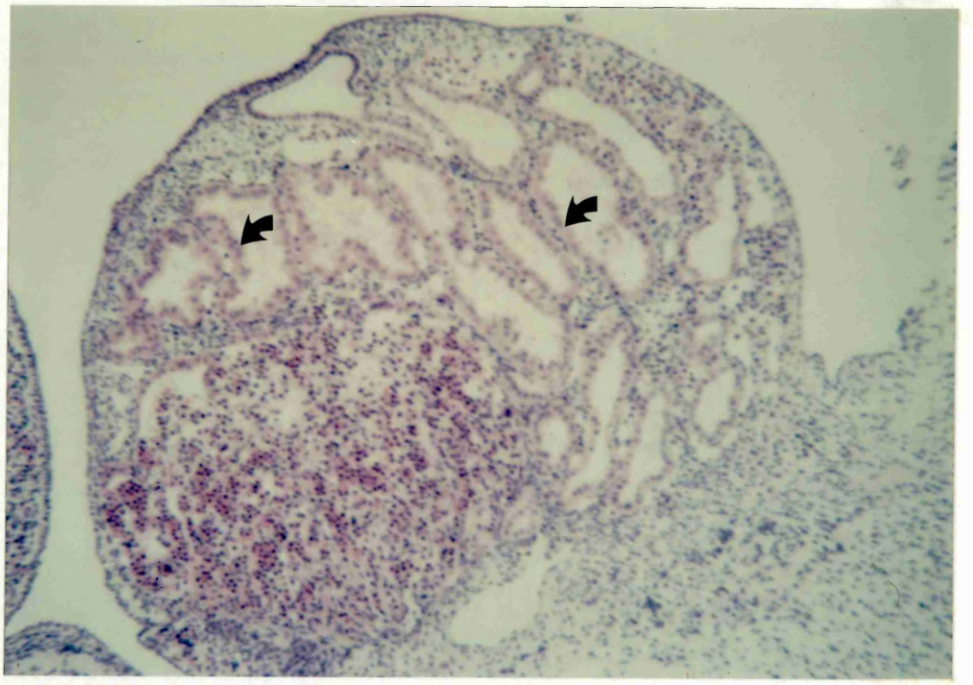


FIG. 34: A normal section of embryonic kidney from Heifer 19. The epithelial lining of the nephrones is intact (arrows).
H & E x100.

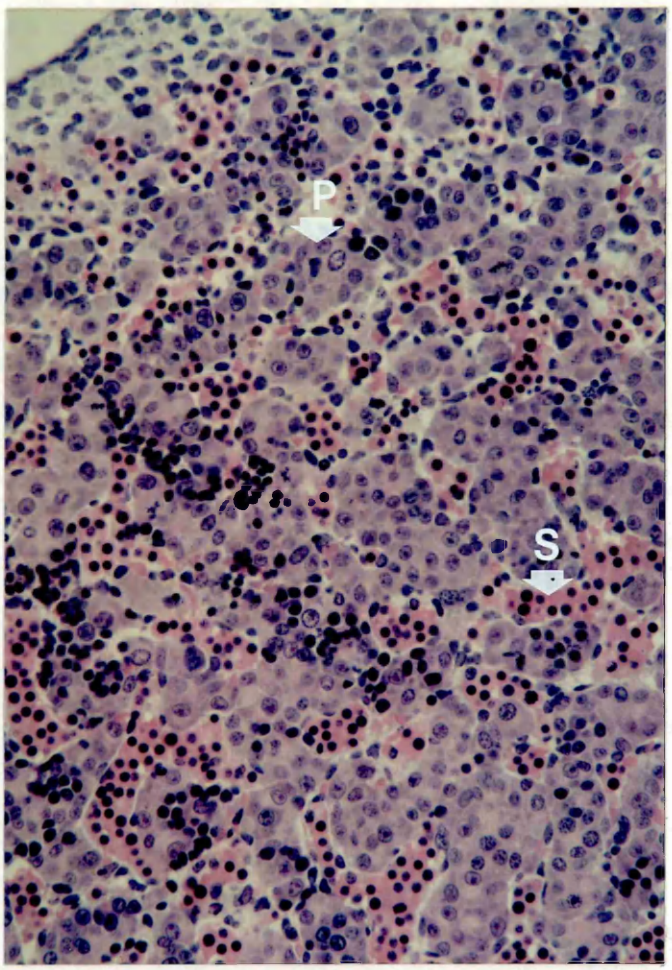


FIG. 35: A section of the liver of the embryo from Heifer 19. It is normal with normal parenchymal tissue, intact liver sinuses (S) clearly differentiated from parenchymal tissue (P).
H & E x250.

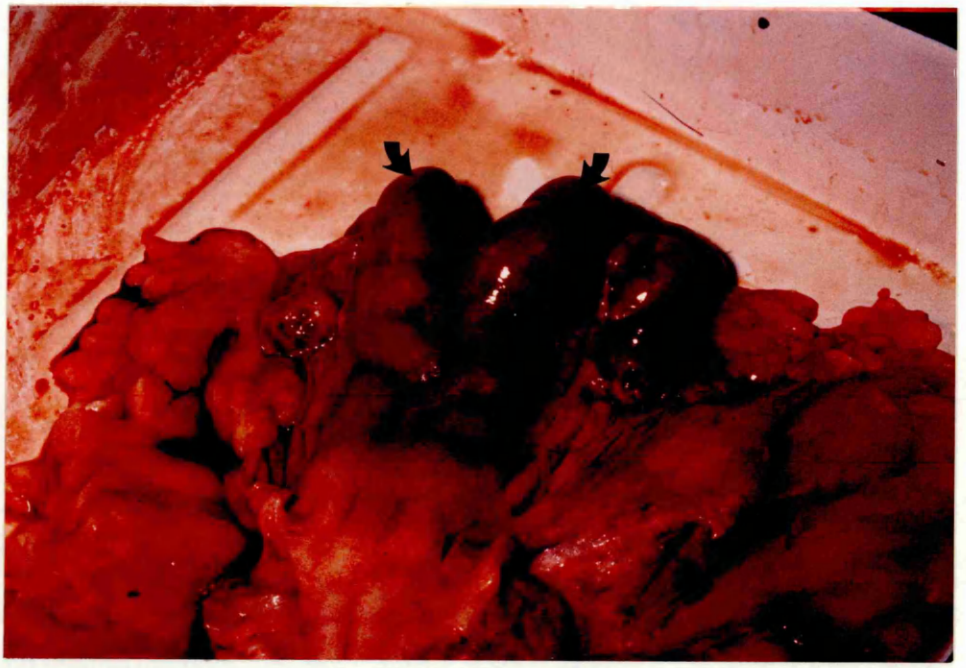


FIG. 36: Gross appearance of the uterus from Heifer 20 which was slaughtered 24 hours after inoculation with live *A. pyogenes*. Marked congestion of both of the uterine horns and the body of the uterus can be seen (arrows).

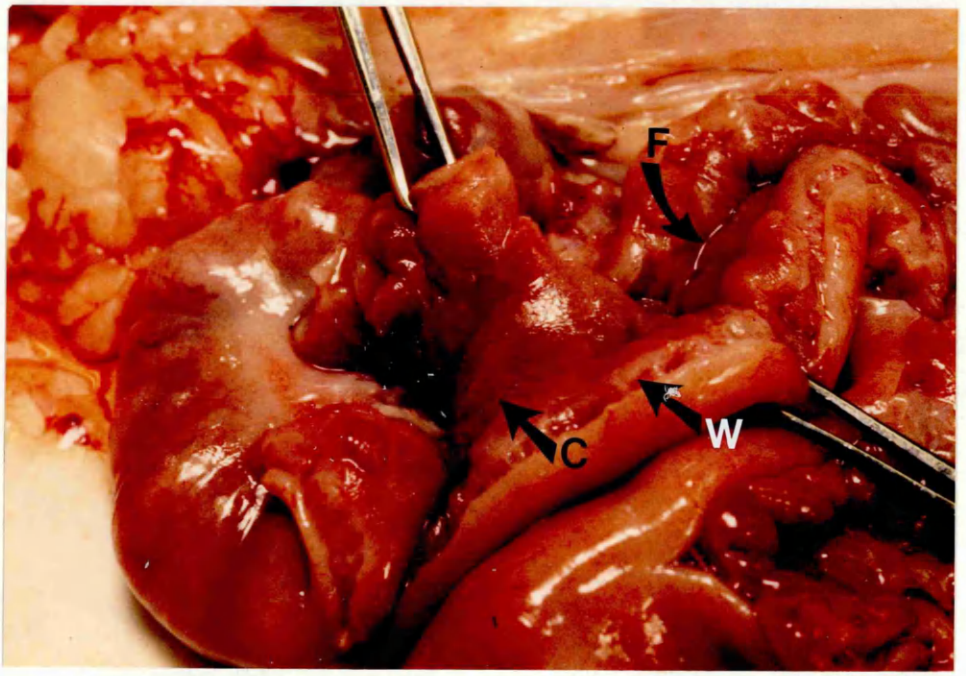


FIG. 37: Internal appearance of the uterine horn from Heifer 20. There is uterine fluid which was cloudy (F), marked congestion (C) of the endometrial lining and a thickened uterine wall (W).

with areas of congestion and necrosis (**FIG. 37**). Caruncles were evident but were not prominent or inflamed. The endometrium was thickened to about 10 mm, considerably more than the 4 mm to be expected.

No cotyledonary areas had yet formed on the allanto-chorion, but there was some mucus and some areas of necrosis covered by pus. There was no apposition between the allanto-chorion and the endometrium. The allantoic fluid was clear. The amniotic membrane was normal and the fluid was clear. The embryo was found to have a crown rump length of 2 cm and was dead at collection as no heart beat was visible and the blood vessels were prominent and contained dark blood (**FIG. 38**).

The L. H. contained fluid but with a smaller amount of pus than the R. H.. The mucosal lining was inflamed but to a lesser extent than in the R. H. and caruncular areas were evident. The wall was about 7 mm thick. The body of the uterus was congested and contained some cloudy fluid. The cervical mucosa was normal with thick mucus. The vagina contained clear, rather thick mucus and had no gross mucosal lesions.

Bacteriological identification. Gram-positive rods and cocci resembling *A. pyogenes* were seen in direct smears from the vagina, cervix, body of the uterus and the uterine horns. Culture of samples of pus from the uterus revealed the presence of grey-white ß-haemolytic colonies along the entire length of the uterus but differing in numbers from one section to another. Maximum numbers were isolated from the uterine horns and the body of the uterus, whereas the vagina yielded the least amount of bacterial growth. The colonies contained Gram positive coccobacilli and were considered to be *A. pyogenes*. No *A. pyogenes* was cultured from the allantoic and amniotic fluids.

Direct smears and cultures of swabs made from the liver, kidney and nasal

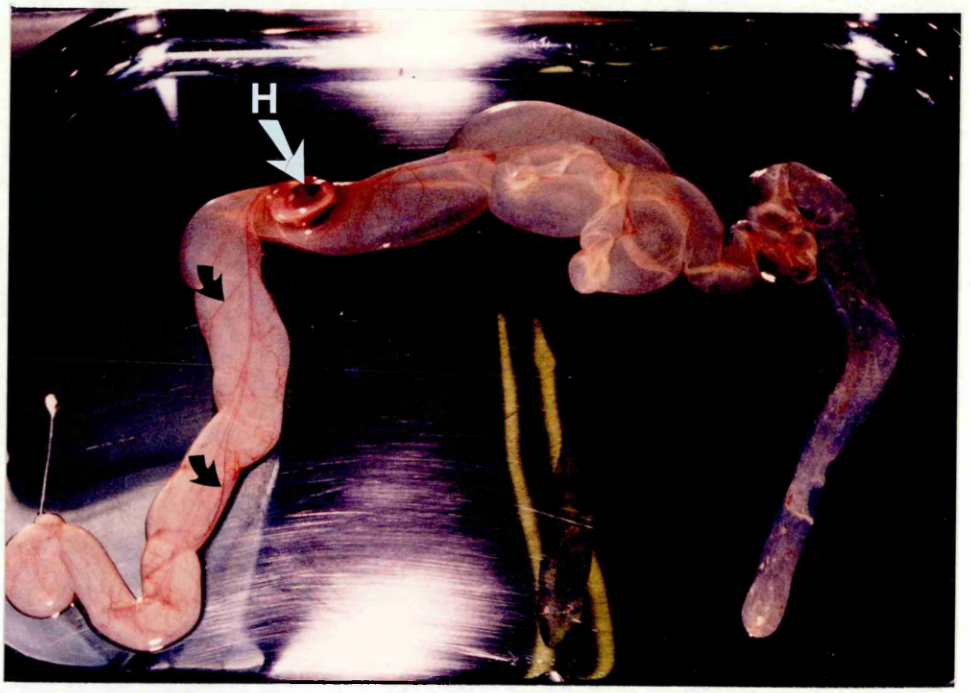


FIG. 38: Embryo collected from Heifer 20 after slaughter. Haemorrhage (H) of the internal organs is present and blood vessels on the allantochorion are prominent.

turbinates revealed no *A. pyogenes*. A few polymorphs including macrophages and neutrophils were seen in direct smears of the nasal turbinates and the liver.

Haemolysin test. Haemolysins were demonstrated in fluid from the body of the uterus at titres of 1:16 and in fluid from the R. H. at titres of 1:2. No haemolysis was seen when the allantoic fluid was tested.

Histology.

(a) The uterus.

Right horn. Major changes were seen in the endometrium in the H. and E. stained sections. The epithelium which was predominantly cuboidal and simple columnar (**FIG. 39**) in the inter-caruncular regions, was eroded in many parts. Tissue debris containing neutrophils, monocytes and free epithelial cells could be seen in the uterine lumen (**FIG. 40**). Inflammatory cell infiltration was prominent in the sub-epithelial layer of some areas of the eroded endometrium (**FIG 41**). The sub-epithelial layer was richly vascularised and contained simple coiled tubular glands. The glands were lined with simple columnar epithelium.

The surface epithelium of the caruncles was composed of cuboidal cells which were intact in some sections (**FIG. 42**). In other sections the epithelium had been completely eroded. The lamina propria of the caruncles was highly vascularised (**FIG. 43**) but devoid of uterine glands (**FIG. 44**) and some of the tissue itself was eroded.

Gram stained sections of the R. H. revealed the presence of Gram positive rods amongst the tissue debris containing epithelial and, connective tissue cells in the uterine lumen in a few areas (**FIG 45**). Tissue debris containing Gram positive bacteria could be seen in the uterine folds (**FIG. 46**). In one area the epithelium was being shed (**FIG. 47a**) and in another area the

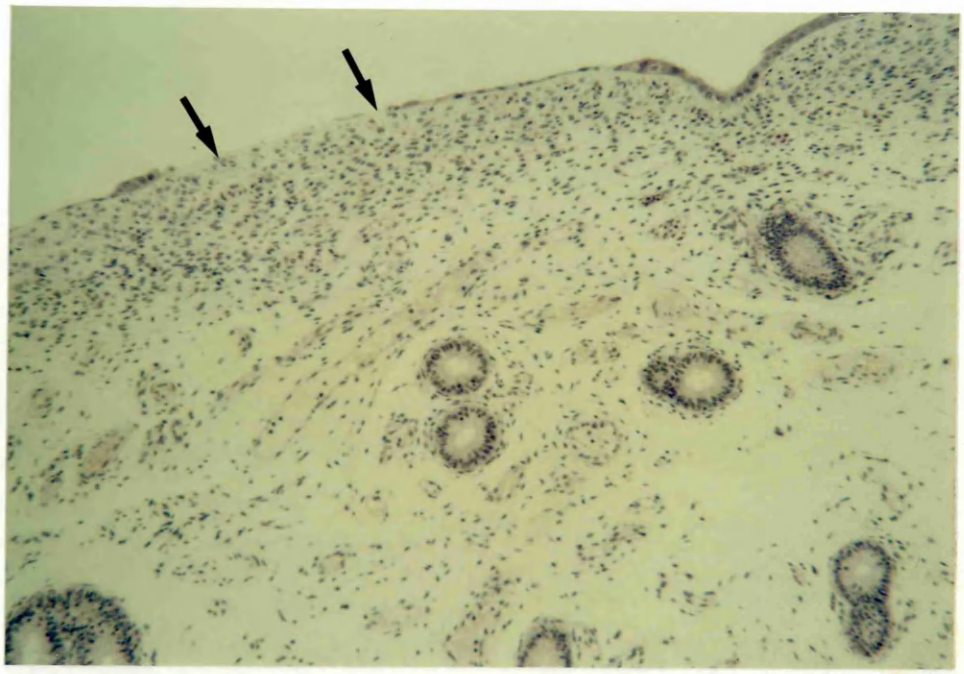


FIG. 39: A histological section of the endometrium of the right horn of the uterus from Heifer 20. Note the eroded epithelium (arrows).
H & E x100.

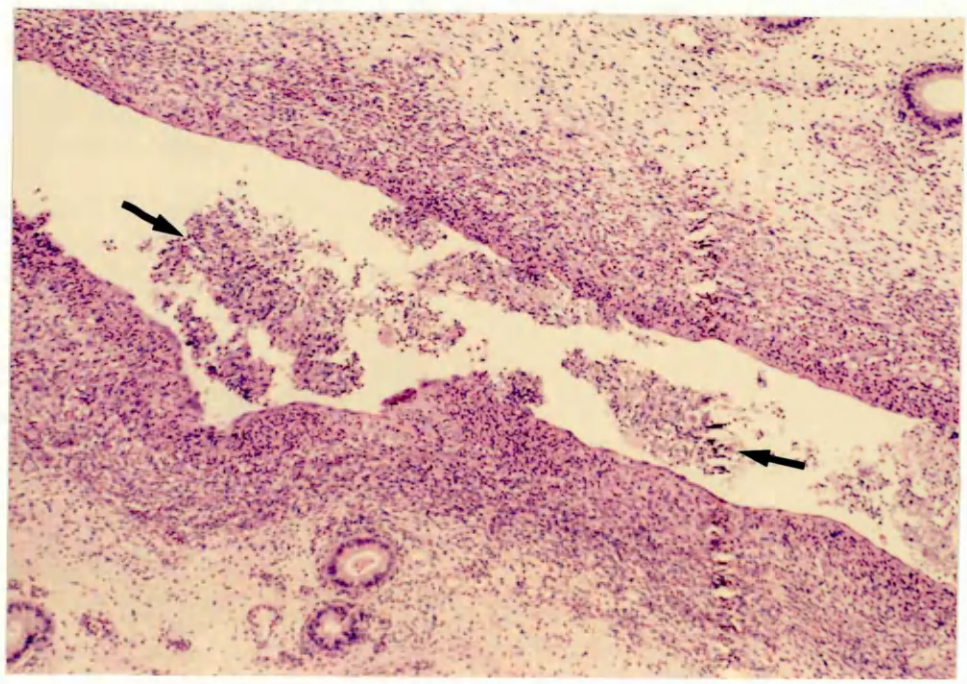


FIG. 40: Tissue debris and pus in the uterine lumen of the right horn (R. H.) of Heifer 20 (arrows). The debris contain large numbers of polymorphs.
H & E x100.

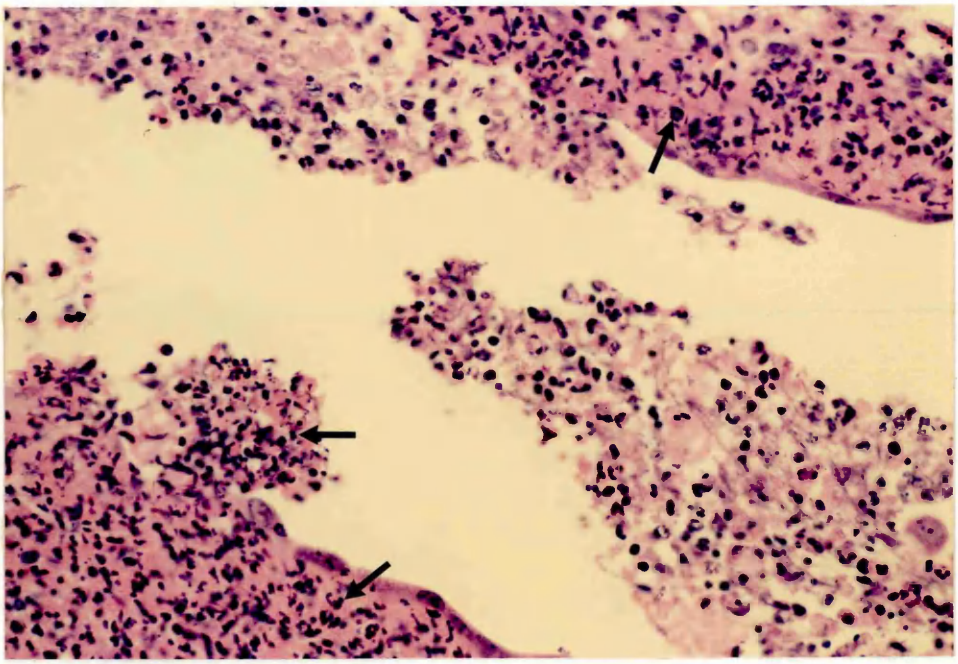


Fig. 41: High power view of endometrium of right uterine horn of Heifer 20. Tissue debris in the uterine lumen and inflammatory cells (arrows) in the subepithelial layer of the endometrium.
H & E x400.

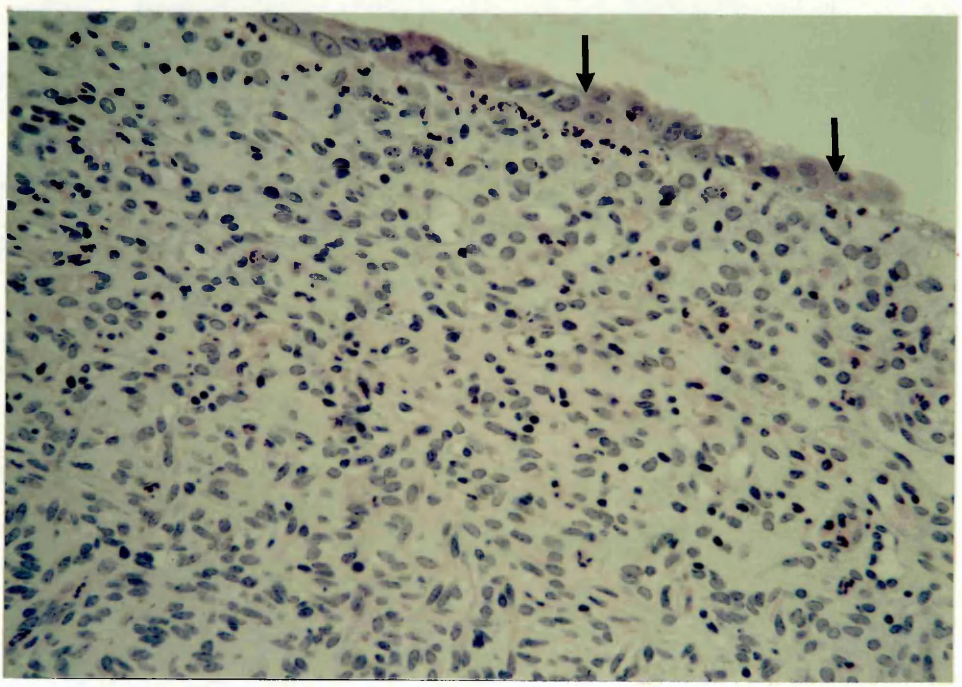


FIG. 42: Endometrium of the right uterine horn of Heifer 20. Intact caruncular epithelium (arrows).
H & E x250.

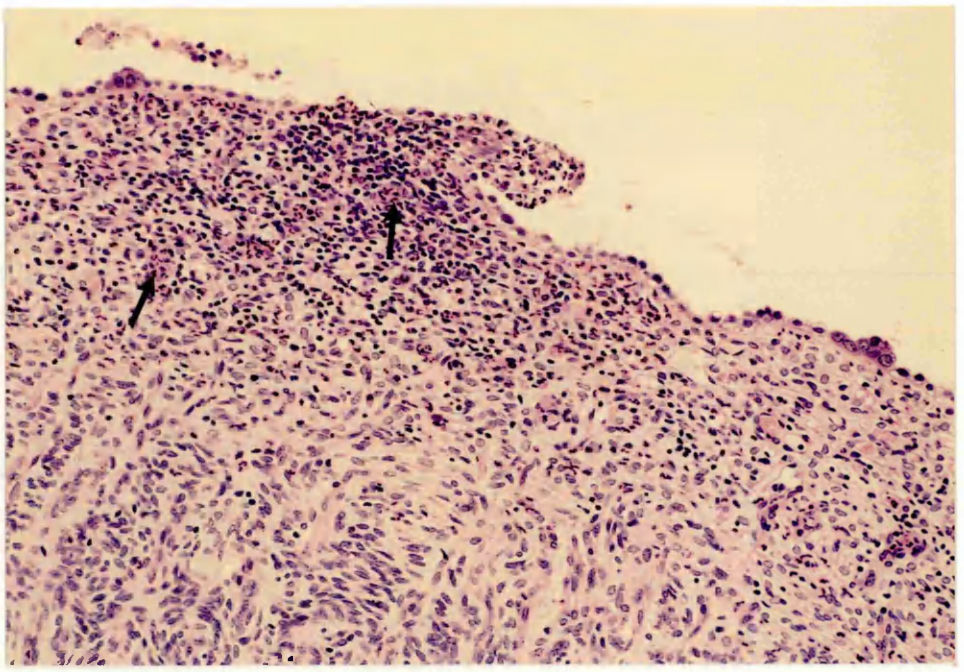


FIG. 43: Caruncular area of uterine horn of Heifer 20. Highly vascularised lamina propria of caruncular epithelial tissue. Blood capillaries contain large numbers of neutrophils (arrows).
H & E x200.

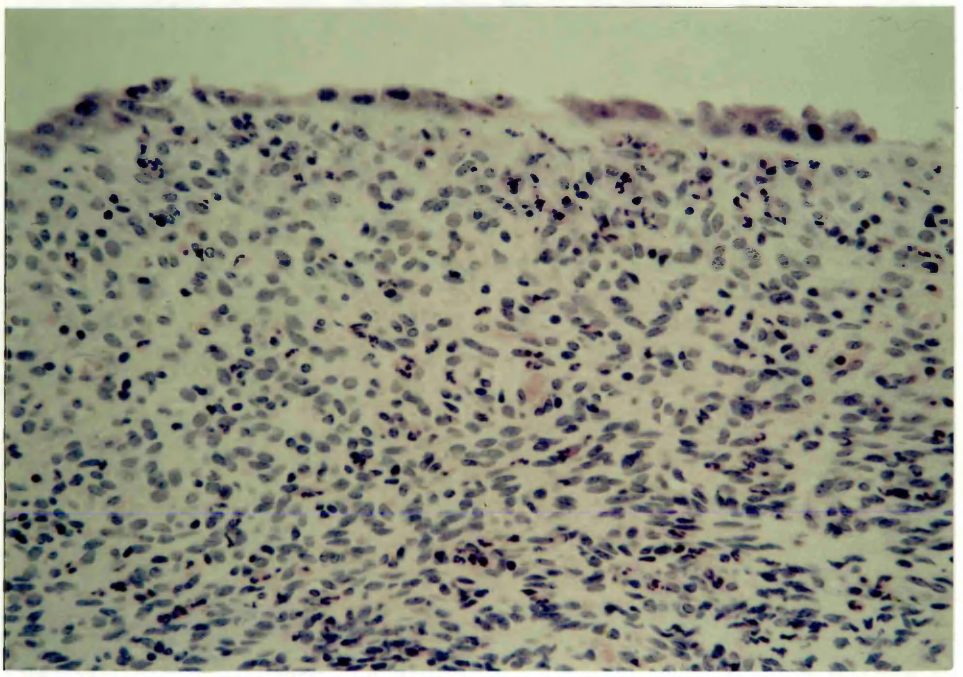


FIG. 44: Endometrium of the right horn Heifer 20. Sub-epithelial caruncular tissue devoid of uterine glands.
H & E x250.

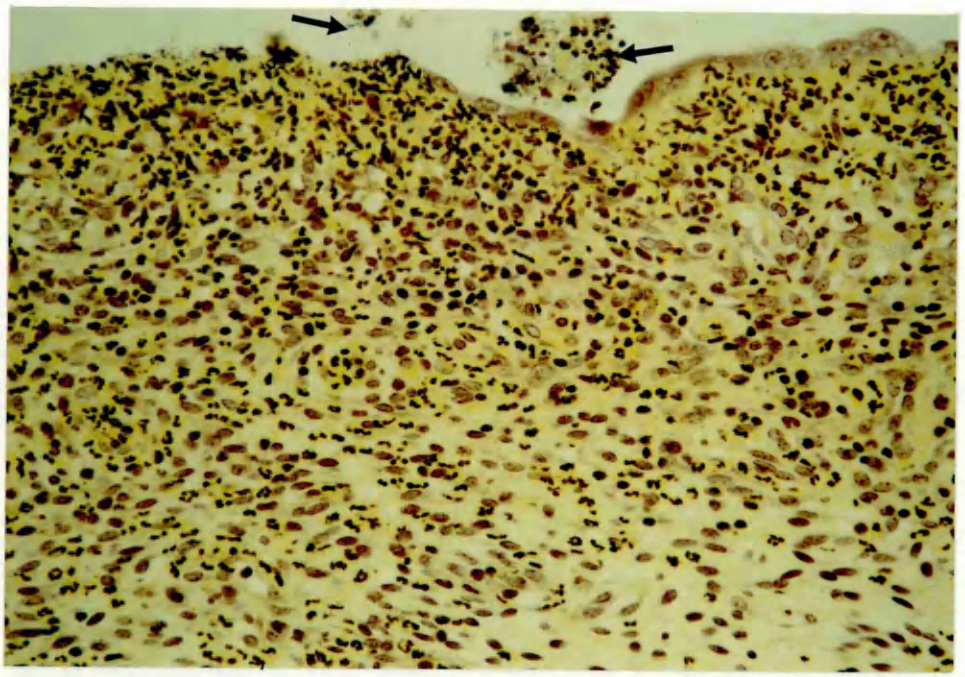


FIG. 45: Endometrium of the right horn of Heifer 20. Gram stained section of uterine lumen with Gram positive rods (arrows) and tissue debris.
H & E x400.

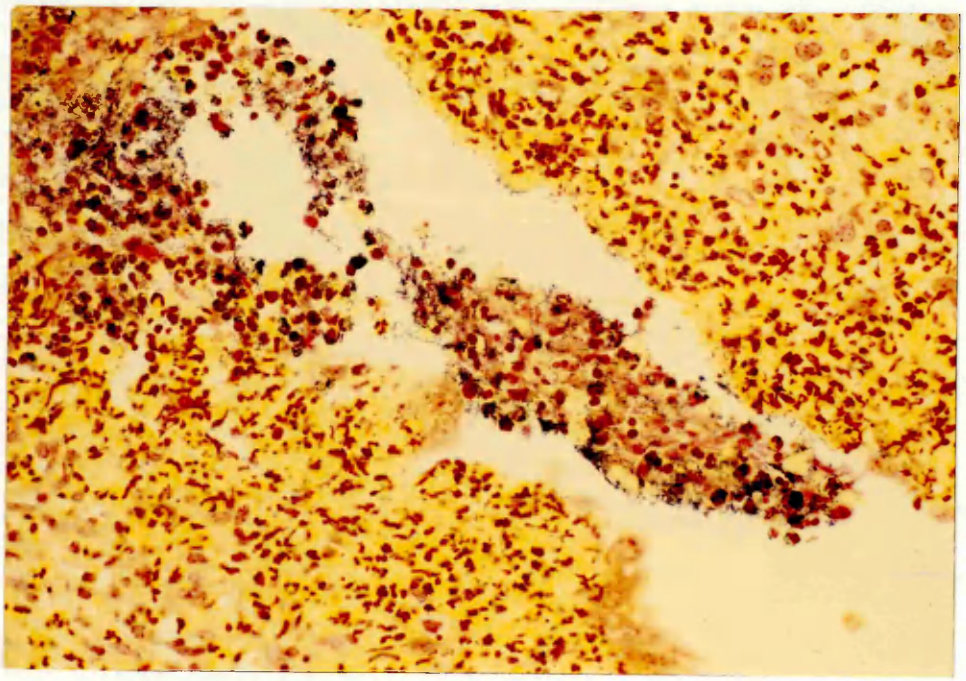
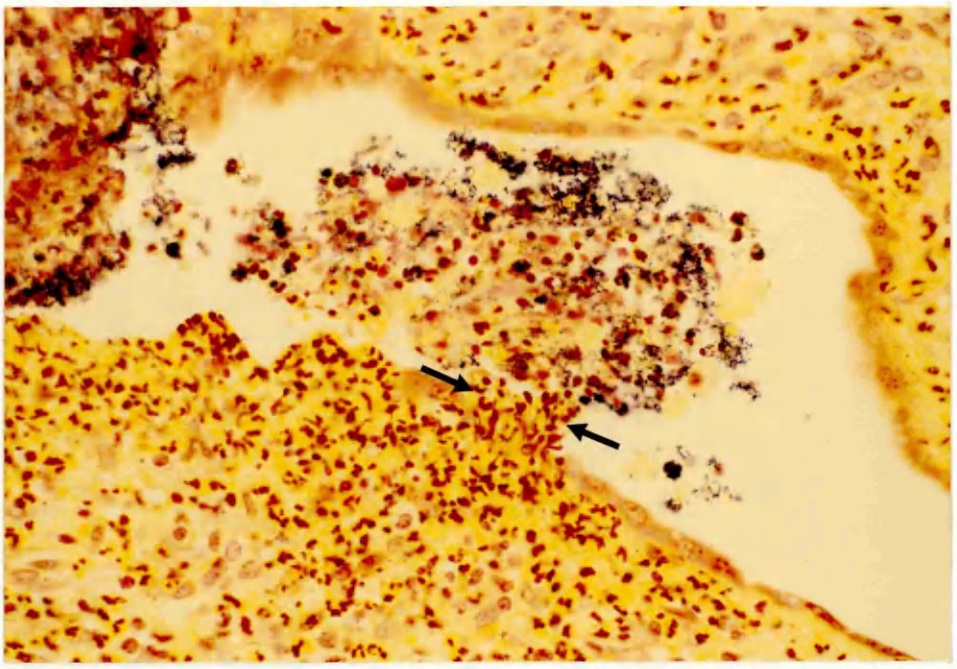


FIG. 46: Endometrium of the right horn of Heifer 20. Gram stained section with epithelium eroded in the uterine fold.
Gram x400.

(a)



(b)

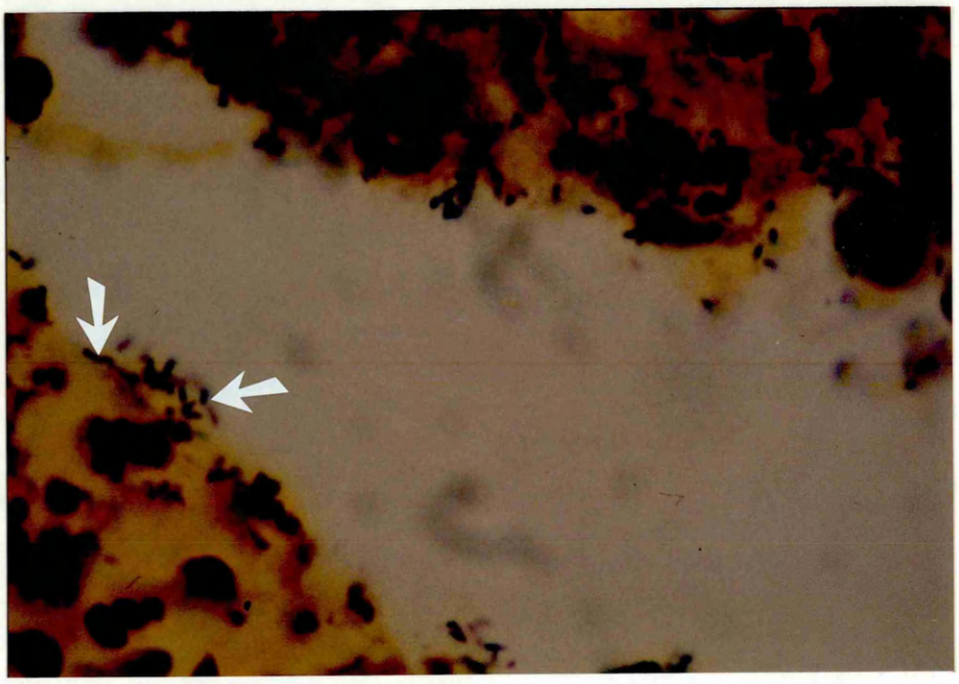


FIG. 47: Endometrium of the right horn of Heifer 20. **(a)** Epithelial erosion (arrows). Gram stain x400. **(b)** Bacterial attachment on the subepithelial tissue (arrows).
Gram x1000.

bacteria were attached to or closely adjacent to the eroded endometrial surface (**Fig. 47b**). In this section the tissue debris in the lumen consisted predominantly of epithelial cells and Gram positive rods resembling *A. pyogenes*.

Left horn. Sections of the uterine wall stained by H. and E (**FIG. 48**) revealed erosion of the epithelium and tissue debris consisting of polymorphs was present in the uterine lumen. Inflammation of the endometrium was present with many polymorphs. There were no glands seen. Fewer bacteria were seen in the tissue debris in the lumen in Gram stained sections than in the right horn. No bacteria were seen attachment to the eroded surface of the endometrium in this horn (**FIG. 49**).

Body of the uterus. In H. and E. stained sections the epithelial surface of the endometrium was extensively eroded with only a few cuboidal cells seen (**FIG. 50**). There was a marked infiltration of the sub-epithelial layer with polymorphs. The sub-epithelial tissue also contained large numbers of uterine glands, branched and mostly coiled distally (**FIG. 51**).
The Gram stained sections resembled the H. and E. sections, no bacterial attachment was evident (**FIG 52**) in those examined from this area.

Embryonic membranes. H. and E. stained sections of the allanto-chorion showed an intact epithelium for long sections of the membranes. The epithelium was predominantly pseudostratified (**FIG. 53**).
The allanto-chorion was almost intact in Gram stained sections with few areas of epithelial erosion (**FIG. 54**). It was very difficult to find *A. pyogenes* in the sections. In one section, however, Gram positive organisms with the morphology of *A. pyogenes* were located within the epithelial cells (**FIG. 55**).

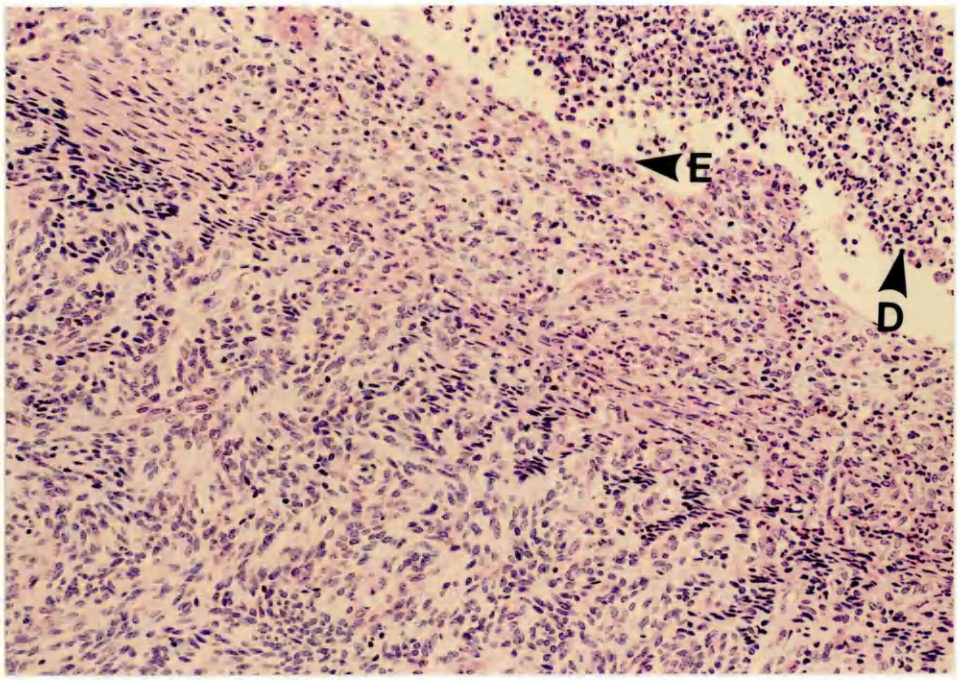


FIG. 48: Endometrium of the left horn of Heifer 20. Caruncular endometrium with less epithelial erosion (E), but tissue debris (D) with polymorphs present in the uterine lumen.
H & E x200.

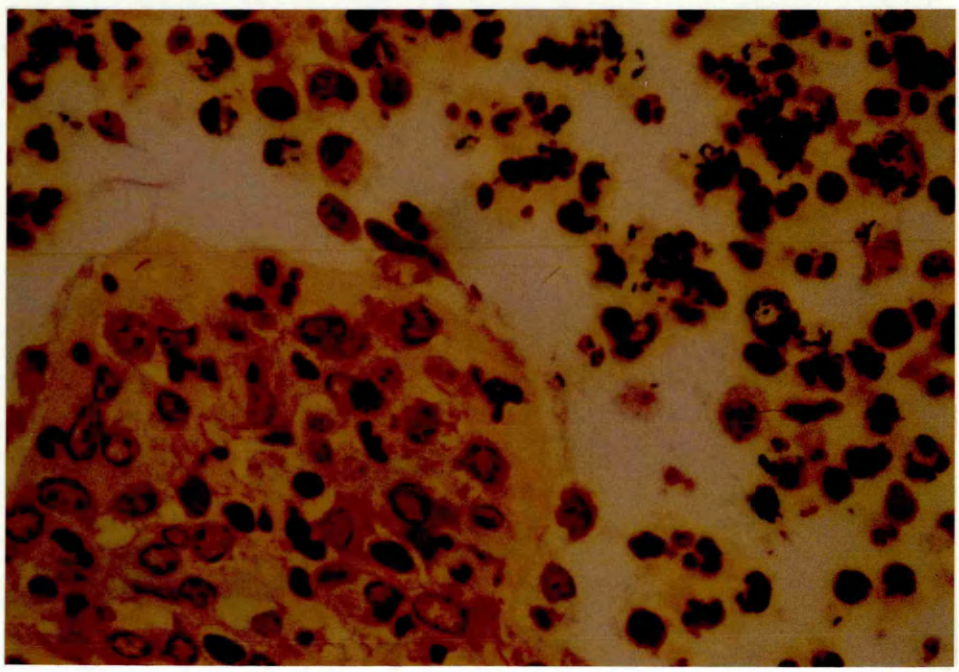


FIG. 49: Endometrium of the left horn of Heifer 20. Gram stained - caruncular endometrium, no *A. pyogenes* attachment is demonstrated.
Gram x500.

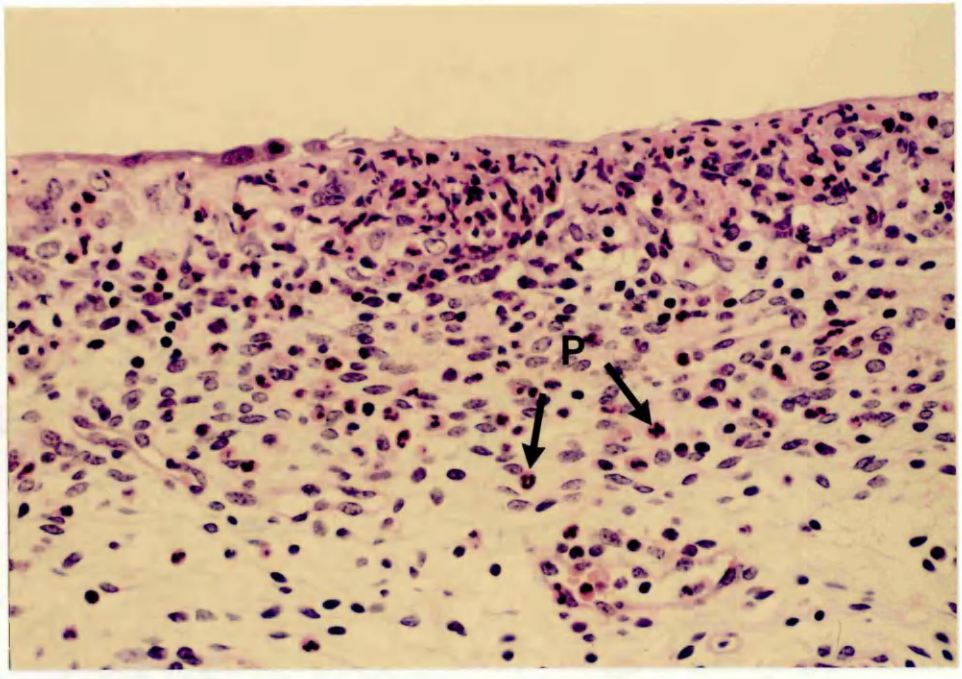


FIG. **50:** Endometrium of the uterine body of Heifer 20. The subepithelial tissue contains fewer polymorphs (P) than in the right horn.
H & E x400.

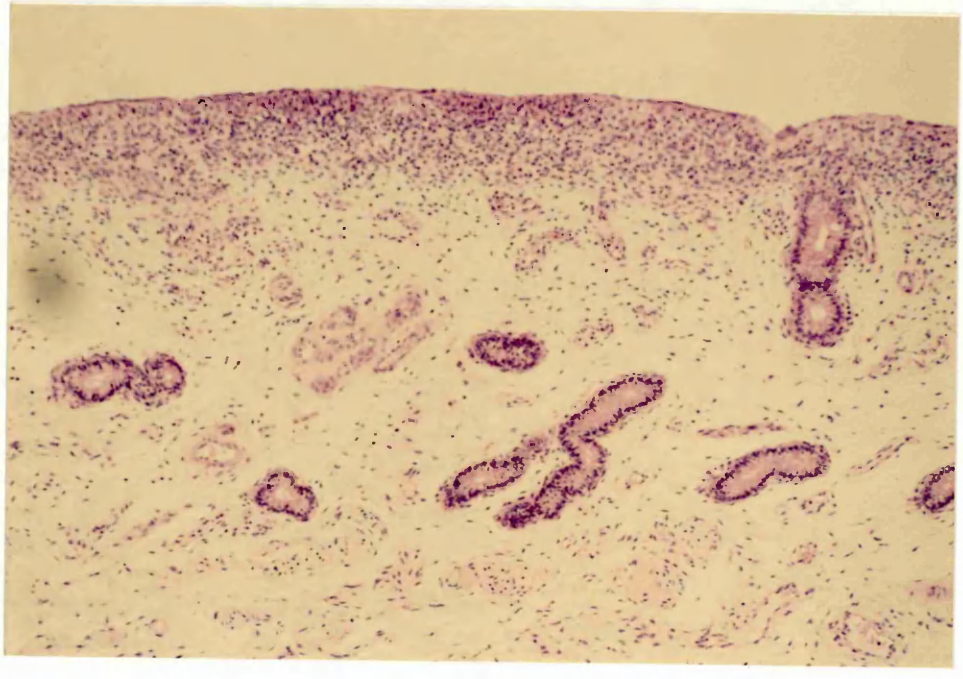


FIG. 51: Endometrium of the uterine body of Heifer 20. Some erosion of the epithelium can be seen and the tubular glands are obvious.
H & E x100.

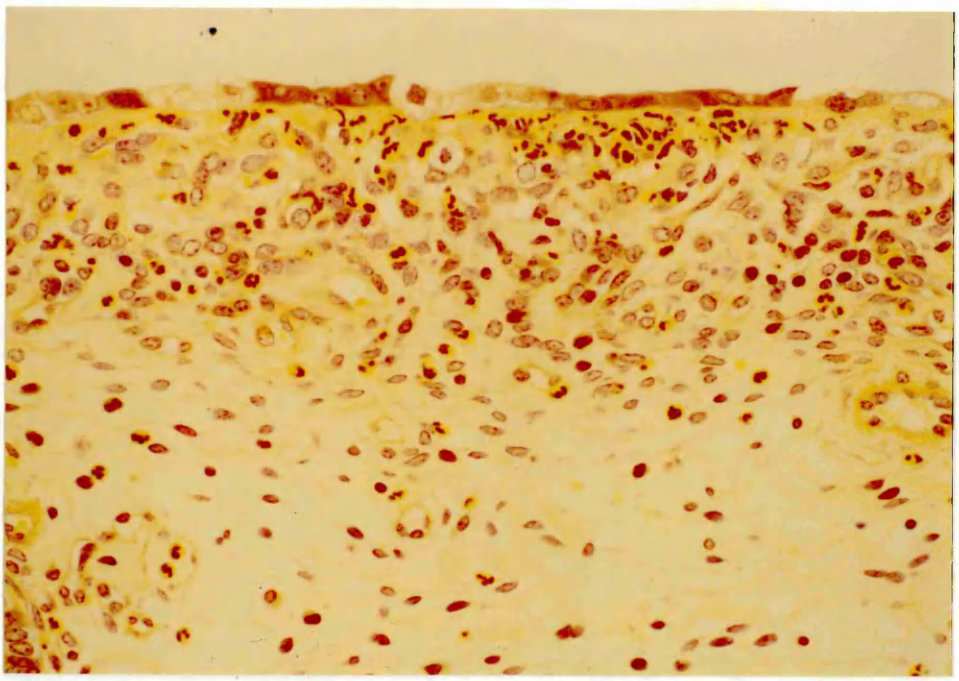


FIG. 52: Gram stained section of the endometrium of the uterine body of Heifer 20. The section contains no Gram positive rods in the lumen and no bacterial attachment to the epithelium.
Gram x400.

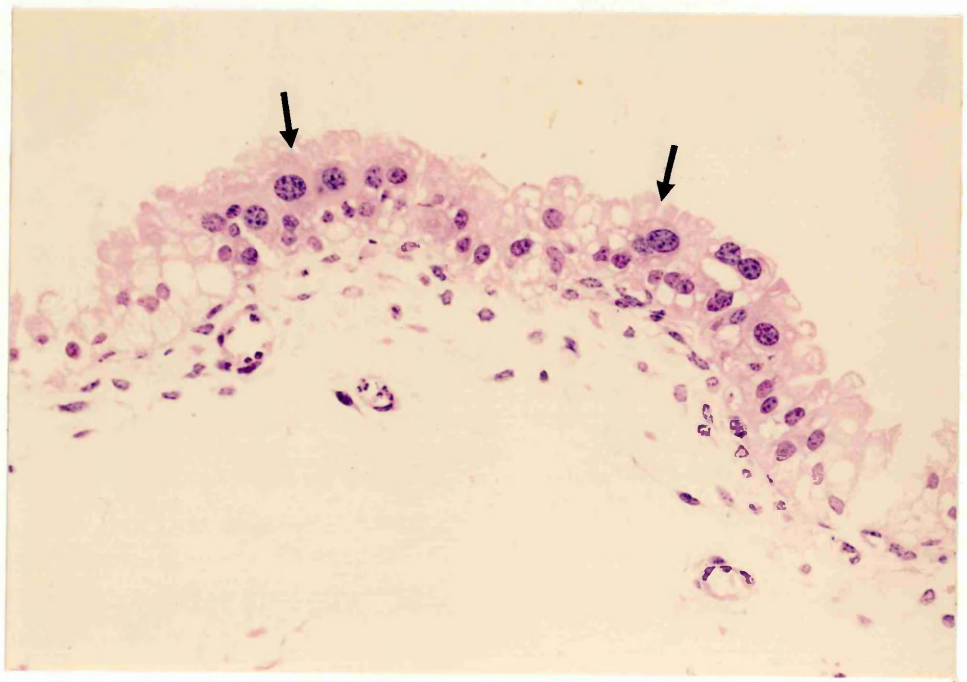


FIG. 53: Allantochorion of the embryo of Heifer 20. The epithelial cells appear intact (arrows).
H & E x400.

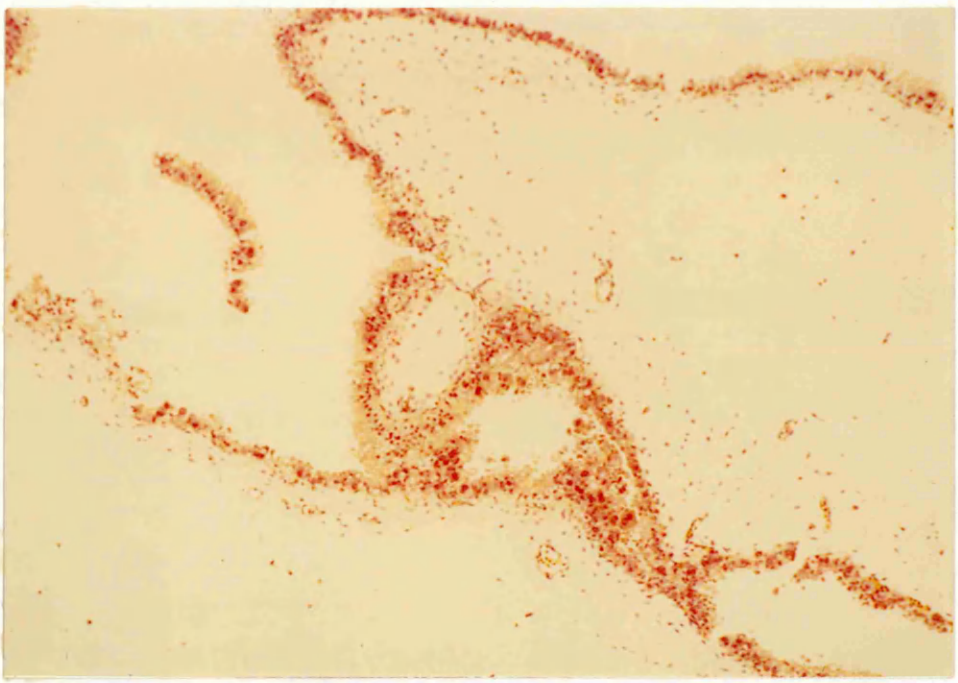


FIG. 54: Gram stained Allantochorion of the embryo of Heifer 20. The epithelial cells are cuboidal, but the epithelium is disintegrating in parts.
Gram x100.

