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A STUDY OF SOME ASPECTS OF THE OVIDUCTS IN NORMAL AND INFERTILE CATTLE

by

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SUMMARY

SUMMARY

Studies were undertaken to obtain more information about the role of the oviduct in normal cows and its contribution to infertility in repeat breeder cows. The investigations involved examination of slaughterhouse material and the evaluation of clinical tests for oviduct patency.

Initial studies, using fluid and a particle suspension to demonstrate the details of patency of the oviducts of cow and heifer genital tracts, suggested that a proportion of oviducts which proved patent to the fluid were blocked to the passage of particles. In the live animal, a modified form of the phenolsulphonphthalein dye test was evolved which proved accurate in diagnosing unilateral and bilateral occlusion of the oviducts. However this test could not be used to diagnose partial occlusion or narrowing of the oviducts.

The use of superovulation, using pregnant mare serum gonadotrophin, and non-surgical embryo recovery was studied as a means of assessing oviduct function. The marked variation in response to the superovulatory drug in individual cows was a limiting factor in this technique. The eggs collected were examined for normality using the light microscope and preliminary studies in preparation and results of the use of the scanning electron microscope are described.

CHAPTER 1

GENERAL INTRODUCTION

1.1 - INTRODUCTION

The oviducts play an essential role in ensuring conception and in the early survival of the embryo. Many details of the periovulatory period in the cow have now been elucidated. The marked behavioural changes constituting prooestrus and oestrus (Williamson, Morris, Blood, Cannon and Wright, 1972) occur once every 18-25 days (Erb, Ehlers and Morrison, 1958). Oestrus comprises a variable period reported as being on average 14.9 hours duration when the cow stands to be mounted (Esslemont and Bryant, 1976). At natural mating, semen is deposited in the anterior vagina and pools at the external os of the cervix (Salisbury, Van Demark and Lodge, 1978). It is estimated that sperm may enter the cervix within 1-2 minutes of mating (Mattner, 1966). The cervix is thought to be the main storage reservoir for sperm after coitus, certainly within the first 24 hours (Mattner, 1968; Thibault, 1973). The composition of cervical mucus is extremely important in relation to sperm storage. After ejaculation the sperm swim between the micelles of mucus which are orientated to the vagina in flow lines, and are thus guided to the cervical crypts (Mattner, 1968; Gibbons, 1969). Although the cervix comprises a reservoir for sperm, some workers have suggested that sperm transport is rapid enough to enable some spermatozoa to reach the oviduct ampulla within 2½-3 minutes of mating (Van Demark and Hays, 1954).

The numbers of sperm progressively decrease as they move upwards to the site of fertilisation in the oviduct There is

approximately a 100-fold reduction in numbers during their passage through the cervix with a similar reduction at the uterotubal junction and isthmus of the oviduct. This results in only a tiny percentage of the original ejaculate reaching the fertilisation site (Thibault, 1973). During the passage of the spermatozoa through the uterotubal junction, the seminal plasma is removed, and it has been suggested that the uterotubal junction works selectively to hold back non-viable spermatozoa in mammals (Cohen, 1967; Cohen and McNaughton, 1974). There is evidence from work in rabbits that the spermatozoa are than stored in the isthmus of the oviduct. From there they are released to the fertilisation site after ovulation. Recently several workers have suggested that the follicular fluid plays a part in the release of sperm from the isthmus (Harper, 1973; Overstreet and Cooper, 1975).

Capacitation must take place prior to the sperm being capable of fertilising the ovum (Austin, 1951; Chang, 1951). Although details of the exact mechanism have not been elucidated, capacitation has been defined as a physiological change occurring in the sperm during residence in the uterus and oviducts. These changes are reflected in increased motility of the sperm and in their ability to release enzymes from the acrosome (Hunter, 1980). In several rodents and man the same changes can be produced by in vitro culture systems (Yanagimachi and Chang, 1963, 1964; Edwards, Steptoe and Purdy, 1970). It has been estimated that spermatozoa require a period of 5-6 hours in the bovine genital tract before they are capable of fertilisation (Trimberger, 1948).

To ensure fertilisation, mating, the transport of sperm and capacitation must be integrated with the time of ovulation.

Ovulation in the cow occurs approximately 24 hours after the onset of behavioural oestrus (Henricks, Dickey and Niswender, 1970; Schams, Schallenberger, Hoffman and Karg, 1977). Therefore following natural mating, sperm are already present in the oviduct when the egg is shed.

Many of the details of the endocrinological control mechanisms governing and initiating the morphological and behavioural changes around the time of ovulation have been elucidated.

Two to 3 days prior to the cow exhibiting oestrus plasma progesterone, produced by the corpus luteum, declines precipitously (Lemming, Hafs and Manns, 1975). This is due to the release of a luteolytic substance from the uterus causing regression of the corpus luteum. Prostaglandin $F_{2\alpha}$ has been shown to be the luteolytic agent in the ewe (Scaramuzzi and Baird, 1976) and is suggested as the luteolytic agent in the cow (Fairclough, Smith and McGowan, 1981). Thereafter circulating concentrations of progesterone remain basal, until the subsequent corpus luteum commences synthesis of progesterone (Hafs and Armstrong, 1968).

Against this background, follicular development culminating in the presence of a mature preovulatory follicle occurs. Although Rajakoski (1960) reports 2 waves of follicular growth during the oestrus cycle, other workers disagree. Donaldson and Hansel (1968), using endoscopy, failed to distinguish any patterns of follicular growth and furthermore stated that on day 16 of the oestrous cycle, the follicle destined to ovulate could not be identified. Associated with this follicular development, oestrogens are secreted and rise

in the peripheral blood (Glencross, Munro, Senior and Pope, 1973; Dobson and Dean, 1974) and reach peak concentrations closely preceding oestrus. The ability of oestrogens to induce the behavioural changes typifying prooestrus and oestrus has been demonstrated (Hassan and King, 1981).