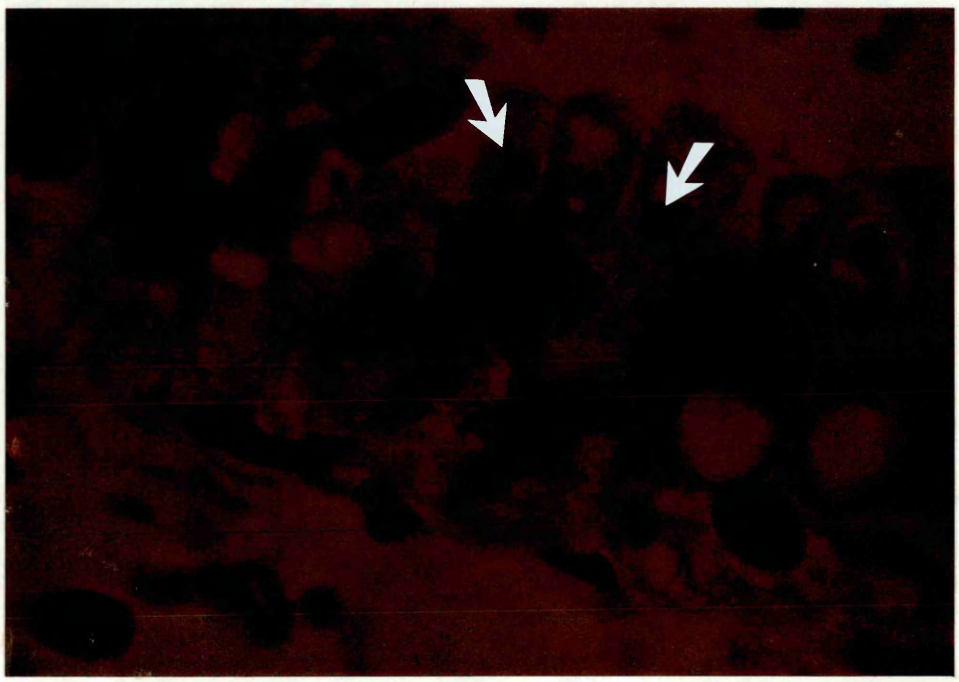


FIG. 55: Gram stained allantochorion of the embryo from Heifer 20. Epithelial cells of the allantochorion containing Gram positive rods (arrows).
Gram x1,000.

(b) The embryo. Post-mortem changes present in several tissues of the embryo. Tissues stained poorly with H. and E. but there were clear signs of tissue degeneration. The neural tube was normal in appearance (**FIG. 56**). There was blood in the lumen of one section of the gut (**FIG. 57**). Similar changes with pooling of blood were present in the kidney tissue (**FIG. 58**). Changes were evident in the mesonephros with areas of blood accumulation in the periphery (**FIG. 59 a and a**). Areas of parenchymal degeneration were present in the liver and there were no intact blood vessels (**FIG. 60**).
No Gram-positive rods or any other bacteria were seen in the Gram stained sections.

Electron microscopy (E. M.).

Scanning E. M. The endometrium of the infected uterus was scanned and a number of findings were made. On the epithelial cells, microvilli were intact in the greater portion of the endometrium, a few cells however were hypertrophied and devoid of microvilli (**FIG 61**). Red blood cells were present on the epithelial surface in some areas. Microvilli on other cells were less dense and rather flattened (**FIG. 62**). Debris and mucus were found to cover the microvilli (**FIG. 63**). Short rods resembling *A. pyogenes* were found to be present in very few areas on epithelial cells of the uterus (**FIG. 64**). Secretion droplets or granules could be seen on cell surfaces (**FIGS 61 and 62**).

Transmission. Completely eroded epithelial cells were seen over the endometrial surface in the tissue debris (**FIG. 65**). Irregular and shortened microvilli on the epithelial surfaces could be seen and parts of the surface were devoid of microvilli (**FIG. 66**). Some epithelial cells were hypertrophied and they contained a large number of mitochondria and some secretory granules (**FIG. 67**). Other epithelial cells contained secretory

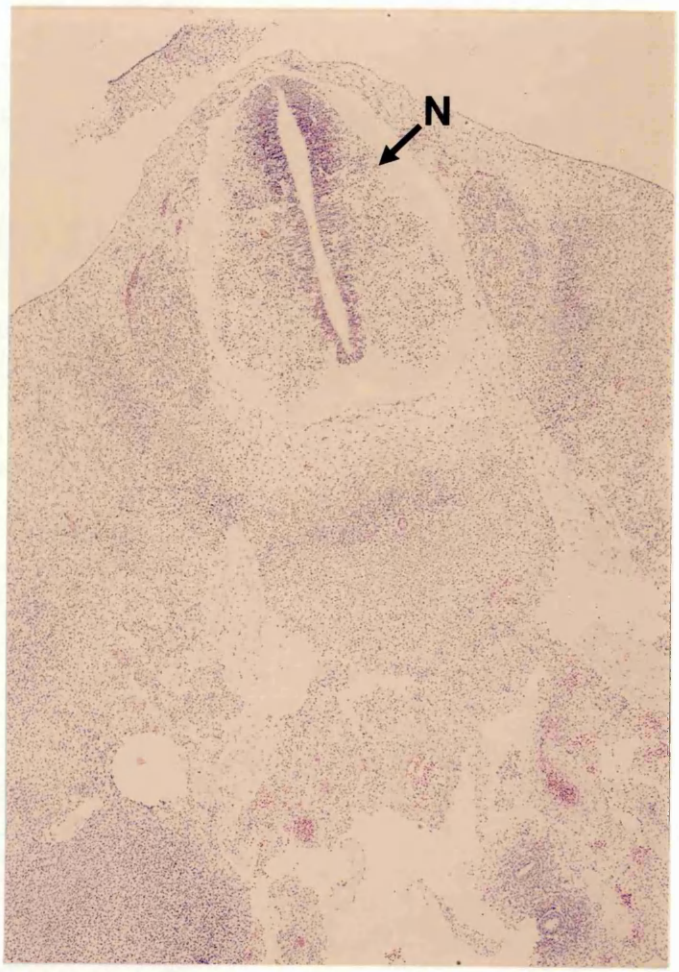


FIG. 56: Neural tube of the embryo of Heifer 20. Neural tube (N) is poorly stained.
H & E x100.

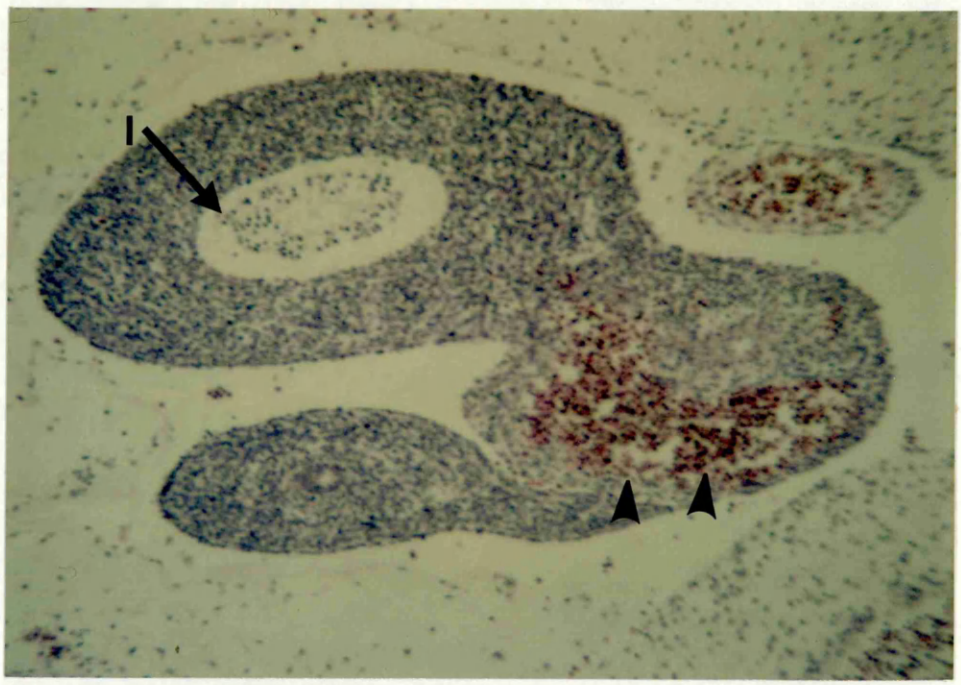


FIG. 57: The gut of the embryo of Heifer 20. The lumen of one section is intact (I) and that of the second half is disintegrated, with blood pooling there in (arrows).
H & E x100.

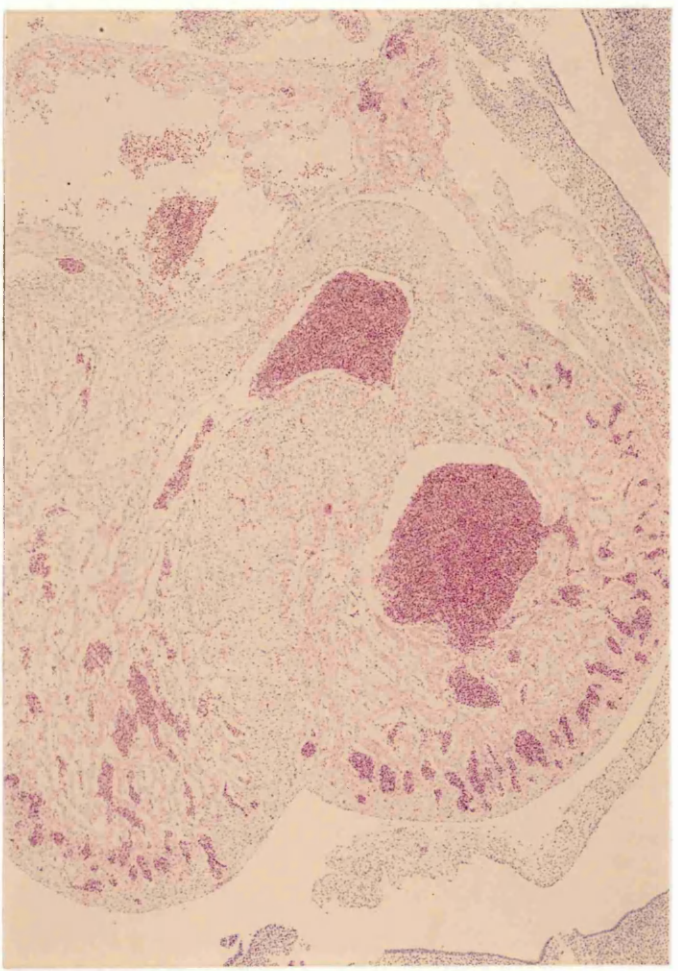
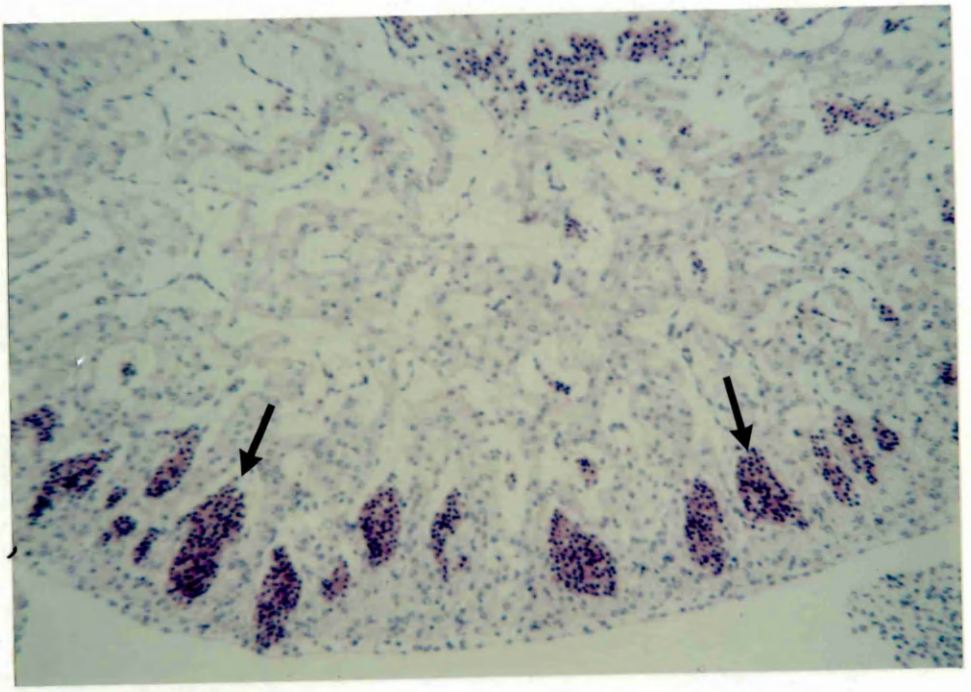


FIG. 58: The kidney of the embryo of Heifer 20. Note the congestion of the nephritic tissue and blood (B) pooling in the cavities.
H & E x100.

(a)



(b)

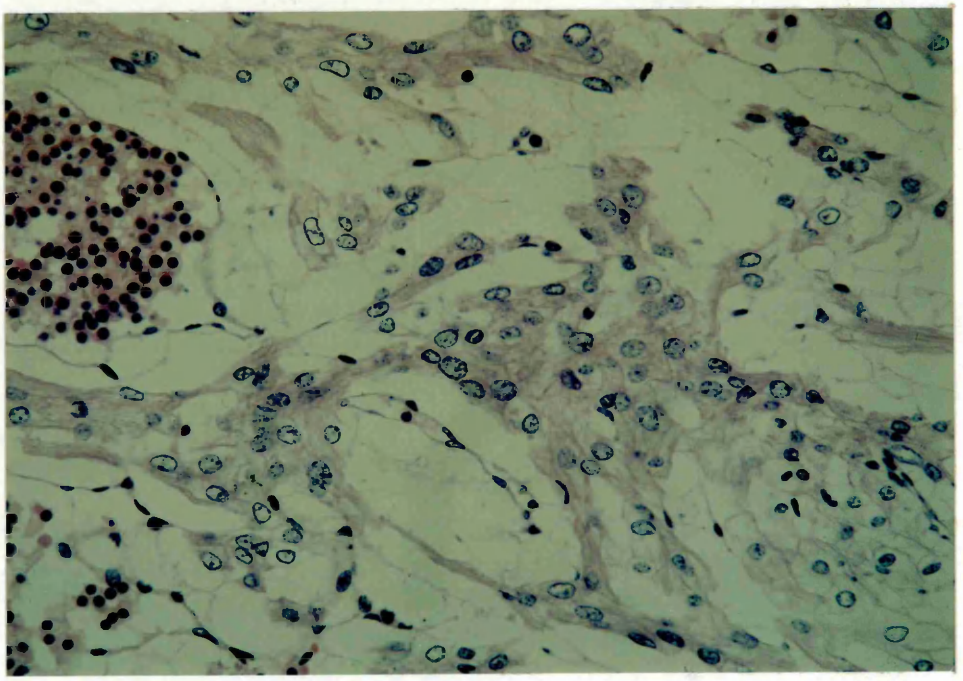
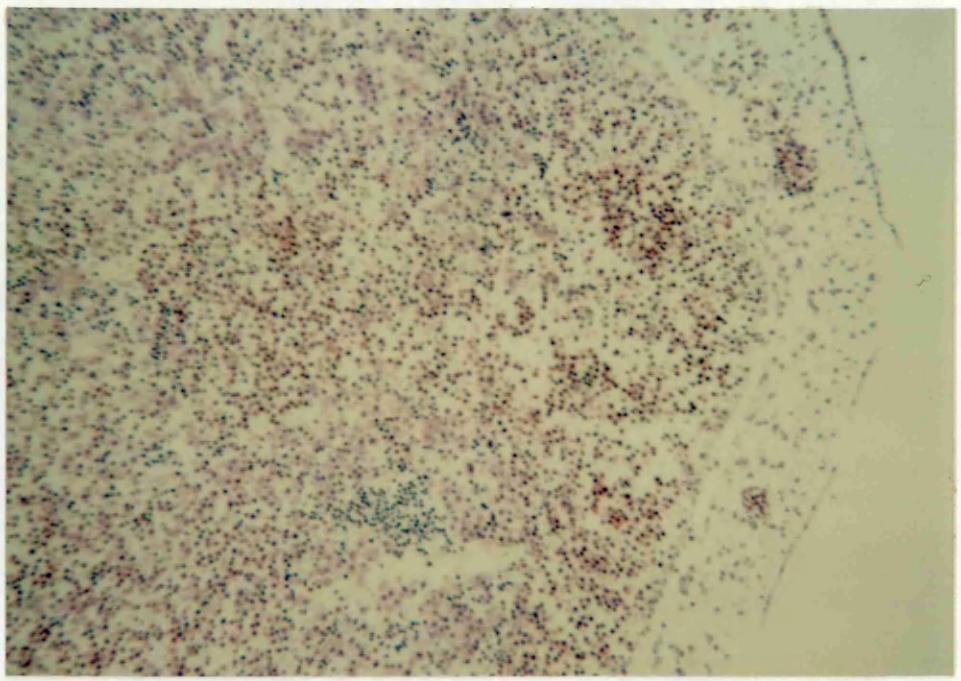


FIG. 59: Kidney of the embryo of Heifer 20. Nephrones of the mesonephros have disintegrated and **(a)** blood (arrows) has pooled in the peripheral tissue (H & E x100) **(b)** A Higher magnification of **a**.
H & E x250.

a)



(b)

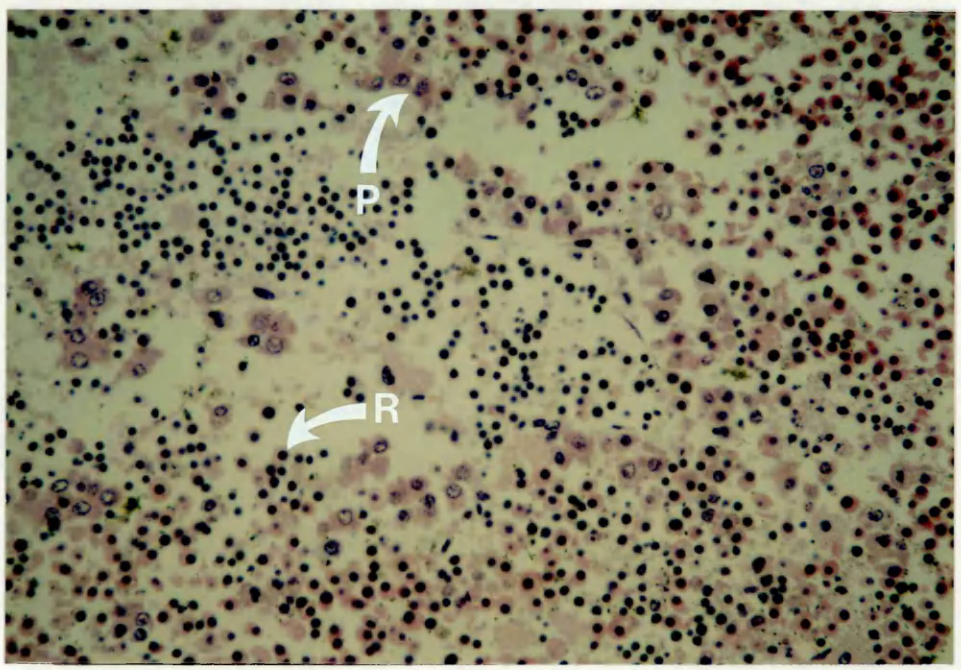


FIG. 60: Liver of the embryo of Heifer 20. **(a)** Low power view of the disintegrating liver parenchymal tissue. H & E x100. **(b)** Higher power view of the liver - red blood cells (R) are seen interspersed with parenchymal cells (P).
H & E x250.

FIG. 61: Scanning E. M. of the endometrial surface of Heifer 20. Epithelial cells (arrows) with microvilli are seen. The red blood cells (R) are probably artefactual.
x1250.

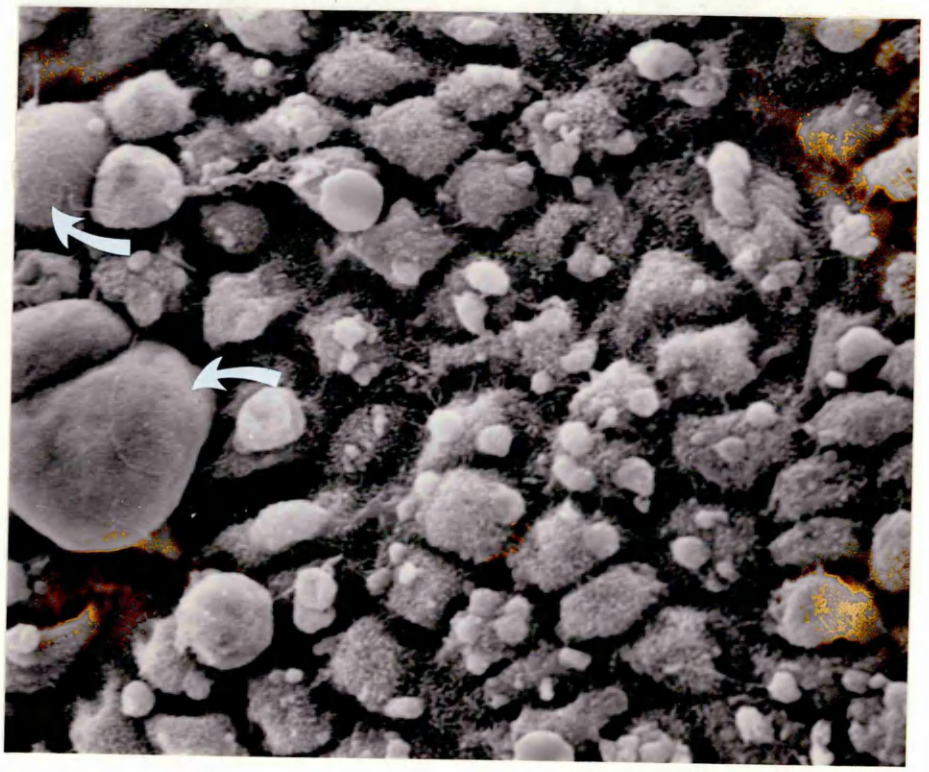


FIG. 62: Scanning E. M. of the endometrial surface of Heifer 20. Epithelial cells with fewer microvilli. Some of the cells are hypertrophied (arrows).
x2500.

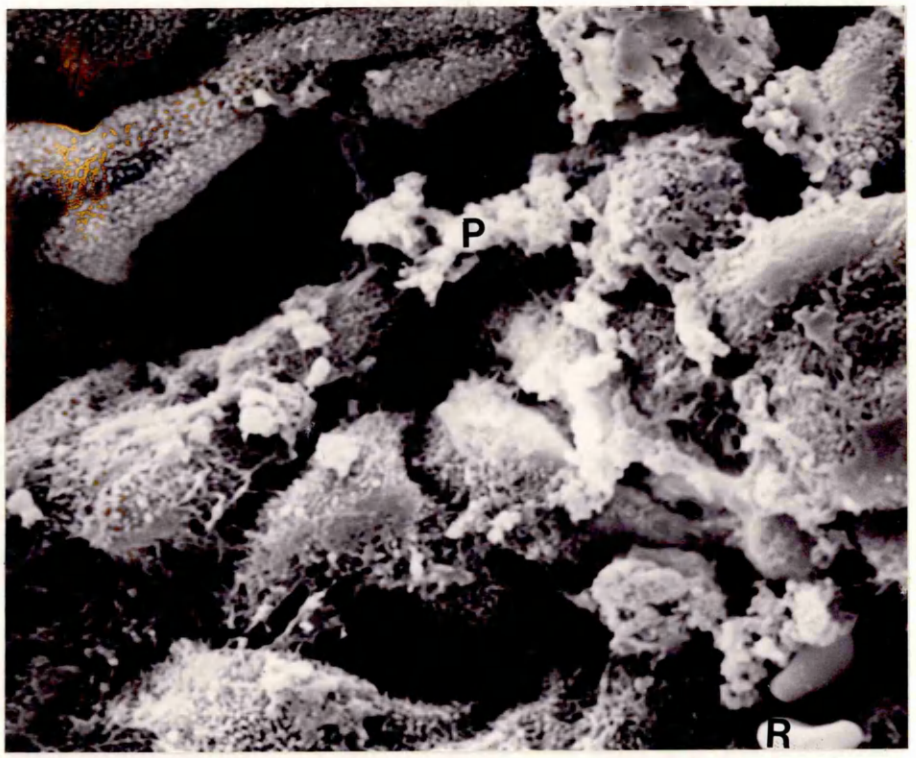


FIG. 63: Scanning electron micrograph of pus on the surface of the endometrium of Heifer 20. Epithelial cells covered with pus (P) and a few red blood cells (R) are present.
x5000.

FIG. 64: High power scanning electron micrograph of the endometrial surface of Heifer 20. Note the presence of organisms like *A. pyogenes* (arrow).
x5000.

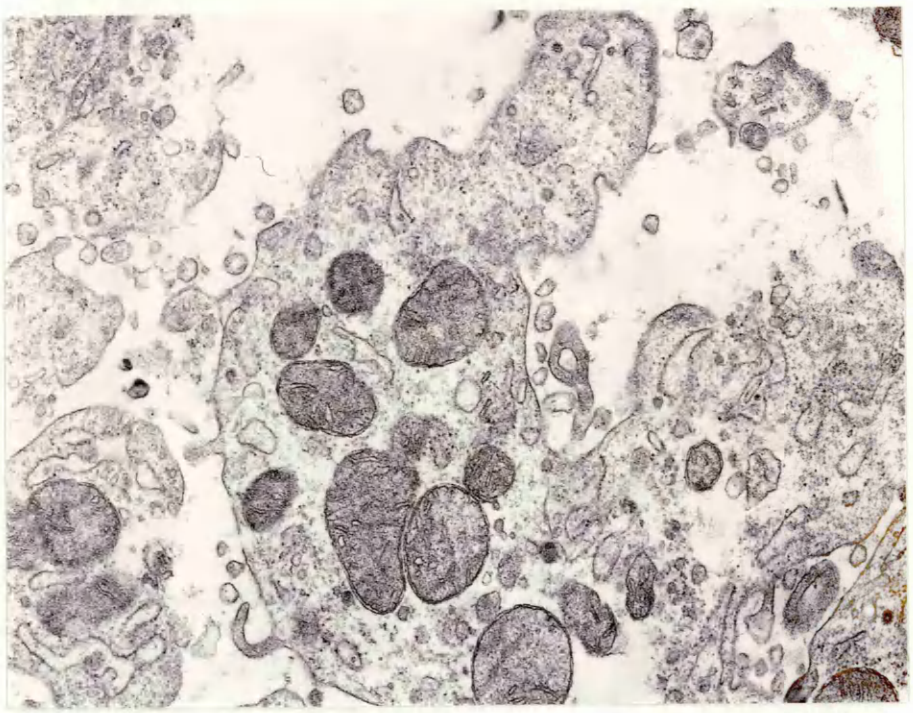


FIG. 65: Transmission E. M. of debris in the uterine lumen of Heifer 20. Tissue debris with disintegrated epithelial cells.
x1400.

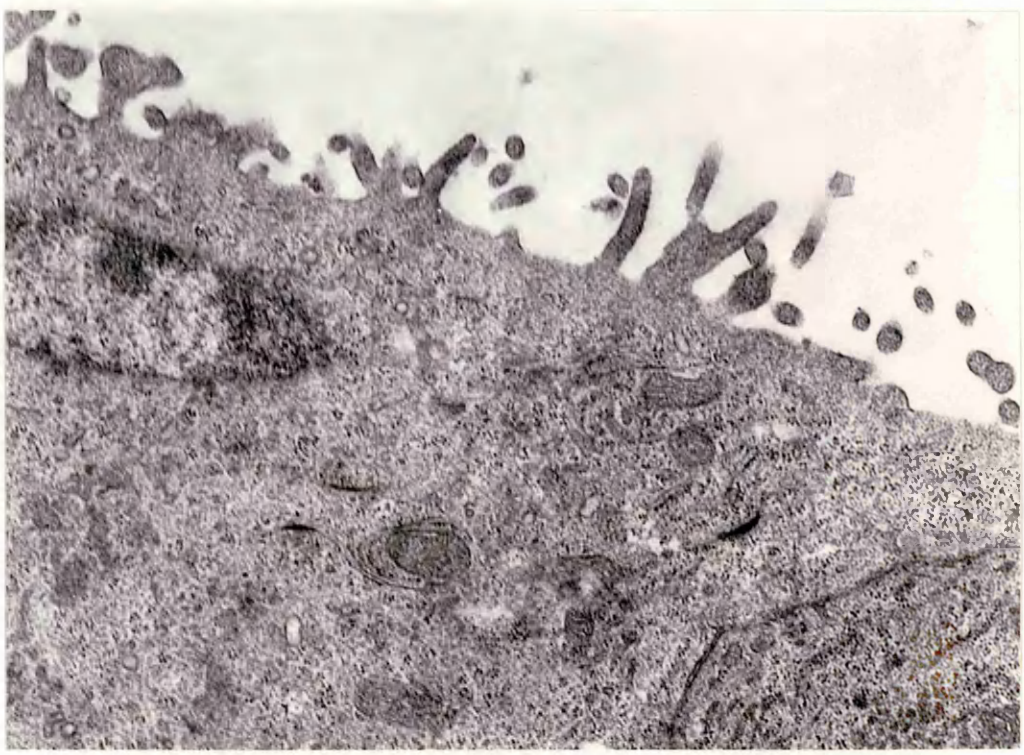


FIG. 66: Transmission E. M. of the uterine epithelium of Heifer 20. Note sparse and irregular microvilli.
x5000.

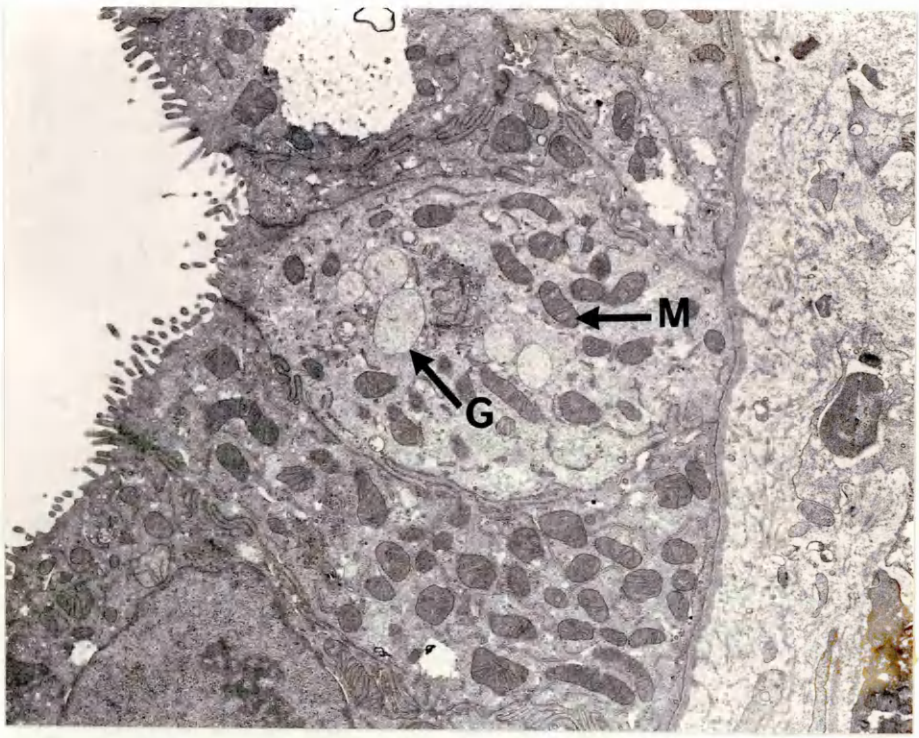


FIG. 67: Transmission E. M. of the uterine epithelium of Heifer 20. Hypertrophied epithelial cells with large numbers of mitochondria (M) and some secretory granules (G) can be seen.
x5000.

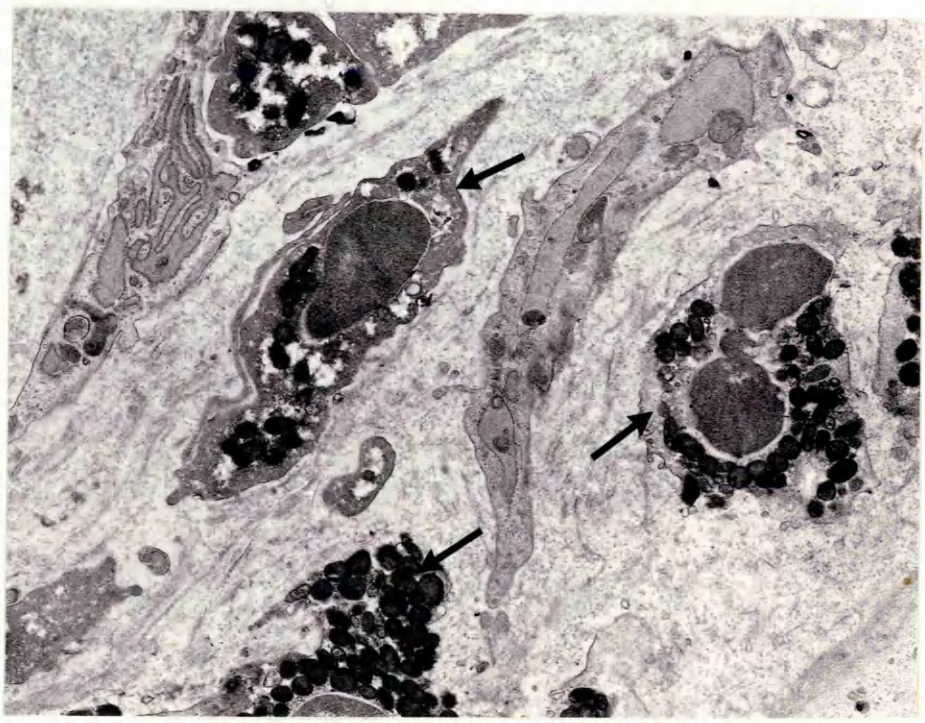


FIG. 68: Transmission E. M. of uterine lamina propria of Heifer 20. Sub epithelial layer with neutrophils (N) but no bacteria.
x5000).

granules and the sub-epithelial layer contained a large number of neutrophils and secretory granules (**FIG 68**). No bacteria were seen in the subepithelial layer.

Slaughter house pregnant uterus.

Control tests for haemolysis were carried out on fluids from the control tract with sheep and horse blood cells were negative. No *A. pyogenes* was isolated.

EXPERIMENT III.

Inoculation with killed bacteria. The findings in the two animals inoculated with the killed bacteria differed. One of the cows (15) maintained a normal pregnancy up to 50 days when cloprostenol was administered to terminate pregnancy. There was no evidence of membrane separation or other signs of pending abortion throughout the period of observation (Appendix TABLE B).

Cow 16 was found to have a normal temperature and normal appetite during the period of examination. It was observed, however, that while using ultrasound, the animal had evidence of cystitis before experimentation. Urine passed by the animal was cloudy. Direct smears made after centrifugation of the urine revealed the presence of streptococci which were found to be haemolytic on culture. No treatment was given and inoculation was carried out as described above. Pregnancy was maintained until 138 hours after inoculation, when the fluid appeared echogenic on ultrasound and membrane detachment became evident. At this point the embryo was still alive. Abortion occurred about 144 hours after inoculation. The animal came into heat 24 hours after abortion (Appendix TABLE B).

Whitish discharge was seen from the vagina at abortion. No bacterial growth was obtained on culture and direct smears revealed abundant white

blood cells especially neutrophils and monocytes but no bacteria. The embryo from aborted cow 16 was intact within its membranes and appeared fresh with no internal haemorrhage.

EXPERIMENT IV.

Bacterial toxin. The two animals (17 and 18) that were inoculated with *A. pyogenes* toxin at 27 and 34 days of pregnancy failed to abort or to show clear signs of pending abortion. Abortion was induced using cloprostenol treatment at 40 and 50 days of pregnancy respectively (Appendix TABLE B). Direct smears and culture from the of aborted material at this stage did not reveal the presence of *A. pyogenes* or any other significant bacteria.

EXPERIMENT V.

The detection of serum antibody to *A. pyogenes*. Assay I. There was an increase in the absorbence (binding) from about 0.5 (at 1/10 dilution) for the first 3 days of infection and this had increased to about 0.78 (at 1/10) 10 days after infection. A similar increase in absorbence was evident at other dilutions 1/30, 1/100, 1/300, 1/1,000, 1/3,000, 1/10,000 (**FIGS 69 and 70**). The dilution of 1/100 was found to be the have least background interference and this was used as the starting dilution in the subsequent assay. Results for animals examined in the second assay are given in **Table 7.**

Animals (1, 2, 4, and 5) which were infected with live *A. pyogenes*. No antibody was detectable at a titre of 1:100 on day 0. By day 5 after infection all the animals apart from cow 2, were positive at a titre of 1:100. From Day 10 to Days 20 or 25 all animals showed positive reaction to the infection. Cow 3 showed a positive reaction (at 1:100) on the day of infection and this

FIG. 69: Antibody response to *A. pyogenes* detected by ELISA between days 0 and 8 after infection in Cow 4.

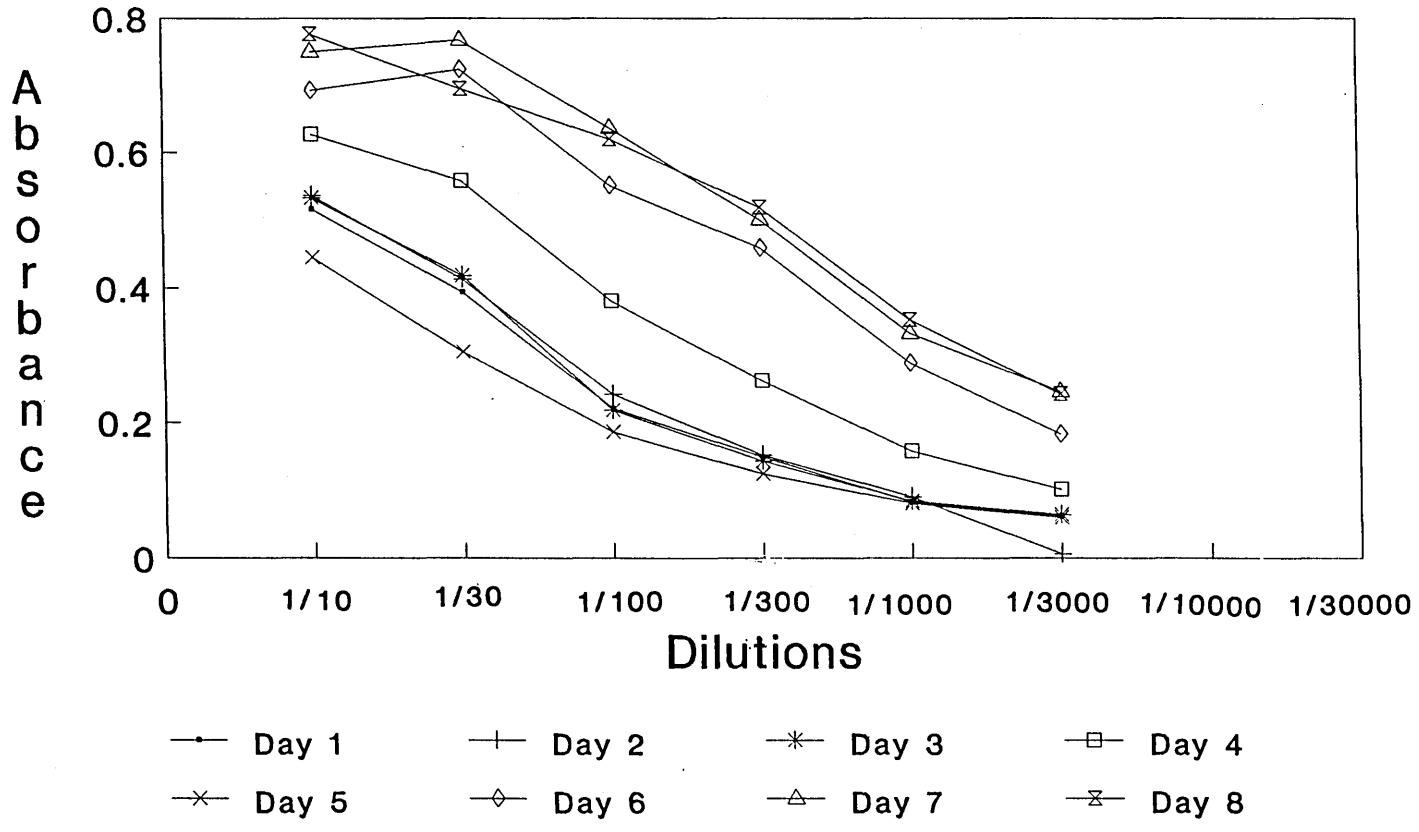


FIG. 70: Antibody response to *A.pyogenes* infection detected by ELISA on days 9 - 10 after infection in Cow 4.

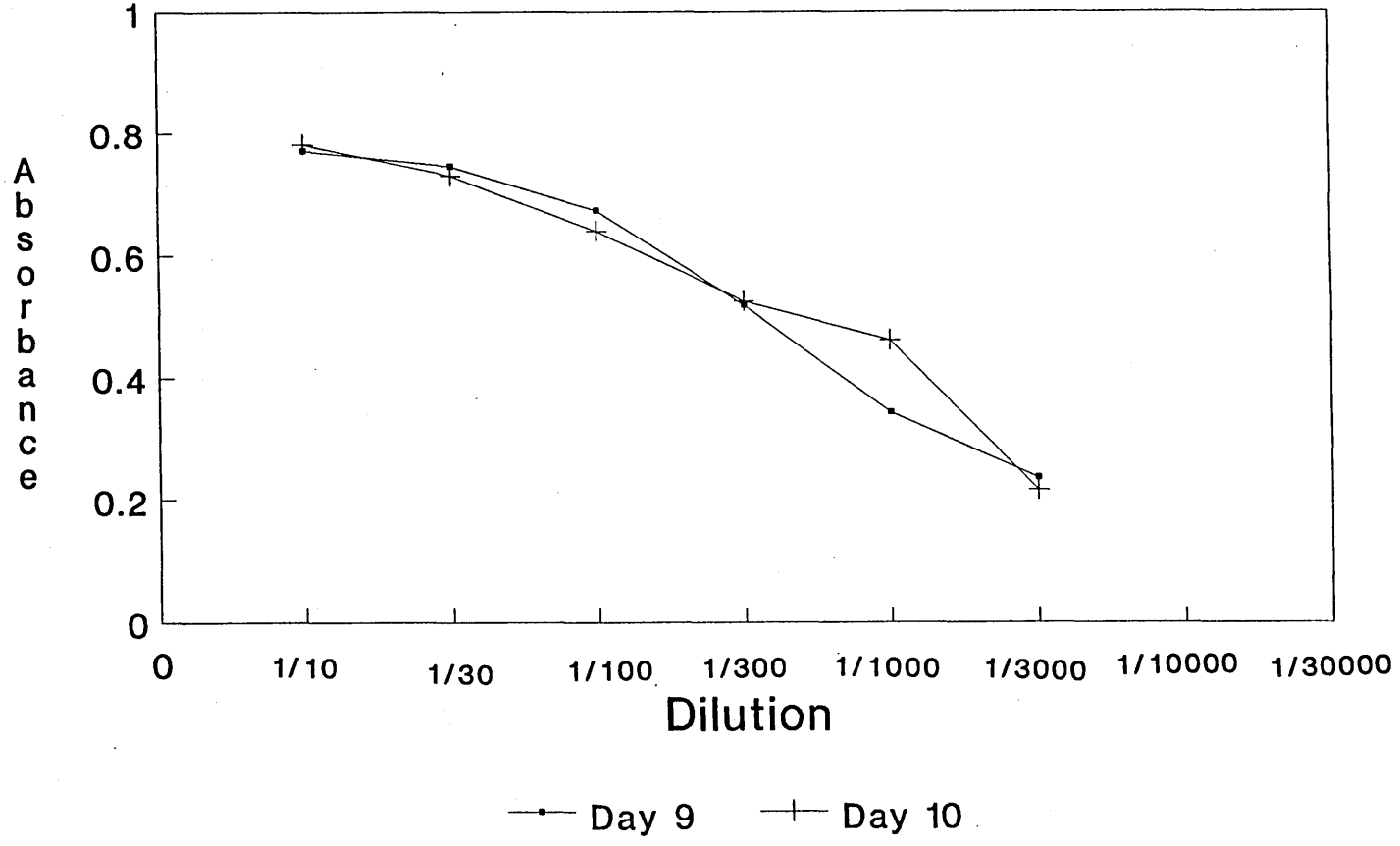


Table 7.
A. pyogenes antibody levels determined by ELISA.

No	Type of Animal	Days 0	5	after 10	15	20	infection 25
1	Cow	- <1:100	+ 1:100	++ 1:100	ND	++ 1:100	ND
2	Cow	- <1:100	- <1:100	+ 1:100	+++ >1:1000	++ >1:1000	ND
3	Cow	++ >1:1000	++ >1:1000	++ >1:1000	+++ >1:1000	+++ >1:1000	++ **>1:1000**
4	Cow	- <1:100	++ >1:1000	+++ >1:1000	+++ >1:1000	++ >1:1000	++ **>1:1000**
5	Heifer	- <1:100	+ 1:1000	+++ >1:1000	++ >1:1000	ND	++ **>1:1000**
17	Heifer	- <1:100	+ 1:100	++ >1:1000	+/- 1:100	ND	ND
18	Heifer	+/- 1:100	+ >1:1000	+ >1:1000	- <1:100	ND	ND
22	Cow	ND	+ >1:1000	+ >1:1000	++ >1:1000	ND	ND
23	Heifer	-2/2 (mean) <1:100					
24	♂	-4/4(mean) <1:100					

Absorbance at 1:100, less than cut off point <1:100 or >1:1000 absorbance above the cut off point. ND = Not done. ♂ = 3 months' old male calf.

was followed by an increase in absorbance by day 25 as was seen in the other infections (at 1:100).

A titre of 1:1000 was found in the serum of cow 22 5 days after infection, but infection did not establish in this cow and pregnancy continued until termination with cloprostenol. There was no significant increase in the level of antibody after infection.

Heifers 17 and 18 inoculated with *A. pyogenes* toxin did not abort after the treatment but developed a positive titre of 1:100 five days after. The animals exhibited a transient rise in antibody titre between days 5 and 15 after inoculation. Heifer 17 had a doubtful response by day 15 but Heifer 18 was clearly negative by that time (**Table 7**)

Heifer 23 and the 3 months old male calf (no. 24) were not infected and their results were negative at titre of 1:100 on examination.

EXPERIMENT VI

Haptoglobin (Hp). In cow 1, examination began 28 days before intrauterine infection which took place at 35 days of pregnancy. During this period the Hp concentration was between 0 and 15 mg/100 ml. At the day of infection the concentration was 10 mg/100 ml, but this had increased to >150 mg/100 ml 72 hours later and embryonic death and abortion occurred 22 and 29 hours after infection respectively (**FIG. 71**). The concentration was maintained at >150 mg/100 ml for 8 days after that, after which it declined for the next 10 days when the concentration varied between 54 and 150 mg/100 ml. At the end of this period the animal came into oestrus, with a steady decline of the Hp concentration to pre-infection levels 8 days after oestrus. Antimicrobial treatment in this cow began 6 days after

abortion.

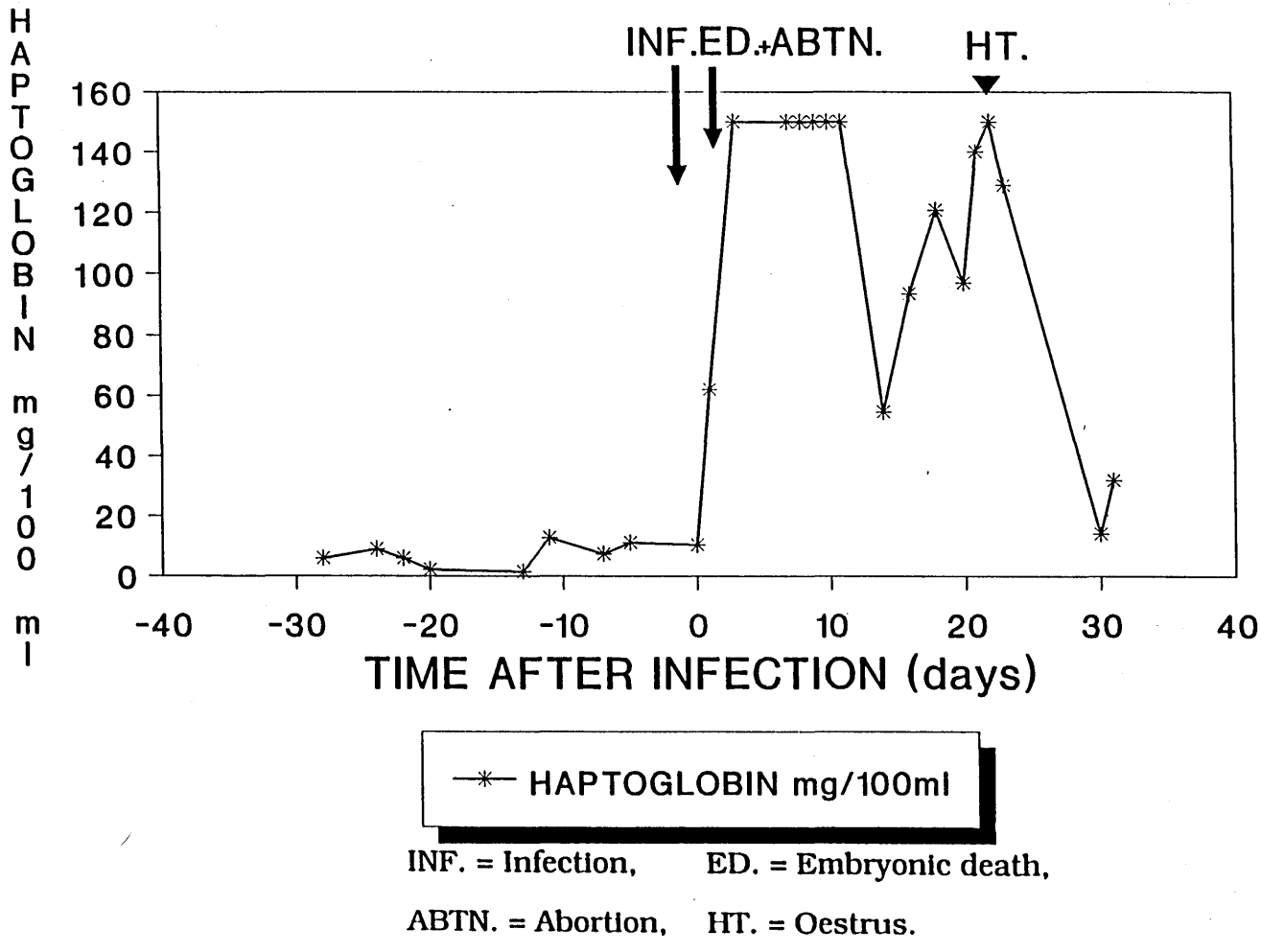
Examinations in cow 4 started before bacterial infection. At infection the concentration was 9 mg/100 ml and it had reached 101 mg/ml 32 hours later and 150 mg/100 ml 48 hours after infection but embryonic death occurred 96 hours infection and abortion 48 hours later. The maximum concentration of >150 mg/100 ml was maintained for 5 days after which it started to decline (**FIG. 72**). The decline was consistent and reached pre-infection concentrations in the next 5 days. This was followed by a temporary rise to 50 mg/ 100 ml and then a second decline to pre infection levels in 7 days. The animal came into oestrus at the end of this 7 day period (20 days after abortion). The pre infection concentration was maintained for 10 days following oestrus. Antimicrobial treatment in the cow started 10 days after abortion.

DISCUSSION.

The studies described in this chapter were intended to determine the possible mechanisms by which *A. pyogenes* causes embryonic death. All the studies involved small numbers of animals and for this reason a number of areas still require further investigation. This discussion evaluates the technical features of the experiments and their validity. The significance of the changes found will be related to the literature in the general discussion in Chapter VIII.

The first experiment in which two infected heifers were killed (Experiment II) represented an opportunity to study the relationship between the cow, the embryo and the organism. Both animals were inoculated and the embryos observed by ultrasound. That in Heifer 19 was still alive but that in Heifer

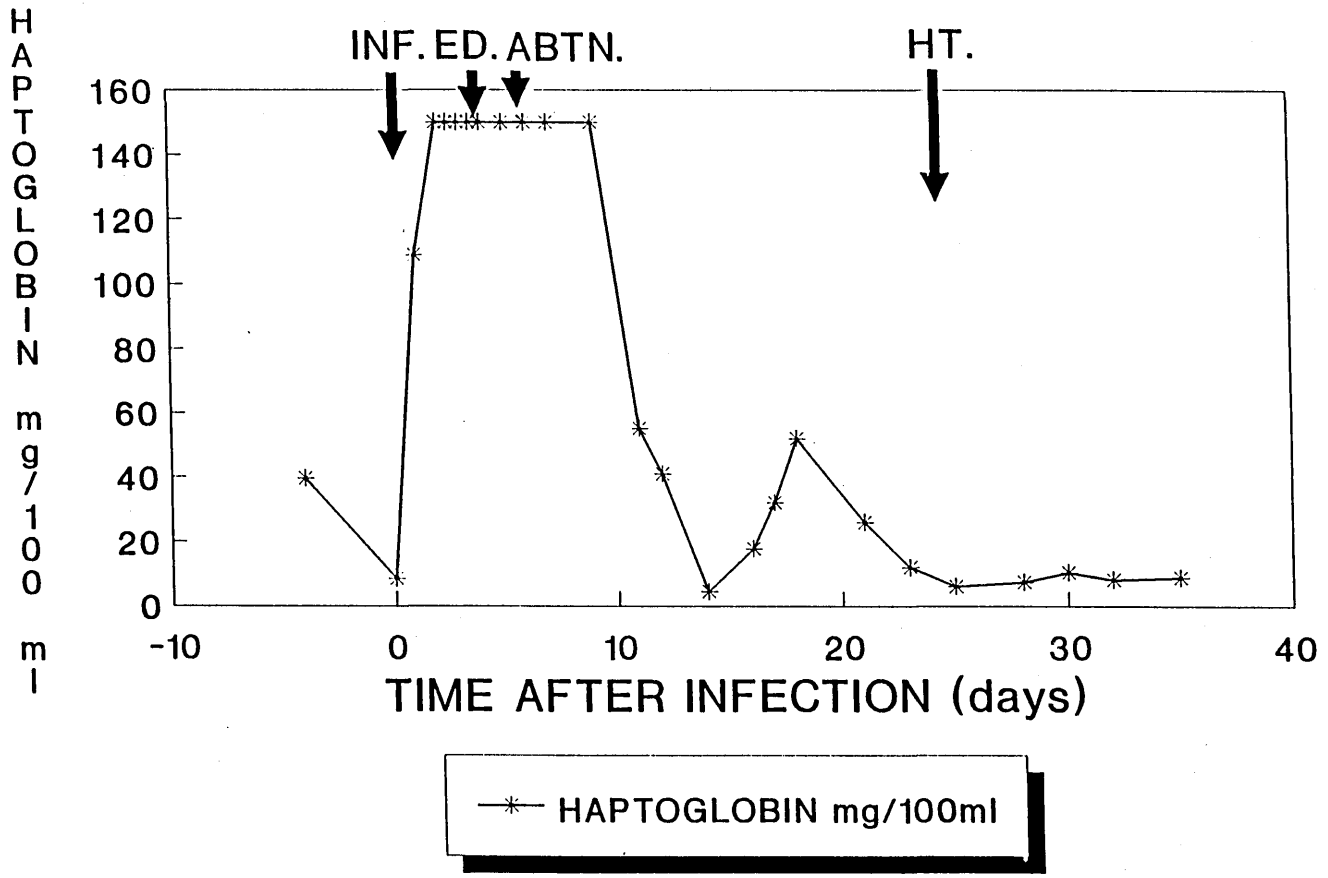
FIG. 71: Haptoglobin (Hp) concentration in Cow 1 before infection, during infection and after abortion.



INF. = Infection, ED. = Embryonic death,

ABTN. = Abortion, HT. = Oestrus.

FIG. 72: Haptoglobin (Hp) concentration at infection and after bacterial induced abortion in Cow 2.



INF. = Infection, ED. = Embryonic death,

ABTN. = Abortion, HT. = Oestrus.

20 was dead after slaughter. It had last been seen alive 30 minutes before slaughter. It is therefore reasonable to assume that the changes seen in that embryo had occurred at or around the time of embryonic death. As Heifer 19 was killed 18 hours after inoculation and Heifer 20 24 hours after inoculation, the changes seen can be considered an accurate reflection of those seen in the early stages of the disease. No clinical abnormalities were detected.

In view of this, the lack of any significant pathological changes and the absence of *A. pyogenes* from the lungs, liver, spleen and kidneys suggests that the bacterium were localised in the uterus. There did not seem to be any immediate effect on the corpus luteum resulting from the presence of the bacterium, granted the time of exposure was short. Finding of a live embryo on opening the uterus in Heifer 19, allowed the changes accompanying embryonic death in Heifer 20 to be interpreted more accurately. The control uterus provided further information about normal embryos. In Heifer 19 it was clear that by day 36 of pregnancy there is a firm relationship between the allanto-chorion and the endometrium. This firm apposition had been destroyed 24 hours after infection by the accumulating purulent uterine fluid in Heifer 20.

Hyperaemia was found at the site of injection and could be due to the inflammation as a result of injection. Injection in both animals alone is not considered to be important in the pathogenesis of abortion since the introduction of saline (Chapter III) did not affect pregnancy. The hyperaemia of mucosa seen particular in Heifer 20 was due to inflammation. This was confirmed histologically for Heifer 20 but was less clear in Heifer 19. The thickening of the endometrium seen in Heifer 20 was due to inflammatory change and the presence of the organism within the lumen

and attached to the eroded surface suggests that inflammation was chiefly due to infection with *A. pyogenes*.

The volume of uterine fluid in Heifer 20 was greater than that in Heifer 19 or the control. This increased volume could be the result of inflammatory change alone or come partly from increased endometrial secretion. It is possible that this increase in fluid resulted in the loss of the allantochorial - endometrial apposition but this is discussed further below.

The extent of the changes differed in the two animals. In Heifer 20 inflammation was present in the left horn but was less than in the right horn suggesting that the infection was spreading slowly. Absence of any gross lesions in the cervix and the vagina confirms the spreading nature of infection, and underlines the fact that inflammation of the cervix is not necessary for embryonic death. In the animals infected in Chapter III the cervix eventually opened and the pus was found in the vagina suggesting that these were later changes.

Isolation of *A. pyogenes* from all sections of the uterus simplex showed that given time infection spreads to the entire organ. As such, isolation of the bacterium from any part of the tract may give an indication to the state of infection of the whole organ. *A. pyogenes* was only present in the allantoic and amniotic fluids in the embry of Heifer 19 and its presence could be due to penetration of the organism through the membranes or contamination by the needle. The numbers of the colonies however were too small for any firm conclusion to be drawn. The severely affected embryo of Heifer 20 yielded no *A. pyogenes* suggesting that its presence within the embryo was not essential for embryonic death.

The insignificant numbers of *A. pyogenes* isolated from Heifer 19 could be due to failure of the organisms to multiply in the short time of incubation between inoculation and killing.

The demonstration of **haemolysin** in both the allantoic and amniotic fluids in Heifer 19, may indicate that the toxin may affect the embryo after crossing the membrane barrier. The failure to detect any haemolytic activity in Heifer 20 should lead to caution as the two results are contradictory. It is however also possible that, failure to demonstrate haemolytic activity in Heifer 20, was due to the very small volume of amniotic fluid available. Failure to detect any haemolytic activity in the allantoic and amniotic fluids from the slaughter house material, confirmed that normal fluids do not cause haemolysis. The haemolytic effect seen in Heifer 19 was therefore most probably due to the presence of *A. pyogenes* toxin.

Histological changes in the endometrium showed that erosion of the epithelium is one of the results of bacterial infection and may contribute to embryonic death. It is possible that during fixation of the tissues some of the evidence of infection was lost as only small amounts of uterine contents were present (**FIGS 45, 46 and 47**). This is thought to be the case because the contents were seen only in the uterine folds and glandular ducts. Accumulation of inflammatory cells, bacteria and tissue debris in the lumen confirmed that the inflammatory changes seen in the lamina popria were the result of bacterial infection. Increased capillary dilatation especially in the lamina propria could also be due to infection. The large amount of glandular tissue in the sub-epithelial tissue could account for the increased fluid in the uterine lumen.

The presence of *A. pyogenes* was confirmed in the Gram stained sections.

The bacteria were best studied in the uterine folds or glandular ducts where uterine contents had remained attached during tissue processing and fixation. There was no bacterial attachment seen on intact epithelial cells but bacterial attachment was clearly seen on the eroded endometrial tissue. So it is possible that by some mechanism the bacterium destroys the epithelial lining for example by toxin and then proceeds to attach to, to attack and destroy the underlying sub-epithelial tissue.

There seemed to be very little destruction of the allanto-chorion by the bacterium at the time of examination. *A. pyogenes* was however seen in small numbers in a few areas of the membranes. Degenerative changes seen in the embryo i.e. the gut, liver and kidney could represent the effects of anoxia and acidosis which could have occurred with the separation of the allanto-chorion from the endometrium. Changes could also be due to the toxin if it can cross the membrane barrier as suggested by the results of toxin tests in the embryo of Heifer 19. Absence of *A. pyogenes* in embryonic tissues rules out any possible direct bacterial action.

Scanning and transmission E. M. confirmed the cultural and light microscopical studies which failed to demonstrate significant numbers of bacteria in the tissue or attached to it. The hypertrophy of some epithelial cells in the infected uterine horn may have been due to the inflammatory reaction which could also have led to the loss of microvilli from these cells. The absence of microvilli and presence of tissue debris complement the results seen in histological sections. Some epithelial cells contained a large number of mitochondria possibly providing the energy required for the secretion of fluids. The secretory granules seen on the cell surfaces and in some cells could be linked to this process. The severity of the inflammatory reaction was confirmed further by the large number of neutrophils in the

sub-epithelial tissue. No *A. pyogenes* was seen in any section of the endometrium suggesting that the bacteria do not produce their effect by invasion and are limited to the surface or exposed layer(s) of the endometrium.

The clear association between the inoculation of the live bacteria and embryonic death described in Chapter III and Experiment II was not found with killed bacteria. In Experiment III one cow aborted (cow 16) and the other did not when inoculated with killed bacteria. The reason for the abortion of one animal are not clear. *A. pyogenes* was not isolated from the products of abortion and active infection with it cannot have played any part. The animal which failed to abort was the one inoculated earliest in pregancy on day 27 while the one which aborted was 36 days pregnant. It is believed that the period between days 36 - 45 of pregnancy, is critical for placentation in cattle (King et al., 1979). So it is possible that the animal inoculated earlier (day 27) was able to clear the dead bacteria and associated reaction and for placentation and normal pregnancy to continue; whereas the second animal inoculated at day 36 failed to overcome the inflammation and abortion occurred. The numbers of the bacteria inoculated does not seem to have been important since the numbers had a narrow range (2.5×10^{10} and 1.2×10^{11} respectively). No serum antibody determination was carried out on these cows and their immune status was unknown. The presence of cystitis in the aborted animal may have contributed to abortion.

The results of Experimant IV suggested that toxin had no effect on pregnancy. This finding needs to be considered with caution since the toxin has been shown to cause haemolysis to red blood cells and to cause death in mice. It is possible that the quantities used in the inocula were too small. Since the toxin was demonstrated *in vivo*, a cumulative effect of the toxin

might occur as when bacteria proliferated in Experiment II, or it may be necessary for it to be absorbed into the allantoic cavity or the amniotic cavity.

The identification of *A. pyogenes* **in pure cultures** from the uterus suggests that the changes seen in the uterus and embryos of Heifer 20 and seen clinically and by ultrasound in Chapter III, were due to the bacterium. The accumulation of fluid and pus in the uterine lumen and the inflammatory reaction may lead to the separation of the membranes from the endometrium and directly contribute to embryonic death through hypoxia and acidosis. The associated damage to cells leading to loss of microvilli, erosion of the epithelium and sub-epithelial tissue may further interfere with maternal - embryonic exchange by causing damage on the maternal side. The bacterium attached to the subepithelial tissue after erosion of the epithelium. The failure to demonstrate attachment of bacteria to epithelial cells of the allanto-chorion was unexpected. It was postulated above that the adherent bacteria had been removed with exudate during processing. This may also have been responsible for the failure to demonstrate bacteria adherent to the allanto-chorion in any numbers. The results obtained here comes from a single case and require repeating before any definite conclusions are reached. If the studies were to be repeated, attention would be paid to retain exudate on these organs or possibly to fixing or perfusing the embryo in the intact uterus. The results which indicate that invasion was not a major feature in either the maternal response to infection or in the death of the embryo are unlikely to have been affected by processing. The studies with killed bacteria need to be repeated as the evidence above suggested that no firm conclusion could be drawn about their role from the results of Experiment III. The negative results of Experiment IV requires to be repeated perhaps with different toxin preparations as there is a possibility

that toxin rather than anoxia may have caused embryonic death.

A. pyogenes **antibody(s).** The results from the two assays indicate that the assay is very sensitive as means of detecting antibody to *A. pyogenes*. Sera from animals 1, 2, 4, 5 and 23 & 24 (controls) were negative at a titre of 1:100 at the Day zero of infection and they may not have been previously exposed to *A. pyogenes* infection. An increase in the antibody levels on day 5 in cows 4, 5 and 18 may indicate the presence of an anamnestic response to the infection. Cow 3 was strongly positive (at a titre of 1:100) for *A. pyogenes* antibody at the time of infection and it is likely that the animal had been previously exposed to the bacterium. Infection however was established and abortion occurred in the animal. The presence of the antibody demonstrated by this test did not appear to be strong enough to protect the animal or did not enter the uterus. After infection in this cow a further increase in the antibody production occurred as shown by a positive titre of 1:1000 between 15 and 20 days after infection (Table 7). Cow 22 was also positive at the time of infection, but unlike Cow 3 infection was not established. The role of the antibody in protection against establishment of infection in this cow is questionable, since:- (i) the antibody present at Day 0 was not determined and (ii) the rise in antibody levels produced after infection was negligible compared to the other infected animals. Thus the reason for failure of infection to establish does not seem to have been affected by the presence or absence of antibody against *A. pyogenes*.

Heifers 17 and 18 which were inoculated with *A. pyogenes* toxin, developed a measurable antibody response (at 1:100 titre) though the toxin did not seem to affect pregnancy. It is possible that the toxic effect was not strong enough to affect pregnancy. The period for antibody production to rise was shorter (0 - 15 days) compared to other infected animals (10 - 25 days or more) and

this may indicate an anamnestic response and suggests partial protection against toxin even in Heifer 17 which was apparently seronegative to the antigen used. The ability of the antibody to protect the host against future infection is doubtfully from this study.

The ELISA developed in this study was very sensitive and may become useful for diagnosis of *A. pyogenes* infection. ELISAs have the advantage of being fast and reproducible. This ELISA used whole bacterial antigen and an extensive series of tests and evaluations would be needed before it could be used as a diagnostic tool in the field. In the study reported here the preinfection sera provided controls against which a rise in antibody could be detected. The use of purified toxin as an antigen might provide a guide to antitoxic antibodies. Their presence in sera containing antibody to whole cells is merely inferred and was not demonstrated here.

Hp. The pre infection concentrations of Hp (0 - 40 mg/100 ml) which increased to >150 mg/100 ml in 24 - 72 hours after infection, demonstrated the acute phase reaction to infection. It confirms results by other workers in *A. pyogenes* infection (Spooner and Miller 1971). The high Hp concentrations (>150mg/100 ml) were maintained for 5-8 days and it was during this time period that abortion occurred. It is not clear from this study whether the acute phase response may have had any direct role in the process of abortion. The relationship between Hp levels and embryonic death (**FIGS 71 and 72**) suggests that acute phase response was already well developed by the time embryonic death occurred and it may well have played a part in the deaths of both of the embryos. The continuation of high Hp levels after both embryonic death and abortion suggests that Hp alone cannot be used to determine the point at which embryonic death occurs. The relationship of Hp levels to post abortion phenomenon will be discused in Chapter V.

CHAPTER V.

STUDIES OF THE POST ABORTION PERIOD AND ANTIMICROBIAL TREATMENT AFTER *A. PYOGENES* INDUCED EMBRYONIC LOSS IN CATTLE.

INRODUCTION.

The production of early embryonic death by infection of the bovine uterus with pure cultures of *A. pyogenes* allowed the changes which follow embryonic loss to be studied clinically and ultrasonically. Ultrasound enabled the changes present in all parts of the tract to be monitored and recorded from abortion to the first oestrus. This record was made without invasion of the uterus and therefore provided a unique means of studying the changes which occurred.

In this chapter the following findings are recorded:

i. clinical observations including rectal temperatures, vaginal examination and vaginal discharge,

ii. a study of the pathological changes occurring in the reproductive tract after abortion,

iii. a comparison of the above changes with those occurring after cloprostenol (PG) induced embryonic loss, and

iv. a study of the effect of systemic antimicrobial treatment of the infected uterus.

MATERIALS AND METHODS.

Five of the six cows/heifers which had aborted as a result of infection with *A.*

pyogenes were examined both clinically and once or twice daily using ultrasound and rectal palpation by the methods described in Chapter II. The sixth cow (cow 6) was treated with cloprostenol 5 days after abortion as the animal had to be returned to stock. No data from this cow is presented here. The portable real time B - mode ultrasound scanner with a 7.5 mHz linear array rectal transducer was used to follow changes in the reproductive tract of the remaining 5 cows from abortion to recovery from infection. Treatment of 4 of the infected animals started 6 - 11 days after abortion using at least 5 doses of penicillin (Dulapen LA, Duphar Veterinary Ltd. U.K.) (1,700 I.U./kg) or oxytetracycline (Pfizer Ltd.) (7.5 mg/kg) intramuscularly (**Table 10**). The fifth animal (Heifer 5) remained untreated as a control for the treated group. The results of abortions made between the day of abortion and day 8 have been described in Chapter III. The findings in all five cows were so similar that those from cow 3 are reported as representative of the group observed from the time of abortion through the time of antimicrobial treatment to the first oestrus. Individual ultrasonographic measurements of the ovaries, uterine horns and cervix for each of these animals aborting after bacterial infection, are reported in Chapter VI in relation to the hormonal studies.

Changes in the reproductive tract after cloprostenol induced abortion were followed using the same methods for one or two oestrous cycles in the cloprostenol - treated group of 6 cows. Findings being reported after cloprostenol induced abortion are for one cow (14) representing findings in the 6 cows/heifers in this control group. Antimicrobial treatment was not undertaken in this group of animals.

RESULTS.

1. Infected animals. The results described below for cow 3 are representative of the changes seen in the infected group

Clinical observation. Abortion occurred 103 hours after infection and was followed by a continuous discharge of small amounts of mucopurulent material from the vagina. Mucopurulent material was observed in the perineal region or on the floor until 29 days after abortion. Examination of the external os of the cervix revealed an open cervix with pus coming through. The mucosa of the cervix was slightly congested. The vaginal wall was mildly congested but there did not seem to be any sign of erosion.

On rectal palpation an increase in the size of the uterine horns from 3 cm to 5 cm in diameter and increased uterine tone (scores 4 -5) were detected. Vaginal temperatures taken between abortion and the first oestrus (33 days after abortion) were between 38.4 and 38.8^{o} C. The animal had a bright demeanour, and the appetite was normal throughout the time of observation. The above observations continued until the animal came into oestrus.

Ultrasonography. Ultrasound allowed observation of the recovery process in the representative cow (No.3). This started 23 days after abortion and was completed 9 days later with re-establishment of the normal cycle.

The endometrium remained thickened (>4 mm) and the uterine lumen contained cloudy fluid and the cervix remained open (2 - 3 mm) and cloudy fluid remained in the vagina until recovery from infection took place. 33 days after abortion the genital tract returned to normal and changes of normal oestrus occurred. On Day 29 an increase in the cervical lumen to 6

mm, total evacuation of the mucopurulent material from the reproductive tract, an increase in the endometrial thickness to >8mm were seen followed by standing heat about 96 hours later. The corpus luteum was maintained until pus disappeared from the reproductive tract. Ultrasonographic changes described are for days 1, 8, 12, 23 and 29 after abortion. A detailed description of observations in the representative animal is given below.

The corpus luteum. One day after abortion the corpus luteum was present and measured 2.7 cm^2 in area (**FIF. 73 A1**). The corpus luteum remained similar in size until day 29. Measurements taken 8 and 12 days after abortion are presented in **FIGS 74 B1 and 75 C1** and show the corpus luteum to have an area of 2 and 2.6 cm^2 on those days. Antimicrobial treatment with penicillin started 10 days after abortion in this cow and was continued for 7 days. Twenty three days after abortion the corpus luteum was still present and was 2.6 cm^2 in area (**FIG. 76**), but it had regressed by day 29 and was then 1.3 cm^2 in area (**FIG. 77 E1**). By day 33 after abortion when the cow was seen in oestrus, the corpus luteum had an area of 0.3 cm^2.

Follicular growth. Follicular development was observed in both the right and left ovaries (R. O. and L. O.) as shown in **Table 8.** Follicles appeared round or oval black and non-echogenic ultrasonically. Between days 1 and 29 following abortion there was little follicular activity in the R. O. (which bore the corpus luteum) and only one follicle at 5 mm in diameter was observed. During the same period three follicles were seen in the L. O. The largest follicle was 20 mm in diameter in the L. O and was seen 23 days after abortion. Between days 29 and 34 after abortion more follicular activity was seen in the R. O.. The activity observed from day 29 coincided with the start of evacuation of pus from the uterus. Three follicles were seen in the R. O. at day 29 and a dominant one developed from 6 mm to 14 mm

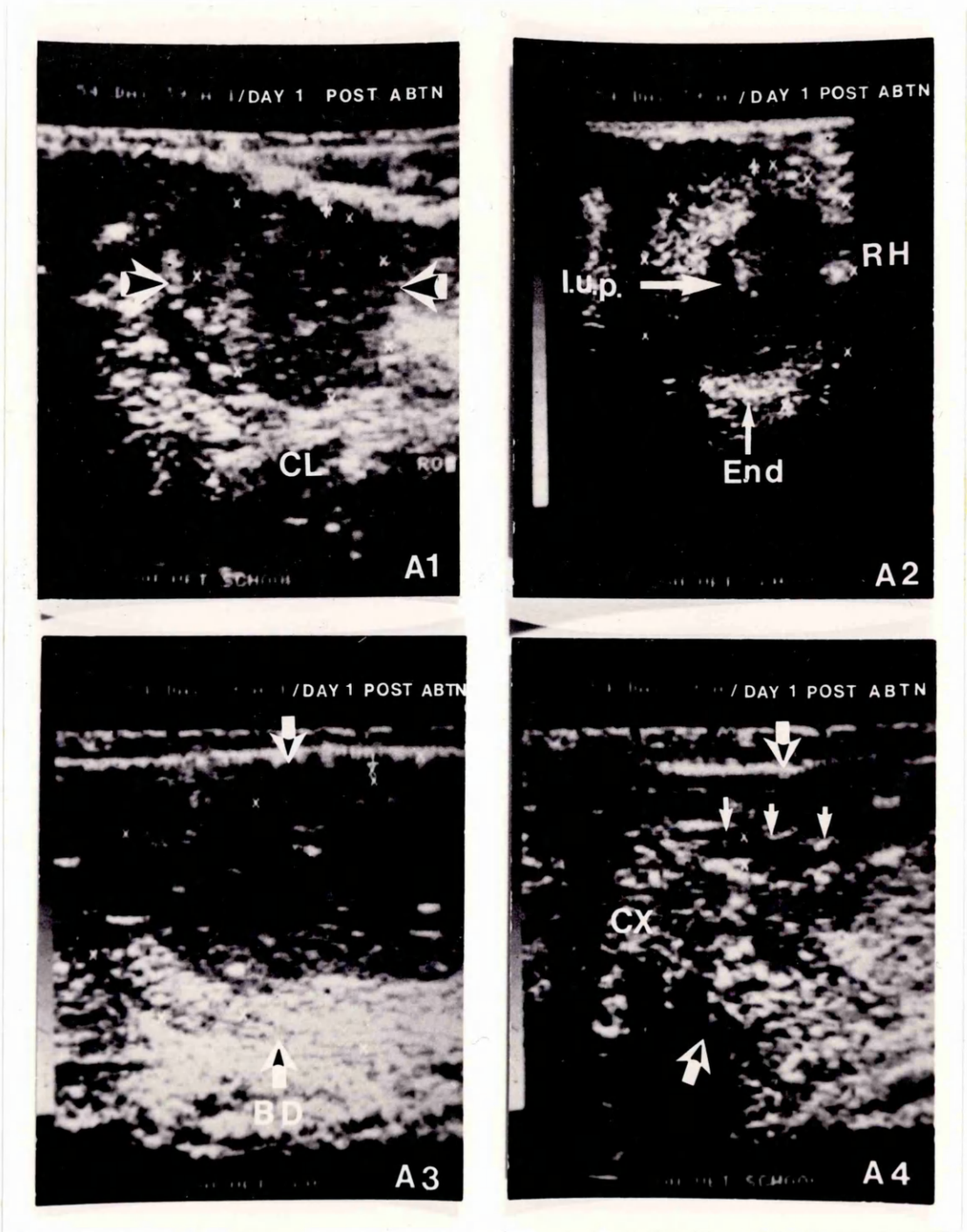


FIG. 73 (A1 - A5): Ultrasonography of Cow 3 one day after *A. pyogenes* induced abortion. **A1** the corpus luteum (CL) was maintained = 2.7 cm^2; **A2** endometrial (End) thickening (5 mm) and intra uterine pus (I.U.P.) seen in the right horn (RH); **A3** pus could be seen in the body of the uterus (BD), **A4** Cervix (CX), and

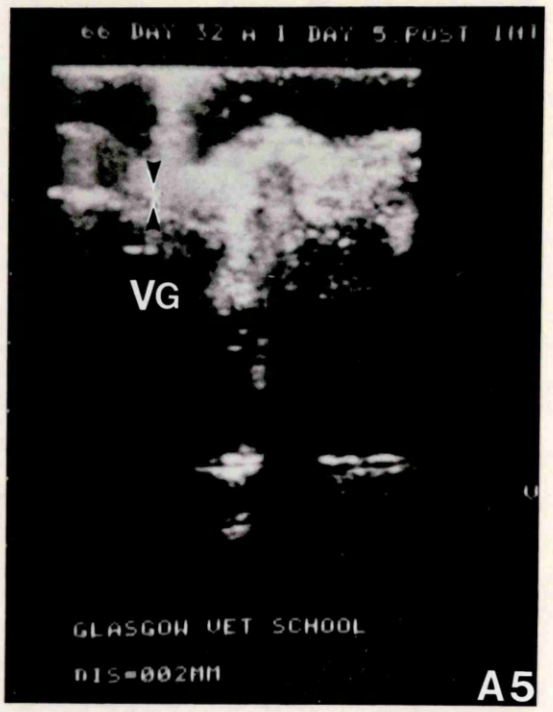


FIG. 73 A5 vagina (VG) contained non echogenic fluid.

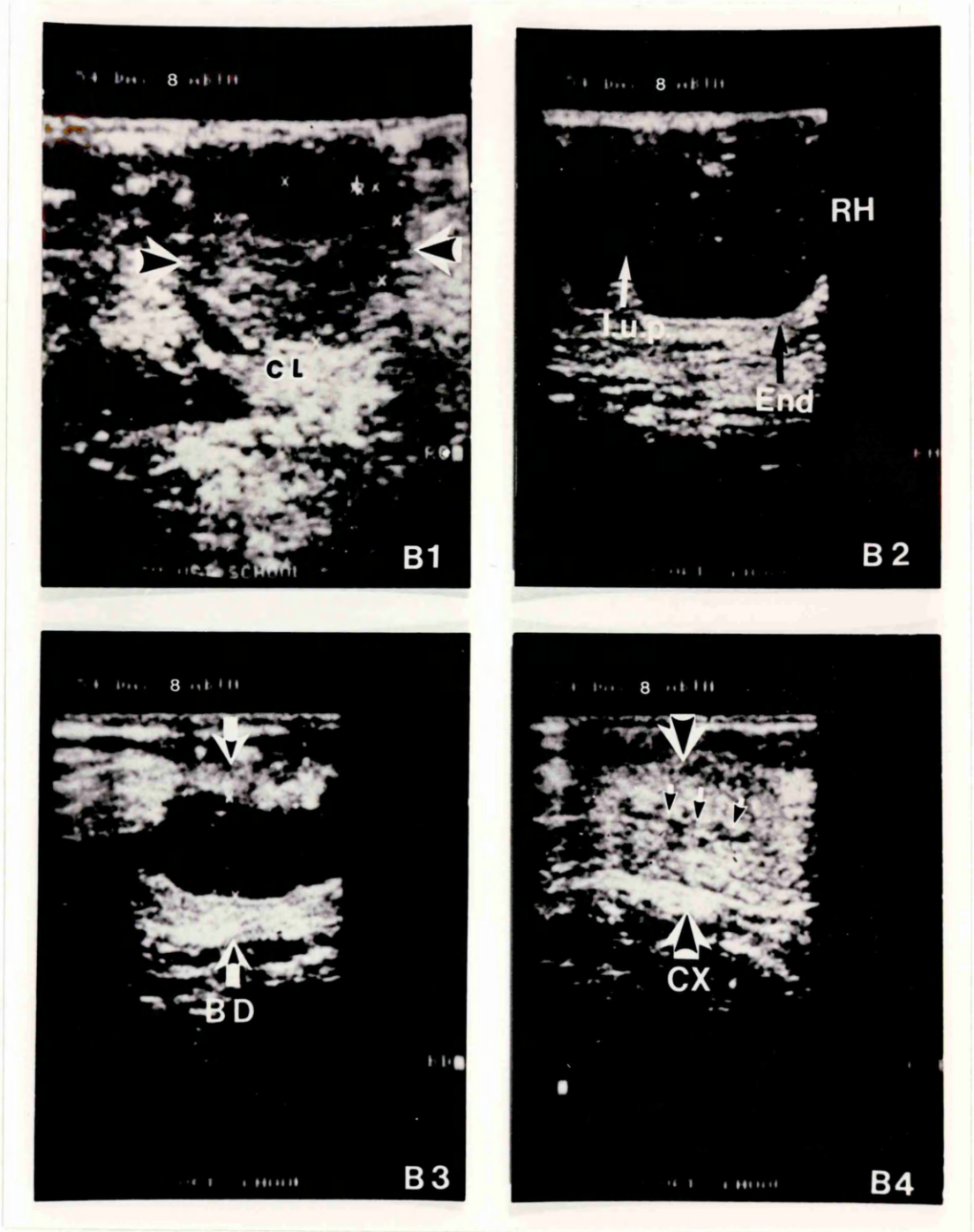


FIG. 74 (B1 - B5): Ultrasonography eight days after abortion in Cow 3. **B1** CL maintained = 2 cm^2; **B2** accumulation of I.U.P.(which is echogenic) in the RH with endometrial (End) thickening (8 mm); **B3** BD filled with pus (echogenic in appearance); **B4** CX open at 3 mm and

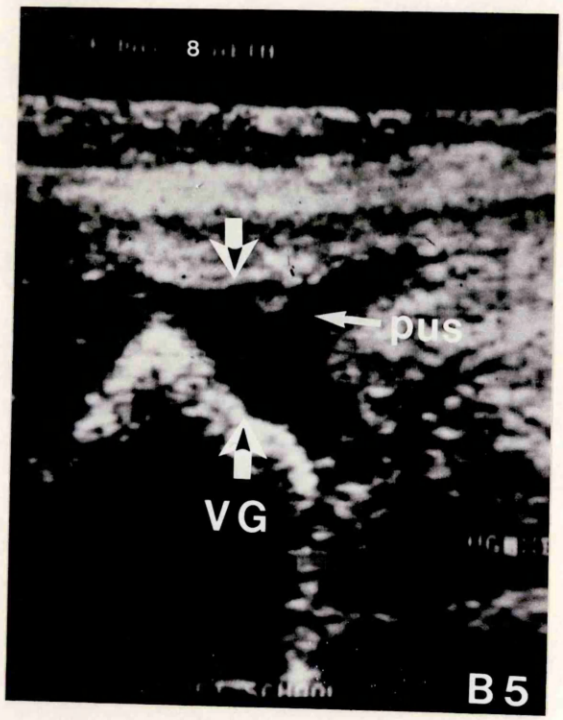


FIG. 74 B5 vagina (VG) filled with mucopurulent material (echogenic in appearance).

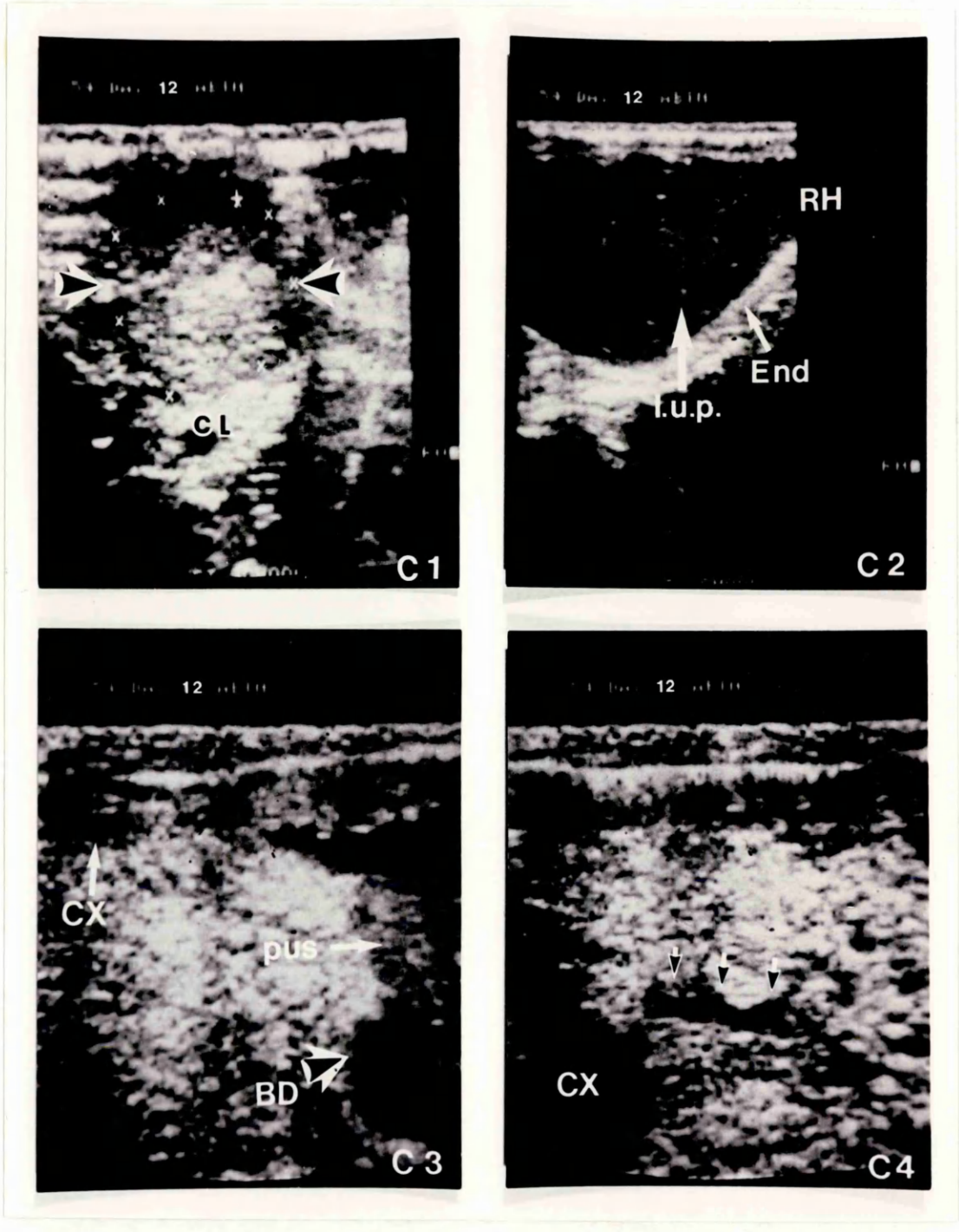


FIG. 75 (C1 - C5): Ultrasonography of Cow 3 twelve days after abortion and two days after antimicrobial treatment started. **C1** CL maintained at 2.6 cm^2; **C2** further accumulation of I.U.P. in the RH; **C3** accumulating pus causing extension of the BD, **C4** the CX (3 mm) and

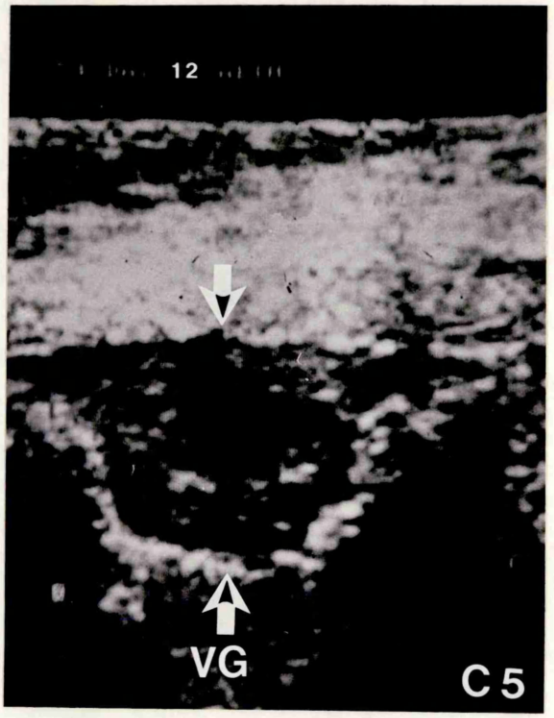


FIG. 75 C5 the vagina (VG) contained the echogenic material. Systemic antimicrobial treatment continued for 7 days.

in diameter 4 days later, when the cow was seen in oestrus. It was this dominant follicle which ovulated during the next 24 hours with formation of a new corpus luteum. There were only two follicles in the L. O. from day 29 and the dominant one developed to 14 mm in diameter but no ovulation was observed in any of these follicles.

Uterine horns and body. The endometrium was 5 mm in thickness 1 day after abortion (**FIG. 73 A2**) and pus was observable in the uterine lumen of both the horns and the body (**FIG. 73 A3**). Uterine findings were still similar 8 days after abortion (**FIGS 74 B3 and 75 C3**). By day 12 post abortion there was a further accumulation of pus in the uterus (**FIGS 75 C2 and C3**). The first changes in the content of the uterus were seen at 23 days following abortion (9 days after the start of treatment: **FIGS 76 D2 and D3**) which were areas of black (non-echogenic) and grey - white (echogenic) appearance of the fluid in the uterine lumen (**FIG. 76 D2**). By day 29 the content of the uterus was a clear - black (non - echogenic) fluid (**FIGS 77 E2, E3 and E4**). The uterine lumen and the body were drastically reduced in size by day 29. The endometrial wall remained thickened to between 4 and 9 mm in diameter until the evacuation of pus from the uterus. The highest endometrial diameter of 14 mm was reached 31 days after abortion and 2 days before oestrus.

Cervix and vagina. The cervix was open with an aperture of 3 mm just a day after abortion and the vagina contained cloudy echogenic fluid (**FIGS 73 A4 and A5**). By day 8 after abortion the lumen of the cervix was still 3 mm in diameter (**FIG. 74 B4**) and the vagina contained echogenic pus (**FIG. 74 B5**). At the start of antimicrobial treatment the cervical lumen was open (**FIG. 75 C4**) and echogenic pus was seen in the vagina (**FIG. 75 C5**). When the first changes were seen in the uterine horns 23 days after abortion, pus was still

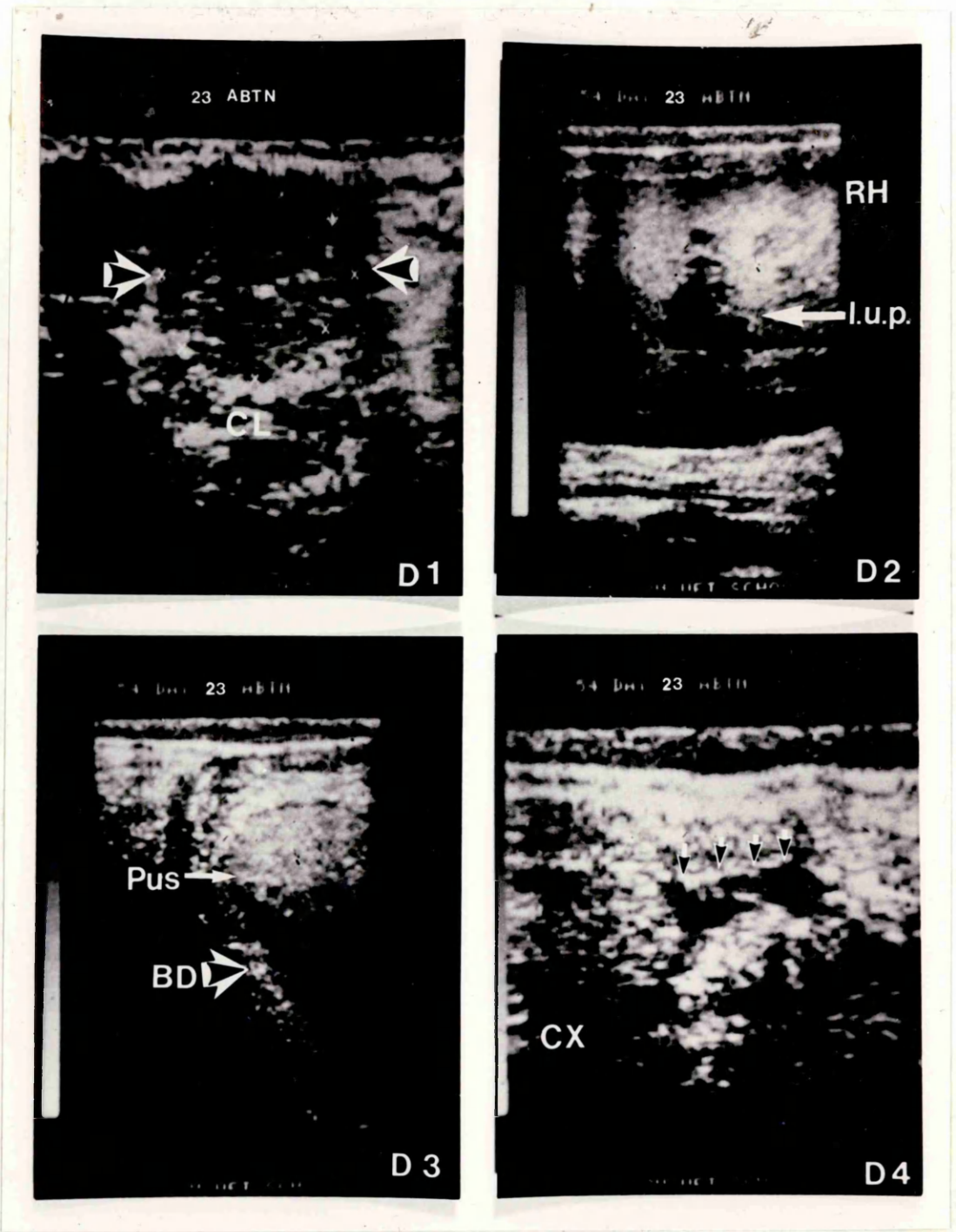


FIG. 76 (D1 - D5): Ultrasonographic changes (Cow 3) 13 days after start of antimicrobial treatment (23 days after abortion). **D1** CL maintained at 2.6 cm^2; **D2** RH containing pus (i. u. p) with echogenic and non echogenic areas; **D3** pus present in the body (BD), **D4** the CX (3 mm) and

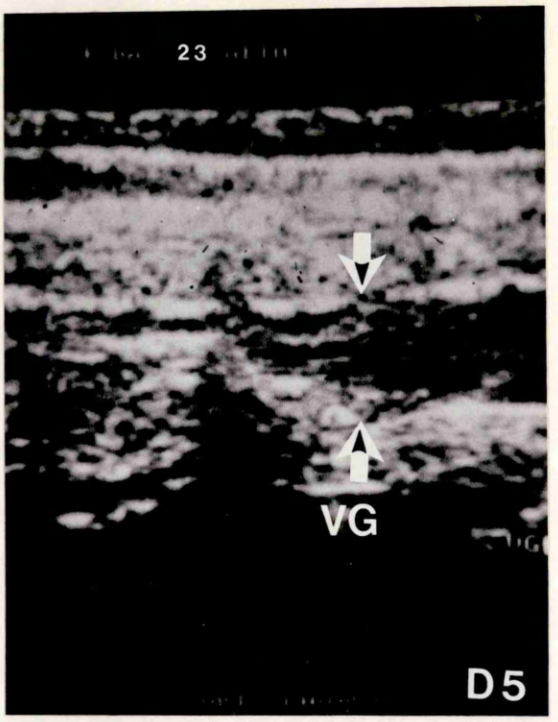


FIG. 76 D5 the vagina (VG) contained the echogenic material.

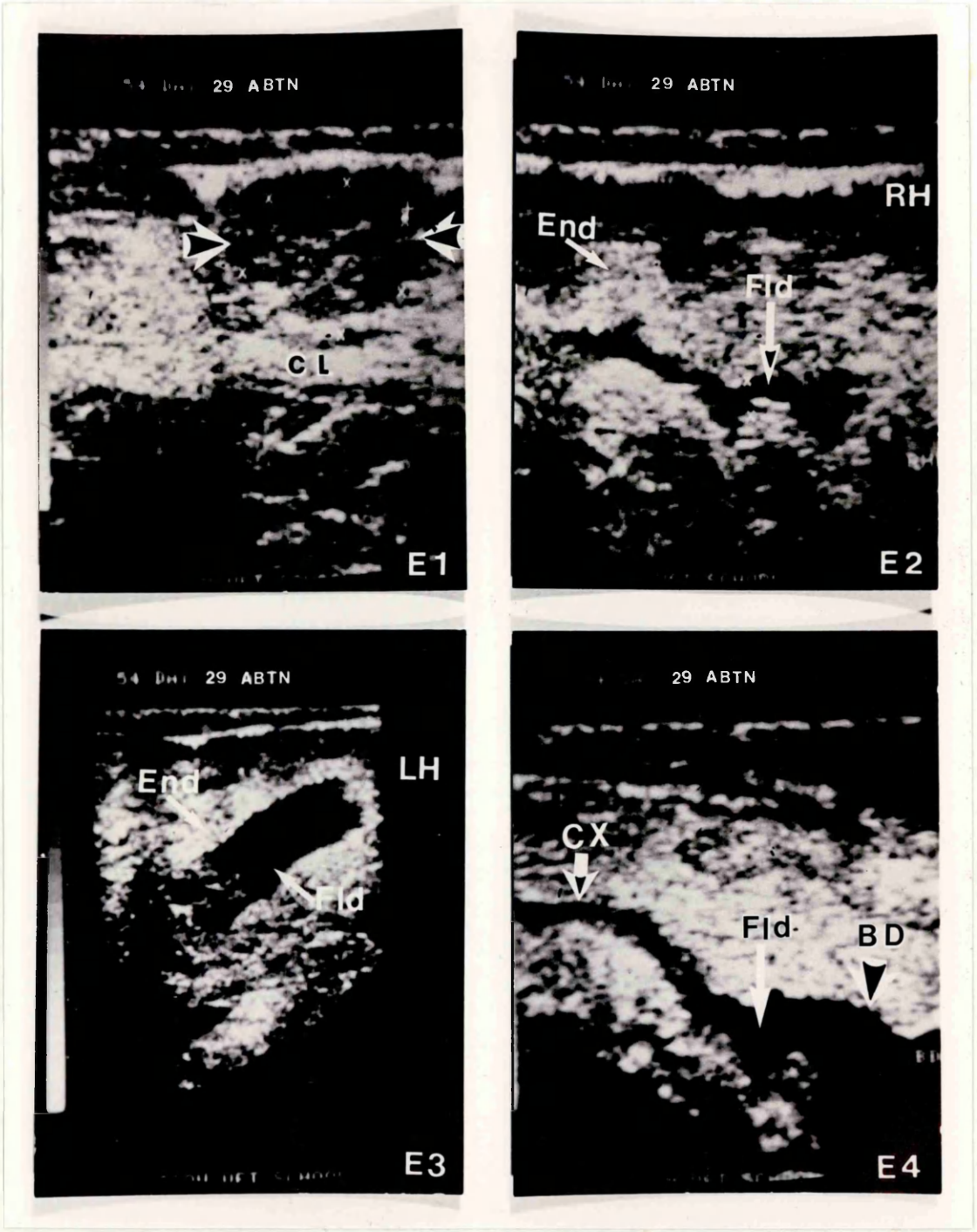


FIG. 77 (E1 - E5): Cow 3 ultrasonography 29 days after abortion, when copious amounts of pus were evacuated from the uterus via the vagina, accompanied by **E1** the CL regression to 1.3 cm^2; **E2** and **E3** with dark non echogenic fluid (Fld) in the right horn (RH) and the left horn (LH) and thickened endometrium (End); **E4** the fluid also present in the body (BD), and cervix (CX) and

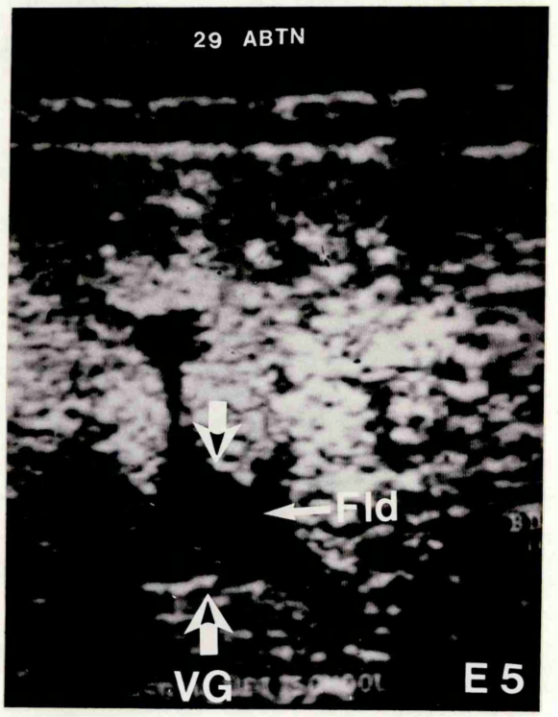


FIG. 77 E5 the vagina (VG) contained non echogenic fluid. The cow came into oestrus 96 hours after elimination of pus.

TABLE 8.

Follicular development (diameter in mm) in the ovaries of Cow 3 after *A. pyogenes* induced abortion. Three of the largest follicles were examined in each ovary. *Follicle which ovulated 33 days after abortion.

Days after abortion	Right ovary			Left ovary		
	1st	2nd	3rd	1st	2nd	3rd
	Follicles (diameter mm)					
1	-	-	-	12	-	-
8	5	-	-	10	7	5
12	-	-	-	10	7	5
23	-	-	-	-	-	20
29	6	7	4	13	9	-
31	10	9	-	10	11	-
32	10	8	-	14	-	-
33	*14	6	-	14	-	-

evident in the cervical lumen and the vagina (**FIGS 76 D4 and D5**). By day 29 after abortion the cervical and vaginal content was a clear - black non-echogenic fluid (**FIG. 77 E5**). The cow came into heat 96 hours after elimination of pus from the uterus.

Pus was collected from the vagina using the vaginoscope on days 2 and 15 after abortion. Cultural results on sheep blood and chocolate agars revealed presence of ß-haemolytic colonies which were Gram positive on staining and resembled *A. pyogenes*. No further cultures were carried out.

2. Cloprostenol treated animals. Cloprostenol induced abortion was followed by regression of the corpus luteum and by oestrus within 24 hours after abortion. Then ultrasonographic changes seen after abortion were given in Chapter III. These included a regressed corpus luteum (**FIGS 27 J1 and 28 K1**) and development of one dominant follicle on the same ovary (**FIG. 28 K2**), presence of small amounts of non - echogenic fluid in the uterine horns and body, an open cervix (**FIG. 29 L1 - L5**), a highly thickened endometrium (14 mm), and a fluid filled vagina. Following heat the dominant follicle ovulated and a fresh corpus luteum was formed, the endometrial size declined, the cervix closed and the fluid disappeared throughout the uterus. A summary of the comparison of the changes following both infected and cloprostenol induced abortion is given in **Table 9.**

3. Antimicrobial treatment. Treatment of cows 1, 2, 3 and 4 of the 5 infected animals examined revealed differing findings (**Table 10**). In two cows (1 and 4) which were treated with oxytetracycline (7.5 mg/kg for 3 days) and penicillin (1,7000 I. U./ kg for 5 days) at 6 and 11 days after abortion respectively, the cows came into oestrus between 18 and 20 days

after abortion (ie 12 and 7 days after start of treatment). Cow 2 received two treatments first oxytetracycline (7.5 mg/kg for 7 days) followed by penicillin (1,700 I.U./kg for 5 days). The animal failed to respond to the treatment and pus was observed in the uterus up to 34 days after abortion when observations were terminated. Cow 3 which received penicillin (1,700 I.U./kg for 7 days) 10 days after abortion came into oestrus 33 days after abortion (21 days after start of treatment). Heifer 5 did not receive any antimicrobial treatment and it came into oestrus 21 days after abortion.