The effects of gonadotrophins in stimulating the growth of follicles are well established (Hackett and Hafs, 1969; Hansel and Snook, 1970). Although a slight increase in luteinising hormone (LH) occurs during the preovulatory period, the characteristic of this hormone is its distinct preovulatory surge, associated with the peak in circulating oestrogens (Chenault, Thatcher, Kalra, Abrams and Wilcox, 1975; Lemon, Pelletier, Saumande and Signoret, 1975). Coincident with this is a peak in follicle stimulating hormone (FSH) (Dobson, 1978). Many of the details of the hypothalamic control of gonadotrophin release, by the action of materials from the hypothalamus on the pituitary, have been determined. LH has been shown to be the ovulatory hormone in the cow (Hansel and Snook, 1970), with the interval between the LH peak and ovulation approximately 24 hours (Chenault et al, 1975; Schams et al, 1977). However, the act of mating is known to cause ovulation to occur earlier, than in animals which are not mated (Marion, Smith, Wiley and Barrett, 1950).

The high levels of oestrogens found in late prooestrus have an effect on the tubular genital tract. Increased glandular secretion occurs in the cervix, with a change in the amount and nature of the mucus (Herrick, 1951; Glover, 1960; Linford, 1974). During prooestrus and at oestrus the cervical mucus becomes stringy and clear, and the change in morphological 'fern' patterns seen

on smears of oestrous mucus in women allows the follicular phase to be identified (Roland, 1952). Oestrogens also have an effect on the uterus. There is a marked increase in the vascularity and oedema of the endometrium (Hansel and Asdell, 1951; Marinov and Lovell, 1968; Hackett and Hafs, 1969). Associated with oestrus, an increase in the spontaneous contractility of the myometrium has been recorded. Oestrogens administered to ovariectomised cows have been shown to increase uterine motility (Hays and Van Demark, 1953a). The act of mating appears to supplement these increases, with a postulated role for oxytocin release (Hays and Van Demark, 1953b).

Ovulation occurs approximately 10 to 12 hours following the end of behavioural oestrus (Christenson, Echternkamp and Laster, 1975). The exact mechanism of ovulation is not yet known. In the last few hours before ovulation, the ovulatory follicle markedly increases in diameter, due to an increase in the accumulation of antral fluid. There is a reduction in follicular pressure. A 'weakening' of the theca externa is produced by an enzyme, thought to be secreted by the granulosa or theca interna cells. An aperture is thus produced and the follicular fluid slowly emerges from this point. The egg, when released, is surrounded by the cumulus cells, but already separation of the egg from the cumulus is beginning (Bedirian and Baker, 1975; Miller and Campbell, 1978; Hunter, 1980). Following ovulation, a blood clot forms inside the follicular cavity due to haemorrhage of the capillaries in the thecal wall. This structure is the corpus haemorrhagicum. Luteinisation of the follicular wall occurs. Further development and maturation of the luteal tissue depends on continuing low levels of LH. The corpus haemorrhagicum becomes yellow

in colour, and is called the corpus luteum. The corpus luteum becomes a secretory gland, producing progesterone which is released into the circulating blood (Hansel, 1966; Hafs and Armstrong, 1968; Hansel Concannon and Lukaszewska, 1973).

Around 4 to 5 days after oestrus, progesterone first increases significantly in the peripheral plasma (Lemming et al, 1975; Schams et al, 1977). The corpus luteum then reaches full functional status at approximately day 10 of the oestrous cycle. During this period a transient increase in plasma oestrogens has been reported (Glencross, Munro, Senior and Pope, 1973). The significance of this is not clear. Under the influence of progesterone, the animal is prevented from showing oestrous behaviour, and changes in the tubular genital tract occur (Hobson and Hansel, 1972). The endometrium enlarges and the uterine glands increase in depth and in their degree of coiling (Asdell, de Alba and Roberts, 1949; Hackett and Hafs, 1969). The motility of the uterus decreases as progesterone has a quiescent effect on the response of the uterus to oestrogens and oxytocin (Hays and Van Demark, 1953b). The composition of the cervical mucus alters, becoming thickened and reduced in volume (Boyd, Gibbons and Tasker, 1972).

Although follicular development can occur in the presence of elevated levels of progesterone, final follicular development is prevented due to the blocking effect of progesterone on the release of large amounts of pituitary LH (Hobson and Hansel, 1972).

To summarise, the establishment of a pregnancy requires a series of events which involve changes in centres situated throughout the female body. Not least of these areas are the oviducts.

1.2 - HISTORICAL REVIEW OF THE OVIDUCT

Although the presence of the uterus and oviducts was known to anatomists in antiquity, they were generally considered initially to be one organ. Later they were described as seminal ducts arising from the female testis (Soranus, 1894). The idea of the oviducts being ducts for the passage of female semen was accepted for many years. However in 1561, Gabriel Fallopius described the oviducts or 'passageways for the female semen' as 'slender and narrow seminal passages arises ..... from the uterine horn ..... becomes gradually broader and curls like the tendrils of a vine until near the end when the tendril-like curls spread out and it terminates in a very broad ending.' The author also described the fimbria of the oviduct as 'this ending is quite shredded and worn as if it were the fringe of a worn piece of cloth.' The oviducts were now known to be seperate entities from the uterus although still connected to it. However confusion still reigned as to their function. In 1621, Fabricius described, in the hen, the role of the oviduct in the production of the egg although his observations were not, at that time, universally accepted. In 1668, Johannis van Horne put forward the suggestion that the oviducts may be the means by which the ovum is carried to the uterus in the mammal. Four years later de Graaf described and defined the ovarian follicle (de Graaf, 1672). At this time there was still dissent over the role of the oviduct in the transportation of the ovum. However towards the end of the 17th century, Nuck carried out an experiment in which he ligated the uterine horn of a dog 3 days after coitus and 20 days later discovered 2 foetuses above the ligature (Nuck, 1691). He assumed that the 2 foetuses had come from

the 'fecundated ova' present in the ovary and they had therefore travelled down the length of the oviduct. By the 18th century most workers had accepted the theory that the oviduct was the means by which the ovum passed to the uterus, although there was some confusion as to how this occurred since the ovary was anatomically separate from the oviduct. At the end of the 18th century, Cruikshank recovered blastocysts from the rabbit oviduct 3 days post-coitus and observed that the number of blastocysts was equal to the number of corpora lutea present in the ovaries (Cruikshank, 1797). This study confirmed the theory that the passage of the ovum to the uterus occurred via the oviduct. Further studies established the motility and histological anatomy of the oviduct and confirmed that the oviduct was the site of fertilisation (Todd and Bowman, 1845-56; Bischoff, 1845; Pouchet, 1847). By the middle of the 19th century the general histological structure of the oviduct was known and its role in gamete transport, fertilisation and secretory function determined.