DISCUSSION.

The clinical and other observations made above indicate that there was a profound difference between the recovery of the animals from abortion caused by *A. pyogenes* infection and from those initiated with cloprostenol injection. Animals which had been infected did not come into oestrus until 18 - 33 days after abortion (**Table 10**) and one animal (Cow 2) did not return to oestrus during the period of observation. This finding was in marked contrast to the situation in the 6 cloprostenol treated animals which came into oestrus 24 - 48 hours after abortion.

The clinical features associated with this delayed return to oestrus have been described above. The most prominent were the presence of purulent discharge from the vagina which on vaginoscopy could be seen to come from the cervix. Rectal temperatures were near the normal for a cow (38.5^{o} C). This, coupled with a bright demeanour and normal appetite suggests that infection was localised in the uterus and suggests that these two parameters may be of no value in indicating intrauterine infection on a clinical basis.

TABLE 9: Comparison of the findings resulting from *A. pyogenes* infection and cloprostenol treatment.

Anatomical structural change	Infected cow (COW 3)	Prostaglandin(Cloprostenol cow (PGF2x) (COW 14)
A. Corpus luteum + Follicles	maintained until evacuation of pus + No ovulation	regression within 24 hours + Abortion and ovulation
B. Uterine pus	remained until treatment	none formed
C. Uterine wall	endometrial thickening until return of cycle	marked endometrial thickening but reducing after heat
D. Cervix	open from time of infection, with pus, until 1st heat	open only during heat and closed soon after
E. Vagina	filled with cloudy fluid (pus) until recovery from infection	containing clear fluid (mucus) for few days after heat
G. *A. pyogenes*	isolated from discharge	none.

TABLE 10.

Results of antimicrobial treatment of 4 cows following *A. pyogenes* abortion.

Number of animal	Antimicrobial used and daily levels	Time of 1st treatment (days after abortion)	Period of treatment (days)	Return to heat (days after treatment
Cow 1	oxytetracycline 7.5 mg/ml.	6	3	20
Cow 2	oxytetracycline 7.5mg/kg & penicillin 1,700 I.U./kg.	9	7 5	>34
Cow 3	penicillin 1,700 I.U./kg.	10	7	33
Cow 4	penicillin 1,700 I.U./kg	11	5	18
5(Heifer)	NONE	NONE	NONE	21

The delay in the return to oestrus and the vulval discharge of pus were associated with distension of the uterus and maintenance of the high levels of tone observed during abortion. The lack of continued enlargement of the affected horn made distinction between the uteri of infected aborted cows and those of normal pregnancies increasingly easy. The persistence of increased tone of the uterus, the presence of a corpus luteum and the absence of large follicles (>14 mm) distinguishes the condition from oestrus. These findings were common to the cows in the infected group and differed from the findings in the cloprostenol treated animals. The pus seen in the vagina was seen to come from the external os of the cervix in all animals of the infected group and was a consistent feature absent from the cloprostenol group. The mild congestion of the cervix and vaginal wall indicated possible inflammation by *A. pyogenes*. As no erosions were seen on the walls it is not clear whether these changes represented inflammation or congestion. In any future study, biopsies from vaginal wall could establish the presence of inflammation at this site.

Ultrasonography of infected animals confirmed the retention of the corpus luteum, the presence of pus in the uterine lumen, cervix and vagina from the time of abortion until the first oestrus. Thickening of the endometrial wall and the accumulated mucopurulent material in the uterine lumen indicated a change due to the inflammatory reaction. The increase in thickness of the endometrium after infection was not as marked (5 to 8 mm) as that after cloprostenol treatment (15 mm) which is similar to that seen at normal oestrus. The ultrasonographic changes in the endometrium of an infected cow were different from those in a cloprostenol treated cow (**FIGS 73 A2 and 29 L2**). Reference to the ultrasonography of the ovaries could confirm the distinction.

The cervix was open from abortion to the first observed oestrus in the infected animal but was also open between abortion and the first oestrus in the cloprostenol treated - group but for a much shorter time and with no evidence of presence of pus. The accumulating pus may be responsible for maintaining the cervix open during infection. The control of cervical opening is discussed further in Chapters VI and VIII.

Changes in the vagina were clearly visible by ultrasound. Echogenic fluid was present in the vagina between abortion and until day 29 post abortion in cow 3 and for a similar period in the other infected animals. The appearance of an infected vagina differed from that of a normal vaginas of the cloprostenol treated cows and differs from that in dioestrus and pregnancy. During dioestrus and pregnancy the vaginal wall is collapsed and the lumen is difficult to see because then the cervix is closed (Omran, 1989). In animals treated with cloprostenol, there was non - echogenic (black) fluid throughout the uterus and vagina only during abortion and oestrus, while in infected animals the echogenic fluid was present in both for many days. The fluid found in cloprostenol treated animals was in smaller amounts compared to the infected animals. Thus the quantity and quality of the uterine and vaginal fluid can act as a good marker of the pathological state of the uterus in this condition.

Whereas the corpus luteum was maintained for at least 18 days after abortion in infected animals, there was lysis soon after cloprostenol treatment. The corpus luteum present during infection was seen to reduce in size after evacuation of pus from the uterus in contrast to the rapid decrease in size seen in the cloprostenol treated group. Recovery of the uterine endometrium after infection may have led to the production of PGF2α and luteolysis.

Follicular activity in the two ovaries was observed during the study using ultrasound. There seemed to be little activity in the R. O. of cow 3 which contained the corpus luteum up to day 29 after abortion compared to the L. O.. Despite there being a dominant follicle (20 mm) in the L. O. 23 days after abortion, the animal was not seen in heat and the follicle was not seen to ovulate. Reason for the failure of this follicle to ovulate may be the presence of a corpus luteum and the aspect is discussed further in Chapter VIII. After day 29 follicular activity in the R. O. returned with development of one dominant follicle (14 mm) which ovulated to form a fresh corpus luteum. Factors responsible for ovulation of one of the follicles in the ovary previously bearing the corpus luteum were not investigated in this study but hypotheses are discused in chapter VIII. With ultrasound however it was possible to study follicular events leading to oestrus after bacterial infection. The possible hormonal interplay during the post abortion observations is described in Chapter VI. Ultrasound was clearly useful in identifying the presence of pus in the uterus and confirming the absence of pregnancy. It seems unlikely that it can distinguish between metritis and pyometra.

After antimicrobial treatment, in two cows (1 & 4) the infection resolved and pus was eliminated from the uterus, with the cycle re - established in 18 - 20 days following abortion. The duration of return of these cows to heat was within the normal range for returning to heat after parturition. It not clear whether the two cows would have returned to oestrus without the antimicrobial treatment given. Results for Heifer 5 which came into heat 21 days after abortion without treatment further casts doubt on the usefulness of the treatment. Both oxytetracycline and penicillin were apparently effective. However in cow 3 oestrus only occurred on day 33 after abortion, 16 days after the end treatment. Cow 2 did not return to oestrus by day 34 in spite of treatment with both antimicrobials. These results casts further

doubt on the usefulness of parenteral antimicrobial treatment alone. The reasons for the failure of this cow to respond to treatment are unclear. It may be that local antimicrobial treatment could have improved the results obtained from treatment or the use of a more appropriate antimicrobial than those chosen. Dose levels given were within the ranges recommended for therapy by the manufacturers of the antibiotics used.

In all the animals however infection seems to disrupt the normal cycle, as maintenance of the corpus luteum in the first >18 days before the first oestrus indicated that there was no silent heat during this time. The observed follicular activity in the ovaries during infection supports the assumption that no oestrus occurred during infection, and there is need to understand the mechanisms involved in re-establishment of normal ovarian activity. Discussion of these findings in relation to the literature available is presented in Chapter VIII.

The concentrations Hp in Cow 1 and 4 were reported in Chapter IV and were seen to decline after embryonic death and abortion (**FIGS 71 and** 72). The period between the first decline in Hp concentration to the first oestrus in both animals was punctuated by a temporary rise in the concentration of the protein. It is therefore possible that as long as infection persisted in the uterus Hp could still be detected. The role of Hp in the process of repair of uterine damage due to *A. pyogenes* is not clear from this study. The very low concentration of Hp maintained after oestrus however strongly indicates absence of infection in the uterus.

The observation that Hp started to decline 5 days after abortion in cow 1, may suggest that treatment was effective (**FIG. 71**). In cow 4 however antimicrobial treatment started 10 days after abortion and about 1 day after

Hp had declined to the basal concentration for the first time. This indicates that uterine repair was already on the way and casts doubts about the efficacy of antimicrobial treatment.

These observations have demonstrated that Hp concentration can be a very good indicator of progress of infection. It was not possible to examine the possible role of Hp in the process of abortion in the study. Whereas recovery from infection could be predicted from Hp concentration, the usefulness of antimicrobial therapy was not clear.

Findings reported here have demonstrated that ultrasound is useful in monitoring the effects of bacteria on the reproductive tract. Endometritis was examined and the efficacy of parenteral treatment was evaluated. *A. pyogenes* caused uterine infection which was accompanied by accumulation of pus and maintenance of a corpus luteum which was most probably functional as this corpus luteum regressed with the elimination of pus from the uterus. Follicular events leading to oestrus after recovery from infection were studied. The usefulness of the systemic antimicrobial treatment of an infected uterus is debatable from the available evidence in this study. A more effective regime of treatment may yield better results. This area may require more extensive investigation. One clear observation however was that infection disrupted the return to the normal oestrous cycle. A detailed discussion of the findings in this Chapter is done on the background of the available literature in Chapter VIII.

CHAPTER VI.

PROGESTERONE AND OESTRADIOL 17 B CONCENTRATIONS IN EXPERIMENTAL EARLY EMBRYONIC DEATH IN CATTLE CAUSED BY *A. PYOGENES*.

INTRODUCTION.

The role of progesterone and oestradiol 17 β in the oestrous cycle and normal pregnancy has been outlined in Chapter I. Changes in the concentrations of the two hormones in the plasma of the cows in the experiments described in Chapters III, IV and V were measured and are described here. The studies were carried out to determine whether changes in their concentrations could be related to the events recorded in the uterus during abortion. Levels of progesterone were determined in samples taken at 15 minutes intervals over important periods such as post - inoculation and abortion for 2 animals in order to detect even transient variations in levels.

MATERIALS AND METHODS.

In this part of the study the plasma progesterone and oestradiol 17 β concentrations from 5 animals nos 1, 2, 3, 4 and 5 infected with live *A. pyogenes* were recorded. Circulating plasma progesterone and oestradiol 17 β concentrations were also examined in plasma from one animal (cow 15) inoculated with the killed bacterium. In another 2 control animals (heifer 17 and cow 14) inoculated with the bacterial toxin and cloprostenol treatment respectively, only the concentration of plasma progesterone was recorded.

In heifers 5 and 13 progesterone was determined in plasma samples collected using an indwelling catheter every 15 minutes after inoculation. In heifer 5 which had been infected with live *A. pyogenes* 15 minute plasma samples were collected throughout the period from embryonic death to abortion. Heifer 13 was induced to abort by the administration of cloprostenol and 15 minute samples were collected for the first 8 hours after administration, then at intervals which varied from 30 minutes to 1 hour for the next 8 hours then daily through embryonic death to abortion. Samples from the remaining animals were taken at the intervals shown in the figures **78 - 87.**

The enzyme linked immunosorbent assay (ELISA, Cambridge Life Sciences) was used to investigate the concentration of progesterone (see Chapter II). A conversion factor of 3.18 was used to convert readings from nmol/l to ng/ml (Eckersall, personal communication). The assay had a sensitivity of 0.5 ng/ml. Inter-assay and intra-assay coefficients of variation were determined using duplicate pooled samples of bovine plasma of known progesterone concentrations used as labaratory standards. Inter-assay coefficient of variation (CV) determined by analysis of control samples in 20 assays was 22.57% at 3.3 ng/ml of progesterone in the low control plasma sample and 14.85% at 6.9 ng/ml of progesterone in the high control plasma sample. Intra-assay coefficients of variation were determined in 20 assays by replicates of the controls with results of 22.64% and 10.88% for the plasma with progesterone at 3.3 and 6.9 ng/ml respectively. The samples which showed concentrations higher than 10 ng/ml were diluted in some studies with blank plasma from 3 month old calves which had progesterone concentrations of <0.5 ng/ml. The samples were diluted at a rate of 1:5.

Oestradiol 17 β concentrations were analysed by radioimmunoassay (RIA; chapter II). The sensitivity of the assay was 0.8 pg/ml. The inter-assay and intra-assay coefficients of variation were determined in 5 assays using replicate low and high control samples. The inter-assay coefficients of variation were 18.8% and 21.3% at 5.1 pg/ml and 19.8 pg/ml respectively. The intra-assay coefficients of variation were 14.7% and 28.8 % at 5.1 pg/ml and 19.8 pg/ml respectively.

The changes in the reproductive tract to which the hormone levels were related are given below. Changes in the cross-sectional area (cm^2) of the corpus luteum, follicles (mm), endometrial thickness (mm) and cervical lumen (mm) were observed using the ultrasound during the studies described in Chapters III, IV and V. When the cervical lumen was 2 mm or less, it was considered to be closed.

RESULTS.

Cows infected with *A. pyogenes*.

Findings from 5 cows infected with live *A. pyogenes* are presented. Results from individual animals are presented because they were infected on different days of pregnancy and there were individual variations in the findings.

Cow 1 was observed from day 7 of pregnancy, over infection at 35 days of pregnancy and until the first oestrus. The progesterone concentrations increased from about 4.6 ng/ml at the beginning of observation to 10.3 ng/ml at the time of infection **(FIG. 78a)**. The concentration was maintained above 4 ng/ml during the time of abortion and treatment, 17 days after abortion. The concentration had declined to 0.5 ng/ml when

oestrus occurred. The levels had increased to 4.9 ng/ml 8 days after oestrus. Oestradiol 17 ß concentration remained about 0.8 pg/ml before infection, during infection, abortion and treatment. The concentration however started to increase 17 days after abortion and was 4.6 pg/ml, 20 days after abortion, when the animal came into oestrus. Oestradiol 17 ß concentration declined 24 hours after oestrus to 1.2 pg/ml.

The area of the corpus luteum was measured as 0.5 cm^2 at 2 days after A.I. and had increased to 3 cm^2 at the time of infection. This was maintained between 1.5 and 3 cm^2 from the time of infection, through abortion and for 18 days after antimicrobial treatment (20 days after abortion). It declined to 0.4 cm^2 at 20 days after abortion when the animal came into oestrus (**FIG. 78b**). Follicles were present in the left and right ovaries during pregnancy (ie before infection) and were 6 to 14 mm in diameter. The diameter of follicles between infection and abortion was between 6 and 10 mm. Follicular activity for 8 days following abortion was reported in Chapter III. On day 20 after abortion the largest follicle (22 mm in diameter) was present in the right ovary coinciding with the observed oestrus. It is this follicle which ovulated 24 hours later with formation of a fresh corpus lutem.

Before infection the endometrial thickness was about 2.5 mm in thickness but 21 hours after infection it had increased to 4.7 mm. This was followed by a gradual decline in thickness which was maintained between 4 and 2.7 mm until oestrus when it declined to 2.4 mm (**FIG. 78c**). The cervix was closed before bacterial infection. 48 hours after infection it opened and the lumen was 3 mm in diameter. It attained its maximum diameter of 6 mm at 4 days after abortion. However the cervix was closed when the animal came into heat (**FIG. 78c**).

FIG. 78 (a - c): A study of progesterone, oestradiol 17 ß plasma concentrations and their relationship with corpus luteum size, endometrial thickness, and cervical lumen diameter in **Cow 1**, after infection with live *A. pyogenes*. INF. = time of infection, ED. = embryonic death, ABTN. = time of abortion, RX. = antimicrobial treatment, HT. = oestrus.

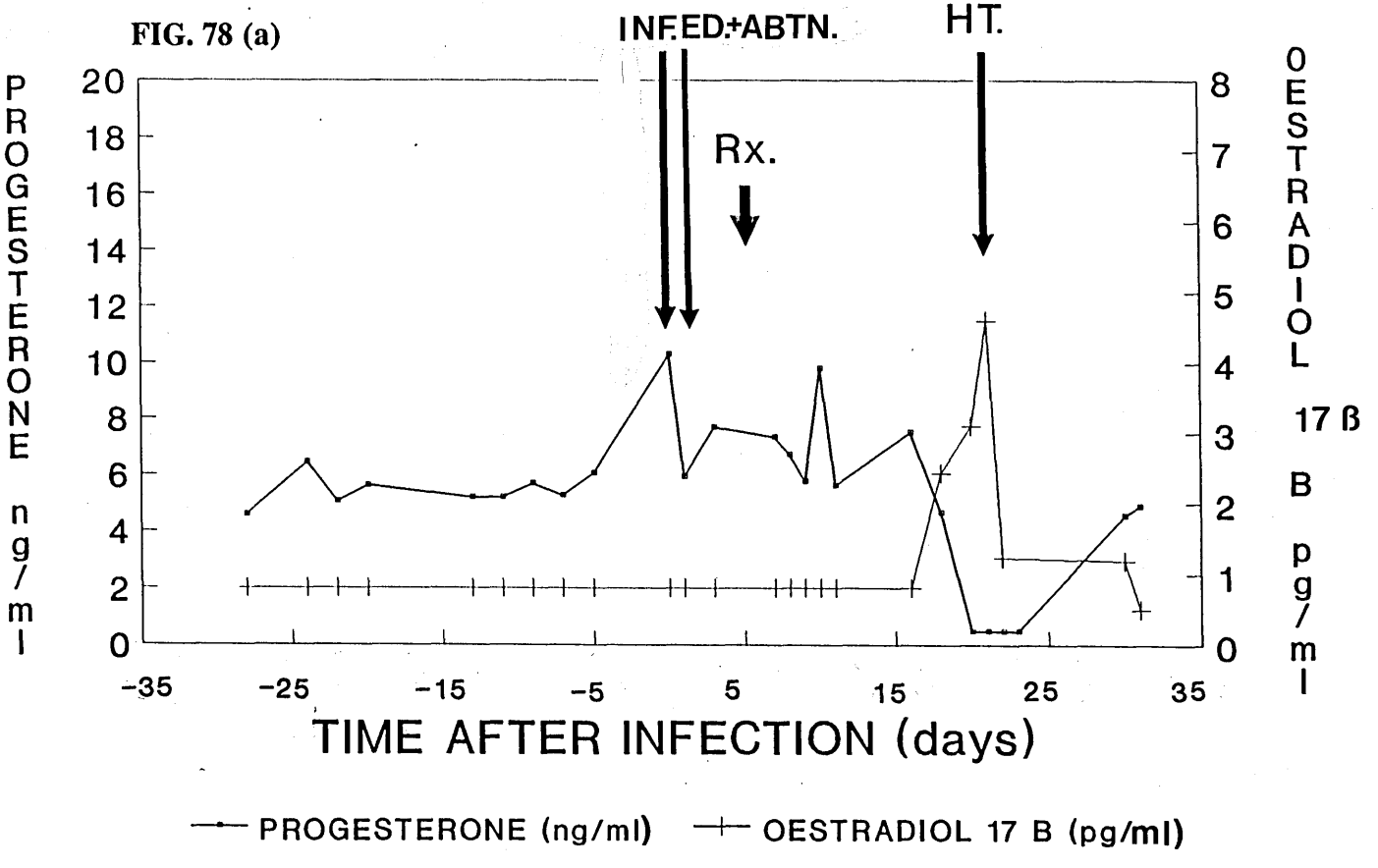
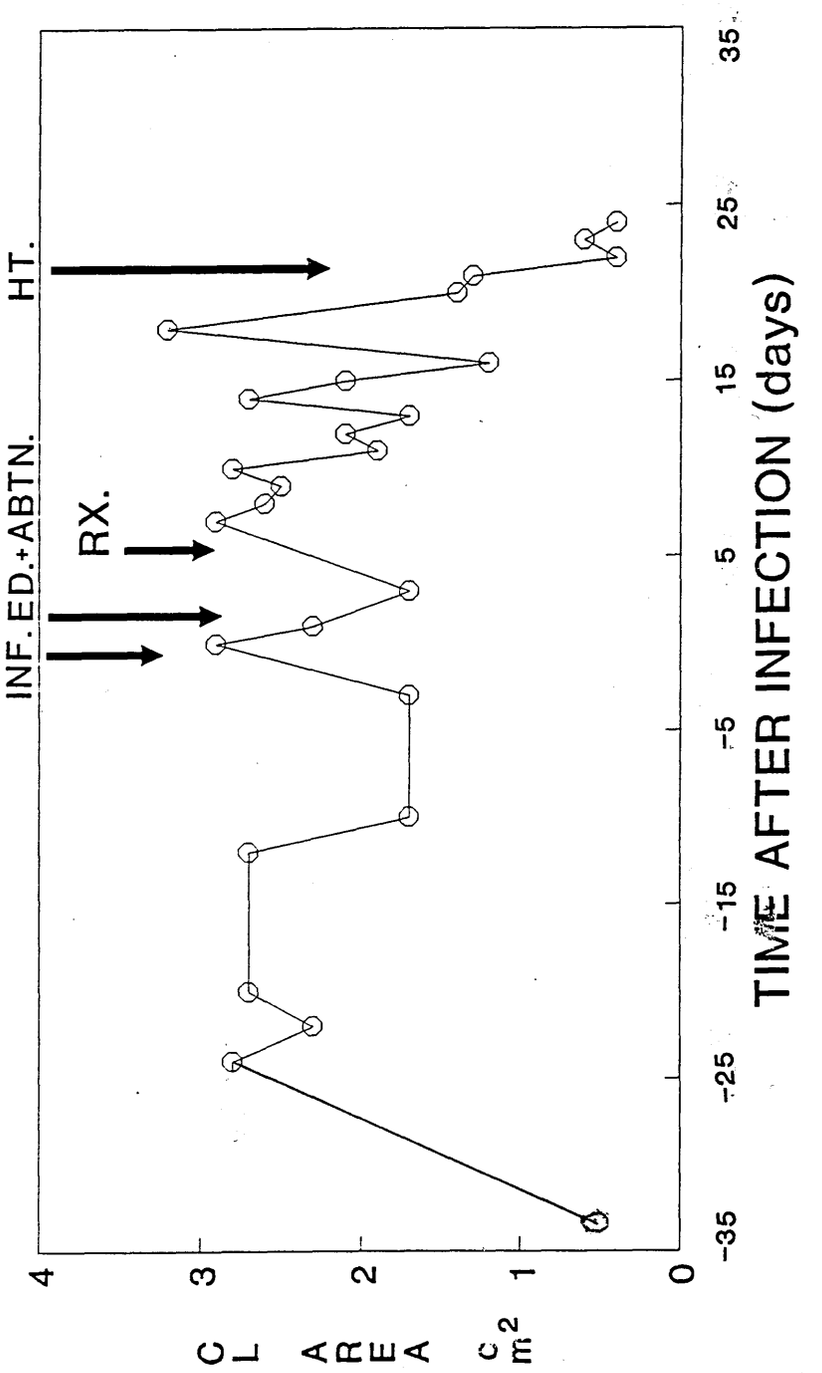


FIG. 78 (b)


INF. ED.+ABTN.
RX.
HT.
CL AREA c_{m^2}
4
3
2
1
0
-35
-25
-15
-5
5
15
25
35
TIME AFTER INFECTION (days)
CL AREA cm²

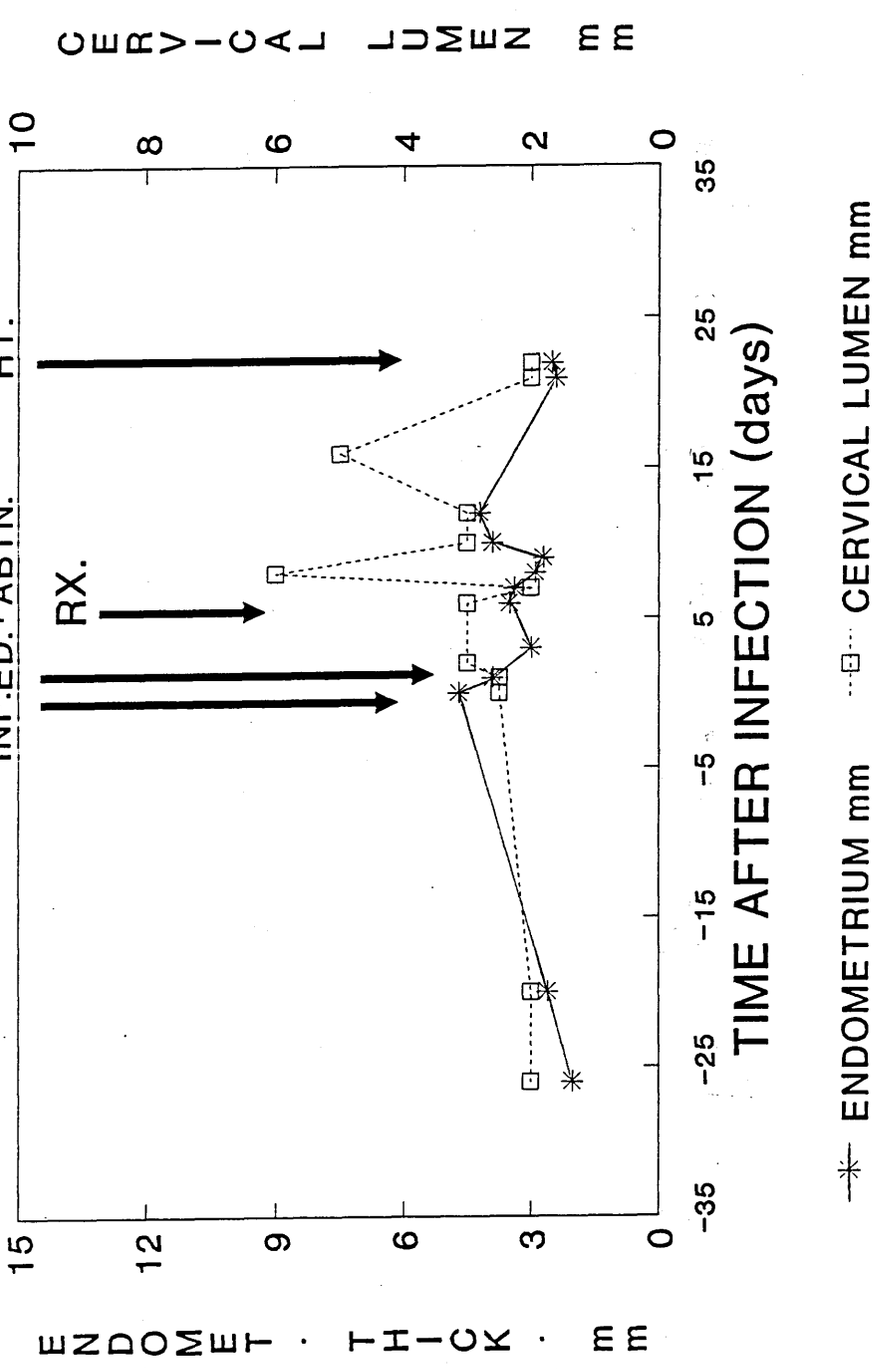

ENDOMET · THICK · mm
15
12
9
6
3
0
CERVICAL LUMEN mm
10
8
6
4
2
0
INF.ED.+ABTN.
HT.
RX.
-35
-25
-15
-5
5
15
25
35
TIME AFTER INFECTION (days)
ENDOMETRIUM mm
CERVICAL LUMEN mm

FIG. 78 (C)

Cow 2 was examined from 14 days before infection and was infected at day 30 of pregnancy (**FIG. 79a**). The progesterone levels increased from 7.5 ng/ml at the start of observation to above 15 ng/ml at the time of infection. The concentration declined thereafter but did not fall below 4.9 ng/ml throughout the time of abortion and antimicrobial treatment. The concentration was above 4.9 ng/ml at 34 days after abortion and the animal was not observed in oestrus during the time of examination. Oestradiol 17 ß concentrations were at their basal levels (0.8 pg/ml) throughout the time of observation.

The area of the corpus luteum (**FIG. 79b**) was more than 3 cm^2 from the time of observation to the time of infection. Examinations between days 0 - 15 after infection the area of the corpus luteum showed it to be between 1.3 and 2.1 cm^2, the rest of the examinations revealed the area to be around 3 cm^2 up to the end of the experiment. There was a consistent decline in the size of the corpus luteum in the last 5 days of examination but the lowest area reached during this period was 2.3 cm^2. Both the right and left ovaries had dominant follicles which were 15 mm on the day of abortion. Growth and atresia of follicles was observed in three waves in the ovaries up to termination of the experiment 35 days after abortion. Three large follicles 14 mm in diameter were observed in the right ovary at different times after abortion and in the left ovary the largest follicle (13 mm) was observed 10 days after abortion. None of these follicles ovulated and the animal did not come to oestrus.

The thickness of the endometrium increased 5 hours after infection from 4 mm to 6 mm (**FIG. 79c**). The thickness was maintained for most of the time of observation between 3.5 mm and 6 mm. The diameter of the cervical lumen was seen to be at its greatest at 6 mm, two days after abortion.

FIG. 79 (a - c): A study of progesterone, oestradiol 17 ß plasma concentrations and their relationship with corpus luteum size, endometrial thickness and cervical lumen diameter after infection with live *A. pyogenes* in **Cow 2.** INF. = time of infection, ED. = embryonic death, ABTN. = time of abortion, RX. = antimicrobial treatment.

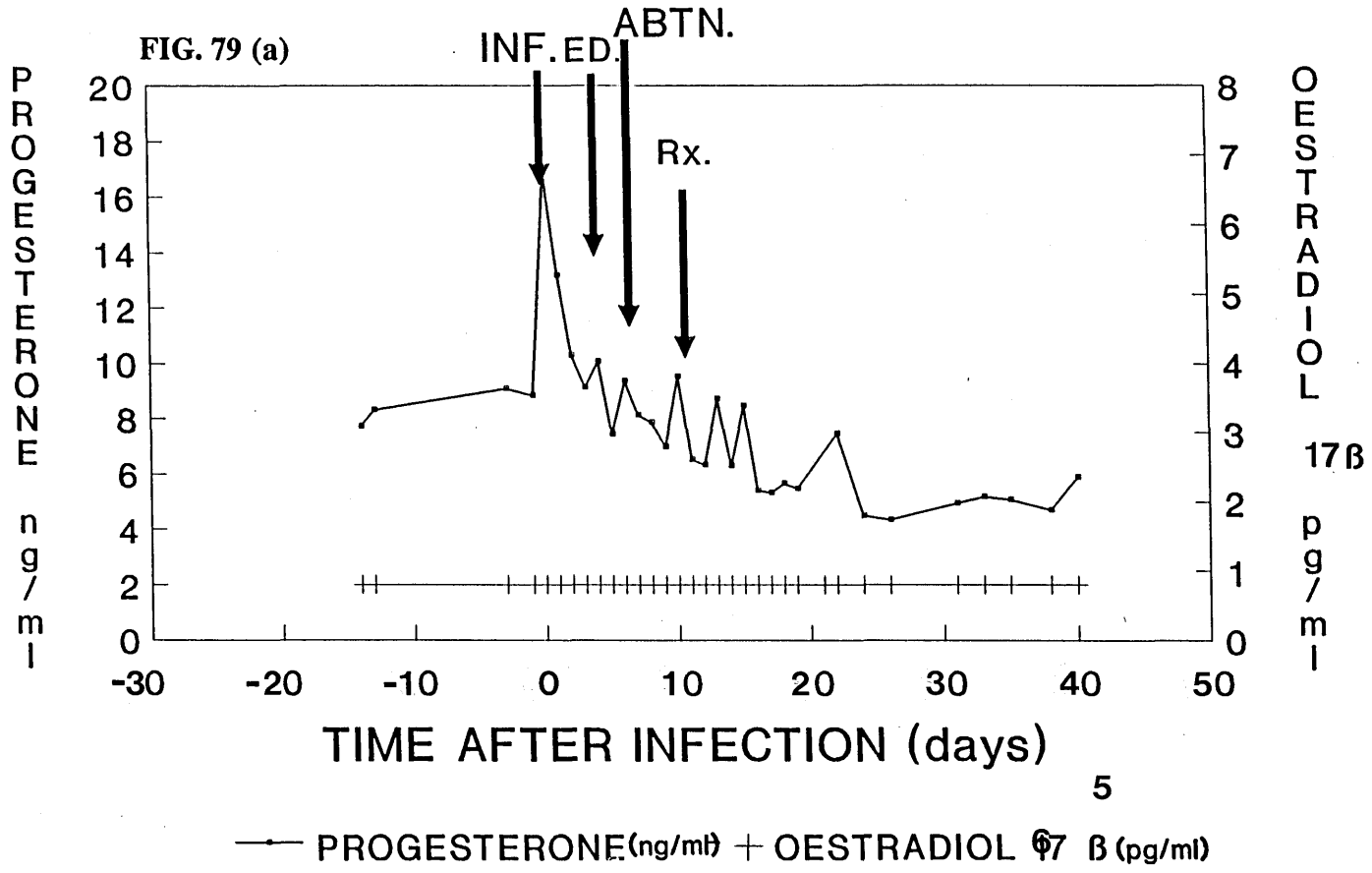


FIG. 79 (b)

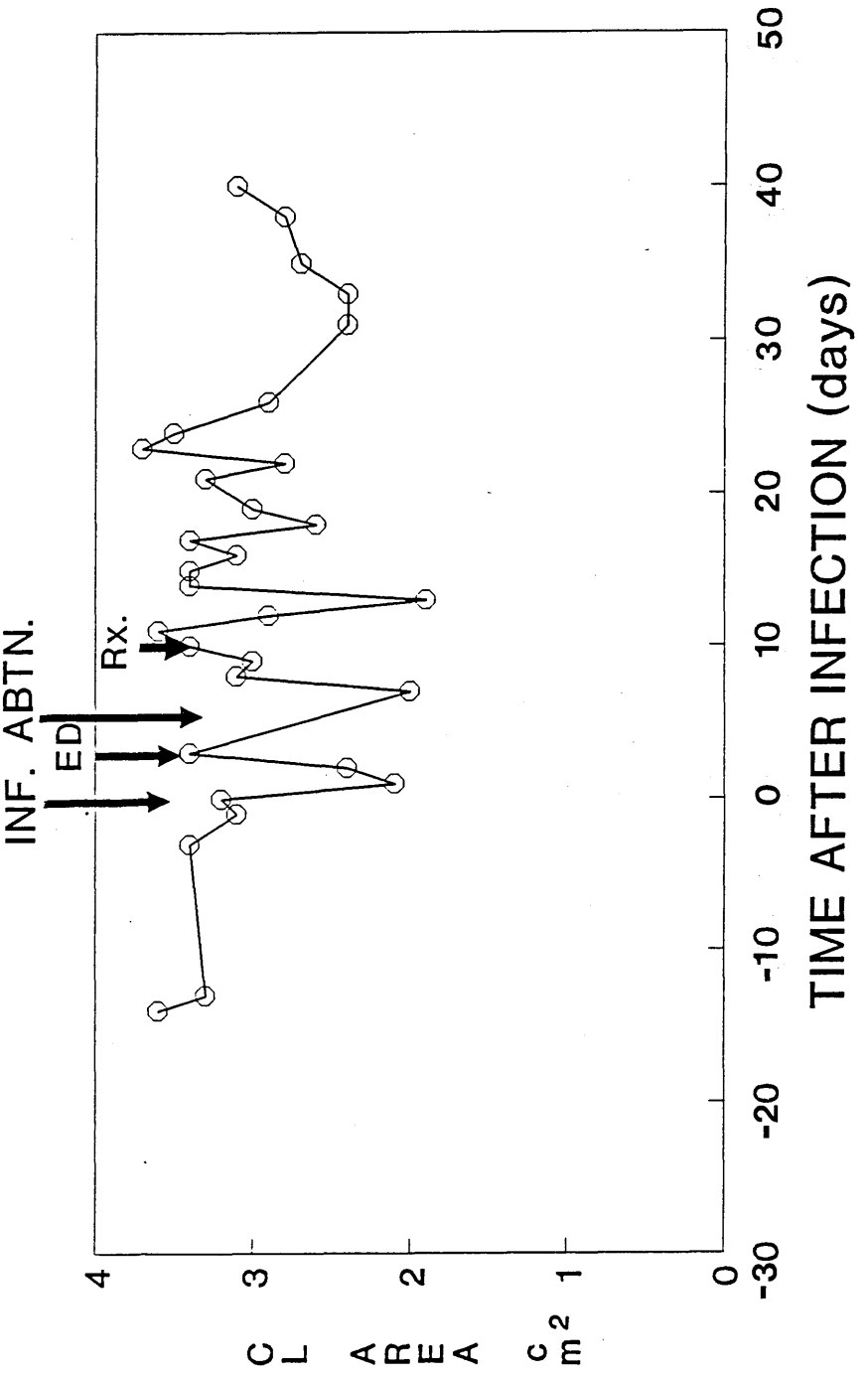
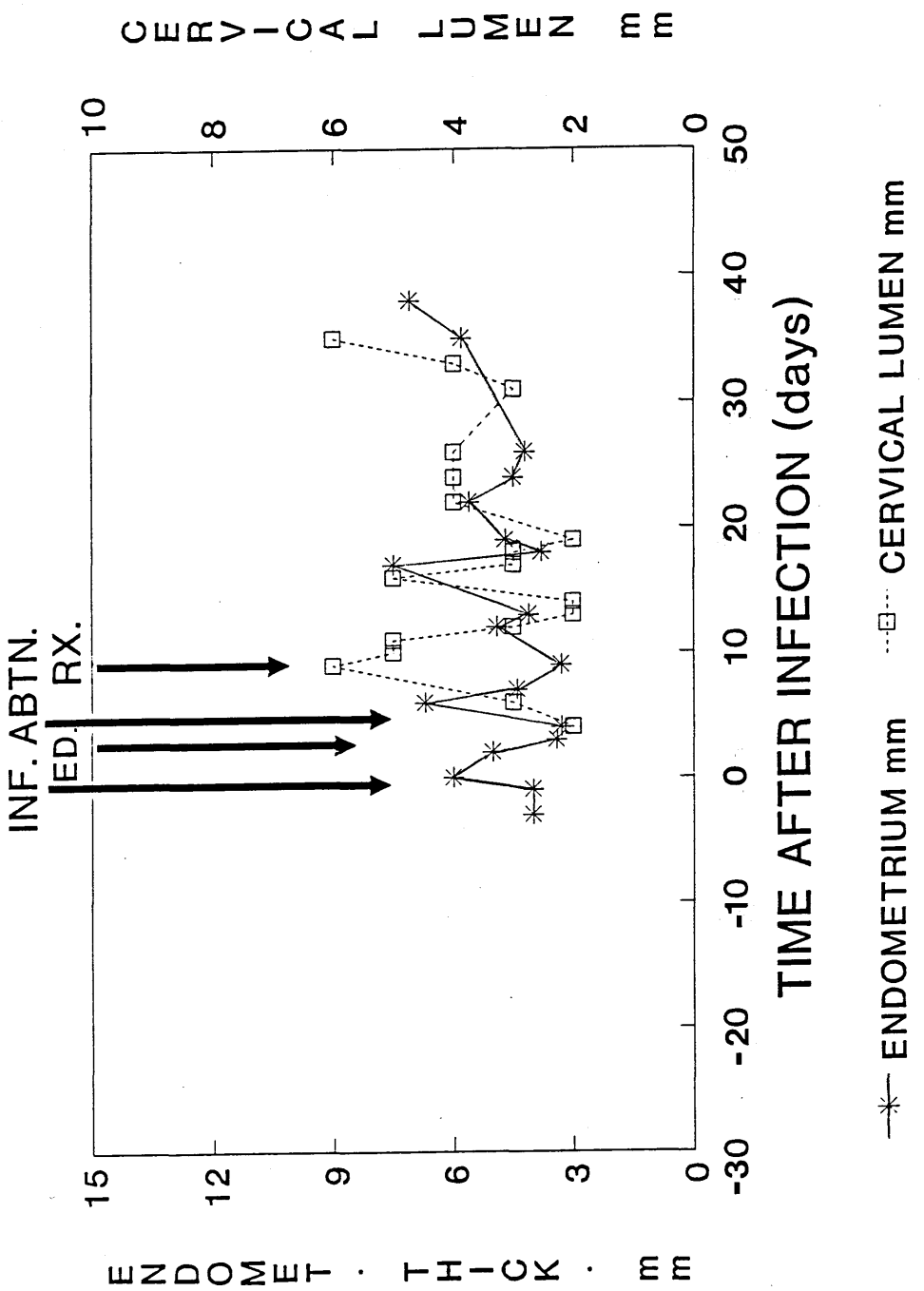

INF. ABTN.
ED
Rx.
CL AREA cm²
4
3
2
1
0
-30
-20
-10
0
10
20
30
40
50
TIME AFTER INFECTION (days)
CL AREA cm²

FIG. 79 (c)


INF. ABTN.
RX.
ED.
ENDOMET · TH-CK · mm
CERV-CAL LUMEN mm
TIME AFTER INFECTION (days)
ENDOMETRIUM mm
CERVICAL LUMEN mm

In cow 3 infected at day 34 of pregnancy aborted 103 hours later. Progesterone concentration increased from 4 ng/ml at the time of infection to 9 ng/ml 2 days after abortion, declined to 4.2 ng/ml between 3 - 6 days after abortion and increased again to 8.2 ng/ml 8 days after abortion (**FIG. 80a**). (**FIG. 80a**). There was a moderate decline of the concentrations up to above 4 ng/ml during further observations. The decline to the basal levels (0.5 ng/ml) occurred at the time of oestrus (31 days after abortion). Oestradiol 17 ß concentration spikes of between 0.8 and 2 pg/ml occurred between 2 and 22 days after infection. A significant increase in concentration to 5.6 pg/ml was however seen at the time of oestrus. The concentration declined to the basal level 2 days after oestrus.

The area of the corpus luteum was 3.4 cm^2 at the time infection (**FIG 80b**), and remained between 2 and 3 cm^2 for most of the time of observation before oestrus. At oestrus the area of the corpus luteum had declined to 0.3 cm^2. There was one 13 mm follicle in the left ovary between infection and and abortion (35 hours after infection). Further follicular activity leading to oestrus in this cow was reported in Chapter IV.

Endometrial thickness (**FIG. 80c**) increased between the time of infection and abortion from 4 to 8 mm and remained between 4 and 9 mm in thickness until 2 - 3 before oestrus. A maximum thickness of 14 mm was achieved days before oestrus. The cervical lumen was open >3 mm from abortion to oestrus 33 days after abortion (**FIG. 80c**).

FIG. 80 (a - b): A study of progesterone, oestradiol 17 ß plasma concentrations and their relationship with corpus luteum size, endometrial thickness and cervical lumen diameter, after live *A. pyogenes* infection in **Cow 3.** INF. = time of infection, ED. = embryonic death, ABTN. = time of abortion, RX. = antimicrobial treatment and HT. = oestrus.

FIG. 80 (a)

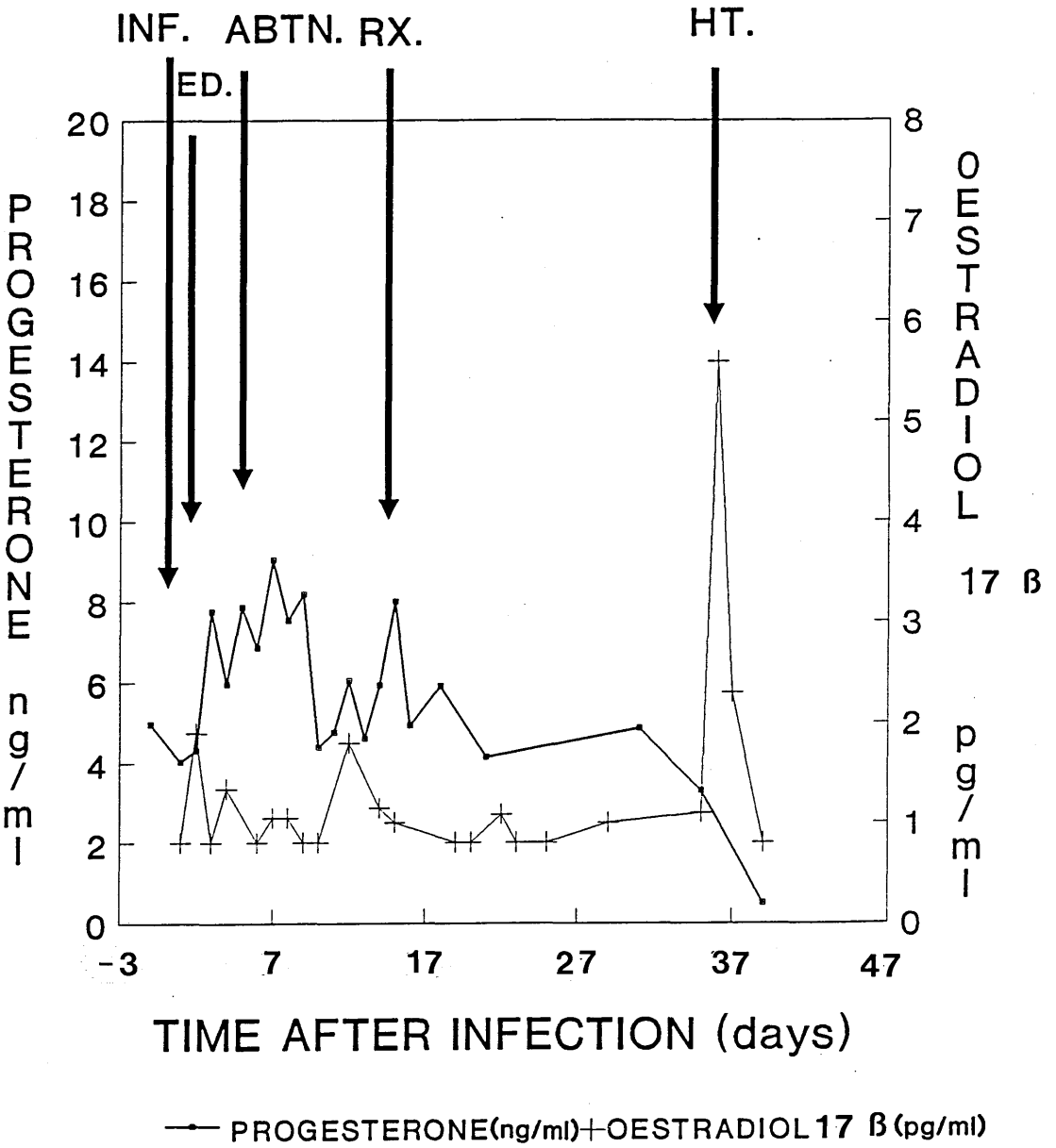


FIG. 80 (b)

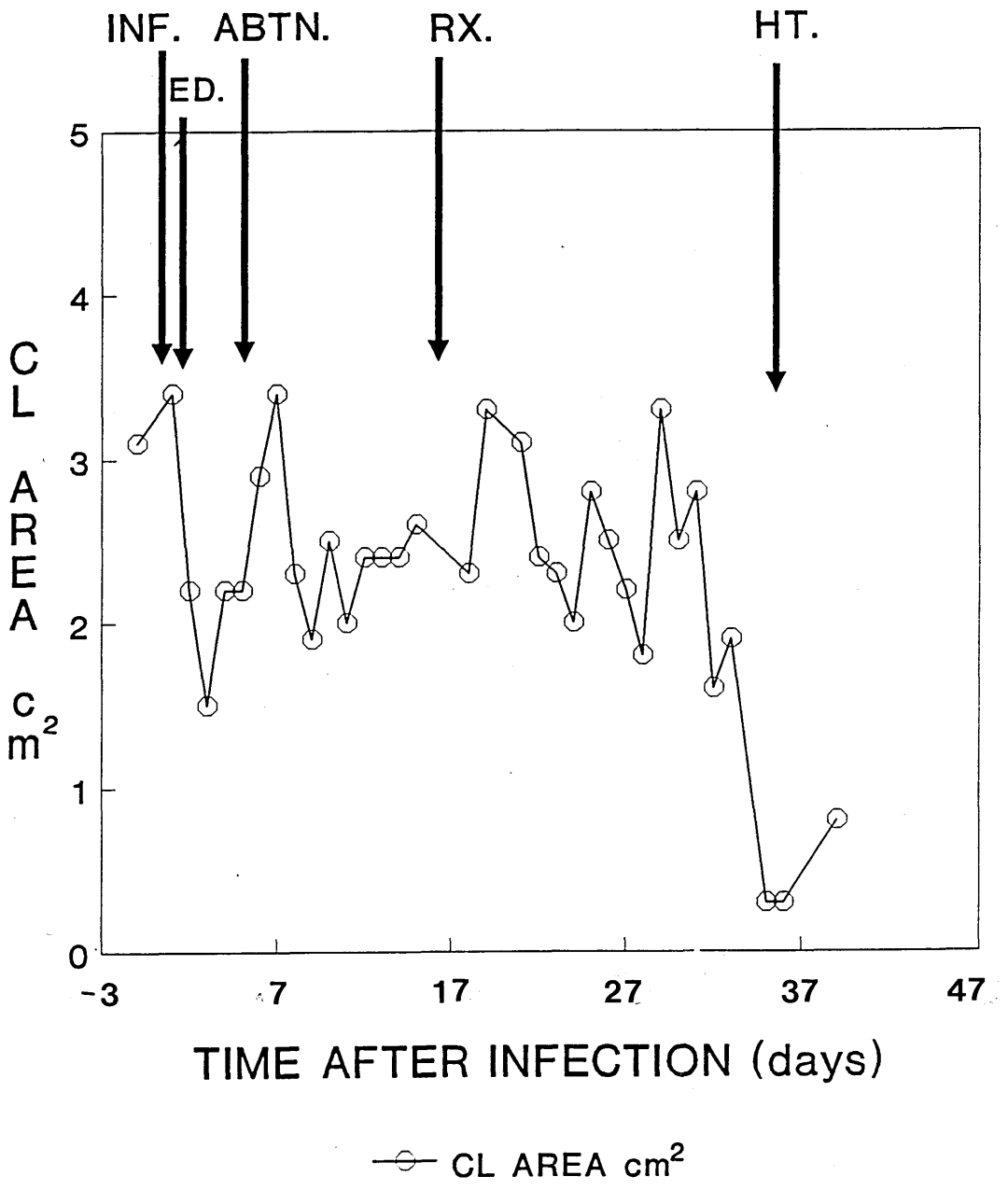
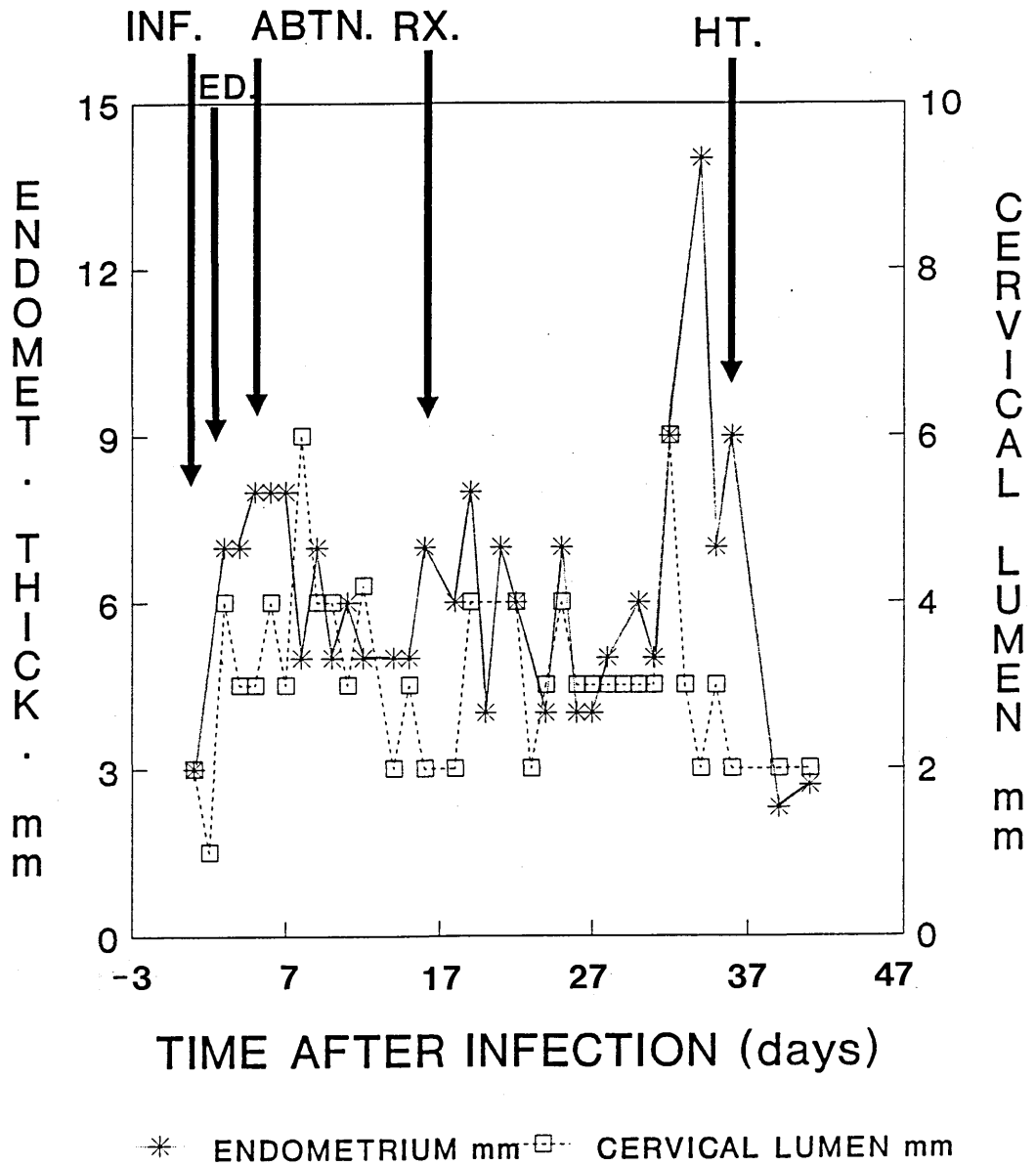

INF.
ED.
ABTN.
RX.
HT.
5
4
3
2
1
0
CL AREA cm2
-3
7
17
27
37
47
TIME AFTER INFECTION (days)
CL AREA cm2

FIG. 80 (c)


INF.
ABTN.
RX.
HT.
ED.
ENDOMET. THICK. mm
CERVICAL LUMEN mm
15
12
9
6
3
0
10
8
6
4
2
0
-3
7
17
27
37
47
TIME AFTER INFECTION (days)
ENDOMETRIUM mm
CERVICAL LUMEN mm

Cow 4 Progesterone and oestradiol 17 ß levels were followed from one day before infection in cow 4 which was infected at 41 days of gestation (**FIG. 81a**). Progesterone concentrations remained above 4 ng/ml throughout the time of infection and abortion. The concentration declined to basal levels (0.5 ng/ml) 23 days after abortion. This fall was accompanied by the signs of oestrus and 10 days after oestrus the concentration of progesterone had risen to 6.24 ng/ml. Oestradiol 17 ß on the other hand was at its basal levels (0.8 pg/ml) for the greater part of the period from infection, through abortion and antimicrobial treatment until heat occurred. There were, however, 2 spikes of 2 and 3 pg/ml between days 11 and 16 after abortion (**FIG. 81a**). The concentration of oestradiol increased on the day of oestrus to 4.3 pg/ml with another spike (2.4pg/ml) four days later.

The size of the corpus luteum was above 2.5 cm^2 throughout the time of abortion, treatment and up to the time of oestrus when it fell to 0.8 cm (**FIG. 81b**). Follicular growth was seen in both the right and left ovaries and the diameters were between 5 and 13 mm between infection and abortion. From abortion to oestrus (19 days after abortion) further follicular growth in both ovaries was observed and it occurred in 4 waves. The activity was predominantly in the right ovary (which bore no corpus luteum) and the follicular diameter was between 5 and 15 mm. The dominant follicle was 14 mm in diameter at oestrus and ovulated a day later with formation of a fresh corpus luteum which was 2.4 cm^2 in area, 6 days after oestrus.

Endometrial thickness was 7 mm 7 days after abortion (**FIG 81c**). It then decreased to between 4 and 6 mm up to the time of oestrus when it increased 13 mm. The thickness declined soon after oestrus to about 5 mm. The cervical lumen increased from 2 mm at the time of infection to about 4 mm at embryonic death and was 6 mm 2 days after abortion. The lumen

FIG. 81. (a - c): A study of progesterone, oestradiol 17 ß plasma concentrations and their relationship with corpus luteum size, endometrial thickness and cervical lumen diameter, after live A. pyogenes infection in **Cow 4.** INF. = time of infection, ED. = embryonic death, ABTN. = time of abortion, RX. = antimicrobial treatment and HT. = oestrus.

FIG. 81. (a)

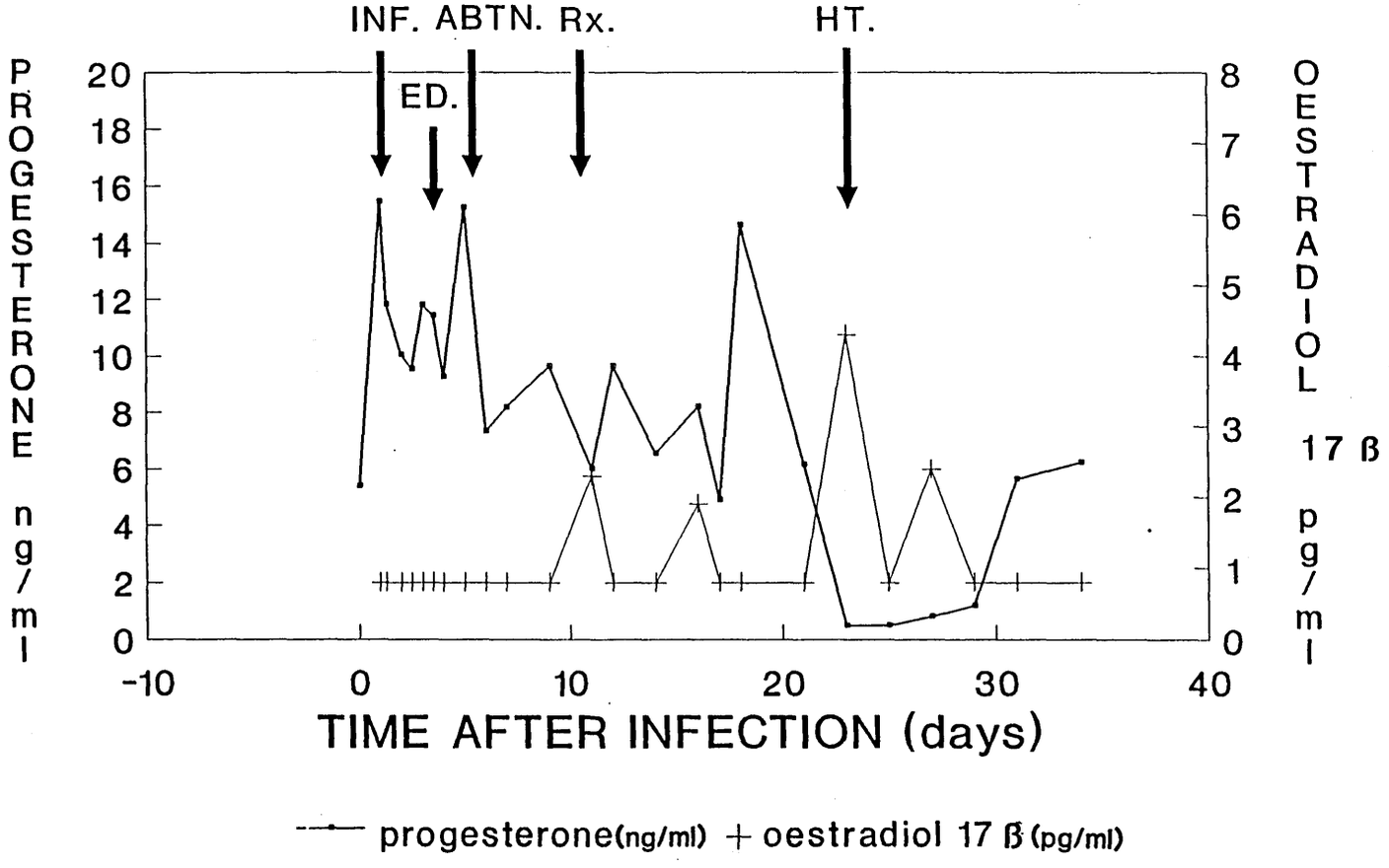


FIG. 81. (b)

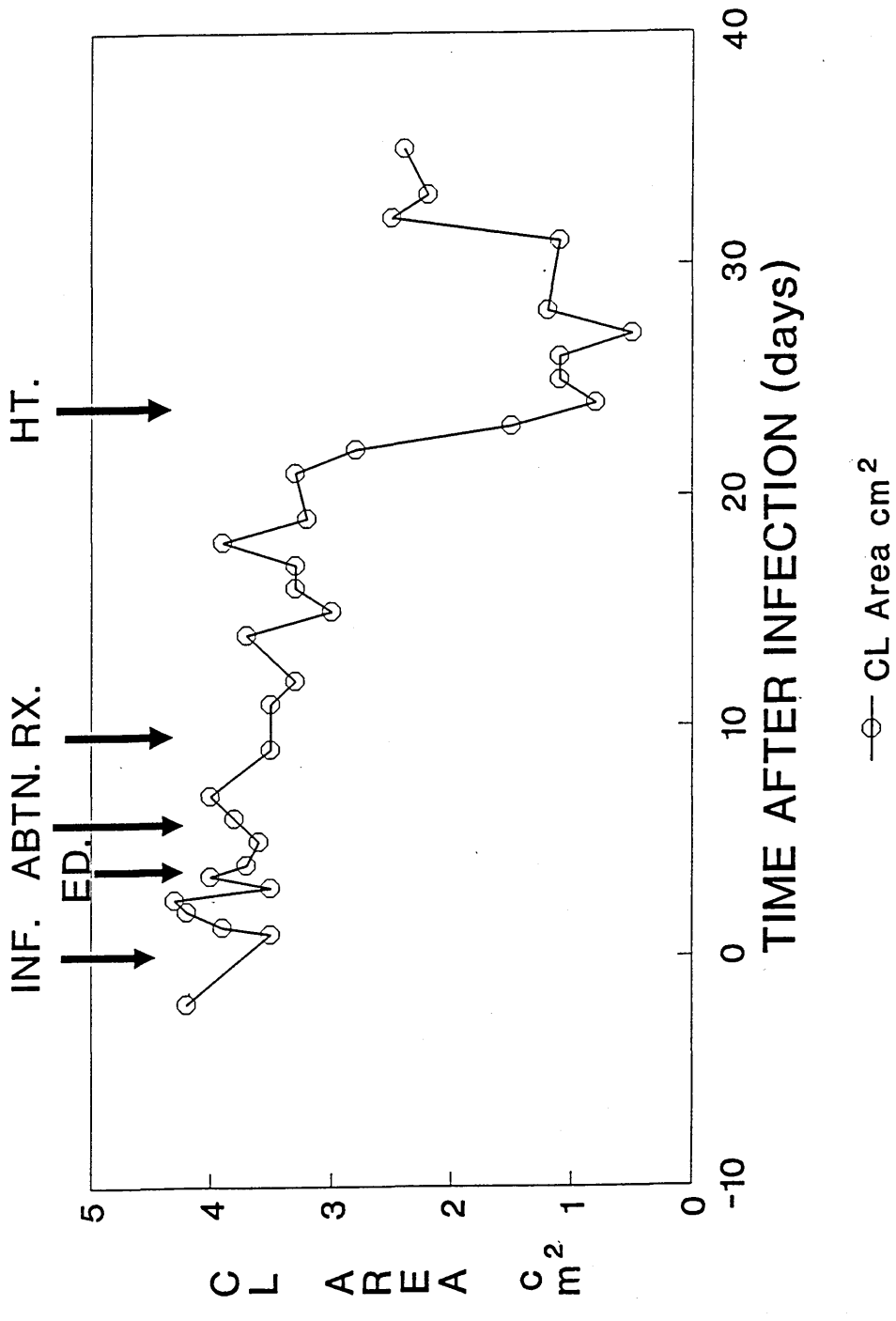
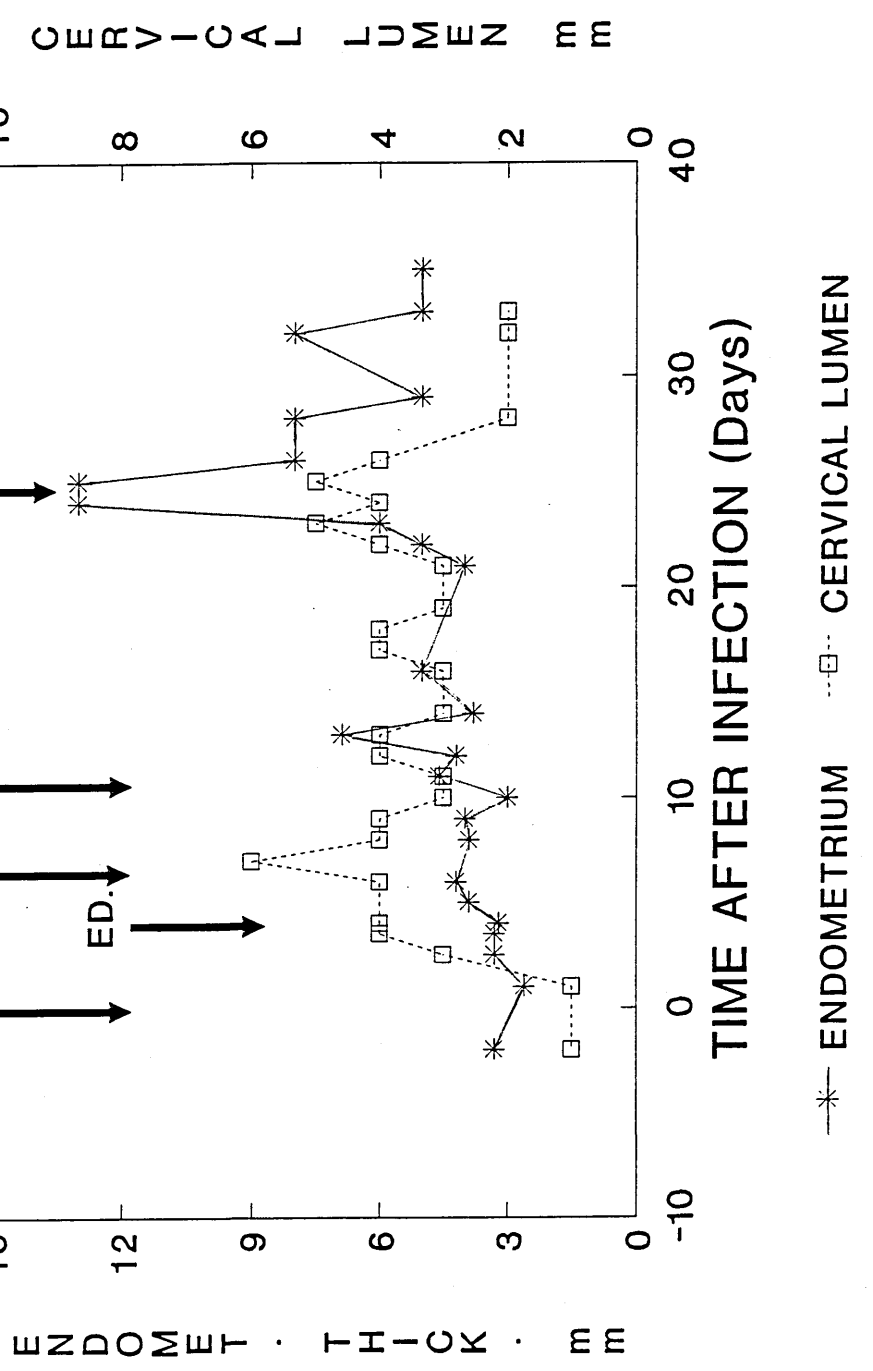

INF.
ABTN.
RX.
ED.
HT.
CL AREA cm²
5
4
3
2
1
0
-10
0
10
20
30
40
TIME AFTER INFECTION (days)
CL Area cm²

FIG. 81. (c)


ENDOMET · TH-CK · m m
15
12
9
6
3
0
-10
0
10
20
30
40
TIME AFTER INFECTION (Days)
CERV-CAL LUMEN m m
10
8
6
4
2
0
INF. ABTN. RX.
ED.
HT.
ENDOMETRIUM
CERVICAL LUMEN

was maintained at 3 to 4 mm for most of the time up to oestrus when it increased to 5 mm and then declined to 2 mm, 10 days after oestrus.

Heifer 5 (FIG. 82a) was infected at 33 days of pregnancy and the progesterone concentration was about 8 ng/ml. Two increases of progesterone above 10 ng/ml were seen between infection and abortion (i.e in 4 days). Though there was a decline after that time, the levels were maintained between 4 and 9 ng/ml until day 19 after abortion when the concentration fell to 1.1 ng/ml. The animal came into oestrus 2 days later with a plasma concentration of progesterone of 0.5 ng/ml. When the new corpus luteum was formed, the progesterone concentration increased to 4.34 ng/ml within 8 days after oestrus. No antimicrobial treatment was instituted during the post abortion period in this animal. Oestradiol 17 ß was at its basal concentration at the time of infection. Between the period after abortion and oestrus, there were 3 episodes of oestradiol increase of 4 to 6 pg/ml. The highest concentration of the hormone however was seen at the time of oestrus and was 7 pg/ml. Oestradiol 17 ß concentration however had fallen to the basal levels by 8 days after oestrus.

The area of the corpus luteum **(FIG. 82b)** was 3.6 cm^2 at the time of infection, and was maintained above 2 cm^2 up to 18 days after abortion. The corpus luteum declined in size after that to 0.6 cm^2 by the time of oestrus. Follicles were observed in both ovaries between infection and abortion, with the largest follicle (10 mm in diameter) in the right ovary. No oestrus or ovulation occurred during this time. Following abortion follicular development was observed in the left ovary (which did not bear the corpus luteum). Four waves of follicular growth were observed in this ovary between abortion and 16 days later when oestrus occurred. However a dominant follicle grew to 15 mm and ovulated in the right ovary.

FIG. 82. (a - c): A study of progesterone, oestradiol 17 ß plasma concentrations and their relationship with corpus luteum size, endometrial thickness and cervical lumen diameter, after live A. pyogenes infection in **Heifer 5.** INF. = time of infection, ED. = embryonic death, ABTN. = time of abortion, RX. = antimicrobial treatment and HT. = oestrus.

FIG. 82. (a)

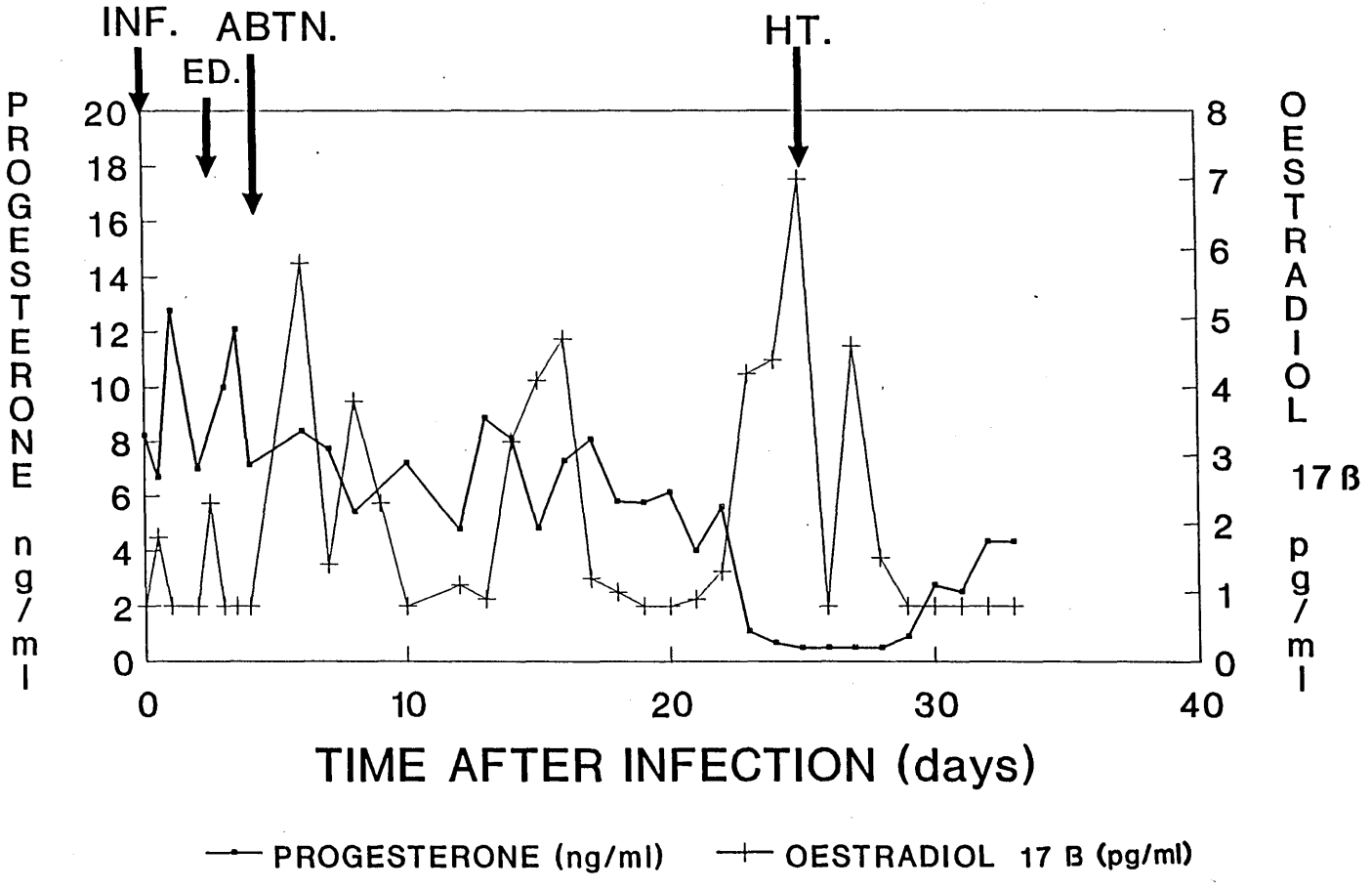


FIG. 82. (b)

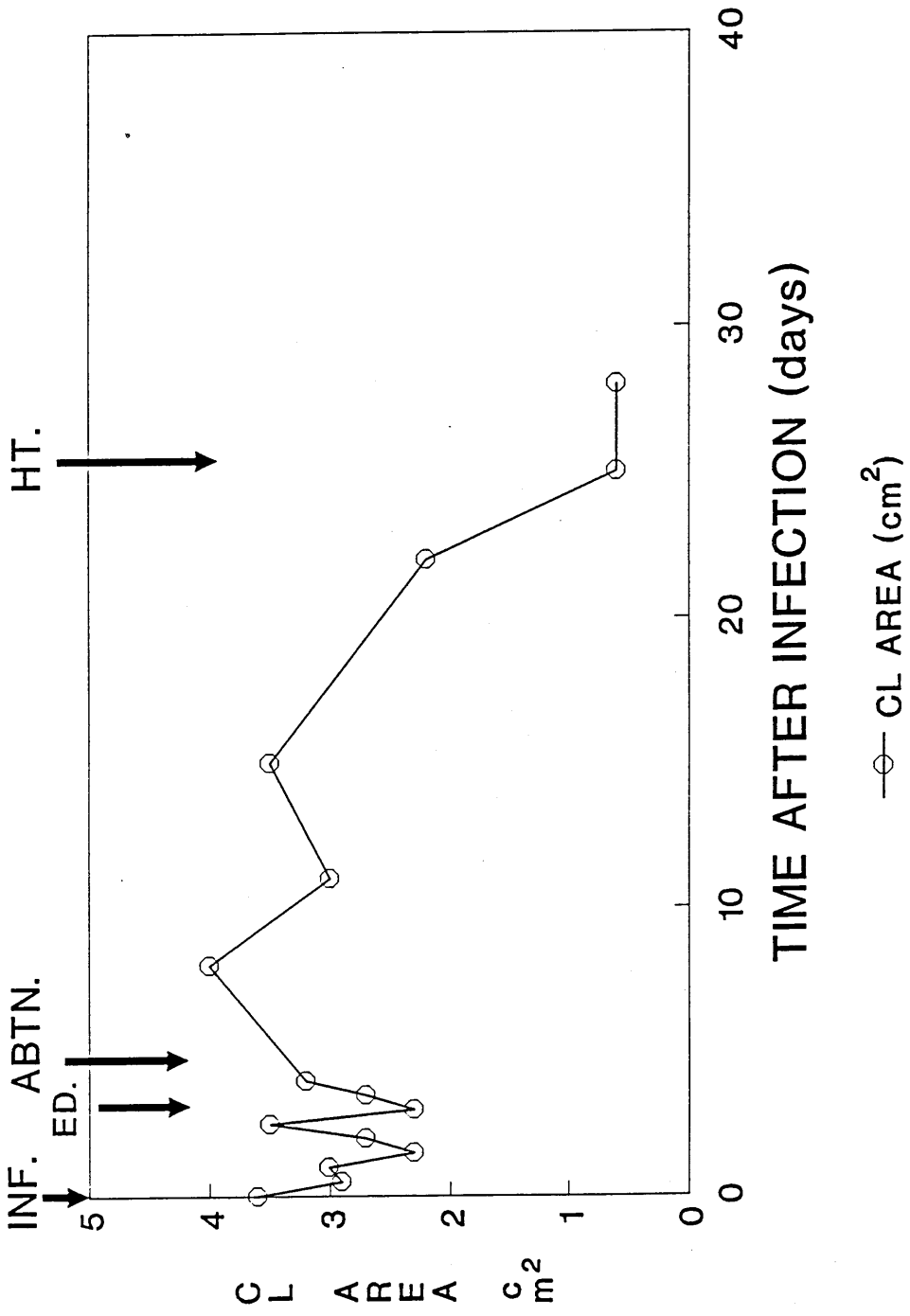
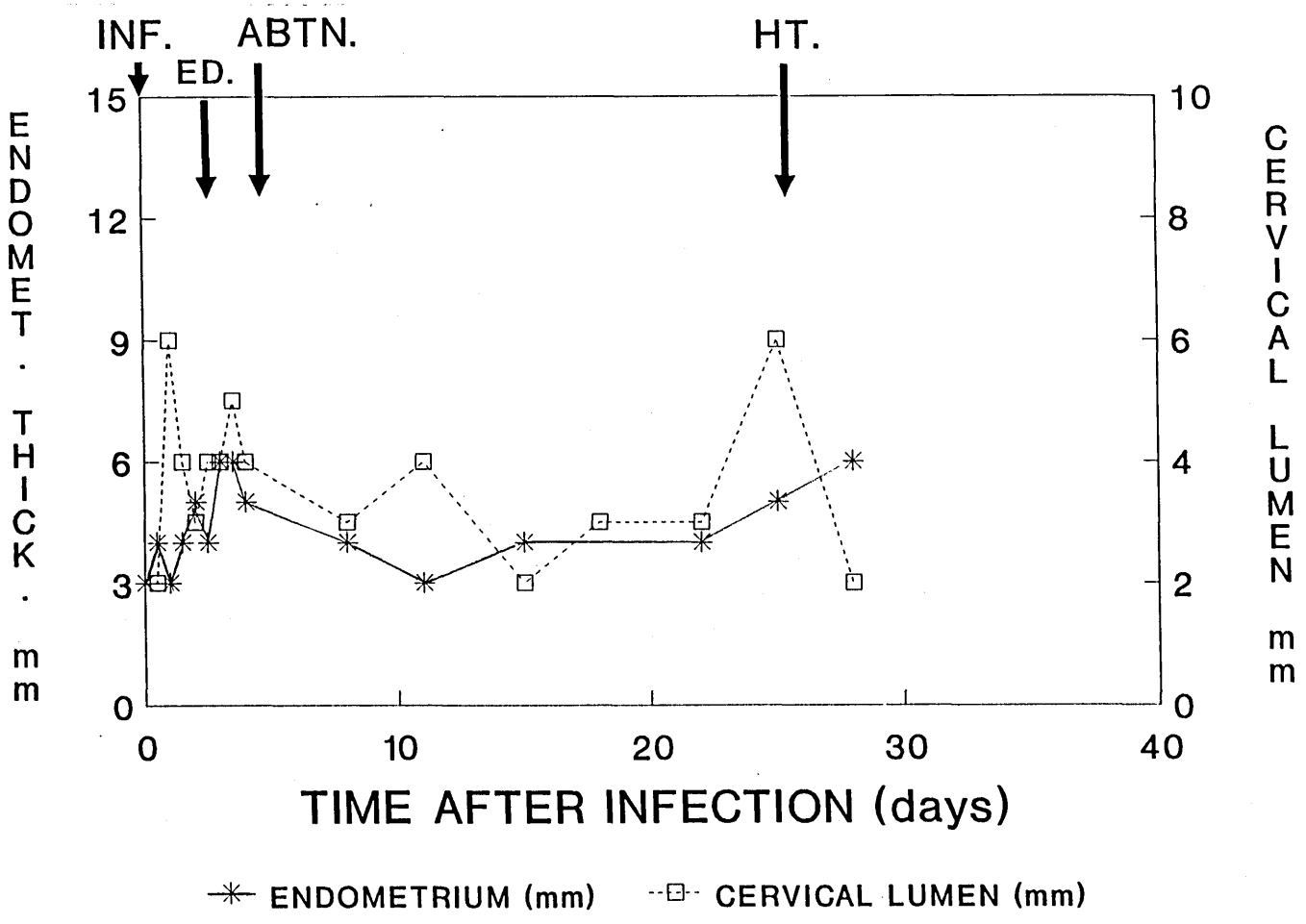

INF.
ED.
ABTN.
HT.
CL AREA cm^2
0
1
2
3
4
5
0
10
20
30
40
TIME AFTER INFECTION (days)
CL AREA (cm^2)

FIG. 82. (c)


INF.
ED.
ABTN.
HT.
ENDOMET. THICK. mm
CERVICAL LUMEN mm
0
3
6
9
12
15
0
2
4
6
8
10
0
10
20
30
40
TIME AFTER INFECTION (days)
ENDOMETRIUM (mm)
CERVICAL LUMEN (mm)

The dominant follicle in the left ovary which was 12 mm in diameter at oestrus did not ovulate.

The thickness of the endometrium had increased from 3 mm at the time of infection, to 6 mm by the time of abortion. There was a transient decline to 3 mm, but otherwise the thickness was maintained at 4 mm until the time of heat when it increased to 5 mm. The thickness was 6 mm by the time of the last examination. There was a drastic increase in the size of the cervical lumen from the time of infection to abortion when it was 5 mm in diameter. The lumen remained open for most of the time between abortion and oestrus. During oestrus however, the lumen was seen at its maximum size at 6 mm but closed 3 days later (**FIG. 82c**).

In cow 15 inoculated with killed *A. pyogenes*, embryonic death was not induced and pregnancy was terminated with cloprostenol treatment at 50 days of pregnancy (Chapter IV). The concentrations of progesterone and oestradiol 17 ß in this animal were as reported below (**FIG. 83a**). By the time of inoculation (27 days after A.I.) progesterone concentration was 12 ng/ml. Between inoculation and cloprostenol treatment, plasma progesterone concentrations were maintained at or above 6 ng/ml and changes during that time were not significant. After treatment with cloprostenol, the concentration fell to 0.6 ng/ml 48 hours after the treatment. Abortion accompanying the treatment also occurred 48 hours later. Oestradiol 17 ß was seen at its basal concentration for most of the time after inoculation to cloprostenol treatment. There were however spikes of the hormone up to 2 pg/ml especially soon after inoculation. The highest concentration of the hormone (4.6 pg/ml) however was reached at 2 days after cloprostenol treatment, at the time the animal came into oestrus.

FIG. 83. (a - c): A study of progesterone, oestradiol 17 ß plasma concentrations and their relationship with corpus luteum size, endometrial thickness and cervical lumen diameter, in **Cow 15** after intrauterine inoculation of killed A. pyogenes. INO. = time of inoculation, PG. = administration of cloprostenol, ED. = embryonic death, ABTN. = time of abortion, RX = antimicrobial treatment and HT. = oestrus.

FIG. 83. (a)

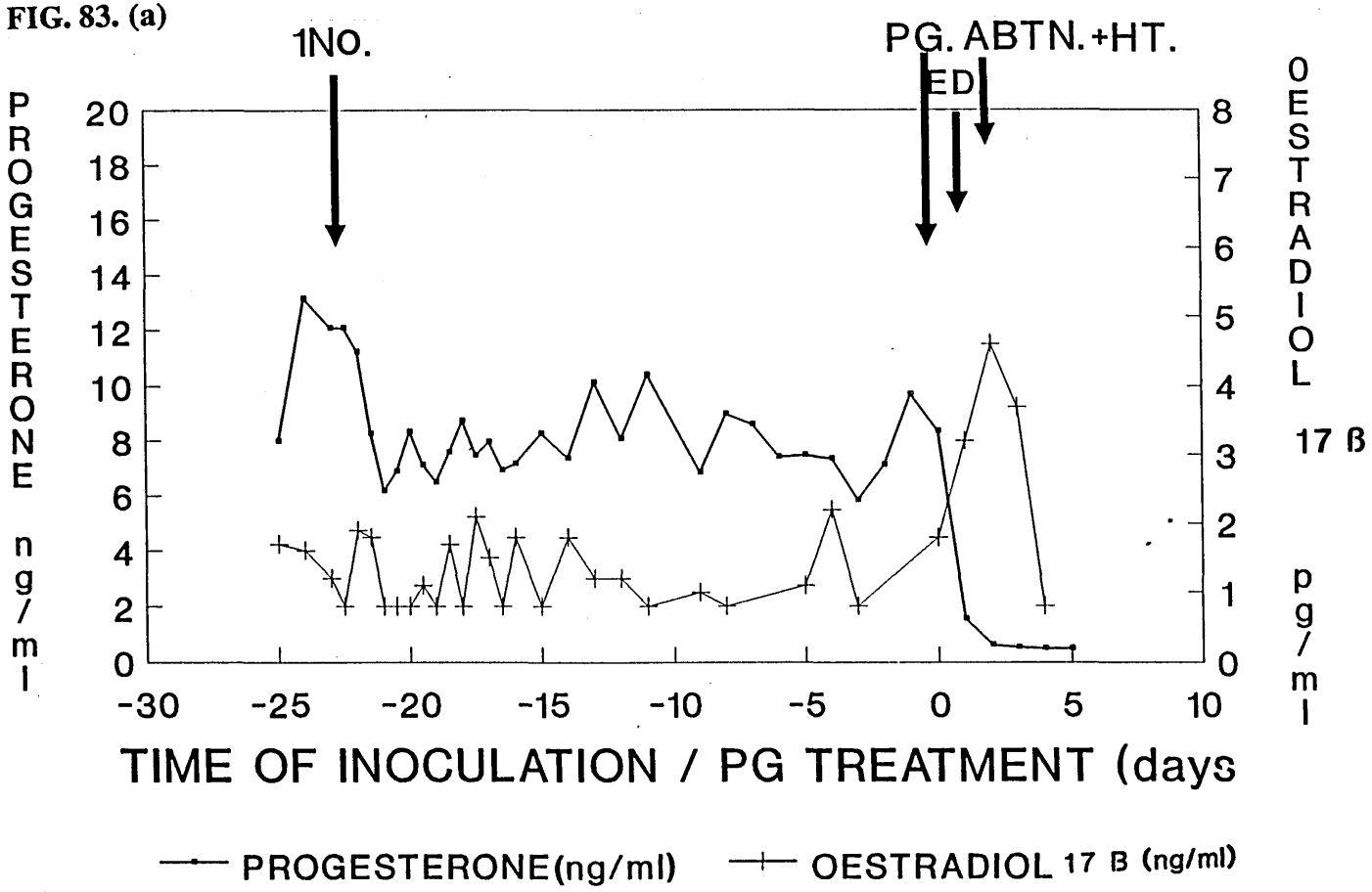


FIG. 83. (b)

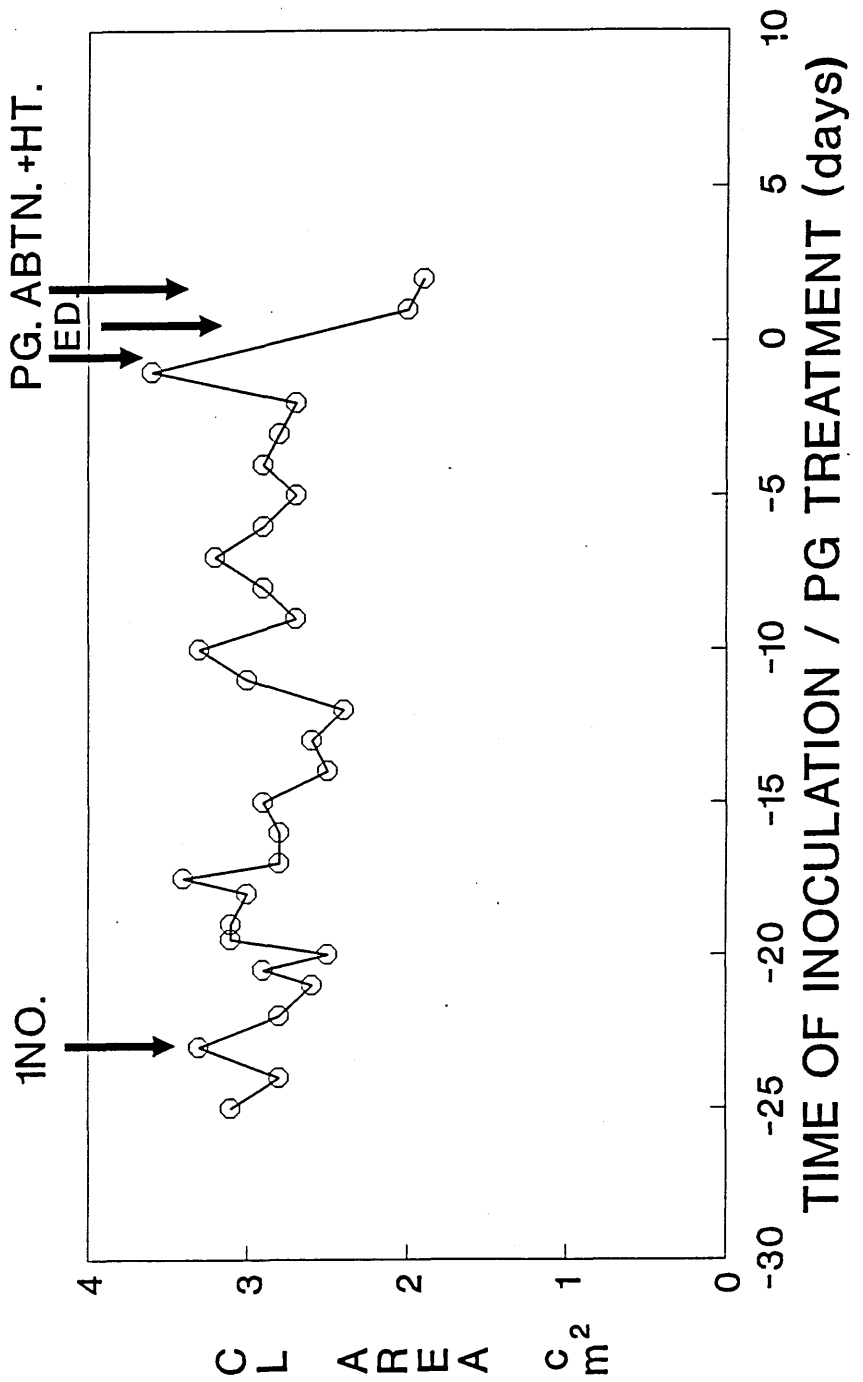
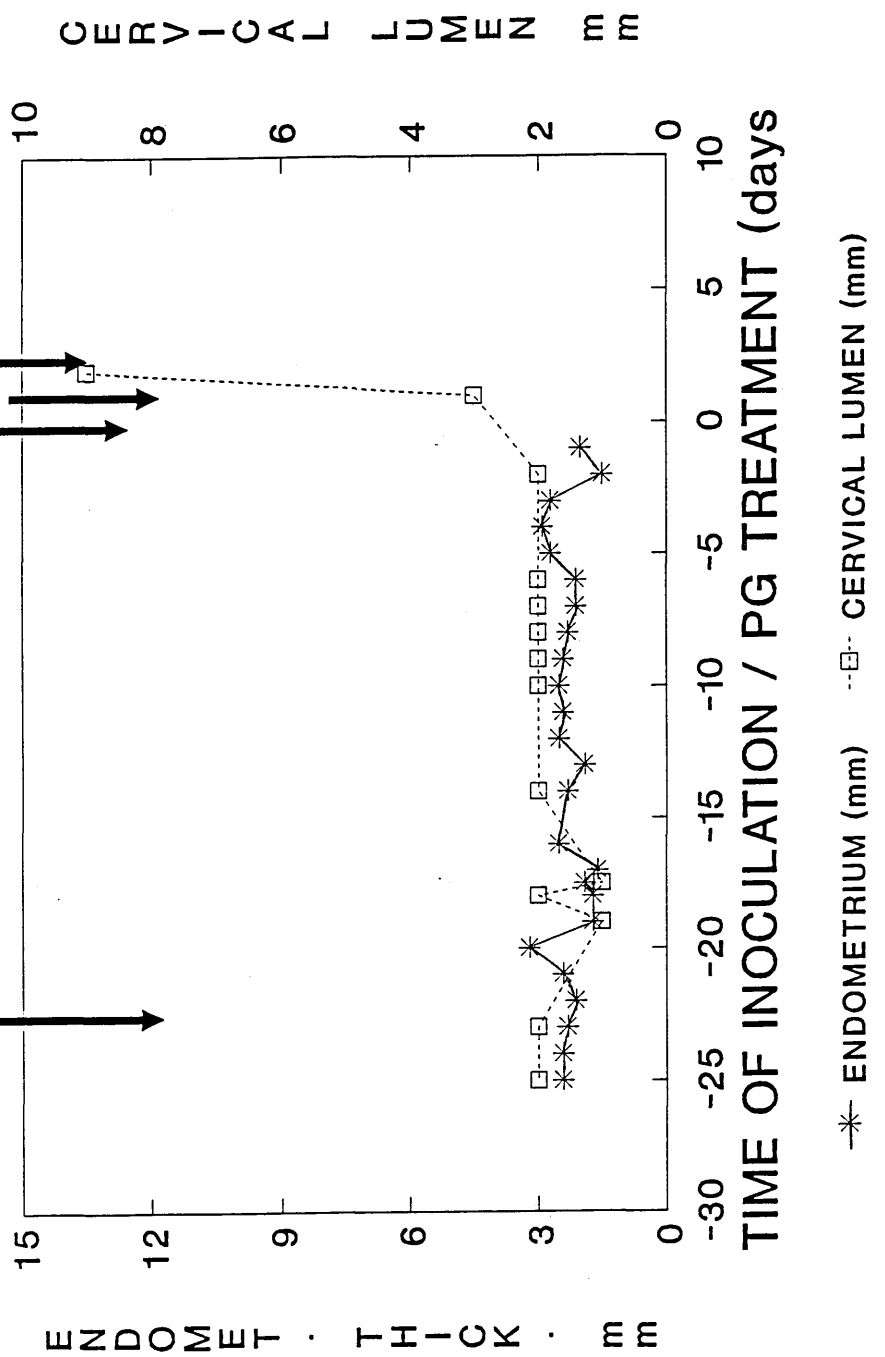

1NO.
PG. ABTN. +HT.
IED.
CL AREA cm²
4
3
2
1
0
-30
-25
-20
-15
-10
-5
0
5
10
TIME OF INOCULATION / PG TREATMENT (days)
CL AREA (cm²)

FIG. 83. (c)


INO.
PG.ABTN.+HT.
ED
ENDOMET · TH-CK · mm
CERV-CAL LUMEN mm
TIME OF INOCULATION / PG TREATMENT (days
ENDOMETRIUM (mm)
CERVICAL LUMEN (mm)

The area of the corpus luteum was seen to be between 2 and 3.6 cm^2 from the time of inoculation of the toxin to cloprostenol treatment (**FIG. 83b**). The area declined after cloprostenol treatment and was about 1.5 cm^2 at the time of abortion. Follicular development was observed in both ovaries from the time of inoculation of the toxin to cloprostenol termination of pregnancy. Follicular diameter during this time was between 4 and 12 mm. The largest follicle (14 mm in diameter) was observed in the right ovary 24 hours after the administration of cloprostenol.

Apart from during one examination when the thickness of the endometrium was seen to be 3.2 mm, the rest of the examinations revealed the thickness of the endometrium to be about 2.5 mm from inoculation to cloprostenol treatment (**FIG. 83c**). The cervix was also seen to be closed from inoculation to cloprostenol treatment. After the treatment with cloprostenol the cervix opened and was about 8.5 mm in diameter during abortion.

Only progesterone concentrations were determined in Heifer 17 inoculated with the *A. pyogenes* haemolysin (toxin). The animal was inoculated with the toxin at day 36 of pregnancy. Toxin alone failed to have any significant effect on the embryo and pregnancy continued to day 50 when it was terminated by cloprostenol treatment. Although follicular growth of up to 11 mm in diameter was observed after introduction of the toxin and up to 50 days of pregnancy, no follicle ovulated. The concentration of progesterone was 7.48 ng/ml at the time of inoculation. Apart from a transient increase to 11.26 ng/ml two days after inoculation, the concentration of progesterone was maintained above 6 ng/ml up day 50 of pregnancy (**FIG. 84a**). Cloprostenol treatment brought the progesterone concentration down to 0.5 ng/ml within two days after the injection when abortion also occurred and the animal came into oestrus and was associated with growth of a

FIG. 84 (a and b): Changes in progesterone levels and the early pregnant uterus after intrauterine inoculation with A. pyogenes toxin in Heifer 17. TX. = inoculation of A. pyogenes toxin, PG. = cloprostenol administration, ED. = Embryonic death, ABTN. = abortion and HT. = oestrus.

FIG. 84 (a)

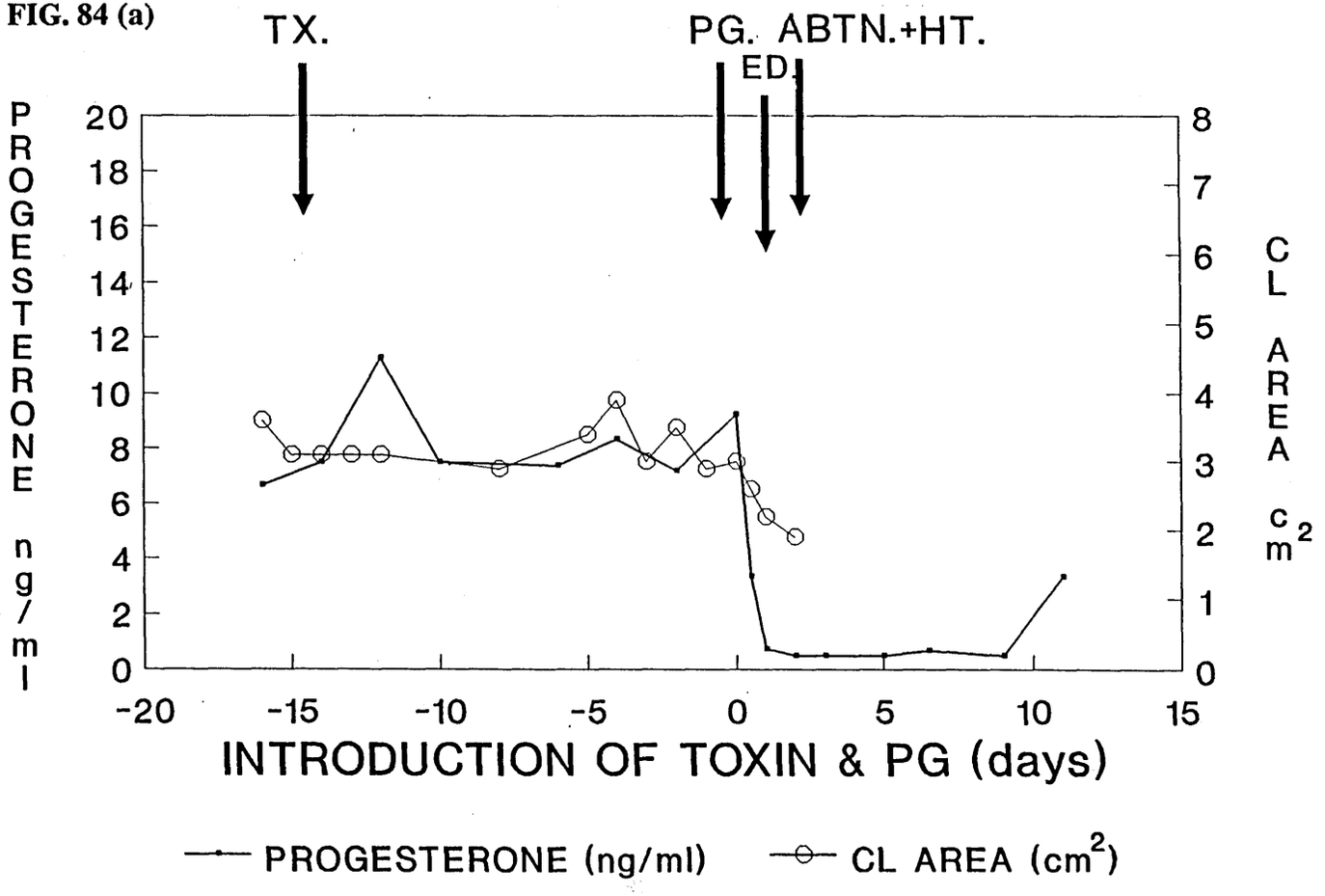
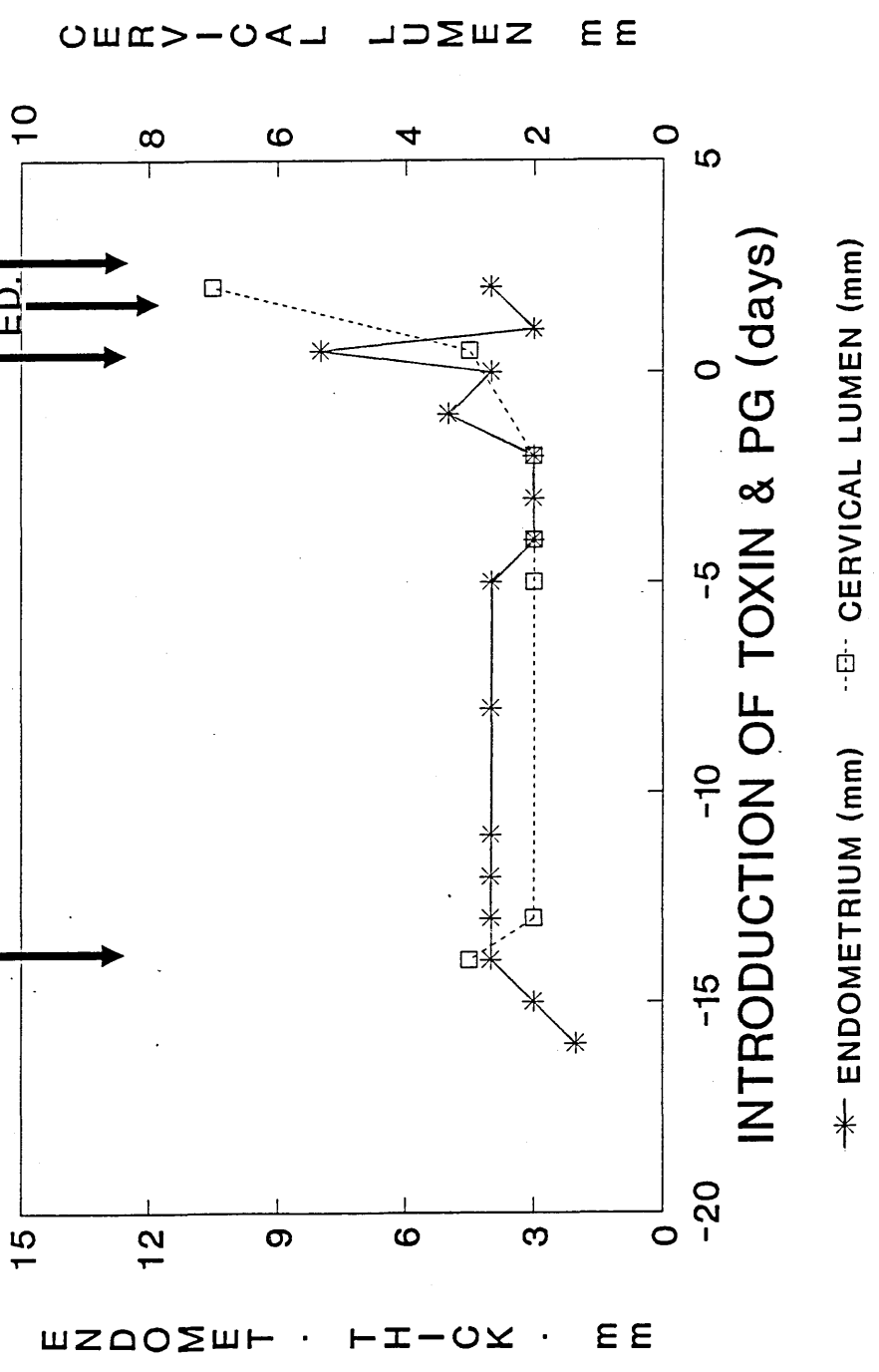


FIG. 84 (b)


TX.
PG. ABTN.+HT.
ED.
ENDOMET · TH-CK · mm
15
12
9
6
3
0
CERVICAL LUMEN mm
10
8
6
4
2
0
-20
-15
-10
-5
0
5
INTRODUCTION OF TOXIN & PG (days)
ENDOMETRIUM (mm)
CERVICAL LUMEN (mm)

dominant follicle (18 mm in diameter) in the left ovary. Five days after start of the new cycle, progesterone concentration had increased to 3.48 ng/ml.