1.3 - GENERAL STRUCTURE OF THE BOVINE OVIDUCT

The anatomy of the bovine oviducts has been described by several workers (Roberts, 1956; McDonald, 1975; Sisson and Grossman, 1975; Hafez, 1980). The oviducts of the cow are paired tubes running between the ovary and the uterus and are derived from the muellarian ducts of the foetus. They are approximately 25-35 cm long and are tortuous and loosely coiled. Each oviduct runs along the free margin of the broad ligament of the uterus and is suspended by the mesosalpinx formed by the broad ligament looping around

the oviduct. The oviduct receives its blood supply from the ovarian artery, the uterine branch supplying the ampulla and isthmus and the ovarian branch supplying the infundibulum (Lamond and Drost, 1974).

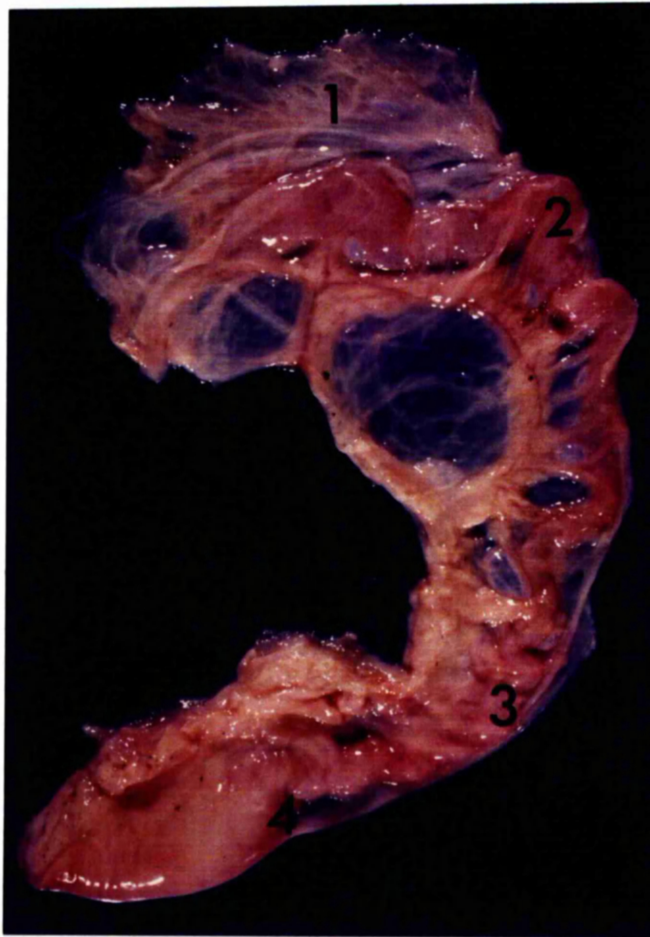
The oviducts are smallest in diameter at the uterine end and largest in diameter at the ovarian end. The wall of the oviduct may be divided into 3 parts; tunica serosa, tunica muscularis and the tunica mucosa. The serosal layer consists of mesothelium continuous with that of the peritoneum and is the outer layer of the oviduct. The tunica muscularis lies inside the serosal layer and its thickness varies as to the region of the oviduct. The muscular layer of the tube consists of 3 layers of smooth muscle; a thick middle layer of circular fibres with a thinner layer of longitudinal fibres on either side (Lombard, Morgan and McNutt, 1950). The tunica mucosa consists of a surface epithelial layer called the lamina epithelialis and a lamina propria. The epithelium is pseudostratified columnar throughout the oviduct and varies in height in different areas and at different stages of the oestrous cycle. The lamina propria is the base for the folding of the oviduct mucosa, which is most pronounced at the ovarian end. The lamina epithelialis consists essentially of 3 cell types, ciliated columnar cells, non-ciliated peg cells and spherical cells at the level of the basal membrane (Lombard et al, 1950). Motile cilia are present throughout the length of the oviduct in varying amounts.

Both functionally and anatomically the oviduct can be divided into 4 parts; the <u>infundibulum</u> (or <u>pre-ampulla</u>), the <u>ampulla</u>, the <u>isthmus</u> and the <u>utero-tubal junction</u> (Hunter, 1977) (Plate 1.1).

Plate 1.1 The oviduct of the cow showing:

1. The infundibulum; 2. The ampulla; 3. The isthmus;

4. The utero-tubal junction



The infundibulum is the ovarian extremity of the oviduct and has the most complex wall with respect to folding. The wall is very thin and consists almost entirely of ciliated cells with few muscular fibres present (Lombard et al, 1950; Shackelford, Dickey, Leland and Hill, 1970; Blankenship, Dickey, Halliday and Ellicott 1975). The infundibulum constitutes the fimbriated end of the oviduct and is wide and funnel shaped. In some species, the fimbria completely envelop the ovary (Westman, 1926; Blandau, 1969) but in the cow the fimbria are attached to the ovary at one point only. The mucosa of the infundibulum has very complex folding, secondary and tertiary folds being thrown backwards on themselves and often extending beyond the centre of the lumen (Lombard et al, 1950). In the infundibulum there is a preponderance of ciliated cells over secretory cells. The infundibulum is mainly concerned with the transport of eggs from the ovary into the ampulla. Due to the scant muscular tissue present in the infundibulum, it is thought that transport of the ovum is brought about by the action of the cilia of the mucosal cells (El-banna and Hafez, 1970a). The exact way in which the ova, at ovulation, are swept from the ovary into the oviduct is not yet clear. At oestrus the fimbriated end of the oviduct becomes oedematous and the epithelial cells markedly increase in height, with scattered epithelial cells protruding into the lumen (Lombard et al, 1950; Nayak and Ellington, 1972). The combination of this increase in thickness and motility at oestrus may cause the sweeping action necessary for transferring the ovulated ova from the surface of the ovary into the infundibulum (Ruckebusch and Bayard, 1975). The cilia of the rabbit oviduct have been shown by stroboscopic observations to be