The area of the corpus luteum was maintained between 3 and 4 cm^2 from the time of inoculation to day 50 of cloprostenol treatment. At the time of abortion the area of the coprpus luteum had fallen to about 1.6 cm^2.

Results from examination of the endometrium and the cervical lumen were as follows (**FIG. 89b**). Endometrial thickness was about 4 mm from the time of inoculation to the time of treatment with cloprostenol. After the treatment, there was a transient increase in thickness to 8 mm one day later but it soon fell to 3 mm. The cervix was closed for most of the time after inoculation. After cloprostenol treatment the cervix opened and was about 7 mm at the time of abortion.

Cloprostenol treatment. Cow 14 treated with cloprostenol at 50 days of pregnancy was examined. Only the progesterone concentrations were determined in this animal and they were related to the size of the corpus luteum (**FIG. 85a**). The concentration was about 4.6 ng/ml at the time of treatment and had reached 0.5 ng/ml 24 hours after the treatment. Abortion occurred 2 days after the treatment and was followed by oestrus. Seven days after oestrus progesterone concentration had increased to about 9 ng/ml. The corpus luteum was 2.3 cm^2 at the time of treatment and had declined to 0.7 cm^2 at the time of abortion. Follicular events in the ovaries were reported in chapter III.

Endometrial thickness and the cervical lumen both had a transient increase after cloprostenol treatment which was sustained for 4 days (**FIG. 85b**). Endometrial thickness had declined to 1.5 mm at 8 days after the treatment

FIG 85 (a and b): Observation of changes in the levels of progesterone and the early pregnant uterus after cloprostenol induced abortion in **Cow 14.** PG. = cloprostenol administration, ED. = Embryonic death, ABTN. = abortion, HT. = oestrus.

FIG 85 (a)

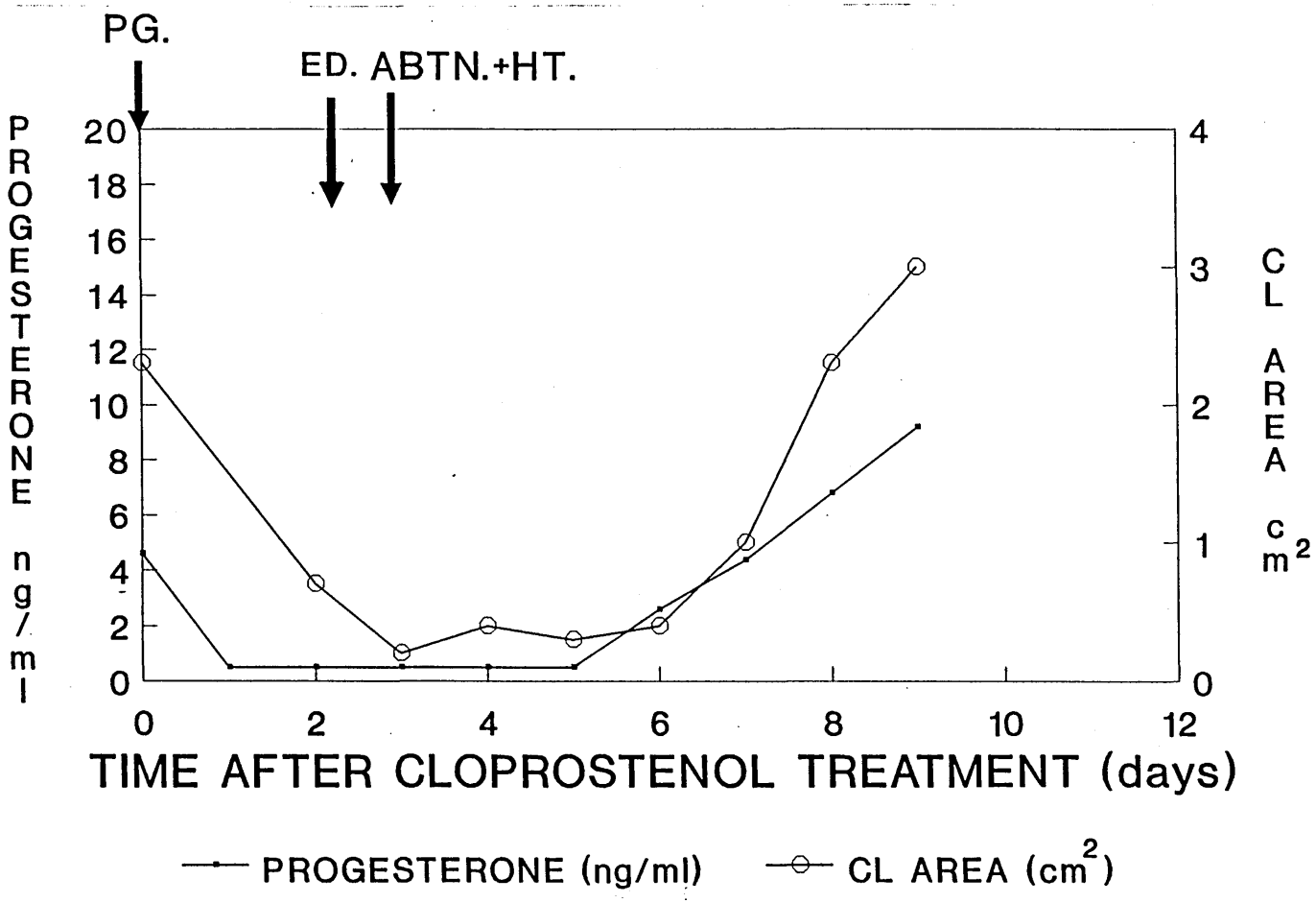
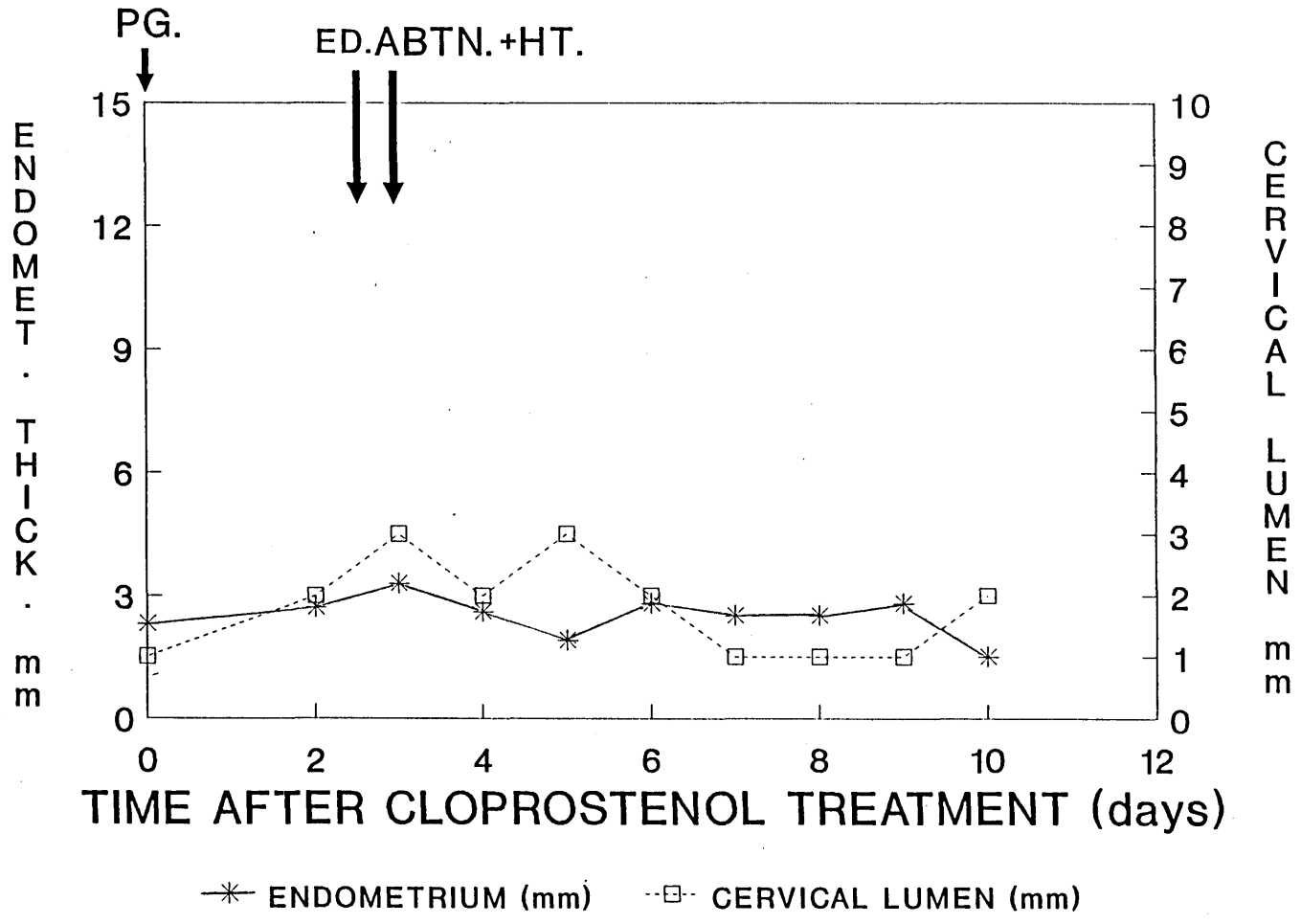


FIG 85 (b)


PG.
ED.ABTN.+HT.
ENDOMET. THICK. mm
CERVICAL LUMEN mm
15
12
9
6
3
0
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
TIME AFTER CLOPROSTENOL TREATMENT (days)
ENDOMETRIUM (mm)
CERVICAL LUMEN (mm)

and the cervix was closed by the fourth day after abortion.

Detailed progesterone examination. Examination of progesterone concentrations in Heifer 5 between infection and abortion revealed that these were above 4 ng/ml while many samples had concentrations of >10 ng/ml (**FIG. 86**). Embryonic death and abortion were observed when progesterone concentration was between 6 and >10 ng/ml. Embryonic death in heifer 13 occurred 40 hours after the progesterone concentration had declined to <2ng/ml from >10 ng/ml at the time of treatment with cloprostenol. Abortion occurred when the concentration of progesterone was 1.3 ng/ml (**FIG. 87**).

DISCUSSION.

The progesterone and oestradiol 17 ß levels found in the plasma of experimental animals are discussed here in relation to the state of the reproductive tract produced by the experiments described in the chapters III and IV and the post abortion observations described in Chapter V. Factors affecting the validity of the results are discussed here, as are the actual findings. These will be related to the general literature in the general discussion.

Animals infected with live *A. pyogenes*.

Progesterone levels. Plasma progesterone levels were measured using the ELISA kit which had an upper limit of detection of 10 ng/ml. Samples which exceeded this level were diluted and retested. The results of this (as the samples were examined 2 or 3 times) dilution and testing were considered to be accurate and the use of controls with the diluted plasma was felt to ensure accuracy. The general criteria for accuracy were given in this chapter.

FIG. 86: Progesterone concentrations during frequent (15 minutes) sampling of plasma progesterone in **Heifer 5** after infection with *A. pyogenes.* INF. = time of infection, ED.= Embryonic death, ABTN.= abortion.

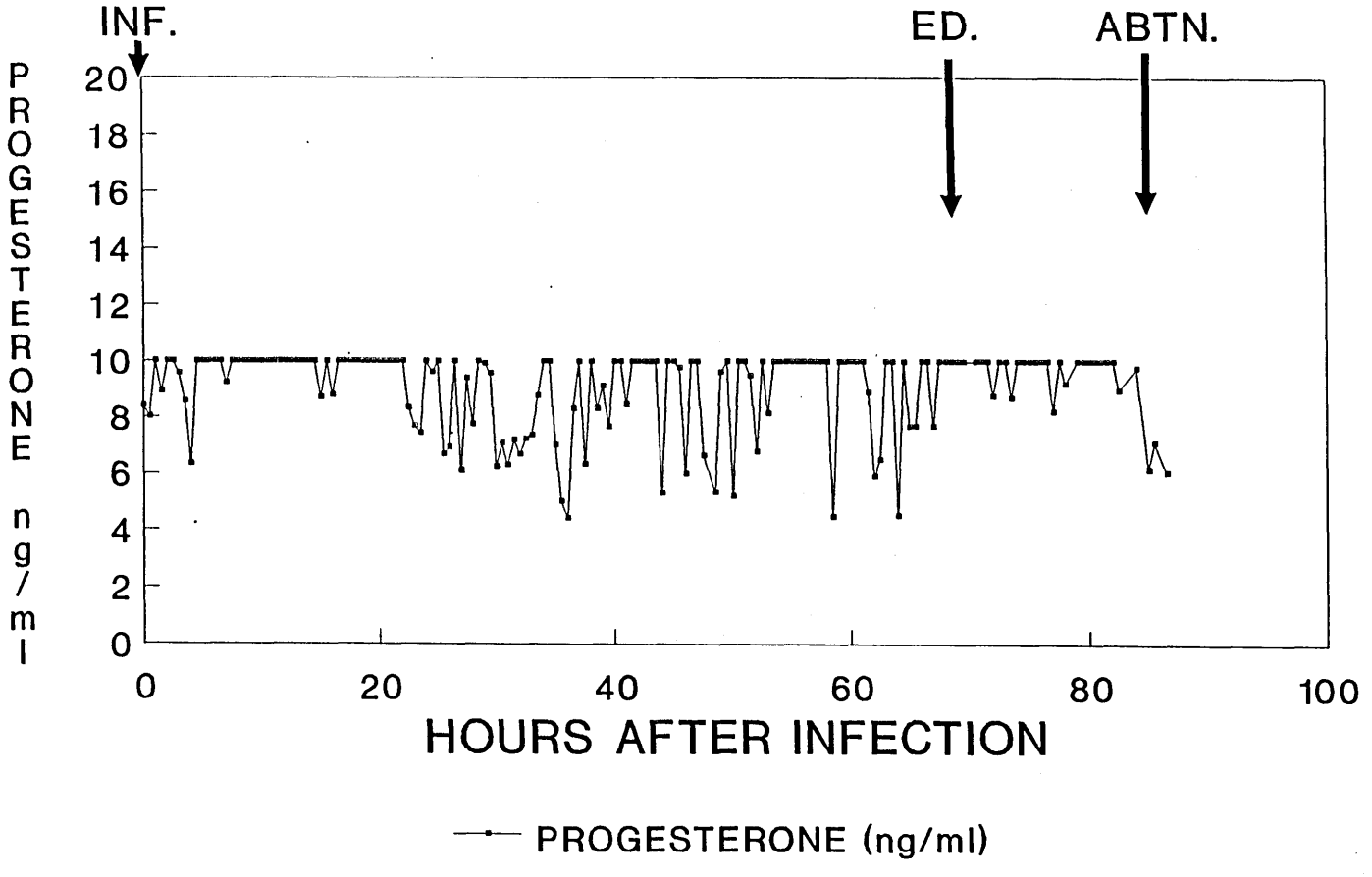
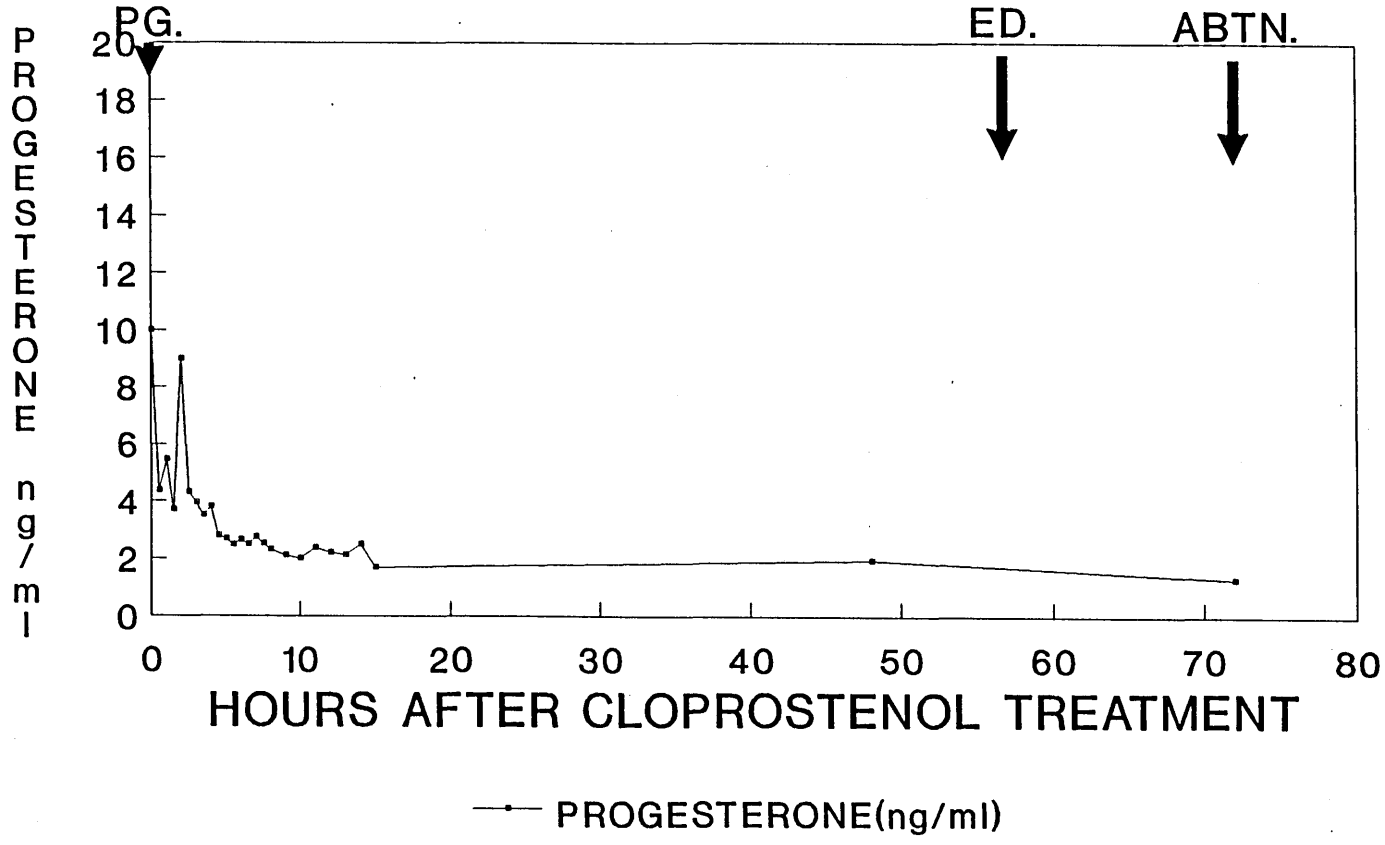


FIG. 87: Frequent sampling for plasma progesterone in **Heifer 13** in cloprostenol induced abortion. PG.=cloprostenol administration, ED.=Embryonic death and ABTN.=abortion.



Concentrations of progesterone remained above 4 ng/ml from before infection to just before the first oestrus (**FIGS 78a, 79a, 80a, 81a and 82a**) and reached levels of 17 ng/ml. The high concentration of progesterone between infection and abortion confirms that the corpus luteum present was functional. It is of interest that soon after infection the concentration of progesterone increased above 8 ng/ml and sometimes exceeded 10 ng/ml. in cow 3 and 5. These figures are higher than those that have been reported for cattle during a cycle or in normal pregnancy as reported by Eckersall and Harvey (1987, from a small number of cows) and Thomson and Dobson (1989, who used the RIA). The reasons for these high progesterone levels was not clear in this study and should be investigated. No previous reports have been made describing this phenomenon. Plasma progesterone concentrations 2 ng/ml or more have been known to occur in normal pregnancy (Thomas and Dobson 1989). It was considered possible that the levels of progesterone might have fallen in intervals between sampling in the infected animals, however, the detailed sampling regime in Heifer 5 (**FIG. 86**) showed that this did not occur and that levels were maintained above 4 ng/ml for the whole period of embryonic death and abortion.

Oestradiol 17 ß

Concomitantly the concentration of oestradiol 17 ß was about 0.8 pg/ml in cows 1, 2 and 4. In the other 2 animals (3 and 5) there was a transient increase in oestradiol 17 ß from 0.8 to 6 pg/ml between 0 and 10 days after infection. These peaks appeared in samples at the same time as other samples from the same animal and must be regarded as genuine. The low levels of oestradiol 17 ß seen in animals 1, 2 and 4 (**FIGS 87a, 79a and 81a**) confirm the lack of cyclical activity in the post abortion period. This differs markedly from the activity which is seen in the absence of pregnancy (Schallenberger et al. 1989). The increase in the concentrations seen in the

other two animals in this group (3 and 5) was of no significance.

Corpus luteum maintenance.

The corpus luteum was maintained from before infection to immediately prior to the first oestrus at 18 - 33 or more days after abortion. The size of the corpus luteum was measured by the use of ultrasound and dimensions were recorded from the image. The size range of the maintained corpora lutea was from 1.5 cm^2 (cow3, 2 days after infection)to 4 cm^2 (cow 4, 3 days of infection). These extremes of size of the corpus luteum were considered to be accurate as ultrasound is known to measure the corpus luteum size accurately (Omran 1989) and physical examination confirms that corpora lutea may have this size range. The variation in size shown in **FIGS 78c, 79c, 80c, 81c and 82c** over the period from infection to immediately prior to oestrus may be due in part to variation in the position of the probe in relation to the uterus.

The minimal size of the corpus luteum observed between infection and prior to oestrus was 1.3 cm^2 (cow 1, 16 days after infection) but this minimal size was associated with a plasma progesterone concentration of 4.3 ng/ml suggesting that it was still fully functional. No attempt to relate echogenicity (density) of the corpus luteum to its function was made although this would have been technically possible (Omran, 1989). As progesterone levels around embryonic death and abortion remained above the minimum considered necessary for the maintenance of pregnancy, the corpus luteum was considered to be functional and such a study (for echogenicity) was not carried out. Regression of the corpus luteum commonly precedes abortion Omran (1989) but this did not appear to occur in any of the infected animals all of which aborted. The observation is extremely important in considering

the aetiology of abortion following *A. pyogenes* infection. The implications of this finding are discused in Chapter VIII.

Follicular activity observed in the ovaries between infection and abortion did not seem to affect the concentration of progesterone and in this respect resembled the position seen during the luteal phase of the oestrous cycle (Omran 1989). Follicles seen in cows 1, 2 and 4 did not significantly affect oestradiol 17 ß concentration during the same time. The transient increase in the concentrations of oestradiol 17 ß in Cow 3 and Heifer 5 could be attributed to the follicular growth observed. Regardless of the concentrations of oestradiol 17 ß in the first 10 days of following abortion, none of the infected animals came into oestrus and none of the follicles ovulated.

Follicles were seen to grow in both ovaries a during the pregnancy - like state, but there was no consistent increase in the concentration of oestradiol 17ß. This may have been due to the progesterone dominant phase and the damaged uterine endometrium. A dominant follicle >14 mm in diameter was seen in one of the ovaries only after evacuation of pus from the uterus (eg Cow 3, **Table** 8). This was preceded by oestrus and ovulation of the follicle with formation of a fresh corpus luteum. Presence of pus in the uterus with the damaged endometrium may have resulted in failure of endometrium to produce prostaglandin F2α to effect luteolysis, and this could have been coupled with the lack of oxytocin receptor formation in the damaged uterine wall.

Follicular growth was observed in control animals 15 and 17 48 - 72 hours after administration of cloprostenol. It is therefore suggested that follicular growth observed during the pregnancy - like state in the infected animals was not important. The factors that trigger follicular growth leading to oestrus after elimination of pus from the uterus were not investigated in this study. Recovery of the uterine wall from infection may have led to re-establishment of its ability to produce PGF2α to cause luteolysis and facilitating follicular growth.

Elimination of pus from the four animals 1, 3, 4 and 5 (**FIGS 78, 80, 81 and 82**) was followed by lysis of the corpus luteum and a fall in progesterone to basal concentrations (<0.5 ng/ml). These were followed by elevation of oestradiol 17ß, oestrus and ovulation of the dominant follicle.

These events appeared to follow exactly those of a normal cycle and to mark the end of the hormonal and other abnormalities associated with infection. It was not clear from this experiment whether elimination of the infection by antimicrobial treatment or the animal's intrinsic defence mechanisms or other hormonal interplay or all were responsible for the re-establishment of the normal cycle. It was however clear that while pus was present in the uterus the animals did not cycle and there were no signs of silent heat during the 18-34 days after abortion (**FIGS 78a, 79a, 80a, 81a and 82a**).

Endometrial thickness. The moderate increase in the thickness of the endometrium (4 - 8 mm) observed after abortion was considered in Chapter IV to result partly from the inflammation. The data presented in **figures 78c - 79c** confirm that endometrial thickening persists until onset of oestrus. Some of the variations recorded in endometrial thickness between abortion and first oestrus may have been the result of the different locations of the probe over the uterine horns. These variations do not appear to be related to progesterone or oestradiol 17 ß levels (Cow 3 **FIG. 80c**). At oestrus the thickness increased dramatically (8 -14 mm) as expected in a normal cycle.

Following oestrus the endometrial thickness returned to low levels of 2 - 3 mm (Cow 3, **FIG. 80c**) or remained higher (4 - 5 mm, Cow 4). Too few animals were followed for too short a time to draw any firm conclusions about it. If the fate of infected cows were to be followed further, this parameter should be studied.

The relationship between embryonic death and hormonal levels. Progesterone levels remained high (>4 ng/ml) between infection and embryonic death and oestradiol 17 ß never increased to levels found at oestrus. The relationship of progesterone levels to embryonic death is reported in more detail in FIG. 91. From the figure the transient reductions in progesterone level never fall bellow 4 ng/ml. Regardless of the possibility of embryonic loss being initiated by a decline in progesterone levels, the failure of its fall below 4 ng/ml suggests that it had no part in embryonic death in this group.

Uterine contents. The accumulation of pus in the uterus reported in Chapters III and IV prior to abortion was associated with a mean thickening in the endometrium (**FIGS 80c, 81c and 82c**) and with embryonic death (Chapters III, IV and V). The hormonal levels were not related to the presence pus and did not change significantly until the onset of oestrus. They therefore appeared to have little influence on abortion but the changes in their concentration coincided with the expulsion of pus from the uterus. It is not clear what part the presence of pus played in the retention of the corpus luteum post abortion and possible mechanisms for this are discussed in Chapter VIII.

The cervix. The observation that in all animals infected with the live bacteria opening of the cervix was accompanied abortion and that it remained open subsequently was an important observation. The data presented in **figures 78c, 79c, 80c, 81c and 82c** shows some variation between daily observations. The cervix was closed prior to infection in every case, opened to 6 mm at abortion and oestrus, but the aperture varied between 2 and 4 mm between abortion and oestrus.

The diameter of the cervical lumen could only be distinguished when it was greater than 2 mm. It may have been patent at or below this diameter but could not be confirmed as such by ultrasound. The vaginoscope observations reported in Chapters III and V suggest that the cervix was patent as pus was seen at the external os and in daily clinical observations pus was seen on the vulva lips and byre floor. It may be that the variation in diameter of the cervical lumen observed and reported here represent periodic opening and closing of the cervix.

The cervix remained open in the presence of a corpus luteum, high progesterone levels, low levels of oestradiol 17 ß and a thickened endometrium (**FIGS 78 - 82**). This important observation will be discussed below in Chapter VIII.

Antimicrobial treatment. There appears to be no clear relationship between the treatment given and any of the parameters recorded in this chapter. Reasons for this will be discussed further in Chapter VIII.

Killed *A. pyogenes*. A study of cow 15 inoculated with the killed bacterium showed that inoculation had no clear effect on any of the parameters recorded here (**FIG. 83**). The only feature noted was spikes in the concentration of oestradiol 17 ß, but these changes were not likely to cause abortion. Changes in the endometrium were not significant and the cervix remained closed. Termination of pregnancy with cloprostenol treatment led to lysis of the existing corpus luteum, a decline in progesterone, an increase in oestradiol 17ß and oestrus associated with cervical opening.

A. pyogenes **toxin.** The bacterial toxin failed to show any significant effect on pregnancy or any of the parameters recorded here (**FIG. 84**). Possibilities for the failure have been discussed in Chapter IV and are discussed further in Chapter VIII. Maintenance of high levels of progesterone at about 8 ng/ml (apart from one increase above 10 ng/ml soon after inoculation) reflected the expected concentrations at that stage of pregnancy. The corpus luteum was functional as reflected by the concentration of progesterone. Termination of pregnancy with cloprostenol treatment was followed by a decline in progesterone, reduction in the corpus luteum size and the occurrence of oestrus. Thickness of the endometrium and sizes of the cervical lumen were significantly increased only after cloprostenol treatment. Findings in this animal were similar to the other 2 uninfected groups and were most probably hormonally regulated.

Cloprostenol treatment. The representative pregnant animal treated with cloprostenol demonstrates what happens naturally at the end of a cycle (pro-oestrus and oestrus) Soon after cloprostenol there was lysis of the corpus luteum and a decline in the plasma progesterone concentration (**FIGS 85a and b**).

The changes observed here were accompanied by an increase in the endometrial thickness, opening of the cervix, abortion and oestrus (**FIG 85b**). Events in this control group depict the changes brought about by prostaglandin F2α and other hormones in the groups of animals described above, when the animals come into oestrus. The detailed monitoring of progesterone levels following cloprostenol administration recorded in **figure 92** confirm this fall in progesterone levels prior to embryonic death.

Embryonic death. Events leading to embryonic death and abortion were different with regards to progesterone concentration after *A. pyogenes* uterine infection and cloprostenol treatment (**FIGS 86 and 87**). The observation of embryonic death and abortion while progesterone concentration was >6 ng/ml, was contrary to that of cloprostenol induced abortion which was <1.5 ng/ml during the same period. This observation confirms the suggestions made above that progesterone plays no role in the death of the embryo and its expulsion during the *A. pyogenes* induced abortion.

Some of the observations reported in this chapter are new. The maintenance of the corpus luteum during embryonic death and abortion is a significant observation. Progesterone, however, seems to play no part in *A. pyogenes* induced embryonic loss as levels remained above 4 ng/ml throughout the period of embryonic death and abortion in contrast to the stimulation in the cloprostenol treated animals. Follicular development was observed between infection and abortion and during the pregnancy - like state, but the oestradiol 17ß produced seems to be of no significance and this may be due to the presence of a functional corpus luteum.

The persistently open cervix was the other major feature of the results reported both in this Chapter and in Chapter V. It is clear from the results discussed above that it was open in the presence of a corpus luteum, high levels of progesterone and low levels of oestradiol 17 β. The implication of these results and for the mechanism of abortion resulting from the *A. pyogenes* infection and the hormonal control of maintenance of the corpus luteum and the cervical opening are discussed in Chapter VIII. More detailed observations of the relationship between progesterone levels and Pregnancy Specific Protein B are reported in Chapter VII below.

CHAPTER VII.

PREGNANCY SPECIFIC PROTEIN B (PSPB) AND PROGESTERONE IN MONITORING VIABILITY OF THE EMBRYO IN EARLY PREGNANCY IN THE COW AFTER EXPERIMENTAL INFECTION WITH *A. PYOGENES*.

INTRODUCTION.

In this chapter the effect of embryonic loss on levels of plasma progesterone and PSPB were studied. Changes in levels of these factors were assessed for their ability to distinguish between normal pregnancy and the abnormal state resulting from embryonic death and abortion caused by *A. pyogenes*. The production of progesterone in early pregnancy in cattle and the use of plasma progesterone levels in the detection of early pregnancy has been reviewed in Chapter 1. PSPB is produced by the binucleated cells of the trophoblast and has been found to be on accurate guide to diagnosis of pregnancy from 28 days after fertilisation (Sasser *et al.*, 1988). PSPB has also been reported to be superior to progesterone in the diagnosis of embryonic loss (Humblot *et al.*, 198 (a)) but no controlled experiments have been reported, and the response of PSPB to embryonic death is not clear. The demonstration that *A. pyogenes* can cause bovine embryonic death and abortion in this study and its identification in time by ultrasound, allowed investigation of the plasma concentrations of PSPB following controlled abortion. PSPB response was related to progesterone profiles and to the time of embryonic death to evaluate the significance of the two in monitoring the viability of the embryo.

MATERIALS AND METHODS.

Eight early pregnant cows/heifers were examined in this part of the study and the animals were bred and managed as described in Chapter II. Four cows which were infected with the live *A. pyogenes* and another 2 cows and 2 heifers induced to abort using cloprostenol were examined for pregnancy specific protein B (PSPB) and progesterone. Heparinised plasma was used for assessment of these analytes. In half of the cows from each group plasma was collected from 20 - 30 days before infection or treatment with cloprostenol, and in the other half collection was from 2 - 4 days before the experimental treatment. Sampling continued through embryonic death and loss until either recovery from infection, or return to oestrus or both. Cow 2 and heifer 11 were specifically examined between infection / cloprostenol treatment and abortion. This was to examine the effect of embryonic death on PSPB concentration.

PSPB assay. Analysis of PSPB was carried out by Dr R. G. Sasser of Dept. of Physiology, University of Idaho, U. S. A.. Plasma samples were lyophilised prior to inter-laboratory transfer and reconstituted on arrival in the U. S. A.. The methods and validations described below and in Chapter II were performed by him. Validation of the radioimmunoassay (RIA) was carried out as described by Sasser et al. (1986) and the assay was performed on samples in duplicate (200 ul). Pregnancy was considered positive at 95.5% of buffer control binding of labelled PSPB, equivalent to 0.21 ng/ml, which was taken as the cut-off point. For values of binding above this point equivalent to lower PSPB levels, cows were considered not to be pregnant. Samples were run in two assays. The inter-assay coefficient of variation of low and high PSPB sera pools (mean = 1.64 and 3.54 ng/ml respectively) were 14.5% and 5.2%.

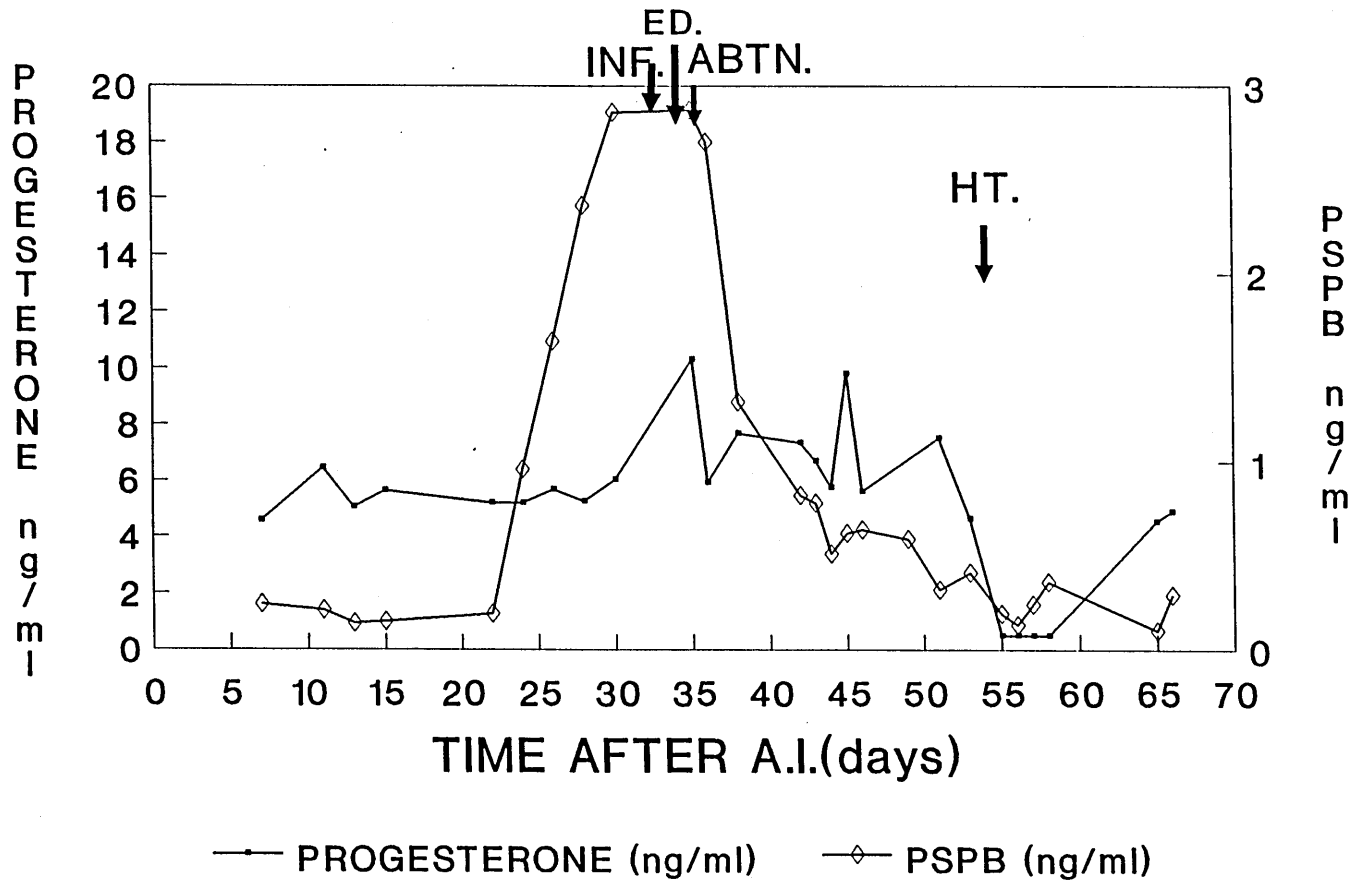
Progesterone assay. The Enzyme linked immunosorbent assay kits were used as described in Chapters II and VI and the inter assay and intra assay coefficients of variation were determined.

RESULTS.

The mean PSPB concentration at the time of infection was 2.22±S.D. 0.57 ng/ml while at the time of abortion was 1.33±S.D. 0.37 ng/ml. At 2-7 days after abortion this had fallen to 0.87±S.D. 0.42 ng/ml. Progesterone was 11.22±S.D. 5.33 ng/ml at the time of bacterial infection and remained high in the four cows through embryonic death, abortion, and until pus was eliminated from the uterus, 18-34 days after abortion when it fell to <0.5 ng/ml. Progesterone only declined to basal levels in three cows (Cows 1, 3 and 4) immediately prior to oestrus but remained >4.04 ng/ml in the fourth for up to 34 days after abortion. There was no marked difference in PSPB levels in the cloprostenol treated animals compared with the infected group as the levels dropped from 1.66±S.D. 0.54 ng/ml to 0.85±S.D. 0.22 ng/ml 5-7 days after abortion. Progesterone dropped from 6.66±S.D. 2.28 ng/ml to <0.5 ng/ml within 24 hours after cloprostenol injection.

The results for the four cows infected with *A. pyogenes*, are described separately. Blood sampling in Cow 1 started 28 days before infection which took place at 35 days of pregnancy. PSPB was undetectable (<0.21 ng/ml) till day 24 of pregnancy, and thereafter there was a rapid increase to 2.87 ng/ml at the time of infection (**FIG. 88**). Progesterone levels were >4 ng/ml on day 7 after service, and peaked at >10 ng/ml on the day of abortion (**FIG. 88**). Embryonic death and abortion occurred 22 and 29 hours respectively after infection and the animal returned to oestrus 21 days after abortion. After abortion PSPB fell to reach 0.86 ng/ml 6 days after

FIG. 88: A comparison of levels of pregnancy specific protein B (PSPB) and progesterone in **Cow 1** infected with *A. pyogenes* on day 35 of pregnancy. INF. = time of infection, ED. = embryonic death, ABTN. = abortion, HT. = oestrus.



abortion, whereas progesterone concentration was >5 ng/ml in all samples taken until 18 days after abortion when it fell to <0.5 ng/ml, coinciding with the onset of oestrus.

In Cow 2 the progesterone concentration was above 7.5 ng/ml 16 days after service while PSPB levels rose to 0.96 ng/ml from 27 days after service (**FIG. 89**) and reached 1.75ng/ml on the day of infection. After infection on day 30 of pregnancy PSPB fell from 1.75 ng/ml to 0.85 ng/ml by 7 days after embryonic death and abortion. Progesterone concentration was maintained >4.3 ng/ml until 34 days (after abortion) when the cow was disposed off.

PSPB concentration in cow 3 which was infected 34 days after service and aborted 103 hours later, had fallen from 2.33 ng/ml at infection to 0.76 ng/ml 4 days after abortion (**FIG. 90**). Embryonic death had occurred 11 hours after infection. Progesterone was maintained at >4.3 ng/ml throughout the period following abortion until 29 days after abortion when the animal returned to oestrus and the progesterone concentration had dropped to <0.5 ng/ml.

Embryonic death and abortion occurred 96 and 144 hours respectively after infection in Cow 4 which was infected at 41 days of pregnancy. PSPB declined from 1.72 ng/ml to 0.42 ng/ml 7 days after abortion. Progesterone concentration was maintained at >5 ng/ml until 20 days after abortion when return to oestrus occurred (**FIG. 91**).

To summarise the results of progesterone and PSPB assays in bacterial induced abortion, PSPB concentration responded to abortion with a gradual decline from the time of embryonic death and abortion, but was still detectable although in very small amounts between days 25 and 33

FIG. 89: PSPB and progesterone concentrations in **Cow 2** infected with *A. pyogenes* on day 27 of pregnancy. INF. = time of infection, ED. = embryonic death, ABTN.= abortion.

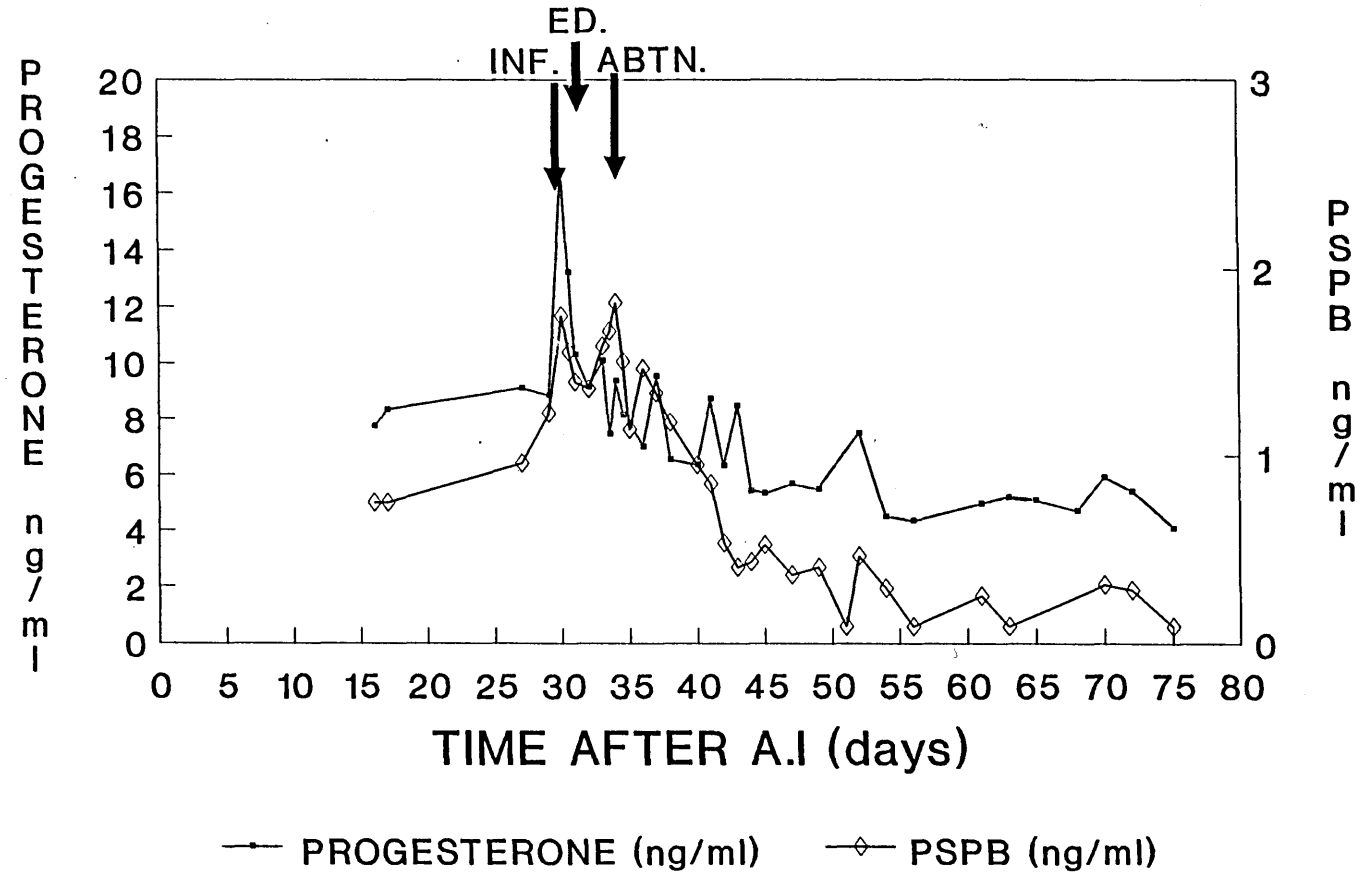


FIG. 90: Concentrations of PSPB and progesterone in Cow 3 infected with *A. pyogenes* an day 34 of pregnancy. INF. = time of infection, ED. = embryonic death, ABTN.= abortion, HT.= oestrus.

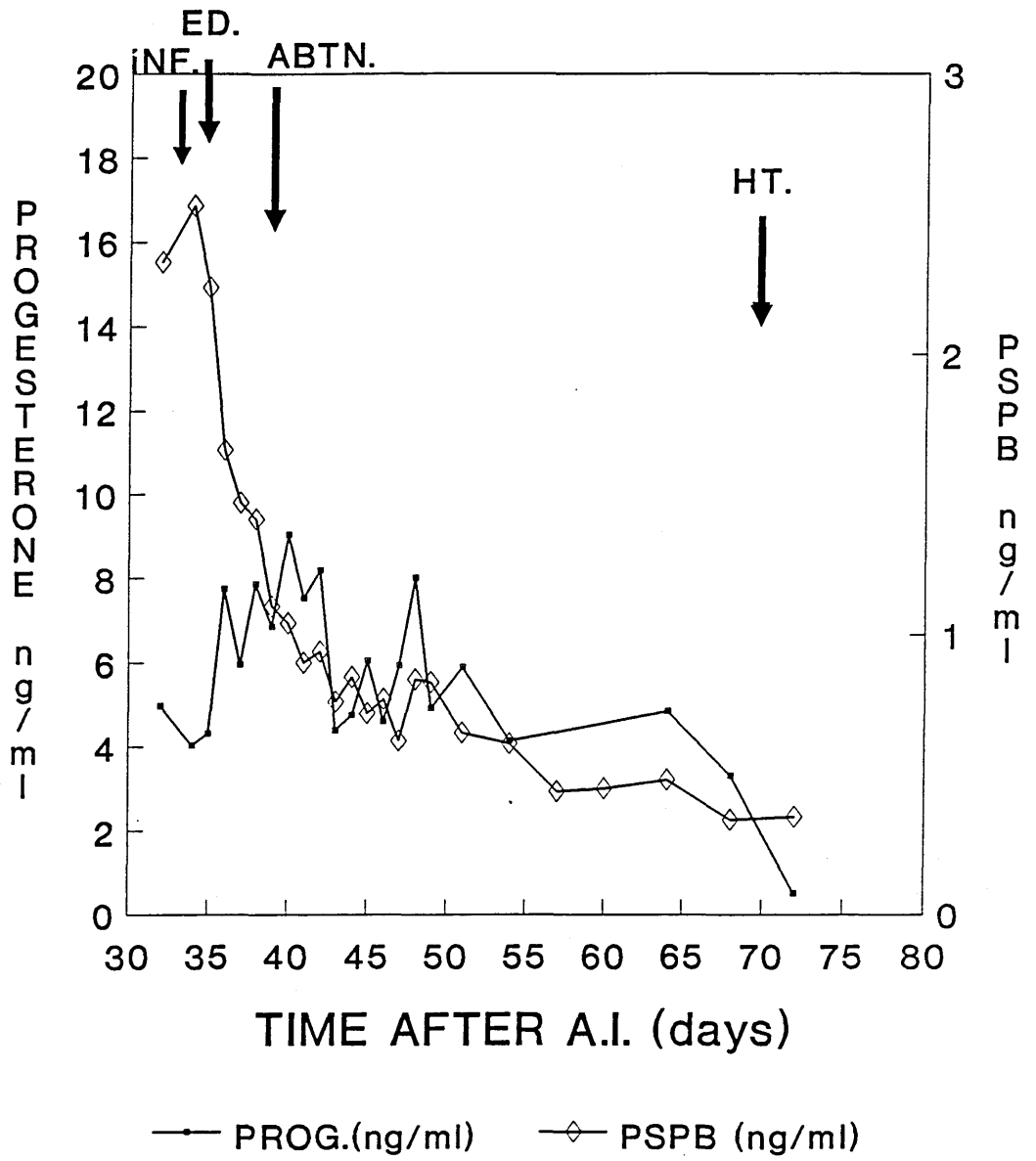
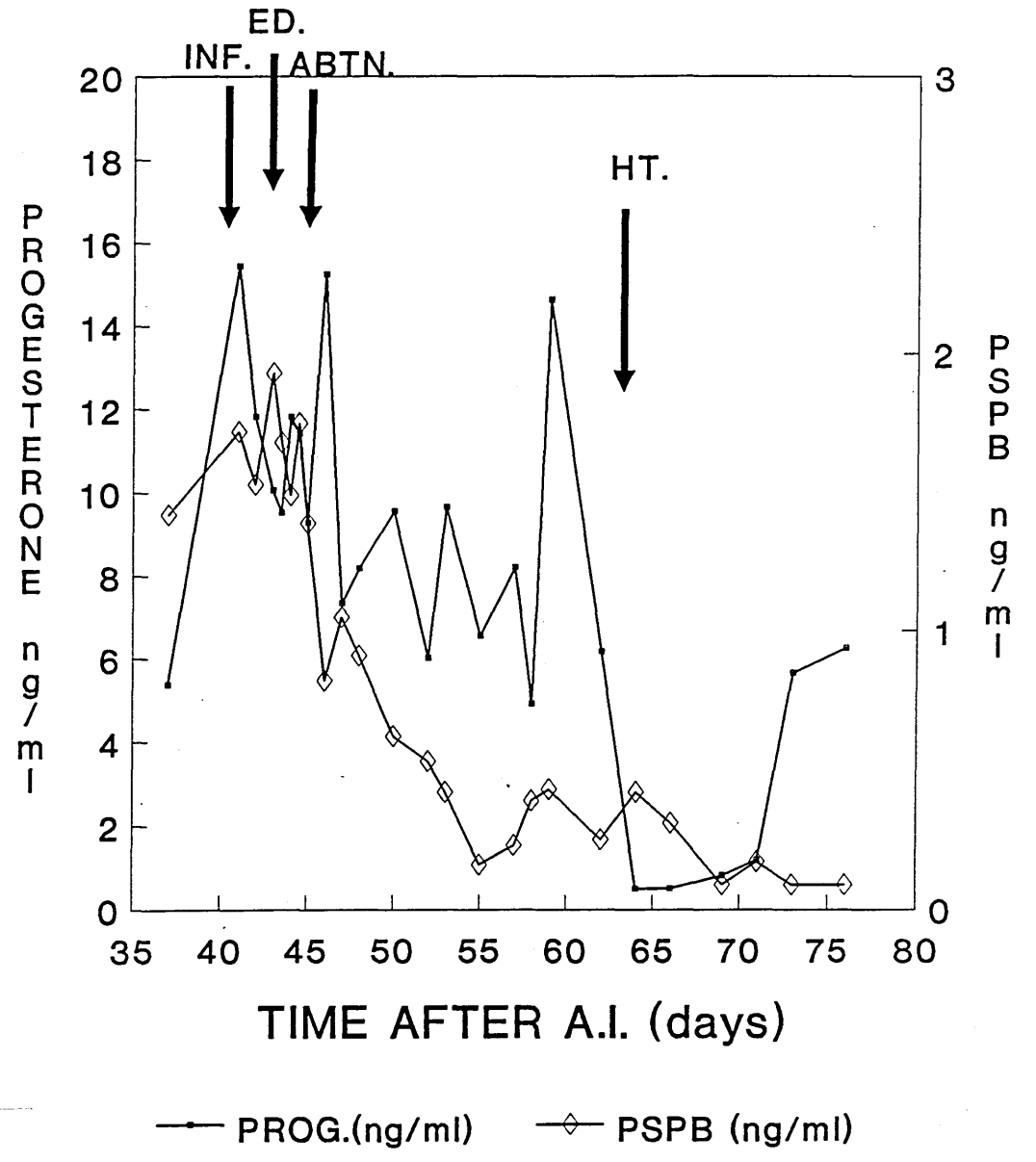


FIG. 91: Concentrations of PSPB and progesterone in Cow 4 infected with *A. pyogenes* on day 41 of pregnancy. INF. = time of infection, ED. = embryonic death, ABTN. = abortion, HT. = oestrus.



after abortion. Meanwhile progesterone concentration was maintained at least >4 ng/ml until elimination of pus from the uterus had occurred. The period between infection and abortion has been plotted separately using a larger scale for Cow 2 (**FIG. 92**) to make the relationships clear.

In the second experimental group, cloprostenol was administered on day 50 of pregnancy and results are shown for means $\pm$S.D. of PSPB (**FIG. 93a**) and progesterone (**FIG. 93b**) concentrations.

In the two Heifers 11 and 13 sampled from day 20 (N=2) the change in concentrations of both PSPB and progesterone 30 days before cloprostenol treatment increased in a trend similar to Cows 1 and 2 above. In the cows and heifers (N=4) that were observed from the time of treatment at day 50 of pregnancy, abortion occurred 66-72 hours later, accompanied by decline of PSPB from 1.66$\pm$S.D. 0.54 ng/ml to 0.85$\pm$S.D. 0.22 ng/ml 5-7 days after the treatment. Contrary to results from infected cows, progesterone concentrations after cloprostenol treatment declined to <0.5 ng/ml within 48 hours and the animals came into heat 24 hours later. The graph for Heifer 11 has been plotted over 5 days from treatment (**FIG. 92**) for comparison with cow 2 from the infected group (**FIG. 94**). The animals (N=3) which were followed to the second oestrus period had concentrations of PSPB and progesterone which fell to 0.09ng/ml and 0.5ng/ml respectively.

The effect of infection on the concentration of PSPB in cow 2 was first observed at embryonic death (**FIG. 92**). After this and throughout abortion a steady decline of PSPB occurred. Progesterone was above 6 ng/ml during this period. PSPB concentration in Heifer 11 started to decline 24 hours after cloprostenol treatment (**FIG. 94**). By the time of embryonic death

FIG. 92: Examination of the concentrations of PSPB and progesterone in Cow 2 infected with *A. pyogenes*, from day zero of infection throughout embryonic death and abortion. INF. = time of infection, ED. = embryonic death, ABTN. = abortion.

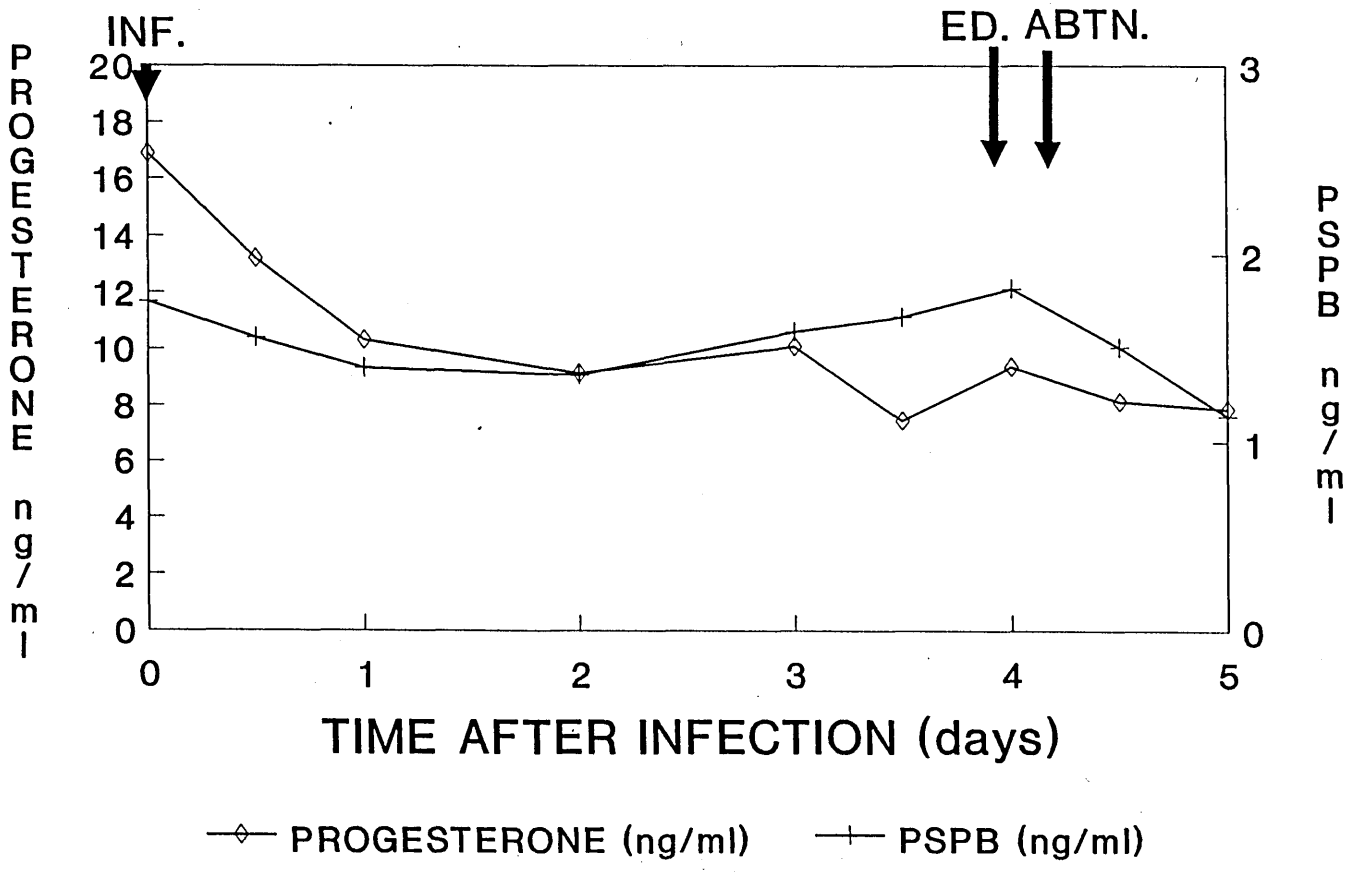
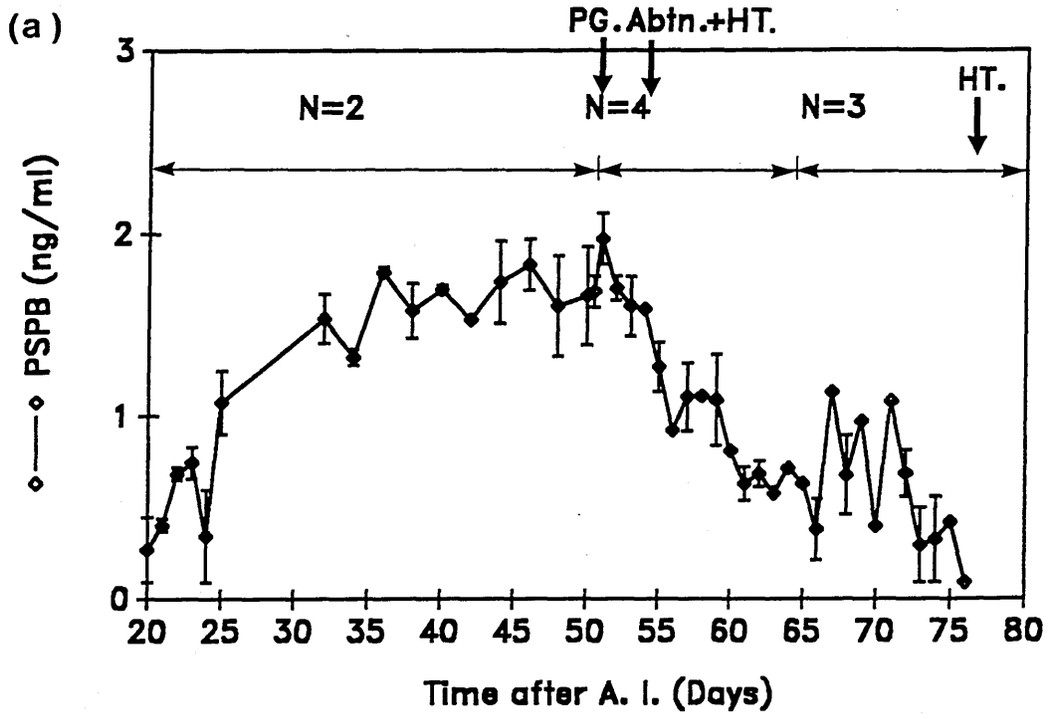
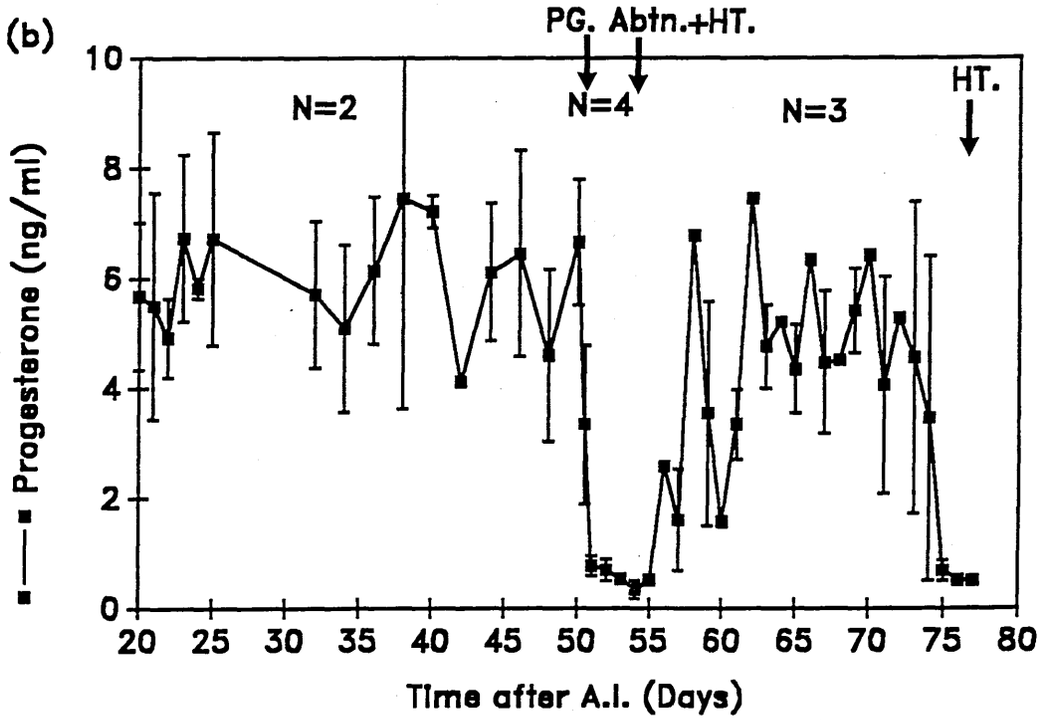


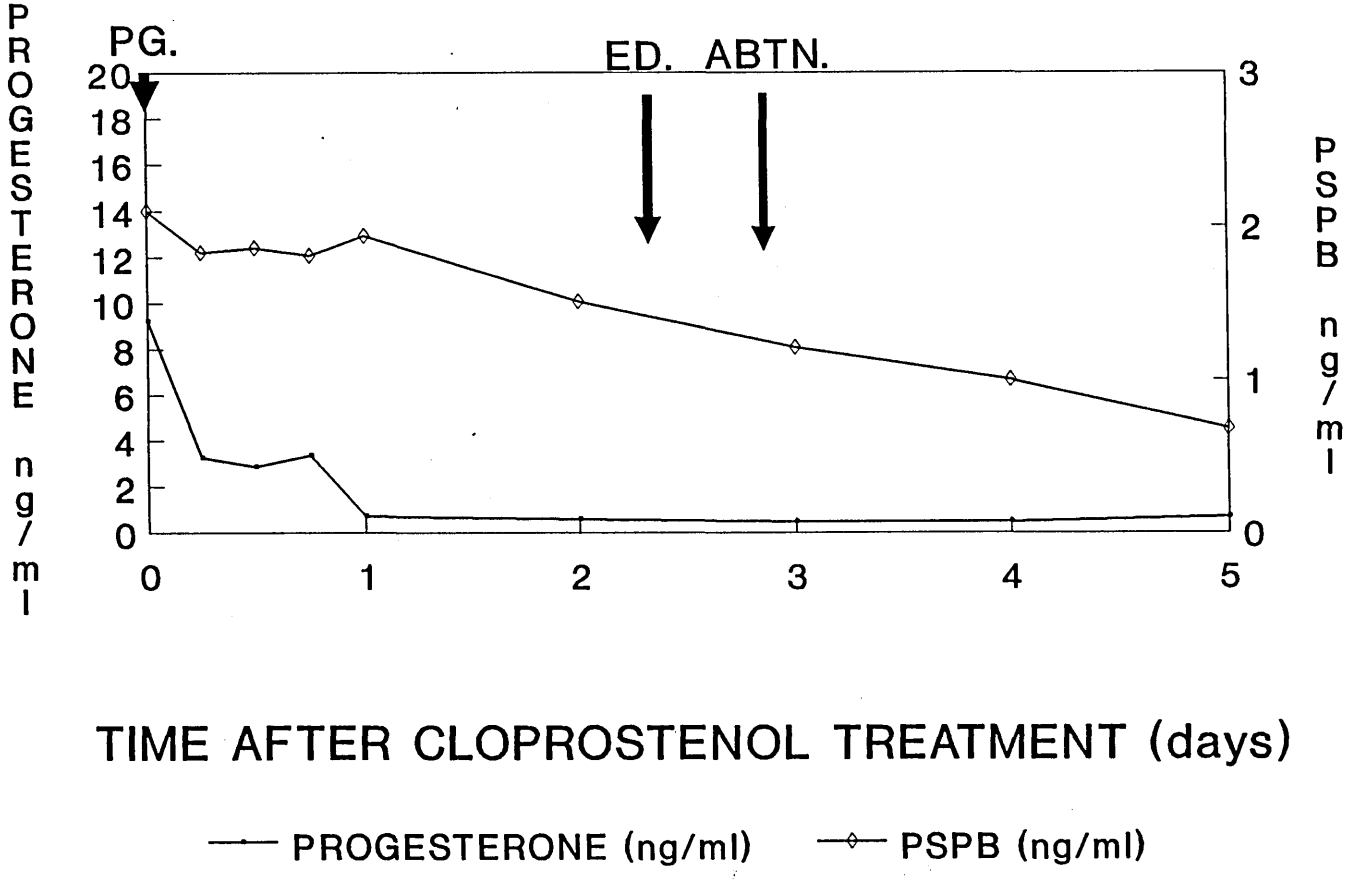
FIG. 93: Mean concentrations of PSPB and progesterone in four animals induced to abort at day 50 of pregnancy. PG.= administration of cloprostenol, ED.= embryonic death, ABTN.= abortion, HT.= oestrus.





■—■ Progesterone (ng/ml) ◇—◇ PSPB (ng/ml)

FIG. 94: Examination of PSPSB and progesterone concentrations in Heifer 11 from the time of cloprostenol treatment through embryonic death and abortion. PG. = administration of cloprostenol, ED. = embryonic death, ABTN. = abortion.



(33 hours later) PSPB had declined to 1.5 ng/ml. A steady decline to 0.6 ng/ml was then observed in the next 36 hours. Progesterone declined from 9.7 ng/ml at cloprostenol treatment to <0.5 ng/ml 24 hours later and for the next 96 hours.

DISCUSSION.

The opportunity to obtain PSPB levels for 8 animals in this study was particularly important of the investigation of abortion due to *A. pyogenes*. Its assay allowed the effect of infection on the binucleated cells of the trophoectoderm to be monitored in terms of their production of the protein. The number of animals used in both groups were small but the results obtained were sufficiently consistent to demonstrate marked differences between the PSPB levels and progesterone levels in the two groups. The differences between the two groups in terms of stages of gestation has been discussed in Chapter III and is discussed more fully in Chapter VIII. The levels of PSPB were similar in both groups before experimental treatment. The responses to bacterial inoculation recorded here may reflect those found in natural infection although there may be some differences due to the route and type of infection. Those recorded in progesterone do, however, provide a useful control for PSPB levels in abortion in the absence of infection.

The elevation of PSPB above the level of detection of 0.21 ng/ml confirmed pregnancy in all animals as early as 24-28 days after service when they rose to >1.3 ng/ml (**FIGS 88, 89, and 93**) (Sasser *et al.*, 1986; Humblot *et al.*, 1988b). The high levels of progesterone found (>6 ng/ml) are also consistent with the establishment of pregnancy (Cavestany and Footes 1985). This was confirmed clinically and by ultrasound (Chapter III). These initial

levels of the two parameters measured in this chapter changed in different ways in the two groups of animals and are discussed below.

The most prominent finding in terms of PSPB levels was that they declined between infection/inoculation and soon after abortion in all animals. The decline appeared to begin soon after cloprostenol injection (**FIG. 93**) but in the infected cows the point at which decline begun was less clear, varying from slightly before embryonic death in Cow 3 (**FIG. 90**) to following abortion in Cow 1 (**FIG. 88**).

The earlier decline of PSPB in heifer 11, 33 hours before embryonic death may be due to separation of the allanto-chorion from the endometrium (**FIG. 94**). This may support the role of binucleated cells of the trophoectorderm in the production of PSPB (Eckblad *et al.*, 1985) and indicates that separation of the membranes may be the starting point of PSPB decline.

The clearance of plasma PSPB from the blood (which took 5 to 10 days after abortion in infection) is more rapid than the post-partum decline in the circulating protein which takes >70 days (Humblot *et al.*, 1988b). The post abortion clearance commenced from a lower initial concentration. The low concentrations of PSPB detectable in cows 2 and 3 between days 25 - 33 following abortion were insignificant compared to the levels in the presence of a live embryo. Humblot *et al.* (1988b), suggested that the prolonged elevation of PSPB in plasma or serum up to 80-100 days post-partum could be due to endometrial production of this protein. In this experiment the time taken for the PSPB to decline to the cut-off point occurred between 13-18 days in both experimental groups. Binucleated cells of the trophoblastic ectoderm (Eckblad *et al.*, 1985) could have been the main source of the

protein since the embryonic membranes were expelled along with the dead embryos and this expulsion was prior to the decline of PSPB concentration.

In contrast to the gradual decline in PSPB levels in both groups following embryonic death or abortion, the progesterone levels behaved very differently. This behaviour has been discussed in Chapter VI and is discussed more fully in Chapter VIII but its relationship to PSPB levels will be discussed here. The maintenance of progesterone levels at >4 ng/ml (**FIGS 88 - 91**) in the infected animals contrasted markedly with the decline to <0.5 ng/ml seen in the cloprostenol treated group (**FIG. 93b**) within 24 hours.

In relation to monitoring the viability of the embryo, it is apparent that a single assay of PSPB or progesterone could give misleading results if embryonic loss has occurred depending on the cause of embryonic death. Thus if a bacterial infection like *A. pyogenes* leads to embryonic loss without corpus luteum regression until the infection is cleared, then progesterone assay would be unsuited to give evidence for embryonic loss. Assay of PSPB would be a better indicator of embryonic viability especially if sequential samples were taken. A downward trend in two or more samples would be the best confirmation of embryonic loss and, if the stage of gestation were known to be as early as that studied here, the absence of PSPB from a second sample taken 7 - 10 days after the first would confirm the absence of a viable embryo regardless of the progesterone level.

There is a need to confirm that PSPB levels decline after infections caused by other bacteria in the same way. The possible role of the endometrium in early pregnancy in the production of PSPB ought to be investigated. From this study the physiological role of PSPB is not yet clear but it probably does

not take part in the maintenance or lysis of the corpus luteum. This is apparent as the drop in the concentration of PSPB after abortion of the embryo did not affect the function of the corpus luteum, which was maintained until infection was eliminated from the uterus.

CHAPTER VIII.

GENERAL DISCUSSION.

INTRODUCTION.

The role of *A. pyogenes* in bovine early embryonic death has been studied in detail. The results of the experimental studies have been presented in Chapters III to VII and their validity discussed in the appropriate chapters. In this chapter the experimental results are discussed in relation to work that has been done in other investigations in the area of early embryonic death in cattle particularly in intrauterine infection with *A. pyogenes*. This discussion is divided into the following sub-sections; reproduction of abortion by *A. pyogenes*, ultrasonographic studies, clinical observations, pathenogenetic studies, immune response to *A. pyogenes* infection, acute phase haptoglobin response to the infection, endocrinology during the infection, the infected uterus after abortion and the final recovery period prior to oestrus.

Throughout this discussion some technical factors which may affect the interpretation of the results should be considered. These have been discused in part in Chapters III - VII but are summarised here. Chief amongst them was the number of animals used. A gross total number of 22 animals were used in the different experiments in the study as follows: 8 = live bacterial infection, 2 = sterile saline, 1 = cloprostenol treatment, 2 = live bacteria and slaughtered, 2 = killed bacteria and 2 = bacterial toxin. These small numbers were a particular problem when numbers of a small group did not behave in the same way. This was a problem particularly when Cow 15 did not abort after inoculation with killed bacteria and Cow 16 did.

The main reason for the small numbers of animals used in the study was their high cost. In Chapter II it was made clear that the animals were purchased from a dealer, used in these studies and taken back to the common pool for subsequent resale to the dealer. The experiments had to be fitted into the periods for which the animals were available and antimicrobial treatments during the experiments had to be curtailed at times in order to ensure the absence of residues by the date of return. These contraints made it difficult to complete treatments and observation periods. Purchase from a dealer meant that it was not possible to use heifers or cows consistently and that cows came without any breeding records or history of disease. Information like repeat breeding in some of the animals would have been useful especially as regards the hormonal effects on the embryo and after abortion. The disease history would have possibly been important in explaining observation such as an antibody titre of 1:1000 in cow 3 on day zero of infection and failure of cows 21 and 22 to abort after live bacterial infection.

Finally, the small number of animals available was responsible for the re - use of 5 animals, Cows 7 & 8 and Heifers 17 & 18 which did not abort after sterile saline and bacterial toxin respectively, and the re - use of Cow 15 after inoculation with killed bacteria. All of these animals were used as part of the cloprostenol control group and were used at 50 days of gestation which accounted in part for the wide range in the stage of pregnancy at which cows were used (27 - 50 days).

A. Reproduction of abortion by *A. pyogenes*.

The results obtained from the infection experiments and described in Chapter III confirmed that *A. pyogenes* can cause death and abortion of the bovine embryo when present in the uterus in pure culture. Thus it can act as a

primary pathogen in the bovine uterus. The observations made in this study support that primary conclusion. Firstly, of the 8 animals infected with the live bacteria, 6 aborted 29-144 hours later (Chapter III). The bacterial culture was pure since it was grown in the laboratory under strictly controlled conditions. Secondly, the clinical signs observed after bacterial inoculation included an increase in the tone of the uterus, an open cervix, mucopurulent discharge from the vagina, and demonstration of the aborted embryo or parts of the embryo all of which suggested an infection - associated abortion. Thirdly, apart from Heifer 5 which had an increase in the body temperature, hyperthermia seemed to have a minimal role to play in the abortion. In the fourth case isolation and identification of *A. pyogenes* in profuse culture during and after abortion confirmed its presence in the abortion. It was absent from the saline inoculated and cloprostenol - treated controls which remained healthy.

The reasons for failure of abortion after infection in Cows 21 and 22 in the present study is not clear. No signs of pending abortion due to the bacteria were seen and there was no evidence of *A. pyogenes* infection when abortion was eventually induced with cloprostenol. The most probable reason for failure of the infection could have been due to the inoculum being introduced outside the uterus. The possible role of antibody protection is discussed in Section E below.

The results obtained by inoculation with sterile normal saline (SNS) and in cloprostenol induced abortion confirmed that *A. pyogenes* was responsible for the abortion seen in the infected group. Inoculation of SNS failed to cause abortion showing that neither the SNS which was used in making up the bacterial inocula nor the puncture of the needle through the uterine wall affected pregnancy. The abortion induced by cloprostenol differed from the

bacterial abortion in that the corpus luteum regressed after cloprostenol treatment whereas it was maintained during the infectious abortion. The embryos aborted after cloprostenol treatment were intact with clear membranes and the embryos were fresh (**FIGS 12 and 13**) as described by Kastelic and Ginther (1989). This was in marked contrast to the embryos from infected cows which had pale membranes covered with pus and embryos showed signs of degeneration or maceration (**FIGS 8 and 9**) .

The embryos aborted after bacterial infection had more thickened membranes than embryos aborted after cloprostenol treatment. Their apparent thickness in the infected embryos could have been due to presence of pus and oedema. The relatively undegenerated embryos after cloprostenol treatment resembled those seen by Omran (1989). In three of the aborting Cows 3, 4, and 6 after bacterial infection, only mucopurulent discharge and foetal parts were collected. The findings appear to agree with the observations by Roberts (1971) of degenerated embryos in the vagina. It is possible that in his case embryonic death was quickly followed by maceration and discharge of the material, as was most probably the case in the 3 animals in this study. Therefore depending upon the cause of embryonic death, the dead embryo may be eliminated with the membranes or it may be macerated. It is thus important that full disease investigations including cultural studies are done to ascertain the causative agent(s).

The only previous experimental reproduction of abortion following *A. pyogenes* infection was carried out by Mohamed *et al.* (1987) who induced abortion by intra-amniotic inoculation with 4.6×10^6 c. f. u. of *A. pyogenes* in a single cow which was followed by abortion and maceration of the foetuses 16 days later. Their study was reviewed in Chapter I where it was shown that they did not emphasize the significance of the *A. pyogenes* infection. The

organism also caused foetal death and abortion in ewes after experimental intra-amniotic inoculation. Their results support those obtained in the study described in chapter III which demonstrated the importance of *A. pyogenes* in embryonic death. The greater interval between infection and abortion in their experiment in cattle could have been due to the lower concentration of the bacterium used. In their study Mohamed *et al.* (1987) also recorded retention of the membranes, a manifestation which did not take place in the study reported here. The retention of membranes in their investigation could be the direct result of the advanced stage of pregnancy in their study (180-200 days) when placentomes are much more developed than in the cows used in this study (20-50 days of gestation).

Isolation of *A. pyogenes* from the products of abortion is a positive indicator that it was responsible for the abortion of the embryos. Other authors have concluded from their observations that *A. pyogenes* is the commonest isolate in endometritis (Savov and Dimitrov 1974, Griffin *et al.*, 1974, Hartigan 1978, Studer and Morrow 1978, Miller *et al.*, 1980, Ruder *et al.*, 1981, Wilson 1984). In his investigation of chronic endometritis in dairy cattle, Anderson (1985) found *A. pyogenes* as the commonest non-specific bacterial isolate. He failed to isolate the organism in 25% of cases.

Anderson (1985) further observed that 75% of *A. pyogenes* isolates were obtained from cows in the pre-service period (before oestrus) whereas enterobacteria, *E. coli* and *Pseudomonas sp* were isolated during oestrus. He proposed then that *A. pyogenes* initiated the infection and the other bacteria were secondary. Similar observations were made by Griffin *et al.* (1974). A number of authors have considered that *A. pyogenes* requires other organisms for initiation of abortion. The results described above suggest that this is not so and confirm the results obtained in a single cow by Mohamed *et al.* (1987).

The inocula used in the present study were prepared from pure cultures and the organisms were recorded in profuse culture after abortion. The only other organisms identified from the vagina in this series were *E. coli*, staphylococci and streptococci. Coliforms and streptococci were also reported as common in the bovine reproductive tract by Studer and Morrow (1978) and David and Bonner (1987) but these were not thought to be potential pathogens and purulent discharges were mainly associated with *A. pyogenes* (Studer and Morrow 1978). The increasing importance given to *A. pyogenes* in the bovine uterus was stressed by Takacs *et al.* (1990) who isolated the bacteria from 90% of uteri of post partum cows. No attempt was made to identify the infected status of the cattle used with regard to other pathogens such as Leptospira, Brucella and B. V. D. (Chapter I). Ideally, all should have been tested for. The use of control animals with similar backgrounds suggested that these agents were not important in this study.

The observations in the present study are significant and differ from previous reports suggesting presence of synergism between *A. pyogenes* and *F. necrophorum* infections (Roberts 1967 and Ruder *et al.*, 1981). In this study no *F. necrophorum* was isolated. Farin *et al.* (1989) subscribed to the view that *A. pyogenes* requires another organism for its effects but also demonstrated that it could cause endometritis when inoculated alone.

Observations in the present study therefore agree with earlier observations by Biancardi *et al.* (1982), Bucharova (1982) and Jerrett *et al.* (1984) who isolated *A. pyogenes* from aborted embryos, foetuses, placenta and vaginal discharge from the field at 5.65%, 39% and 10% respectively and suggested that the bacteria could have been the primary cause of abortion. These results indicate the primary role of *A. pyogenes* as an abortifacient and the earlier work seeming to support the theory of synergism of *A. pyogenes* with some

anaerobes is not borne out by the findings presented in this study.

B. ULTRASOUND.

The ability to follow events with the use of ultrasound formed a major and novel part of the study described. Apart from the work done by Omran (1989) to follow events accompanying cloprostenol induced bovine embryonic death and abortion ultrasonically, very little experimental work has been carried out on early embryonic death in such detail. The scarcity of such studies could be due to difficulties in learning the technique and the fact that it is relatively new. In this study the changes which occur at the onset of bacterial contamination of a pregnant bovine uterus were monitored and the findings were compared with events following cloprostenol induced embryonic loss in cattle.

Pregnancy was confirmed in all the animals before any experimental intervention and the observation of a corpus luteum (> 2 cm^2), a pregnant uterus with non-echogenic fluid and a live embryo within the amnion, with a closed cervix and a vagina with no content agree with earlier observations by Hanzen and Delsaux (1987), Hughes and Davies (1989), Boyd *et al.* (1990), Khan (1990) and Pieterse *et al.* (1990).

After inoculation with *A. pyogenes*, changes that led to abortion of the embryos were clearly observed. The most important benefit of the use of this technique was that it was possible to view and record the death of the embryo and to fix it in time. This parameter has never been established previously in studies of infectious abortion and forms the basis for the interpretation of all other examinations recorded. The ultrasound studies provided two sorts of other information. They provided a means of monitoring the development of

the changes in the embryo and its supporting structures and provided information about the possible mechanisms of infectious abortion. These are discussed below in order of their appearance. The value of ultrasound as a diagnostic tool in investigation of early embryonic death is then discussed.

Ultrasound observations and pathogenesis.

The first abnormal observation was the separation of the allanto-chorion from the endometrium begining 8 hours after inoculation. This was at about the same time as in the group with cloprostenol induced abortion where separation was first observed at 7 hours (Chapter III). Similar observations were made in all animals in the two groups. Separation of the allanto - chorion from the endometrium after cloprostenol treatment could be firmly related to the reduced ability of the corpus luteum to produce progesterone due to luteolysis. This would lead to stimulation of GnRH and FSH production by the pituitary which would in turn facilitate follicular growth, oestradiol 17ß production increased endometrial mucus secretion and uterine tone. These destroy the quiescent state of the uterus leading to the membrane separation (Sauer 1979, Omran 1989). However, in contrast to the animals given cloprostenol treatment, the corpus luteum was maintained throughout the period of embryonic death and abortion after infection and progesterone concentrations were high (Chapter VI), so it seems that separation of the membranes was due to different causes in the two groups. Two factors that could have contributed to the membrane separation in infection could have been the formation of pus between the allanto - chorion and the endometrium (see below) and the increased uterine tone probably resulting from inflammation. Progesterone and oestradiol 17ß do not seem to play a significant part in the separation of the allantochorion from the endometrium as their concentrations in pregnancy remains largely unchanged throughout infection embryonic death and abortion.

The accumulating echogenic mucopurulent discharge was clearly distinct from the non-echogenic allantoic and amniotic fluids. The echogenic fluid was not seen in the saline - treated group. The accumulating discharge continued to displace the membranes from the endometrium and this echogenic fluid could readily be distinguished from the non - echogenic uterine fluid seen after cloprostenol induced abortion (Omran 1989). Fissore *et al.* (1986) described a similar snowy image of the echogenic fluid in non-pregnant animals with uterine infection and pus formation. The separation of the allanto - chorion from the endometrium could have been a major contributor to the death of the embryo due to a cessation of the gaseous and nutritional exchange. Cessation of the heart beat of the embryo was considered to indicate death of the embryo as in cloprostenol treatment although the accumulating mucopurulent material and its echogenic nature sometimes made it difficult to visualise this cessation in infected animals.

Thickening of the membranes was seen in embryos observed by ultrasound in infected animals. This thickening was absent from embryos in the cloprostenol treated animals. The thickening may have been due to oedema or coating of the allantochorion by pus. When aborted embryos were collected, pus was found on them and no evidence of oedema was found in the embryo of Heifer 20 in the pathogenicity study (Chapter IV) suggesting that pus was responsible for this thickening.

After infection the dead embryos were seen to float between the two horns and the body of the uterus for longer than in the cloprostenol induced abortion (Omran 1989). This could have been the reason for the generally longer time taken to abort in the infected animals (29 - 144 hours) compared with cloprostenol treated animals (66 - 72 hours), but in the later group follicular growth and oestrogen production may have contributed to the earlier abortion due to the oestrogenic effect on the uterine wall. Although

the cervix was seen to open before abortion in both bacterial infection and cloprostenol treatment (Omran 1989), the positive effect of cloprostenol on uterine contractility (Day 1977, Youngquist *et al.*, 1977, Lindell *et al.*, 1981, Schultz and Copeland 1981) could have led to an earlier abortion. In their study Chaffaux *et al.* (1986) predicted embryonic mortality in 23% of the 100 cows examined. Their conclusions were obtained indirectly from one ultrasonic observation of 16 of a hundred animals which were diagnosed to be pregnant and had high plasma progesterone levels 30 days after service. Rectal palpation of these 16 animals at 60 days revealed the animals to be empty and embryonic loss was presumed to have occurred. In this study 7 cows were found to have low progesterone but were identified as pregnant by ultrasound around day 23, and were found to be empty by day 60 after service. In a more systematic ultrasonographic study Kahn and Leidl (1989) observed pregnancies between 15 and 40 days in cattle. They identified retarded embryonic growth which was followed by reduction of the heart rate about 5 days later, and subsequent cessation of the heart beat. The process of embryonic resorption was observed and was accompanied by resorption of placental fluids and gradual disappearance of embryonic contours. None of the authors described intrauterine echogenic material of the type described in this study. These studies demonstrated the value of ultrasound in cases of embryonic loss, but the cause of embryonic death and resorption was not investigated fully, since cultural studies were not reported. It is evident that events following the above studies were different from the death due to *A. pyogenes* presented in this study.

The use of ultrasound enabled accurate measurements of ovarian structures such as corpora lutea and follicles to be made and related to the changes seen in the uterus in a way that is not possible by rectal palpation. In all the

animals aborting after bacterial infection the corpus luteum was maintained throughout the period of abortion and for 18 to 34 days after abortion. In contrast the corpus luteum regressed soon after administration of cloprostenol, from > 2 cm^2 to 0.4 cm^2 4 - 5 days later (Lindell *et al.*, 1981, Guilbault *et al.*, 1988, Knickerbocker *et al.*, 1988, Omran 1989). The lysis of the corpus luteum after cloprostenol administration has been attributed to the direct effect on the cells of the corpus luteum. The maintenance of the corpus luteum during an abortion has not been reported before and the possible endocrinological mechanism(s) responsible for the phenomenon will be discussed later in the chapter. This is the first report of a corpus luteum of pregnancy maintained after abortion with luteolysis only seen to occur after elimination of pus from the uterus. Ultrasound has confirmed for the first time that, the corpus luteum of pregnancy can be maintained for many days after abortion resulting from *A. pygenes* infection. The observations also confirm earlier reports of presence of the corpus luteum in endometritis and pyometra (DuBois and Williams 1980, Markusfield 1984).

Although follicles were seen in both ovaries during infection embryonic death and abortion, no follicle capable of ovulating was seen in any of the animals during this period (Chapters III and V). The presence of follicles in ovaries during pregnancy is normal in cattle (Thomas and Dobson 1989) and was confirmed in this study. Lack of follicular growth leading to oestrus after infection was in contrast to the observation after cloprostenol treatment where one follicle grew and ovulated after oestrus in all the treated animals. The follicles present in the ovaries in infected animals did not appear to be of any significance in embryonic death or abortion. Ultrasound was able to demonstrate that follicular development leading to oestrus 18 to 34 days after abortion in infected animals was only after pus had been eliminated from the uterus thus suggesting an inhibiting effect of pus on the onset of oestrus. No

previous report has been made of the follicular activity in infection yet it seems to be important as it may explain changes of some hormone concentrations during this time.

The change in thickness of the endometrium after infection seen by ultrasound was confirmed to be due to endometritis from the results of the pathogenicity study (Chapter IV). Thickening of the endometrium was consistently seen after abortion. This observation is in agreement with the one made by Fissore *et al.* (1986) who in addition to the snow - like echogenic particles in the uterine fluid, observed thickening of the uterine wall. They concluded that changes in the uterine wall were due to endometritis. After the administration of cloprostenol, abortion was also accompanied by endometrial thickening (**FIG. 31. J2**). Omran (1989) attributed this drastic increase in endometrial thickness to the direct action of the drug, causing uterine contraction. He also suggested that oestradiol 17 ß (produced by the developing follicles) might contribute to the thickening. Since no significant follicular growth was seen following the bacterial infection, oestradiol 17 ß is not expected to have been a major mediator in the endometrial thickening observed and the increase in endometrial thickness was not considered to be associated with oestrus (Chapters III and V).

Changes in the lumen and wall of the body of the uterus after bacterial inoculation closely followed those discussed for the uterine horns, but were seen a few hours later. No emphasis has been given to changes seen in the body of the uterus in the literature for pathological studies to date. Yet from observations in this study, pathological changes in the body of the uterus can be a very useful guide to the pathological state of the uterus. Changes in the body of the uterus after cloprostenol administration were marked by accumulation of non-echogenic fluid and thickening of the uterine wall. This

was in agreement with the observations of Omran (1989). The appearance of the fluid was clearly distinct from that of the mucopurulent material seen with infection.

In the animals observed closely by ultrasound after bacterial infection and cloprostenol administration there was fluid accumulation in the vagina. There was a difference in the appearance of the fluids, that in infection was echogenic and that in cloprostenol induced abortion was non-echogenic (Omran 1989). It has been reported that mucus cells of the cervix and cranial vagina can produce mucus under the influence of oestradiol 17 ß (Salisbury *et al.*, 1978). This may contribute to the fluid in the vagina after use of cloprostenol in addition to the fluid from the uterus. Since there is little oestrogenic effect to be expected at the time of abortion in infection (Chapter VI), the fluid seen in the vagina most probably comes from the infected uterus and was purulent in nature when examined by vaginoscopy (Chapter III).

Ultrasound observations and the diagnosis of *A. pyogenes* abortions.

The studies described and discussed in this thesis in Chapter III provide the basis for diagnosis by ultrasound of embryonic death and abortion due to bacterial infection. In the field, the repeated examinations performed here may not be possible. It is therefore possible to use the information described above to evaluate ultrasound in diagnosis. Recommendations of its use in the field for investigation of early embryonic death and fertility control are therefore justified. These recommendations are based on the Real - time B mode ultrasonographic findings and are intended primarily for users of that and other similar scanners (Chapter I).

Ultrasound examination allows a tentative diagnosis of uterine abnormality to be made by systematic examination of the ovaries, horns of the uterus, body of

the uterus, cervix and the vagina. The state of the embryo can be examined and significant changes in the appearance of the membranes may help to detect early cases of abortion. Examination of any fluid present in the pregnant horn or any other part of the uterus during pregnancy and its echogenicity may indicate infection. Following abortion, ultrasound can distinguish between pregnant and non pregnant infected uterus at this early stage of pregnancy better than manual or hormonal techniques (reviewed below). It does this primarily by demonstrating the absence of the embryo, the presence of echogenic uterine content and the presence of an open cervix.

Features of the uterus and its supporting structures viewed by ultrasound and considered to be important in the diagnosis of bacterial infection of the early embryo and investigation of fertility are listed below in order of importance.

1. Presence or absence of the embryo.
2. Appearance of the embryo.
    a) Presence or absence of the heart beat.
    b) Definition of the membranes.
    Thickened membranes and poor definition indicate the presence of infection. Occasionally (Cow 6) echogenic material (pus) may make this difficult.
    c) Movements of the embryo (from 35 days of pregnancy).
    Their absence may indicate abnormality.
    d) Separation of the allantochorion from the endometrium.
    e). Size of the embryo (demonstrated by Khan and Leidl, (1989) but not detected here because of the short incubation period of infection).
3. Echogenic fluid in the uterine lumen.
4. Presence and size of the corpus luteum ($> 1{,}5\ cm^2$)
5. Thickening of the endometrium (in excess of 3 mm in the presence of the

corpus luteum as in 4).

6. Patent cervical lumen. The limit of detection was 2 mm in this study and more than one observation may be necessary or vaginoscopy for pus at the external os of the cervix may be necessary to confirm functional potency (Chapters III and V).

7. The presence of echogenic fluid in the vaginal lumen.

Earlier diagnosis of infection using ultrasound may be useful in the future to enable the institution of appropriate treatment to save the embryo. In this thesis a systematic study of the changes accompanying bacterial infection in early pregnancy is reported for the first time. Times of embryonic death and abortion were determined. Ultrasound could be useful in future investigations of the pathogenetic role of other non-specific bacterial infections. The technique could also be used to study the pathogenesis of established specific infections in pregnancy in cattle.

C. OTHER CLINICAL OBSERVATIONS.

The identification of the exact time of embryonic death and abortion in this study and described in Chapter III above, made it possible to evaluate the clinical signs recorded in terms of their value in contributing to the understanding of the pathogenesis of infection and in the diagnosis of early embryonic death and infertility. Similarly it allowed the relationship between hormonal levels which were monitored in this study to be related to embryonic death. The interaction between these factors and embryonic death is discussed in full in the next section and their role alone considered here.

The contribution of other clinical observations to understanding the pathogenesis of embryonic death and abortion.

1. Demeanour and appetite were unaffected, suggesting that systematic involvement was unlikely. This was confirmed by the absence of fever (Biberstein and Chung Zee 1990).

2. Inspection demonstrated the presence of vaginal discharge and the presence of abortion. The appearance of the discharge and of aborted embryos suggested the involvement of bacteria. *A. pyogenes* was isolated and the demonstration of its presence is important in confirming that the changes seen were related to infection.

3. Rectal temperature did not rise consistently. In Heifer 5 however the rectal temperature rose to 40.3 ^{o}C and this may have contributed to the death of this embryo (Atkins and Bodell 1972, and Miller 1986).

4. Rectal palpation did not contribute greatly to the understanding of the pathogenesis of embryonic death. The presence of increased uterine tone was distinctive and may have contributed to or resulted from embryonic death and abortion (Chapter III). The persistence of the corpus luteum and the failure to detect follicles were distinguishable and would have been important in the absence of the ultrasound data. No information of the state of the embryo was obtained by this means.

5. Vaginoscopy only confirmed the presence of pus at the external os of the cervix and hence its probable uterine origin but could not contribute to further information about intrauterine changes.

6. Plasma sampling allowed the detection of levels of progesterone, oestradiol 17ß, haptoglobin and PSPB. The high progesterone levels confirmed the presence of a functional corpus luteum and low levels of oestradiol 17ß the minimal follicular activity. The haptoglobin levels were sufficiently high to indicate infection but could not identify this as occurring in the uterus. The PSPB levels detected did not contribute to understanding of the pathogenesis of embryonic death and abortion.

The value of the other clinical parameters in diagnosis of embryonic death, infertility and persistent post abortion uterine infection. These are listed as follows in order of importance:

1. Finding the aborted embryo or recognisable portions of it on the ground or in the vaginal lumen by vaginoscopy was the most important parameter. Maceration of the embryo and the presence of pus would suggest a bacterial abortion and culture could confirm presence of *A. pyogenes* but not rule out other pathogens.
2. Demonstration of vaginal discharge around the perineal region or the tail. This and the presence of pus in the vaginoscope suggest an infectious discharge. The presence of *A. pyogenes* might be confirmed on culture. Its occurrence in early pregnant animals may be suggestive of early embryonic death or abortion.
3. Detection of increased uterine tone by rectal palpation in the presence of a corpus luteum may indicate the presence of intrauterine infection but could not identify the nature of the infection or the intrauterine status of the embryo.
4. Slipping of the membranes was not performed in this study but might distinguish between pregnant and non pregnant enlarged uteri.
5. **PSPB** levels taken 7 days post abortion are low and in animals with known service dates these would suggest that embryonic death had occurred without revealing the time and the reason for the death.

In the present study PSPB proved to be a good indicator of the presence of a viable embryo. Elevation of PSPB above the cut off point as early as 24 - 28 days after service confirmed pregnancy in all the animals (**FIGS 88, 89, and 93**)(Sasser *et al.*, 1986, Humblot *et al.*, 1988a), while the high levels of progesterone found (> 6 ng/ml) are also consistent with the established pregnancy (Cavestany and Footes 1985).

The decline PSPB concentration to the baseline after embryonic death and abortion in the present study was faster when compared to the period observed post parturition (Humblot *et al.*, 1988b). At term complete removal of the protein from the circulation takes a long time (70 - 100 days) because of the very high circulating levels of PSPB reached i.e. 700 ng/ml (Sasser *et al.*, 1986). Ruder and Sasser (1986) showed that clearance of PSPB from sera of cows between days 22 and 53 post partum occurred with a half life of 8.4 days for intact and 7.3 days for hysterectomised (surgery on day 21 post partum) post partum cows (differs at $P<0.5$). These data suggested that residual placental tissue contributed to delay in clearance but that there was also an extrauterine source and/or a long term requirement for metabolic clearance.

Investigation of PSPB and progesterone concentrations in *A. pyogenes* induced abortion and that caused by cloprostenol have demonstrated that the embryonic loss can be best diagnosed using both parameters. Future application of the investigation in other bovine uterine infections and also other animals like the pig may prove to be beneficial. It would be interesting to find out what the levels of PSPB were in the experiment of Kastelic and Ginther (1989), where embryonic loss was induced by rupture of the embryonic membrane in heifers which was followed by expulsion of degenerated conceptuses a few weeks after embryonic death. The physiological role of PSPB is not yet clear (Beckers *et al.*, 1988, Sasser *et al.*, 1989) but it probably does not take part in the maintenance (Packham *et al.*, 1990) or demise of the corpus luteum (Humblot *et al.*, 1988a).

The relationship between PSPB levels and progesterone in diagnosis is given in **Table 11.**

6. The onset of oestrus. Animals infected with *A. pyogenes* fail to come into

oestrus >8 days following abortion in contrast to cows aborting after cloprostenol treatment. Failure of the infected animals to come into oestrus could be associated with the disturbed oestrus cycle that is associated with endometritis (Anderson 1985) and may indicate that infection is still present after abortion has been observed.

7. Other parameters such as demeanour, appetite, rectal temperature, progesterone and oestradiol 17ß levels do not clearly indicate that embryonic death or abortion have occurred following *A. pyogenes* infection. The first indication that the animals are not pregnant, other than those listed above may be the appearance of oestrus.

It is clear from the above discussion that the only readily available clinical method of identifying the time of embryonic death is ultrasound and that it is particularly valuable for monitoring intrauterine changes. Confirmation of the exact nature of infectious causes of embryonic death has to rely on microbiological or serological examination.

D. THE PATHOGENESIS OF *A. PYOGENES* INFECTION IN THE EARLY PREGNANT COW.

The remainder of this chapter comprises a discussion of the mechanisms by which *A. pyogenes* infection causes embryonic death, abortion and the features of the period between abortion and the first oestrus. The evidence from the experiments described in Chapter IV is supplemented by the clinical and ultrasound observations of the animals described in Chapters III and V. The hormonal and other observations presented in Chapters VI and VII are also discussed.

Table 11. PSPB and progesterone levels and their clinical importance in embryonic death and related conditions.

Observations	Tentative diagnosis
1. Low PSPB - High progesterone	a) Luteal phase - heifers b) <24 days of pregnancy, c) >7 days 2nd plasma sample in early embryonic death due to infection.
2. High PSPB - High progesterone	a) Pregnancy b) 1 - 2 days after infection/ embryonic death/ abortion, c) <70 days after parturition. (Luteal phase)
3. High PSPB - Low progesterone	a) Abortion following non - infectious induced luteolysis. b) <70 days after parturition (oestrus)
4. Low PSPB - Low progesterone	a) Oestrus - >70 post partum b) Oestrus - heifers c) cows in anoestrous.

The observations made in the experiments described in Chapter III have been discussed here and above. They are summarised here and with the remaining information on the pathogenesis of *A. pyogenes* infection. It was clear from the results of the transmission experiments described in Chapter IV that *A. pyogenes* caused embryonic death and abortion. The involvement of the organism was confirmed by its isolation from the products of abortion and the vaginal pus from the infected animals. The failure to isolate it from saline or cloprostenol treated controls confirmed its involvement.

The mechanism by which it produced its effects was clearly unconnected with inoculation alone as the controls inoculated with saline alone were unaffected. Minor damage and inflammation was present at the point of inoculation in the two animals killed and described in Chapter IV but clearly did not affect the outcome of the pregnancy in the controls. The absence of any alteration in demeanour or appetite and the general absence of fever were not responsible for the embryonic death or abortion. It was clear from both rectal palpation and particularly from ultrasound examination that the corpus luteum remained present throughout embryonic death in contrast to the cloprostenol treated group, suggesting that luteolysis was not a factor in embryonic death. Both rectal palpation and ultrasound failed to demonstrate the presence of significant follicular growth at this time in contrast to the situation in the cloprostenol treated group.

The other changes seen by ultrasound included separation of the allantochorion from the endometrium and the formation and accumulation of echogenic material between them prior to embryonic death. This echogenic material was considered to be pus. The endometrium was thickened but the reason for this was not clear until Heifers 19 and 20 were killed and examined post mortem.

The inference was that infection with *A. pyogenes* caused embryonic death by a mechanism which was not clear but may have involved the separation of the implanting embryo by the accumulation of pus between the membranes and the endometrium. The remaining changes of abortion and the post abortion phase followed from this embryonic death. The mechanisms of embryonic death were studied further by the experiments described in Chapter IV. Most important of these were the studies carried out with the two heifers killed following intrauterine inoculation with *A. pyogenes*.

Post mortem studies of the gross changes in the slaughtered animals revealed no infection in the other organs due to *A. pyogenes* and confirmed that infection was localised in the uterus. Biberstein and Chung Zee (1990) reported that *A. pyogenes* infection may be local, but he also said that it may be regional or metastatic. There was no evidence of the immune status of these heifers as serum was not tested for antibodies.

Other authors have suggested that *A. pyogenes* infection is restricted to the uterus. Addo and Dennis (1979) experimentally reproduced *A. pyogenes* abortion in 6 pregnant sheep after an intravenous introduction of the bacterium. Apart from pulmonary abscesses and serous atrophy of fat in three ewes and oedematous lymph nodes near the uterus in three ewes, the lesions were confined to the uterus. Bacterial culture of *A. pyogenes* was not reported from organs apart from the uterus, and animals were seen to show no premonitory signs before abortion. Similar observations were made by Smith *et al.* (1971). From their study and from this study, it may be concluded that *A. pyogenes* infection may cause local infection in the uterus even after systemic infection. In the only previous experimental *A. pyogenes* infection in

pregnancy in cattle changes in the uterus were not recorded (Mohamed *et al.*, 1987). The changes seen in Heifer 20 slaughtered 24 hours after abortion were most obvious. They included the presence of echogenic fluid in the uterine lumen which was confirmed as pus on post mortem, hyperaemia of the endometrial lining and thickening of the uterine wall were also seen and suggested that the endometrial thickness seen by ultrasound was caused by inflammation. These changes were similar but milder than those following experimental infection in the sheep (Addo and Dennis 1979). The difference in severity of the gross lesions could have been due to the longer times of exposure to the bacterium in sheep (> 12 days) compared with one day in cattle. The mode of entry of the bacteria was different in the 2 studies, haematogenous in the sheep study and directly into the uterine lumen in the cattle study reported here. This may have contributed to the differences recorded.

Schiefer *et al.* (1974) described the pathology of bovine abortion due to *A. pyogenes* in 101 cases of abortion. Thorough examination of foetuses and foetal tissues was done but no examination of the dam was carried out and therefore no lesions were recorded in the uterus. The foetuses they described had degenerated and their study did not provide any information about the way in which *A. pyogenes* affects the embryo. In Heifer 20 the embryo was found to have died recently (at the time of slaughter), but the changes seen with cloudy membranes were similar to those seen in aborted embryos in this series. The presence of clear extraembryonic fluids suggests that infection may have been restricted to the surface of the allantochorion. Haemorrhages seen in the embryo may have been due to the toxin or other non invasive causes.

The presence of pus in the bovine and sheep infections cited above may have

contributed to the separation of the allantochorion and represent one non invasive cause. The separation was complete in this study, but some sheep infected by Addo and Dennis (1979) had retained the placentae. The reason for retained placenta could have been due to the more advanced stage of pregnancy. Similarly this could have been part of the reason for placental retention in the *A. pyogenes* induced abortion in cattle by Mohamed *et al.* (1987). The above observations though differing in degree, indicate that the changes described contribute directly to the death of the embryo and subsequent abortion.