beating at approximately 1200 times per minute and this is powerful enough to transport the ova into the ampulla (Borell, Nilsson and Westman, 1957). Although there are difficulties in the cow in determining the precise time of ovulation, it has been estimated that the ovum travels approximately one third of the length of the oviduct in 6 hours (Gerasimova, Potapova, Solovei and Hvatov, 1940). Therefore egg transport in the cow would appear to be very rapid. At oestrus the height of the non-ciliated epithelial cells of the infundibulum are at their maximum size, and the increase in height is thought to be due to the cell becoming filled with granules (McDaniel, Scalzi and Black, 1968; Nayak and Ellington, 1972). These granules have a secretory function which is maximum at oestrus since greater amounts of oviduct fluid may be collected at this time (Stanke, Sikes, Deyoung and Tumbleson, 1974; Roberts, Parker and Symonds, 1975). The ciliated cells also increase in height at oestrus (Lombard et al, 1950; McDaniel et al, 1968; Stalheim, Gallagher and Deyoe, 1975).

The ampulla of the cow is widest at its junction with the infundibulum and tapers down to approximately 1-2 mm in diameter at its junction with the isthmus (ampullary isthmic junction - AIJ). As in the infundibulum there is a preponderance of ciliated over non-ciliated epithelial cells, but the folding in the ampulla is far less complex than in the infundibulum. The muscular coat becomes increased in thickness towards the AIJ (Lombard et al, 1950; El-banna and Hafez, 1970b). At oestrus, the most pronounced increase in height of epithelial cells throughout the oviduct occurs in the

ampulla, although there are differing reports as to the length of time around oestrus when this occurs (Lombard et al, 1950; McDaniel et al, 1968). The granules contained in the non-ciliated cells also increase in size at this time, staining far more intensely with acid fuchsin (Lombard et al, 1950). Some workers have observed the cilia in the ampulla to be more motile around the oestrous period (Stalheim et al, 1975). Others have found them very motile at all times with no appreciable difference at oestrus (Lombard et al, 1950). The diameter of the lymphatic vessels supplying the ampulla doubles at oestrus, and this is thought to be connected with the obvious oedema which occurs around this part of the oviduct, and also with the increased amounts of oviduct fluid present at this time (Kayak, Ellington and Zimmerman, 1974; Roberts et al, 1975). Initially transport of the ovum through the ampulla is thought to be caused by the cilia beating towards the AIJ and forcing the ovum downwards (El-banna and Hafez, 1970a). Progress is then assisted by peristaltic contractions of the muscular coat of the ampulla (Harper, 1961; El-banna and Hafez, 1970a; Rukesbusch and Bayard, 1975). The ovum reaches the physiological barrier of the ampullary isthmic junction approximately 6-10 hours after ovulation (El-banna and Hafez, 1970a). Experiments using planimetric measurements of the oviduct have suggested that the AIJ is located $^{6}/10$th to $^{7}/10$th along the length of the oviduct from the infundibular end (El-banna and Hafez, 1970b). The ovum is held at the AIJ for a period reported as ranging from 72-120 hours (El-banna and Hafez, 1970a; Thibault, 1972; Zerobin and Sporri, 1972) and in the cow this is the site for fertilisation. The 'locking' mechanism at the AIJ appears to be a physiological, as opposed

to an anatomical, sphincter. Brundin (1964, 1965) demonstrated that this area of the rabbit oviduct has a large distribution of adrenergic innervation and this supports the theory of other workers that in the cow the sphincter effect is produced by a constriction of the circular muscles supplied by these nerves (El-banna and Hafez, 1970b). These same workers suggest that the narrowing of the oviduct lumen varies depending on the amount of circulating plasma oestrogens.

The <u>isthmus</u> is the narrowest region of the oviduct but has the thickest wall. In contrast to that found in the infundibulum and ampulla, the epithelium is predominantly non-ciliated cells and the muscular layer is far more developed. At oestrus the epithelium of the isthmus shows only a slight increase in height (Lombard et al, 1950; McDaniel et al, 1968). Transport of the ovum through the isthmus of the oviduct is accomplished by peristaltic and antiperistaltic contractions. This has been suggested to be due to the dual effect of oestrogens and progesterones acting on the adrenergic receptors present in the isthmic wall; oestrogens increase α-adrenergic activity producing a sphincter effect around ovulation, whereas progesterone acts on the β-adrenergic receptors producing isthmic relaxation as progesterone rises with increasing time after ovulation (Howe and Black, 1973; Hunter, 1980). Administration of exogenous oestrogens have been demonstrated to cause a 'tube locking' of eggs in the oviduct of the rabbit and conversely, acceleration of ovum transport into the uterus (Burdick and Pincus, 1935; Greenwald, 1961). Prostaglandins have also been implicated in the control of

ovum transport. Work in rabbits suggests that prostaglandin F may cause constriction of the isthmus, and thus retention of the ovum, while prostaglandin E have the opposite effect (Spilman and Harper, 1973; Spilman, 1976). The fertilised ovum in the cow enters the uterus 66-72 hours after ovulation. At this time, it has reached the 8 to 16 cell stage.

The utero-tubal junction (UTJ) is the region where the oviduct enters the uterus. In the cow, the UTJ enters the tip of the uterus and does not project into its lumen. The UTJ of the cow is not an anatomical sphincter. However both in the cow and ewe, there is a distinct flexure at the junction and it is thought that the degree of this flexure varies with the stage of the oestrous cycle (Edgar and Asdell, 1960). Hafez and Black (1969) have extensively reviewed the anatomy and functional significance of the UTJ in several species. They suggest that in the cow, the supporting ligaments of the UTJ have major significance in determining its mobility and flexure. A physiological barrier is formed at the junction at special times during the oestrous cycle; the barrier is greatest when eggs are present in the oviduct and non-functional during the rest of the cycle. During oestrus in the cow, the tip of the uterine horn curves more than 180°, producing a marked flexure at the UTJ. The UTJ has been suspected of being a barrier to sperm transport and of being able to select against foreign and dead material. The physiological changes in UTJ structure apparently do not prevent sperm transport, since transport may occur at all stages of the oestrous cycle in the ewe and rat. In addition, rapid sperm transport may depend on the increased

motility of the reproductive tract produced at mating. However the role of the UTJ in sperm transport, sperm selection and control of the time the zygote enters the uterus has not been fully clarified.

1.4 - REVIEW OF METHODS OF ASSESSING OVIDUCT PATENCY

The most commonly used method of assessing the structure of the bovine genital tract in the living animal is rectal palpation. This is of value in determining the normality of the ovaries and uterus but is limited in diagnosing abnormalities of the oviduct and the non-pregnant uterus. Although gross lesions of the oviducts and their adnexae may be picked up on rectal examination, any lesser abnormalities are liable not to be detected (Hancock, 1962; Dawson, 1975).

Determination of oviduct patency was first described in 1914 when separate papers by Rubin and Cary were published. These described the test in women known as hysterosalpingography. This technique involves the injection of radioopaque material into the uterus and monitoring its progress by radiography. It provides information about both the patency of the oviducts and the peritubal adnexa where adhesions are present. Accumulations of media may be seen on follow-up films. This test is widely used in investigation of infertile women. However it is impractical in the cow. Improvement of this test was made in 1919, by the addition of a pelvic pneumogram which provided more information on the ability of the oviduct fimbriae to envelop the ovary (Stein, 1919).

In 1920, Rubin published a paper on the 'Non-operative determination of patency of fallopian tubes by means of intra-uterine

inflation with oxygen and the creation of an artificial pneumoperitoneum.' The principle behind this technique was that oxygen, injected under pressure via an airtight cervix into the uterus, could only escape into the abdominal cavity by means of patent oviducts. Determination of patency depended on the recording of a drop in pressure on a device attached to the gas flow. Other methods of detecting the escape of gas were by auscultation of the gas at the fimbria or by the shoulder pain experienced by the patient after the procedure due to the collection of gas under the diaphragm. More recently carbon dioxide has been utilised instead of oxygen as it is more readily absorbed by the peritoneum (Sweeney, 1962). However pitfalls have been reported in the use of this test, for example, the recording of false positives or negatives, risk of infection and risk of emboli formation (Sweeney and Gepfert, 1965).

The Rubin test has been used in cattle. Rowson (1942a) reported an attempt to assess oviduct patency using air insufflation on the genital tracts of cows after slaughter. Freshly removed uteri were placed in water and inflated using a catheter placed through the cervix, which was sealed by a rubber bung. Bubbles appearing at the ovarian end of the oviduct indicated patency. However, in 25% of the tracts endometrial rupture occurred before any bubbles appeared and Rowson concluded that the test would be dangerous if used in the living animal.

In 1945, Spriggs experimented with air insufflation in animals immediately prior to slaughter. He injected approximately 600 cc of air into the uterus of 5 animals using an enema pump. The

animals were slaughtered within 3 hours and the genital tracts examined. In 4 of the 5 cows, rupture of areas 0.5-1 cm long were present in the endometrium of the lesser curvature of the uterus and air had escaped into the broad ligament. In no case did the rupture include the muscular coat and could have been missed on cursory examination.

Hanley (1953) stated that the Rubin test is suitable for use in cattle provided suitable modifications are used. He used a modified catheter with a balloon, which sealed the internal os of the cervix. Carbon dioxide was injected into the uterus, and kymograph tracings used to determine the drop in pressure within the uterus. He reported that the test required to be divided into stages and that the gas had to be introduced gradually. He believed the 25% of cases where rupture of the endometrium occurred in the study of Rowson was due to the rate of gas inflow being too high. He considered that 80 mm Hg was the maximum pressure to be used with safety in cattle and that the test was useful. On the other hand, Kothari (1977) injected air into 15 freshly removed bovine genital tracts. Several showed endometrial rupture at a wide variation in pressure (96-180 mm Hg) and he concluded that this test was not suitable for use in the living animal, since the endometrium of the cow is too friable to permit any build up of gas in its lumen prior to the gas entering the oviduct.

In the human field, other tests used for determination of oviduct patency are the methylene blue test (Ansari, 1968) and the tubal scan (Pertynski, Jakubowski, Stelmachow, Graban and Zurowski, 1977). Neither is practical for use in the cow.

The basis for all these tests carried out in both humans

and cattle is that a substance injected into the uterus passes upwards through the patent oviducts and is detected in the peritoneal cavity. In several species, attempts have also been made to assess tubal patency from the other direction, that is, by injecting materials, ranging from Indian ink and ascaris eggs to starch grains, around the ovary (Gyarmati, 1934; Decker and Decker, 1954; Ansari, 1979). This material, harvested by the fimbria is detected in the vagina, following its passage downwards. The 'starch grain' test, described by Decker and Decker, was used in humans but not widely, due to the patient's dislike of the procedure. The detection of the starch grains in the vagina was by using the properties of starch in staining dark blue when treated with iodine. McDonald (1954) used the technique in cattle with favourable results, but since then others have reported difficulties in demonstrating the presence of starch grains in the vagina (Dykstra, 1955; Murray, 1959; Johari and Sharma, 1964). More recently, Kessy and Noakes (1979) reported the test as effective in detecting unilateral and bilateral oviduct occlusion in 3 cows in which surgical ligation of 1 or both oviducts had been carried out.