Histological examination of sections of the uterus revealed erosion of the epithelial lining of the endometrium and confirmed that the thickening seen was due to inflammation and was mostly confined to the pregnant uterine horn. Increased endometrial height has also been reported in uterine infection by Bonnett *et al.* (1988). The degree of necrosis was not as marked as in the experimental studies in sheep by Smith *et al.* (1971) and Addo and Dennis (1979). The haemorrhages seen microscopically in sheep endometrium following abortion were not seen in this study. This was possibly due to a shorter time of exposure to infection in cattle. *A. pyogenes* attachment was only present on eroded uterine endometrium and was not demonstrated on intact uterine epithelium. It was not possible to determine the cause of epithelial damage in this study. Thus it is possible that bacterial toxin causes the initial damage to the epithelium after which the bacteria attach and damage the underlying tissue. The inflammatory response characterised by accumulation of neutrophils and monocytes in the subepithelial layers was a common finding in all the three studies. Whereas damage to the uterine wall was restricted to the endometrium in cattle in this study, it was more extensive in sheep (Addo and Dennis 1979) reaching the myometrium and this may also have been due to the longer exposure time.

These histological findings suggest that the damage to the endometrial epithelium could also contribute to the death of the embryo by reducing its ability to produce nutrients and transfer oxygen.

Examination of the embryo confirmed the presence of the haemorrhages described above and revealed the presence of damage mainly to the liver, kidney, lungs and the gut (Chapter IV). This may have resulted from the action of the toxins or from anoxia caused by accumulation of pus or deprivation of contact with the endometrial epithelium. No histological evidence was found for the presence of *A. pyogenes* within the embryo itself and only a few organisms were seen on the allantochorion. Bacterial isolation was made in foetal tissue by Smith *et al.* (1971) and Addo and Dennis (1979) in contrast to the results in this study where no significant bacteria was identified in either of the embryos. Their study was carried out on foetuses and aborted material.

Scanning and transmission electron microscopy (EM) confirmed the presence of neutrophils on the uterine surface and erosion of sections of epithelial cells after *A. pyogenes* infection. There was hypertrophy and ultrastructural damage to some of these cells. Though a few bacteria could be seen by scanning EM on the uterine epithelial surface, the evidence for adhesion was not convincing. The investigation of this point could be improved by altering the methods of fixation to prevent possible loss of pus and the damaged epithelium.

The supposition that toxin produced by *A. pyogenes* might have been responsible for embryonic death was investigated in the study in which Cows 17 and 18 (Chapter IV) were inoculated with *A. pyogenes* toxin. The failure of toxin to affect the embryo or pregnancy suggests that it was not responsible

for the changes seen in Heifer II. This is the first study of its type with *A. pyogenes* toxin and the early embryo in cattle. There is little doubt that toxin prepared in the way used in this study is pathogenic in mice (Lovell 1944) and lyses red blood cells (Chapter IV). Its failure therefore to affect the embryo may have been genuine and not a consequence of the quality of the preparation used. Only one volume of toxin (6 ml) was used in this study and only two cows were inoculated. It is possible that further studies using different preparations of toxin and larger numbers of animals might have revealed some effect. It is possible that antitoxin may have been present in these animals but their serum was negative for antibody to *A. pyogenes* at the beginning of the study, therefore clearly more work should be done.

The study using killed *A. pyogenes* led to abortion in Cow 10, 144 hours after inoculation, whereas pregnancy was maintained in cow 15 also inoculated with killed *A. pyogenes*. The absence of *A. pyogenes* from the discharge, the separation of the allantochorion from the endometrium and the accumulation of echogenic fluid reported in Chapter IV suggest that *A. pyogenes* killed cells may have initiated embryonic death and abortion. If so the mechanism may have been mechanical resulting from pus formation or from a cell associated toxin. The possible role of the cystitis in the abortion was not investigated. However failure to abort was also seen in the two control pregnant sheep, exposed to 4×10^{12} c. f. u. of killed *A. pyogenes* intravenously, in their last trimester by Addo and Dennis (1979). Maintenance of pregnancy in the sheep could have been due to the advanced stage of pregnancy when the dead bacterium was inoculated. Advanced pregnancy then would have been least affected by any inflammatory response due to the bacterium. It is also possible that by the time the dead bacteria used in their study reached the pregnant uterus, most had been removed from the circulation. The immune status of these cows was not determined.

E. IMMUNE RESPONSE TO *A. PYOGENES*.

Antibody to *A. pyogenes* developed after infection in 4 cows. Antibody was already present in Cow 3 and may have been present in Cow 22. It was clear from these results reported and discussed in Chapter IV that abortion could occur in seronegative animals and that antibody developed after infection. The ability of this antibody to protect against abortion is not clear. Cow 3 aborted in spite of high levels of antibodies (with a titre of 1:1000) at day 0 of the experiment. The observation that antibody levels in this animal increased even higher suggested the second stage of an immunological response to infection. Fekadu et al. (1979) used a similar ELISA to detect antibody to the organism in chronic mastitis. The antibody present on day 0 could have been due to disease in another organ. The level of antibody present at the time of infection did not offer sufficient protection of the animal to the fresh infection. This may be in agreement with earlier workers who despite observations of antibody formation after immunization trials, failed to protect the animal against *A. pyogenes* infection (Weitz 1949, Lovell *et al.*, 1950, Derbyshire and Matthews 1963, Cameron 1966). Weitz (1949) who carried out vaccination trials in *A. pyogenes* mastitis, and attributed failure of protection to a number of factors. These included insufficiently high antitoxin levels for protection, the antitoxin level not being maintained for long enough, low level of antitoxin at the site of infection and the possibile absence of other antibodies required for protection, other than the antitoxin under study. Cameron (1966) attributed failure of protection to infection in the mice to a possible hypersensitivity reaction.

The reasons for the failure of protection from infection in cow 3 was not investigated in this study. It is possible that the organism that primarily

sensitized the animal, was of a different antigenic type from the organism used in the experiment and that antibody to it provided no cross protection. It is also possible that the concentration at the start of the experiment was lower than that required for protection. Antibody may have been absent from the uterine lumen and finally the antibody detected in this assay may not have been protective. It would have been interesting to re-infect the cow after day 25 and examine the effect of the bacterium since by this time the level of antibody was higher than at the start of the experiment (**Table 7**).

Failure of infection to cause abortion in Cow 22 is of interest. As discussed in Chapter 5, the presence of high level of antibody detected by the ELISA used could not protect Cow 3 against the consequences of infection. In Cow 22, however, no changes in the embryo could be detected by ultrasound and it is possible that protection against the infection occurred. The failure to demonstrate any rise in antibody level after inoculation of the bacteria is not a firm indication of protection. This study does not allow any firm conclusions to be made about the protective value of the antibody detected. This finding agrees with the conclusions of Cameron *et al.* (1976) who tested vaccines to *A. pyogenes*. The role of antibody in protection against primary infection due to *A. pyogenes* requires further studies.

F. ACUTE PHASE HAPTOGLOBIN (Hp).

The haptoglobin results recorded in Chapter IV represent the first determination of the protein in cattle aborting from experimental bacterial infection. The high levels of Hp in this study has clearly demonstrated the presence of tissue damage caused by the bacterium to the uterus, thereby

confirming results that are seen both with ultrasound and post mortem examination (Chapters III and IV).

The low levels of Hp concentration before infection in this study confirmed previous observations that the protein is in very low levels in cattle in the absence of infection (Spooner and Miller 1971, Richter 1974). Twenty four hours after bacterial infection, the level of Hp in cows 1 and 4 had increased to 62 and 109 mg/100ml respectively (FIGS 76 and 77). This rapid increase in the protein was similar to that observed by Conner *et al.* (1989) in 2 - 4 month old calves after experimental infection with endotoxin for *E.coli*, *Pasteurella haemolytica* and *Ostertagia ostertagi*. The highest concentration of Hp reached in this study was higher (>150 mg/100ml) than that (110 mg/100ml) reached in the studies by Conner *et al.* (1989). The peaks reached in their studies differed between infections. The differences in the timing and extent of the peaks after the initiation of infection might have resulted from the response to different infections. This possibility is worth further examination and if true, then it could be an indicator of different infections, and this might greatly aid in diagnosis. Peak levels of haptoglobin were not measured in this study, but levels of haptoglobin were already rising when embryonic death occurred, perhaps indicating that damage to the maternal tissue was important in the pathogenesis of the infection.

G. ENDOCRINOLOGY DURING *A. PYOGENES* INDUCED EMBRYONIC LOSS.

In this study it was significant that high concentrations of progesterone and low levels of oestradiol 17ß were present in the plasma of infected animals during the period of embryonic death and abortion (Chapter VI). The levels of progesterone were maintained above 4 ng/ml until the evacuation of pus

from the uterus, when the level fell to 0.5 ng/ml with manifestation of oestrus. During this period the corpus luteum was observed and was > 2.0 cm^2, its size only reduced to <2.0 cm^2 at oestrus. It is known that adequate circulating progesterone concentration is vital for maternal recognition of pregnancy since without it, embryonic signals fail to protect the corpus luteum from uterine luteolytic mechanisms (Heap *et al.*, 1989).

Progesterone.

The observation of high concentrations progesterone during and after embryonic death and abortion is an intriguing event, as the embryo was seen to die and the abortion was monitored by ultrasound (Chapter III). This event is contrary to previous observations of embryonic loss. In their study, Lamming *et al.* (1989) used concentrations of milk progesterone as a measure of luteal activity in the investigation of 24 to 33% of embryonic deaths in dairy cattle between 21 - 48 days of pregnancy. Their observations were similar to those of Shemesh *et al.* (1981). Lamming *et al.* (1989) did not investigate the actual time and cause of embryonic death, but death was followed by resumption of cyclicity contrary to the finding in this thesis. The results in this thesis stress the importance of determining the cause of infection and abortion. The findings in this study suggest that progesterone concentrations are not influenced or they do not influence the embryonic death or abortion. Even the intensive study of progesterone levels at short intervals reported and discussed in Chapter VI (FIGS 91 and 92) failed to reveal any pronounced fall associated with embryonic death.

The maintenance of high concentrations of progesterone in this study during and after embryonic loss requires further detailed examination. This is discussed further in the sections on abortion and abortion - oestrus period in the context of the failure of the corpus luteum to lyse in the way seen in

cloprostenol treated animals.

The evidence reviewed and discussed above does not indicate precisely the way in which *A. pyogenes* infection kills the embryo. It is clear that the maintenance of the corpus luteum, the high levels of progesterone and low levels of oestradiol 17ß, rule out any direct effect on the embryo due to luteolysis or even transient functional failure of the corpus luteum. The cause of death must therefore be associated with events in the uterus. These may be the result of direct effects of the bacterium on the embryo. Invasion and attachment are not demonstrated but some evidence for the toxic effects was seen even though the demonstration of toxin failed to produce any effects in the study.

The inference is that embryonic death may have resulted from the failure of the endometrium to supply nutrients or from the physical effects of the accumulation of pus. These factors remain to be elucidated.

The pathogenesis of abortion in cows infected with *A. pyogenes*.

The process of abortion and the factors affecting it were not studied specifically in the work described here. The results obtained, however, demonstrated unequivocally that the abortion produced following infection by *A. pyogenes* infection varied from that resulting from cloprostenol treatment.

Controlled induction of abortion using cloprostenol was followed by regression of the corpus luteum and return to oestrus 66 - 96 hours after the treatment in this study (Chapter III) as in studies of Omran (1989)). In his studies, Omran induced abortion by the administration of cloprostenol between 10 - 55 days of pregnancy. Although there were differences in the time taken for corpus luteum regression to occur, the time being shortest

within group 1 (10 - 20 days of pregnancy) at 44 hours and longest in group 3 (44 - 55 days of pregnant) at 72 hours, all the animals responded to the treatment. Similarly Kastelic and Ginther (1989) observed lysis of the corpus luteum 2 -5 days after administration of prostaglandin between 28 and 42 days of pregnancy. In contrast, the infected animals in this study had not come to oestrus by 8 days after abortion and the corpus luteum could be palpated and demonstrated by ultrasound throughout this period.

Maintenance of the corpus luteum has been reported in other bacterial infections including *Streptococcus sp*, *Staphylococcus sp* and *Enterobacteria* which have been isolated in cases of chronic endometritis (Miller *et al.*, 1980, Dubois and Williams 1980, Markusfield 1984, Arthur *et al.*, 1989d). Most of the observed cases of maintained corpus luteum in bacterial infections have been in the absence of the embryo. There is little experimental evidence of the phenomenon observed in this study and few observations of the corpus luteum have been made during bacterial induced abortion of the type described in this study.

On the other hand *E. coli* has been reported to cause lysis of the corpus luteum through the action of its endotoxin leading to short cycles (i.e. before 21 days) (Peter *et al.*, 1987). In their experimental infections of 10 cycling cows with *Trypanosoma congolense*, Luckins *et al.* (1986) observed prolonged maintenance of the corpus luteum in 4 of the animals infected. The reason for the maintenance of the corpus luteum was not clear to them. However the lower percentage (40%) of cows with a persistent corpus luteum in their work compared to the 100% corpus luteum persistence presented in this work may be due to the different pathogens used in the studies. Further work that has been done with regard to the maintained corpus luteum in trypanosomiasis will be discussed below in the section on the post - abortion period.

In other studies, however, Ogwu *et al.* (1984) demonstrated that infection of Zebu heifers with *Trypanosoma vivax* before first mating resulted in infertility. Infection of pregnant heifers during the first trimester resulted in abortion in 1 of the 4 animals infected, but pregnancy continued to term after infection during the second trimester, whereas infection of 4 heifers in the third trimester led to three calves born prematurely and one death at full term (Ogwu *et al.*, 1985). Foetuses of dams infected at this stage were anaemic with no evidence of specific intrauterine infection and with no significant placental lesions, thus attributing the pathogenetic effects to the maternal hormonal imbalance from pituitary damage, the stress of infection or hyperthermia (Ikede *et al.*, 1988). The pathogenesis of *Trypanosoma vivax* induced abortion seems to differ from that caused by *A. pyogenes* in this study. This is because in the latter *A. pyogenes* was identified from the products of abortion by culture and staining methods.

Embryonic death clearly occurred before abortion following *A. pyogenes* infection. Abortion does not inevitably follow embryonic death as some embryos may be resorbed (Kahn and Leidl 1989). In the present study , however, embryonic death was accompanied by the opening of the cervix and abortion.

The mechanisms involved in the opening of the cervix and abortion will be discussed below. From the above discussion, oestradiol 17ß, oxytocin and prostaglandin F2α do not seem to take part in the mechanism leading to the opening of the cervix. This is thought to be so because of the maintained corpus luteum. There are four possible mechanisms. **Firstly**, soon after infection there was an increase in the uterine contractions (Chapter III), these were most probably due to the inflammatory response of the host to infection.

However the contractions may have also been elicited by the production of oxytocin. These contractions may have contributed to the opening of the cervix. **Secondly**, the spread of infection from the site of inoculation was confirmed by identification of the bacterium throughout the reproductive tract in the slaughtered heifers (Chapter IV). The spreading bacteria may have led to breakdown of the cervical plug thus destroying the barrier and opening the cervix. **Thirdly**, there was an accumulation of pus in the infected uterus and this was observable ultrasonographically (Chapter III). As pus accumulated it could have led to an increase in uterine pressure which may have forced the cervix open. **Lastly**, There is a possibility that relaxin may have acted directly on the cervix causing it to open. Relaxin is known to cause cervical dilatation, pelvic relaxation and separation of the pubic symphysis near parturition (Bagna *et al.*, 1991). Its role in these experiments may have been minimal since the hormone has only been demonstrated in the corpus luteum of late pregnant cows (Fields *et al.*, 1980, Musah *et al.*, 1988).

A. pyogenes infection of the early pregnant bovine represents an opportunity to study further the factors involved in non luteolytic abortions.

H. THE INFECTED UTERUS AFTER ABORTION.

Factors involved in the pathogenesis of the period between abortion and oestrus following infection.

The period following abortion in *A. pyogenes* infection was characterised by the presence of the corpus luteum, increased uterine tone (scores 2-4), the uterus contained echogenic fluid, the cervix was patent and pus was present in the vagina. This period ended in return to oestrus after 18 to more than 34 days. The details of the findings during this period have been described in Chapter V and discussed there in the context of the experiment performed.

The study of the pathogenesis of this condition was limited because it was not possible to kill any of the affected animals for detailed examinations. It was possible, however, to interpret the results obtained from the clinical, ultrasound and hormonal findings.

Clinical observations in the infected animals following abortion revealed no generalised signs indicative of a systemic infection in this study. Continued discharge of pus and mucus from the vagina were an important feature and suggested that infection might be important. Anderson (1985) described a similar endometritis characterised by a steady escape of mucopurulent material from the uterus. He also described pyometra with pus in the uterus and increased uterine size and pus in the vagina. It seems that infection after abortion in this study, progressed from endometritis to pyometra as all the signs described by that author were seen.

Previous investigations have also implied the presence of a persistent corpus luteum during prolongation of the bovine uterine luteal phase especially after parturition (Andriamanga *et al.*, 1984). Arthur *et al.* (1989d) also reported that pyometra may occur following embryonic death and is accompanied by maintenance of the corpus luteum as in this study. Results obtained ultrasonically in this study support the above observation in that the corpus luteum was maintained in all the animals as long as there was pus in the uterus. Unlike the report made by Arthur *et al.* (1989d), *A. pyogenes* appeared to be the primary rather than the secondary cause of the condition in the present study.

The relationship of the post abortion - phenomenon with pyometra was not clear. The cervix was seen to be continuously open (about 3 mm) in the infected animals, though to a much lesser extent compared to oestrus period

(about 6 mm). This finding is contrary to the closed cervix which occurs in the development of pyometra (Arthur *et al.*, 1989d). It is however in agreement with the report of continuous discharge of pus from the uterus, which is found in the vagina in endometritis and pyometra (Anderson 1985). Therefore though it is widely believed that in pyometra the cervix is closed, the ultrasonographic studies presented here seem to suggest otherwise. Also due to the fact that pus is normally found in the vaginal floor on vaginoscopy, it may be incorrect to talk of a closed cervix, and it may be better to talk of the degree of openness. This area however requires further investigation to explain why during infection the opening is much smaller (leading to accumulation of pus) compared to oestrus when the bulk of the pus is evacuated. Some of these aspects are further discussed in the section of endocrinology.

The importance of inflammation was strengthened because the endometrium was seen to be thickened during infection, a sign of the presence of endometritis when viewed by ultrasound. The uterine lumen was seen to be filled with echogenic material (pus) which increased in amount with the increase in uterine size. Similar observations have been made by the use of ultrasound in endometritis and pyometra in cattle by Fissore *et al.* (1986) and Kahn and Leidl (1989). The echogenicity of the uterine fluid depended on the consistency of the mucopurulent material. It is of interest that echogenicity of the uterine fluid decreased prior to its disappearance at oestrus (Chapter V).

In the present study, unlike in any previous one, it was possible to follow the progress of infection by ultrasound until normality returned.

No samples of the endometrium were taken during the this period but the purulent vaginal discharge contained *A. pyogenes*. There is therefore no direct

evidence for the state of inflammation of the endometrium. At embryonic death it was severely inflamed. Thickening of the endometrium persisted and may be assumed to be due to inflammation. This proposal is supported by the evidence from haptoglobin levels.

The concentrations of Hp were in detectable concentrations as long as there was pus in the uterus and only fell to pre-infection levels after oestrus (presumably after uterine repair). It is not clear whether Hp has any role in repair of the uterus after infection and not such role has been attributed to it in the literature. Skinner *et al.* (1991) reported that the concentration was elevated in the infectious conditions and low in non-infectious conditions and chronic endometritis. They confirmed the high levels to be due to acute phase reaction and the low levels to occur in non-infectious conditions like normal parturition, suggesting that Hp concentration rises as a result of infection and inflammation and not trauma *per se*. Some workers (Shim 1976, Lee Tong-Ho *et al.*, 1977) have suggested that Hp may inhibit PGE_2 synthesis by affecting glutathione peroxidase activity. This is thought to be through the Hp-Hb complex indirectly affecting the PGE2 levels by inhibiting glutathione peroxidase. On the contrary Hamberg *et al.* (1976) has suggested that glutathione peroxidase could stimulate PGE2 synthesis by catalysing the conversion of PGG2 into PGH2 and conversion of 15-hydroperoxy-PGE2 into PGE2. More work therefore needs to be done to examine the physiological role of Hp in infection. It does, however, suggest that inflammation was gradually becoming less during this period.

The apparent persistence of inflammation accompanies the persistence of a functional corpus luteum. High levels of progesterone (> 4ng/ml, Chapters VI and VII) confirm its functional nature and form a marked contrast to the findings in the cloprostenol treated group in which endometrial inflammation

and purulent discharge were absent. The persistence of the corpus luteum appears to be critical in this period. The reasons for its failure to lyse are not clear.

Maintenance of the corpus luteum and PG2α. The maintenance of the corpus luteum during this period could be due to a number of factors. It is known that PGF2α, which is synthesized and released from the endometrium, causes regression of the corpus luteum at the end of a cycle (Kindahl *et al.*, 1984, and Basu 1985) and at parturition. The observation of persistent high concentrations of progesterone and the corpus luteum in the present study seem to point to lack of adequate concentrations of PGF2α to effect luteolysis. When conception occurs PGF2α synthesis is decreased (Basu *et al.*, 1988). In their study Kindahl *et al.* (1989) were able to demonstrate the presence of an inhibitory factor for PGF2α synthesis in endometrial preparations including microsomes, cytosolic fractions and homogenates. They found the potency of inhibition 8 times higher in cytosolic fractions from Day 17 pregnant as compared to Day 17 non-pregnant cows. They then suggested that bovine endometrium possesses both a PG-synthetase system and potent inhibitor(s) that control prostaglandin biosynthesis. The potency of inhibition was thought to change according to the stage of the cycle and in pregnancy probably from a signal from the embryo. It is possible that in abortion due to *A. pyogenes* the system of PG inhibition was in place and prevented PGF2α synthesis. It is also possible that some of the bacterial products act as inhibitors to the biosynthesis of PGF2α. Endometrial erosion was seen after bacterial infection (Chapter IV). It is therefore possible that apart from temporarily destroying the ability of the endometrium to synthesize PGF2α, *A. pyogenes* may also destroy precursors like phospholipids and arachidonic acid. Arachidonic acid is an important precursor of prostaglandins. One hypothesis in endometritis in the mare is that failure of

luteolysis may be due to failure of the damaged uterine endometrium to synthesize PGF2α in adequate amounts (Stabendfeldt *et al.*, 1980). It is possible that some substances produced by the *A. pyogenes* destroys arachidonic acid or other precursors and thus reduces the production of uterine PGF2α.

Bacterial products may affect the luteal tissue or the endometrium leading to an increased production of prostaglandin E 2 and I 2 which are said to be luteotrophic (Hansel and Dowd 1986). The noted transient increase in in progesterone levels in animals 3 and 5 after infection may be a reflection of the increased secretion of PGE2 and PGI2. The use of transmission E M to evaluate the quality of the two cell types of the corpus luteum (large and small) and their secretory granules and of immunocytochemistry to identify hormonal activity was considered but could not be carried out in the time available. Since the manifestation of a corpus luteum in abortion due to infection was common to all the 5 animals studied, it is significant finding and requires further investigation.

Another possible factor responsible for the failure of regression of the corpus luteum may result from the exocrine production of PGF2α i. e. instead of the production being endocrine with PGF2α gaining access to the corpus luteum via the ovarian artery from the ovarian vein, there is a reversal of secretion with PGF2α being secreted into the uterine lumen. Such a mechanism has been reported in the sow when pregnancy is established and is due to the production of oestrogens (Bazer *et al.*, 1989).

Schallenberger *et al.* (1989) observed early embryonic death in 12 heifers which returned to oestrus after A.I. between 22 and 34 days. In their study embryonic death was accompanied by increased circulating in concentrations

of oxytocin, prostaglandin F2α and oestradiol-17ß and a decrease in progesterone. They suggested that the inhibition to the production of oxytocin, PGF2α and oestradiol-17ß is removed after embryonic death. It is not clear from their study whether dead embryos were collected after death and the cause of death of the embryo is not reported. From the findings in this study on embryonic death due to *A. pyogenes*, it seems the fate of the corpus luteum and hormones produced depends on the cause of embryonic death. This is thought to be so since *A. pyogenes* induced embryonic death was not immediately followed by corpus luteum regression and oestrus of the type observed by Schallenberger *et al.* (1989). A similar conclusion was made by Watson *et al.* (1984).

The toxin of *A. pyogenes* not seem to affect pregnancy when inoculated alone in experiment III (Chapter IV) but the potential effect of the toxin should not be ruled out. Peter and Bosu (1987) and Peter *et al.* (1987) carried a survey of uterine infection in post partum cows. They observed that *E. coli* causes short cycles of 6 - 14 days after parturition and this was found to be due to the synthesis of PGF2α stimulated by the bacterial endotoxin. Findings from the above studies are contrary to the observation in the present study which showed a delayed return to oestrus after embryonic loss. This may be because *A. pyogenes* does not produce endotoxin.

Infection with trypanosomiasis has been known to cause infertility and to cause abortion in ruminants but the pathogenesis seems to differ depending on the degree of infection. Firstly, acute infection with *T. congolense* has been shown to prolong the luteal phase in a few cases (Luckins *et al.*, 1986, Llewelyn *et al.*, 1988). The persistence of the corpus luteum was accompanied by high levels of progesterone, similar to the present studies of *A. pyogenes*. Similar

observations were made in *T. congolense* infection in goats although in 3 goats the persistent corpus luteum was accompanied by half the expected progesterone concentration (Llewelyn *et al.*, 1987). In the goat, corpus luteum prolongation was attributed to impaired uterine PGF2α synthesis or transport to the ovary supporting the theory advanced above for *A. pyogenes* infection. Secondly in chronic infection of goats with *T. congolense*, Mutayoba *et al.* (1988) showed a significant decline in plasma progesterone and oestradiol-17ß and elevation of PGF2α (Mutayoba *et al.*, 1989). These observations suggest that chronic trypanosomiasis affects fertility in a different way compared to the infection of *A. pyogenes*, but in a manner similar to *E. coli* infection.

From observations of other workers and in the present study PGF2α does not seem to be produced in *A. pyogenes* infection. Alternatively if it is produced then it does not have an immediate effect on the function of the corpus luteum. This may explain the maintenance of the corpus luteum for 18-34 days after abortion. It remains to be established whether PGF2α is produced during *A. pyogenes* infection.

Oestrogen. There were periodic increases in the plasma concentration of oestradiol-17ß after infection and abortion (Chapter VI), but the concentration did not seem to have been high enough to potentiate oxytocin and the synthesis of PGF2α to elicit luteolysis as proposed by Flint *et al.*, 1989. The only significant increase in the concentration of oestradiol-17ß was seen after evacuation of pus from the uterus, followed by luteolysis and a decline in progesterone concentration. The oestradiol peaks were followed by oestrus.

Oxytocin. The observation that there was no substantial increase in the levels

of oestradiol-17ß enough to bring about the onset of oestrus raises a query as to what part (if any) is played by oxytocin. In this study it was not possible to measure the concentrations of oxytocin due to the little time available, however the possible role of oxytocin in the post abortion period in this study is discussed here. Administration of oxytocin shortens the luteal phase of the cycle in cattle and goats (Cooke and Knifton 1981) and synchronous secretion of oxytocin and PGF2α at luteolysis has been demonstrated (Flint and Sheldrick 1983). It has also been shown that immunization against oxytocin prolongs corpus luteum function (Sheldrick *et al.*, 1980). It has not been evaluated in the present study, but it is possible that the products of *A. pyogenes* have an inhibiting effect on the role of oxytocin during and immediately after abortion.

It has been suggested that the contribution of ovarian oxytocin to ovarian cyclicity is likely to be systemic involving other organs like the uterus other than the ovary (Flint *et al.*, 1989). That oxytocin may act systemically in luteolysis has been supported by the observation that the non-pregnant uterus becomes increasingly sensitive to oxytocin around the time of luteolysis. This has been associated with an increase in the oxytocin receptors in the caruncular and intercaruncular endometrium, and a fall in circulating progesterone (Sheldrick and Flint 1983, Sheldrick and Flint 1985, and Flint *et al.*, 1989). Two experiments have been performed suggesting that the increased number of uterine oxytocin receptors plays a causative part in the release of PGF2α from the uterus. In the first, the role of oestrogen was examined since it is known that administration of high doses of oestrogen during the mid luteal phase results in luteal regression in ruminants (Hixon and Flint, 1987). When the uterus was examined after oestrogen administration, oxytocin receptors in the uterine wall appeared within 12 hours and this preceded prostaglandin release and the onset of luteolysis

(Hixon and Flint 1987). From these studies, it is possible that with *A. pyogenes* infection, destruction of the uterine endometrium by the bacterium prevents the formation of oxytocin receptors and thus PGF2α production. So even though oytocin was produced by the corpus luteum, it would fail to elicit production of PGF2α.

Early in pregnancy oxytocin concentration in the corpus luteum declines and this happens despite the continued progesterone secretion (Flint and Sheldrick 1986). Low levels of oxytocin receptors have consistently been found during early pregnancy and this is thought to be one of the ways to reduce uterine response to oxytocin (Sheldrick and Flint 1985, Flint and Sheldrick 1986). Since the abortions following *A. pyogenes* infection occurred at a time when oxytocin receptors in the uterus are expected to be low, this could have contributed to lack of PGF2α synthesis, and permitting maintenance of the corpus luteum.

From the discussion above, oxytocin may have a limited role on the corpus luteum function in *A. pyogenes* induced embryonic loss. A number of studies however should be carried out to substantiate the assumption, and these should include the measurement of circulating oxytocin during and after the induced abortion and examination of the uterus at different stages after infection and abortion until resumption of cyclicity for oxytocin receptors. It would also be interesting to culture the cells of the corpus luteum in infected cows and to examine them for production of oxytocin (Wathes and Swann 1982). Finally it would be useful to do similar investigations using other uterine infections like *E. coli* and chronic trypanosomiasis which seem to have a different pathogenesis as far as the hormonal interplay is concerned during the same period.

Other possible mechanisms which may be involved. From 16 days of pregnancy, embryos have been shown to produce substances which are lipid - soluble (Hansel *et al.*, 1989) and others to be proteins like α - fetoproteins, some allantoic fluid proteins and others in foetal cotyledons (Hickey *et al.*, 1989, Izhar and Shemesh 1989). Bovine trophoblast protein (bTP - 1) is also produced by the early embryo and has been found to influence pregnancy by inhibiting PGF synthesis. These substances have been found to be important in maternal recognition and maintenance of pregnancy, preventing lysis of the corpus luteum. It is highly possible that effects of these substances are still operational during the time of embryonic death and abortion seen in this study and may thus be responsible for the presence of the functional corpus luteum. It would thus be beneficial to measure these substances during an infectious abortion and up to the first oestrus.

Finally, in cattle the end of a cycle is characterised by endometrial production of PGF2α, lysis of the corpus luteum and decline of progesterone followed closely by increased secretion of FSH and LH from the anterior pituitary, follicular growth, oestrogen secretion and oestrus. Due to endometrial damage caused by *A. pyogenes* infection, there seems to be a disruption in the production of PGF2α and the events that lead to oestrus. This may be the main reason for the corpus luteum maintenance seen during *A. pyogenes* induced abortion.

I. FINAL RECOVERY PRIOR TO OESTRUS.

The supposition that inflammation of the endometrium and the presence of pus in the uterus were involved in the maintenance of the corpus luteum is confirmed to some extent by the simultaneous disappearance of echogenic

fluid or pus from the uterine, the decrease in area of the corpus luteum and concurrent growth of the follicle of ovulation. These were accompanied by falling progesterone levels and rising oestrogen.

There seemed to be little association between antimicrobial treatment and recovery. The results after antimicrobial treatment varied. In Cows 1, 3 and 4 recovery leading to oestrus was observed 18 - 34 days after abortion. In Heifer 5, on the contrary, without antimicrobial treatment oestrus was manifested 21 days after abortion. It is therefore doubtful whether antimicrobial treatment had any significant effect on the infection. In Cow 2, which was treated with both penicillin and oxytetracycline, infection persisted to the end of its observation. Sensitivity tests for the best drug which were not carried out in this study should be done in future investigations. Animals should be observed for longer times than those reported in this study and only single antimicrobials should be used in each case.
In this study local antimicrobial treatment of the infected animals was not undertaken, thus findings can not be conclusive. To try to understand the role of pus in maintenance of the corpus luteum after abortion, it would be interesting to introduce antimicrobial uterine pessaries (Dobson and Noakes 1990) soon after abortion. These might help to eliminate infection from the uterus. This study clearly demonstrates that infection due to *A. pyogenes* interferes with resumption of normal cycle after loss of the embryo. This is confirmed by manifestation of oestrus 24 - 48 hours after embryonic loss induced by the administration of killed bacteria in Cow 16.

CONCLUSION AND OUTLOOK.

1. The controlled experimental infection of the early pregnant bovine uterus with *A. pyogenes* has demonstrated that the bacterium can cause embryonic death and abortion. The study strongly supports the view that *A. pyogenes* is a

primary pathogen in the uterus and may be responsible for some abortions in cattle (Anon 1988). Although *A. pyogenes* is commonly grouped with non-specific infections, the present study suggests that, in view of some signs which are characteristic of the infection, the overwhelming evidence of its frequency in bovine abortion in the field and the economic implication of the infection, the disease be classified as a specific infection.

2. This controlled study has established a model that can be used in pathenogenetic studies of other so called non-specific infections of the uterus. Future experimental infections should be carried out using a larger number of animals.

3. Use of ultrasound to study the effects of the bacteria on the embryo and the actual time of embryonic death and abortion has been performed for the first time. Determination of embryonic death and abortion adds another important dimension to methods like measurement of progesterone and PSPB for confirmation of presence of a viable embryo. These methods can therefore be used more reliably in the future to determine presence or absence of a viable embryo.

4. The pathogenesis of *A. pyogenes* in abortion has been investigated in depth. Embryonic death is thought to occur most probably due to anoxia as a result of separation of the allantochorion from the endometrium. Damage to the endometrium may also contribute to the death of the embryo. The direct action of the bacterium on the embryo is doubtful since no organisms were identified in the embryonic tissue. The role of the bacterial toxin in embryonic death is possible but the aspect requires more investigation. Acute phase haptoglobin has proven to be sensitive in detecting infection. There are possibilities of employing the assay for diagnosis of different infections.

5. Results of antimicrobial treatment in this study were not conclusive. Other treatment regimes could be adopted for future studies and local treatment is highly recommended.

6. The fact that the corpus luteum of pregnancy is maintained during embryonic death and abortion and for at least 18 days is the first report of its experimental demonstration which is very interesting. This is particularly so since in other uterine infections like *E. coli* and chronic trypanosomiasis the corpus luteum is lysed. Progesterone plays no part in *A. pyogenes* induced embryonic loss. The corpus luteum was found to be functional and accompanied by very low levels of oestradiol-17ß until the first oestrus. These findings lead to questions which require further investigation in the hormonal interplay during *A. pyogenes* induced abortion and the post abortion period. The questions are :-

i) What is the hormone (s) involved in the opening of the cervix?

ii) What is the role of prostaglandins during the abortion?

iii) What is the role of pus in the pregnancy-like state produced after abortion?

iv) What leads to the onset of the first oestrus following abortion?.

7. Preliminary antibody detection of *A. pyogenes* confirmed ELISA to be a potentially useful technique in the disease investigation. More assays than those performed in this study should be run to give significant results. It may also be beneficial to improve the assay so as to determine the different immunoglobulins produced during the disease and their relative importance. Immunological studies were not convincing as to whether protective immunity develops after infection. The possibility of development of a vaccine for *A. pyogenes* infection should be explored.

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APPENDIX

Figures illustrating principles of the operation of the ultrasound equipment.

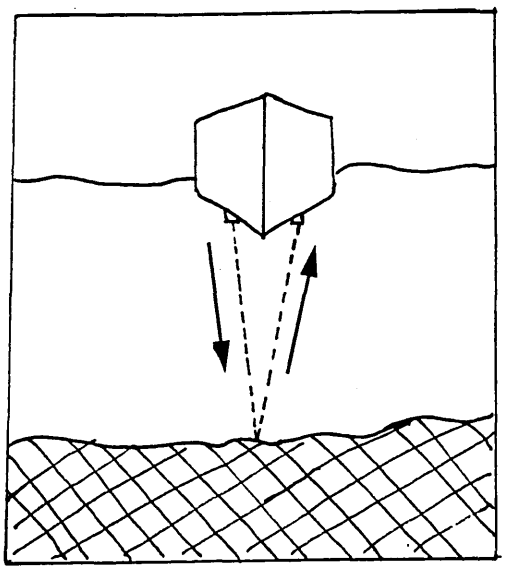

Fig. 1: The principle of ultrasound. Use of ultrasound to measure the depth of water beneath the sea, used in ships or submarines.

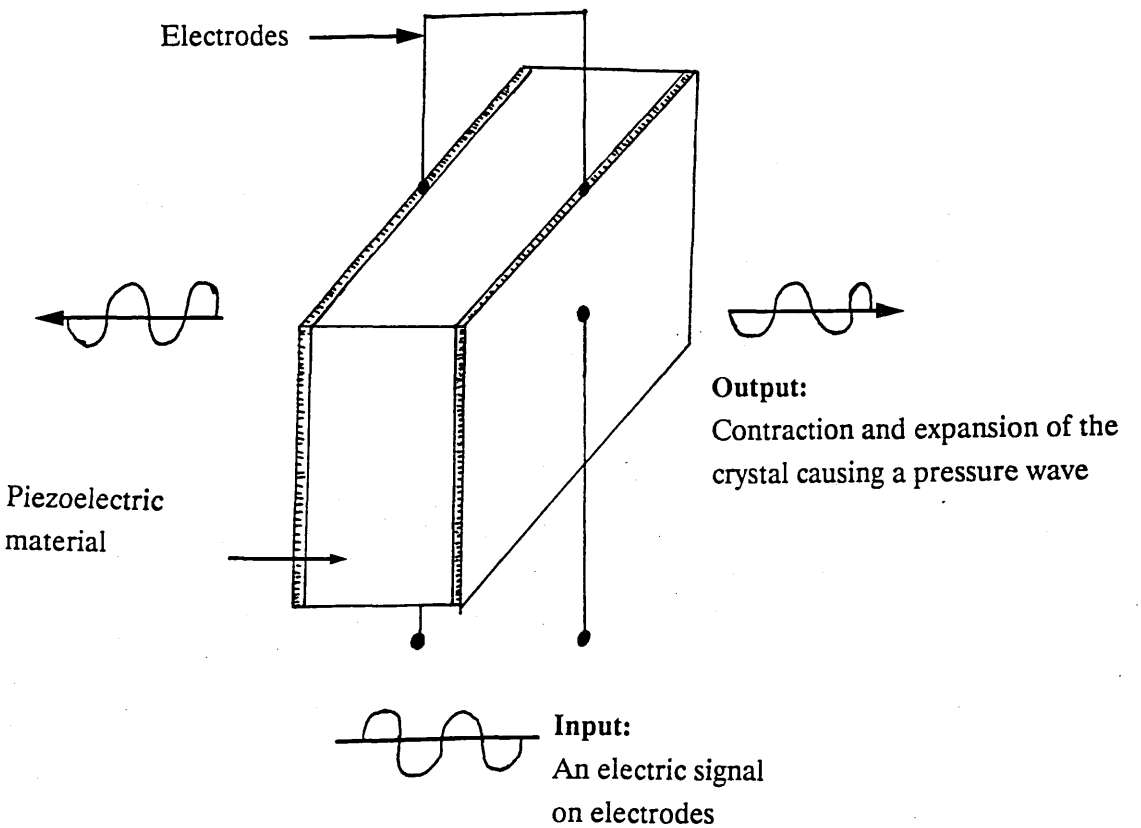

Fig. 2: The principle behind the transducer. Piezo electric material for generation of sound waves used in clinical ultrasound.

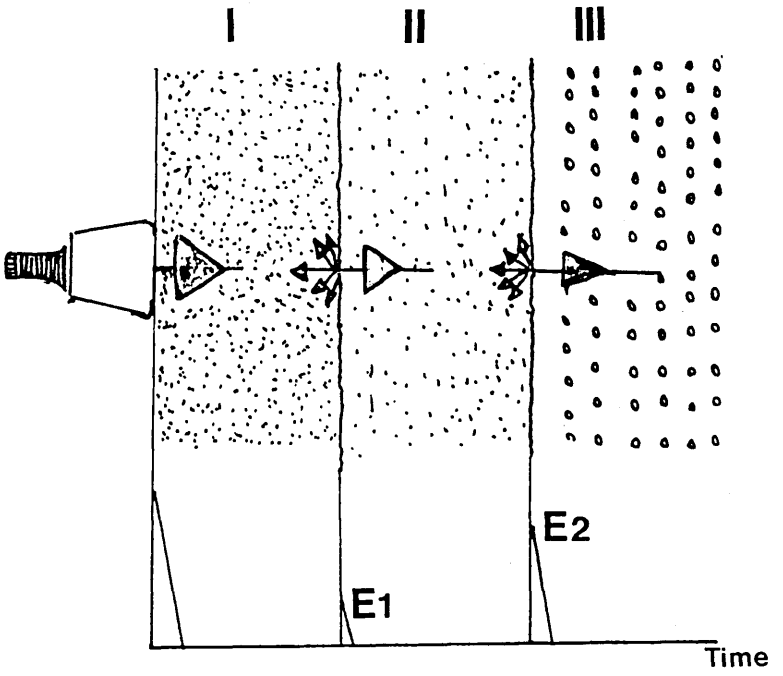

Fig. 3: Reflection of sound waves (echos) by different boundaries in the A -

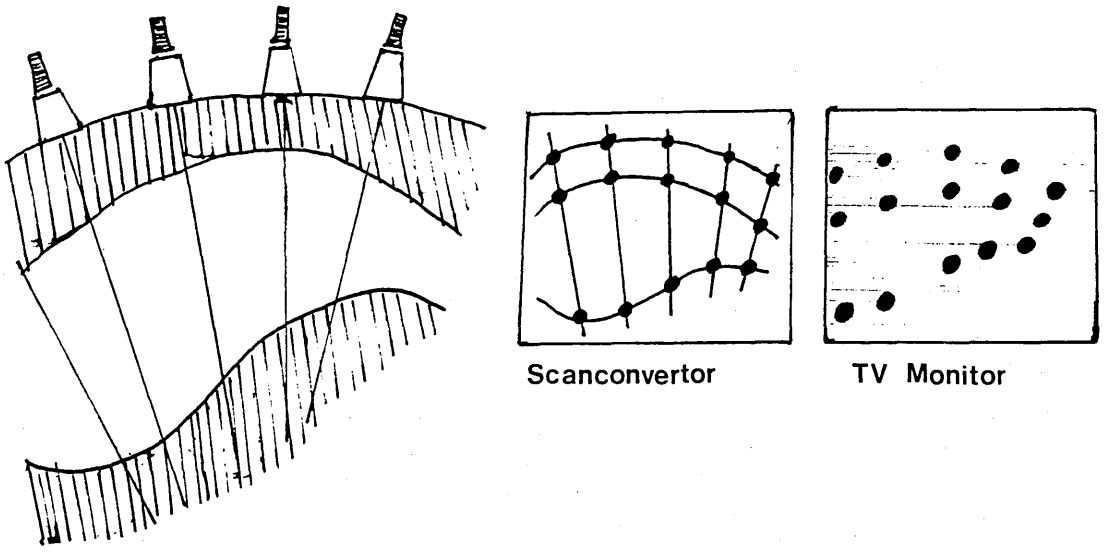

Fig. 4: A display technique for the B - mode or Brightness mode which is two dimensional and produces two dimensional cross - sectional images on the T. V. screen.

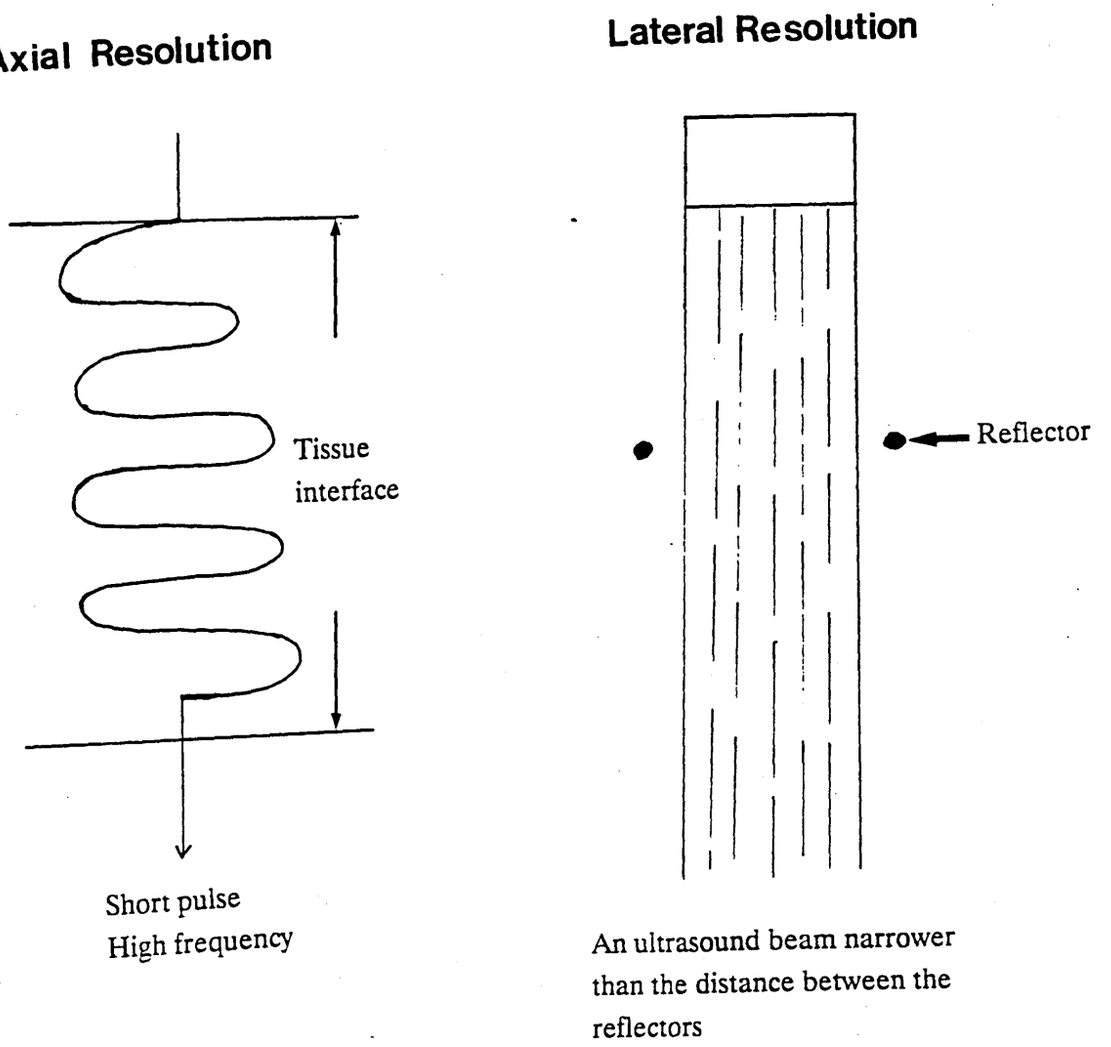

Fig. 5: The principle of resolution. Demonstrates the way in which two adjacent reflecting surfaces, are distinguished from each other.

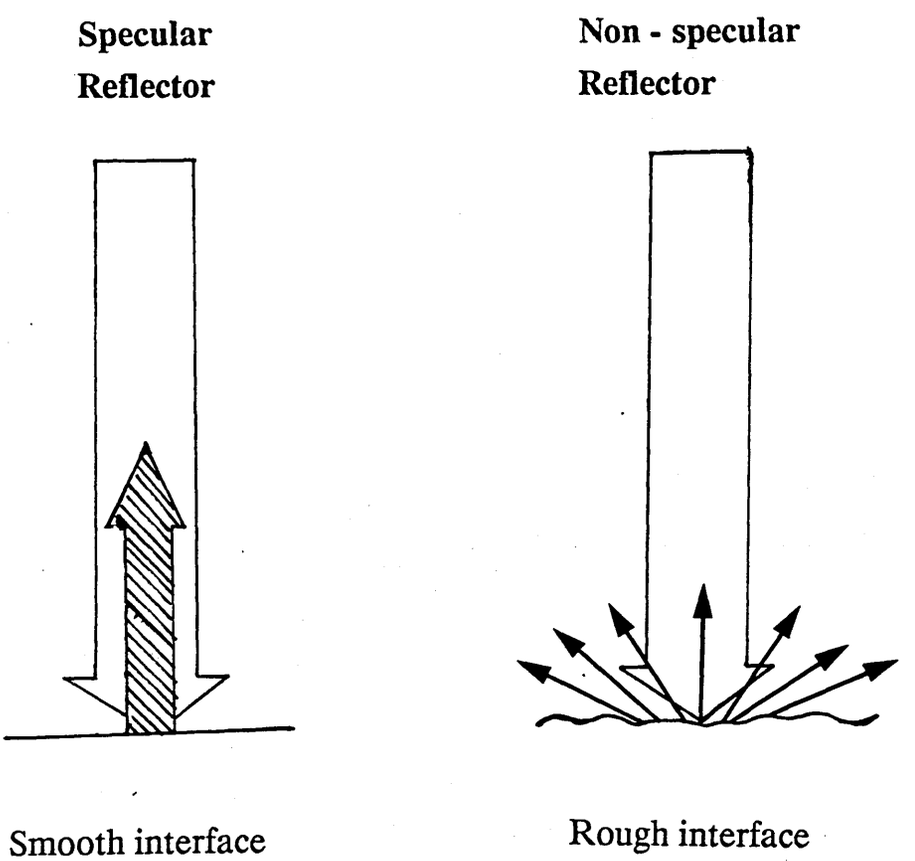

Fig. 6: The mechanism of specular and non - specular reflection of sound waves.

SECTION 2. BIOCHEMICAL TESTS.

Biochemical tests.

The tests to identify *A. pyogenes* were as outlined below:-

1. Catalase test. This was used to demonstrate possible production of catalase by the organism using a 3% solution of hydrogen peroxide. Pure colonies of the organism were taken from horse blood agar by wire loop and dipped in 5 ml of 3% hydrogen peroxide. Production of gas would indicate a positive reaction.

2. Litmus milk which is red in appearance was inoculated with a pure culture of the bacterium and incubated for 48 hours aerobically at 37^{O}C. A positive result for the bacterium was be characterised by change of colour to pale pink or white. The changes in the litmus was brought about by production of lactic acid and digestion of the coagulate caused by proteolytic enzymes if the result was positive.

3. Urease test. This was carried out using a "urease test medium" prepared by the formula of Stuart *et al.* (1945). The medium was inoculated with a pure culture of the bacterium and was incubated at 37^{O}C in a warm water bath for 60 minutes. The presence of urease would be shown by its activity of producing alkali from the urea - with a colour change from yellow to red in positive test.

4. Gelatin liquefaction test. Nutrient gelatin which is a gel at room temperature was used. The gel was inoculated with a pure culture of the bacterium and incubated at 37^{O}C for 48 hours, then placed in a refrigerator overnight. A positive result was shown by a liquefied gel after the cold storage.

5. Sugar test. These were done to find out if fermentation was caused by the growth of the bacterium. The sugars were prepared in "Broth base medium" to which Bromothymol blue (an indicator) was added. The sugars used in the test were (a) glucose, (b) lactose, (c) sucrose, (d) maltose, (e) trehalose, (f) soluble starch and (g) salacin.

Prepared solutions were inoculated with pure colonies of the bacterium and incubated at $37^{O}C$ for 48 hours. The broth base contained peptones which were brokendown during bacterial growth producing substances with alkaline reaction. So if acid was produced from sugar fermentation it had to be in excess of the alkaline to cause colour change of the indicator. A positive result was indicated by colour change from blue to yellow.

Observations from the above tests were recorded.

TABLE A.

RESULTS OF THE BIOCHEMICAL TESTS FOR *A. PYOGENES* FROM THE INFECTION EXPERIMENT COMPARED TO THE RESULT AFTER CARTER *ET AL.*, (1979).

BIOCHEMICAL TEST	RESULTS AFTER *A. PYOGENES* INFECTION	RESULTS AFTER CARTER *ET AL.*, (1979).
1. Catalase	-	-
2. Litmus milk	+	+
3. Urease	-	-
4. Gelatin	+	+
5. Sugars		
(a) Glucose	+	+
(b) Lactose	+	+
(c) Maltose	+	+
(d) Sucrose	+	+
(e) Trehalose	-	-
(f) Starch	+	+
(g) Salacin	-	-

(+) Positive

(-) Negative

TABLE B.

RESULTS OF ANIMALS USED IN THE PATHOGENICITY STUDY.

COW NUMBER	STAGE OF PREGNANCY AT INFECTION OR TREATMENT (days after A. I.)	INOCULATION OR TREATMENT	TIME AFTER INOCULATION OR TREATMENT (hours)	
			EMBRYO-NIC DEATH	ABORTION
EXPERIMENT II	LIVE *A. PYOGENES*			
19	35	$5.5X10^9$	KILLED FOR PM STUDY	
20	36	$3.5X10^9$		
EXPERIMENT III	KILLED *A. PYOGENES*			
15	27	$2.55X10^{10}$	NONE	
16	36	$1.2X10^{11}$	138	144
EXPERIMENT IV	*A.PYOGENES* HEAMOLYSIN (TOXIN)			
17	27	6ml HAEMOLYSIN	NONE	
18	34	6ml HAEMOLYSIN	NONE	