Another method of determining oviduct patency which has been the subject of much discussion in the human field is the use of the 'Speck' test (Speck, 1948). This involves the use of phenolsulphon-phthalein(PSP) dye as the monitor of patency. The dye is injected into the uterus via the cervix, following emptying of the bladder, and 30 minutes later the bladder is catheterised. On alkalinisation of the urine collected, the presence of a pink/red discolouration

demonstrates that the dye is present. PSP dye is not readily absorbed from normal vaginal mucosa, endometrium or endosalpinx, but readily absorbed from the peritoneum. Thus, it may be concluded that the dye has reached the peritoneal cavity via the patent oviducts. Speck reported the test as accurate in 16 cases and strongly recommended it as safe and easy to use.

However, no one since then has been able to match Speck's results. Rosset in 1950 carried out the test on patients prior to their undergoing hysterectomy, and found a lack of correlation between the results of the test and the conditions of oviducts after surgery. He believed that a blocked, but dilated and diseased, oviduct may have the ability to absorb the dye.

Further investigations were carried out by Hoffman in 1951. He demonstrated that the dye was indeed rapidly absorbed through the peritoneum. He used a more concentrated solution of dye than Speck and in 33 cases of known tubal patency, he had 32 positive dye tests. However, he next tested 41 patients known to have blocked oviducts following sterilisation procedures and in every case a positive result was obtained, suggesting oviduct patency. He concluded that the test was unreliable and that the dye is absorbed by the human endometrium. In 1958 Speck and Halter repeated these experiments, again using women who had previously undergone tubal ligation and obtained contradictory results. They hypothesised that the inaccuracies following tubal ligation are due to leakage of dye, or to inversion of the mucosa at the ligation site, allowing absorption of the dye.

However, in 1965, Gromadzki, Lukasik, and Papierowski compared the PSP dye test with the Rubin test and found it the better method of evaluating patency of

the oviduct. Due perhaps to these contradictory results, and/or the availability of other methods, the Speck test has never enjoyed great popularity in the human field.

A test, using indigo carmine dye, was reported by Otel and Drume (1968) to have a use in cattle. The technique was basically the same as the Speck test in women, with the urine being examined for the presence of dye 2 hours following its instillation. They had the advantage of being able to examine the tracts at post-mortem and confirmed all 23 cases where the test had indicated oviduct patency, and 8 out of 10 where oviduct blockage had been demonstrated.

A further experiment was done by Berchtold and Brunner in 1966, using PSP dye, in 21 animals. They were able to confirm their results in 20. There was no evidence of damage to the endometrium of the cows following either of these experiments and it was concluded that the dye test appeared a safer, but as reliable, technique as the use of carbon dioxide by Hanley in 1953. However inaccurate results have been found by some workers (Johari and Sharma, 1964; Ozhurova and Ushev, 1974). A high incidence of false positive reactions was obtained by Von Schneider and Rusch (1976) which they suggested could be due to absorption of the dye by a damaged endometrium.

Kothari (1977) carried out the test in a number of animals with very accurate results confirmed by post-mortem. He reported that the dye test could be used with accuracy at any stage of the oestrous cycle and demonstrated by endoscopy that the dye did pass from the oviducts into the peritoneum. He suggested that unilateral blockage of the oviducts would be diagnosed using this technique, and showed that normal fertility resulted following a succession

of dye tests in a number of animals. Other workers disagree that the stage of the cycle at which the dye test is performed makes no difference (Kessy and Noakes, 1979). They obtained a high number of false negative results when the test was carried out on cows in oestrus. They suggested that this may be due to the 'tubal locking' of the oviduct at this time. However they obtained accurate results when the test was carried out at other times of the oestrous cycle and concluded that the test had given encouraging results in the determination of patency or occlusion.

1.5 - REPEAT BREEDER

One of the more common syndromes associated with reduced fertility amongst cattle in Britain is the 'repeat breeder'. The repeat breeder is a cow which cycles at regular intervals but when mated with fertile sires fails to conceive. On clinical examination, they show normal ovarian function by the presence of a palpable corpus luteum at the recognised normal period of the cycle. They also show no palpable evidence of adhesions between ovary and oviduct, or between oviduct and uterus. Uterine environment is apparently normal. On rectal palpation and when cervical examination is carried out, there is no evidence of abnormal uterine discharges. Obviously any one of the integrated events which culminate in pregnancy could be the cause of the reduced fertility in this group of cattle. According to some workers, conception rate to first service in repeat breeders is only 48.5% (Olds, 1969) compared to a conception rate to first service of 85.8% in heifers (Kidder, Black, Wiltbank, Ulberg and Casida, 1954). There are several reports in the literature of relatively

high incidences of oviduct abnormalities amongst repeat breeders. Most of this evidence has been gathered from post-mortem examinations (Tanabe and Casida, 1949; Lombard et al, 1951; Dawson, 1958; Hoare, 1969; Summers, Campbell and Dennett, 1974; Bowen, Elsden and Seidel (1978).

Many of the cases referred to the hospital environment are repeat breeder cows, whose value encourages their owners to persist in trying to reach a diagnosis for their reduced fertility, with the hope that certain manipulations can be undertaken and a conception achieved. During the period of the studies referred to in this thesis, approximately 40 repeat breeders were admitted to the department for investigation. In certain areas of the work described, results from these cases will be included which add additional useful material for discussion.

The first part of this thesis describes in vitro and in vivo studies undertaken on patency of the oviducts in normal and abnormal cows and heifers.

CHAPTER 2

OVIDUCT ABNORMALITIES IN AN ABATTOIR SURVEY OF BOVINE REPRODUCTIVE TRACTS

2.1 - INTRODUCTION

Besides simply observing the gross structure of the oviducts, both the passage of fluid and/or air have been tried in attempts to assess their patency in vitro (Dawson, 1958, 1964a; Kothari, 1977; Duchateau and Whitmore, 1978). Both of these techniques, while indicating patency of the oviducts to fluid, do not take into consideration the necessary degree of patency required of the oviduct in each species in its role of transport of the ovum. There is little reference in the literature to work done in assessing the patency to fluid, and to particles of a size similar to that of the bovine ovum (Dawson, 1958, 1964a). Studies were therefore undertaken, using slaughterhouse samples of female bovine genital tracts, to investigate the ability of the oviducts to allow the passage of both fluid, and a suspension of pollen grains similar in size to the ovum. In addition, the types of abnormality both macroscopic and microscopic which impeded the passage of these materials was assessed. Since the oviducts are recognised as varying in structure throughout their length, attention was paid to the particular site of the various abnormalities.

2.2. - MATERIALS AND METHODS

A random sample of non-pregnant cow and heifer genital tracts were collected immediately after slaughter from a local abattoir. Tracts were removed as soon as the carcase was suspended and cut open after skinning. An incision was made as close as possible to the attachment of the broad ligament to the abdominal wall

and the vagina cut posterior to the cervix. Tracts were obtained from animals of various breeds and age. All tracts were examined within 2 hours of slaughter. Careful scrutiny of the entire reproductive tract was carried out and any gross abnormalities affecting the oviducts recorded. The structures present on the ovaries were noted.

Separation into cow and heifer tracts:

The genital tracts were divided into those from cows and those from heifers, a heifer being taken as an animal which had never borne a calf. The separation was done by one of, or a combination of, the following 3 methods: 1) The mammary development of the carcase was examined prior to removal of the tract. Those with obvious mammary development were considered to be cows. 2) Both ovaries were examined for the presence of corpora albicantes. In those considered to be from heifer carcases, no corpora albicantes were present on either ovary. 3) By the use of radiography, the tortuosity of the arteries supplying the uterus was determined as follows: A 1:1 water barium mixture, warmed to reduce viscosity, was drawn into a 20 ml syringe and a 20 gauge 1 inch needle attached. Two or 3 prominent uterine arteries, lying in the uterine broad ligament, were injected with approximately 10 ml of the mixture. Blood vessels supplying both horns were injected. The genital tract was then X-rayed at 44 kv, 10 mAS, using a grid height of 36 inches. After developing, the uterine blood vessels were examined for coiling.

Determination of oviduct patency using dye:

Within 4 hours of slaughter, the genital tracts were cleaned of debris using water, and unwanted material such as fragments of rectum and excess fat dissected away.

PSP dye solution (prepared by the method of Kothari et al, 1978) or methylene blue (BDH 1% aqueous solution) was used.

The genital tract was placed on a white enamel tray. The dye was drawn into a 20 ml syringe to which a 20 gauge 1 inch needle was attached. One horn of the uterus was clamped off approximately 3 cm from the uterotubal junction (UTJ) with care being taken not to damage the oviducts. This was accomplished by one of 3 methods: 1) A ligature was inserted through the broad ligament and tied around the uterine horn, or 2) Artery forceps were placed across the uterine horn, or 3) By applying pressure with the finger and thumbs across the uterine horn, below the point of insertion of the dye.

The needle, with the syringe attached, was then inserted through the uterine wall, between the constriction and the UTJ, with its point directed towards the junction. Care was taken to ensure that the point of the needle remained in the lumen and did not pierce the opposite uterine wall. The syringe plunger was gently pushed downwards and the dye instilled. In cases where the oviduct was patent, the dye could be seen moving through the oviduct and emerging at the fimbrial end. Where initially the dye was not seen to be moving, the pressure exerted on the syringe plunger was increased, until the resistance was overcome. Where increased pressure did not overcome the resistance, the injection of dye was halted. The procedure was

repeated on the opposite oviduct.

Determination of oviduct patency using pollen:

A proportion of the tracts in which one or both oviducts had proved to be patent to the dye injection was used in this experiment. To avoid the original puncture wound, a second constriction was placed nearer the UTJ. Air was flushed through the oviduct to remove the dye previously instilled.

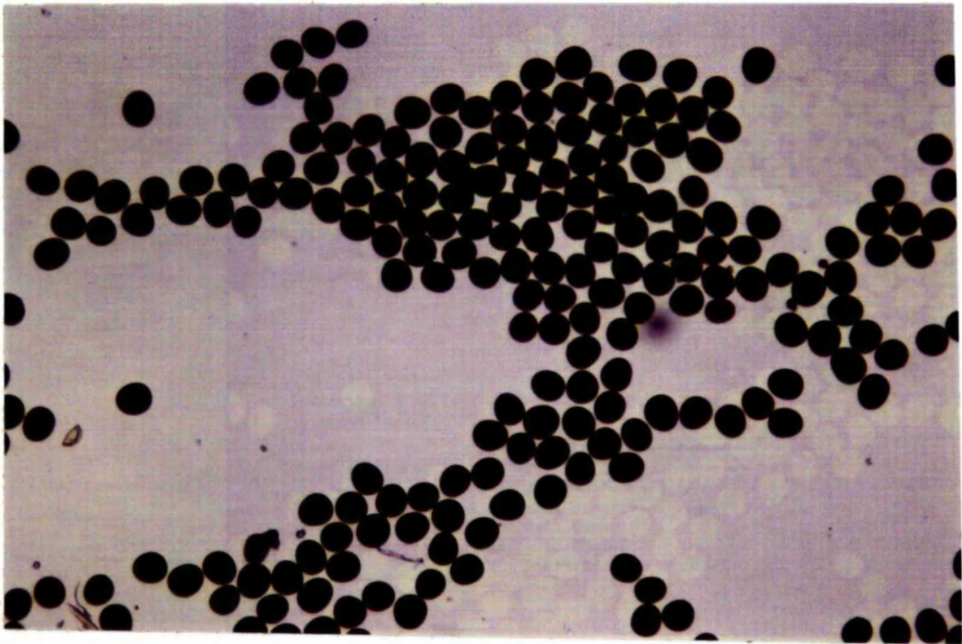
A suspension of pollen grains (Coulter Electronics, Harpenden), of 80 μ in diameter, was prepared. (6 mg pollen grains:50 ml saline). The suspension was shaken frequently in order to disperse the pollen grains uniformly. The suspension was drawn into a 20 ml syringe and the concentration of grains present checked by examining a drop of the fluid from the filled syringe in an embryo dish under a stereo-dissecting microscope. (Plate 2.1).

The suspension was injected from the uterine tip to the oviduct in exactly the same manner as described for the dye. As the flushings emerged from the oviduct, they were collected in an embryo dish and examined under the stereomicroscope for the presence of the pollen grains. In those samples in which no pollen grains were visible in the collected fluid, the procedure was repeated.

Passage of dye from the fimbria to the uterus:

In a proportion of the original samples, and in all cases where flow of dye had been impeded from the uterine to the fimbrial end of the oviduct, the patency to dye in the opposite direction was attempted.

Plate 2.1 An example of a pollen grain suspension as seen under the stereodissecting microscope.



The oviduct, ovary and tip of the uterine horn were dissected free of the body of the uterus by cutting the mesovarian ligaments. A blunted needle (20 gauge 1 inch) was inserted into the fimbrial end of the oviduct and secured there by a ligature. Dye was injected through the needle as before.

Passage of dye from the isthmus to the fimbria:

The UTJ was severed, leaving the lumen of the isthmus visible. A blunted needle (20 gauge 1 inch) was inserted into the isthmic lumen and secured by a ligature. An attempt was made to inject dye towards the fimbria, so bypassing the UTJ. The procedure was repeated on the opposite oviduct.

Examination of repeat breeder cow genital tracts:

A sample of 10 genital tracts from cows with a known history of infertility were available. These were examined grossly and the oviducts tested for patency using dye and pollen grains, exactly as described for the slaughterhouse samples.

Histology:

Sections of approximately 1 cm square were taken from the ampulla, isthmus and UTJ from samples where the dye or pollen had been impeded, and from apparently normal oviducts for comparison.

The sections were fixed in Bouin's solution for approximately 24 hours. They were then dehydrated in a series of upgraded alcohols and impregnated with 1% colloidon in methyl benzoate. Clearing was done using amyl acetate followed by impregnation with paraffin wax. They were blocked out in paraffin wax. This procedure was carried out

using an automatic tissue processing machine (Shandon Elliot).

Sections were cut at 5 μ thickness, placed on to a slide and stained with Mayers haematoxylin and eosin. They were dried on a hot plate and placed in an oven at 56°C overnight, for examination the following day.

A proportion of samples was serially sectioned at 5 μ intervals and every 10th section stained as described above.

2.3 - RESULTS

Plate 2.2 shows the radiograph of the uterus and broad ligament from a heifer tract following the injection of barium into the uterine arteries. There is no coiling of the blood vessels. Plate 2.3 shows the results in a cow tract for comparison.

The number and incidence of gross abnormalities of the oviducts of 260 genital tracts are shown in Table 2.1. The adhesions recorded ranged from delicate strands of connective tissue lying alongside the oviduct and bursa to thick fibrous tissue completely enveloping the ovary and fimbria (Plate 2.4). Hydrosalpinx was apparent as a fluid filled distension of the oviduct with thinning of the wall (Plate 3.2.3).The cysts were white, thin or thick walled structures ranging in size from 0.5 - 2 cm and invariably contained white granular material. They were found lying in the fimbriae or near the wall of the oviduct (Plate 2.5).

The incidence of oviduct lesions present in cows (18%) is greater than that present in heifers (3.3%).

Table 2.2 shows the results obtained in A - heifers and B - cows when in vitro assessment of oviduct patency was done using

Plate 2.2 Radiograph of the uterus and broad ligament of a heifer tract, following the injection of barium into the uterine arteries. The arteries are straight.

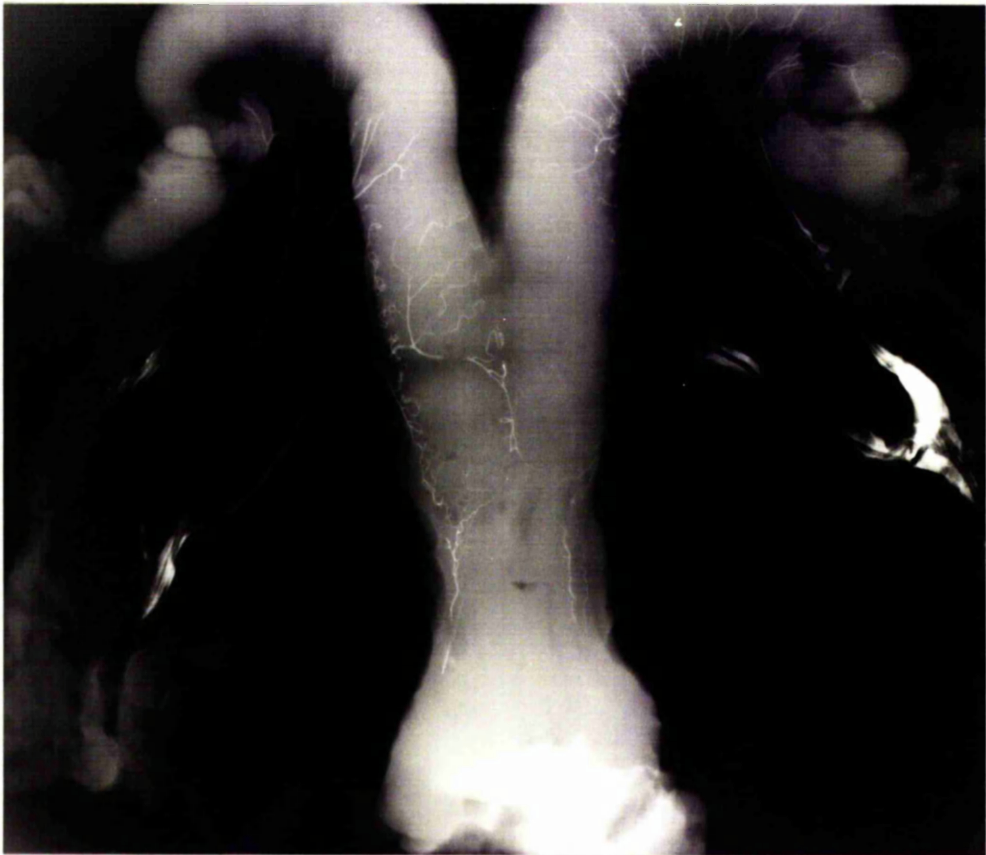


Plate 2.3 Radiograph of the uterus and broad ligament of a cow tract, following the injection of barium into the uterine arteries. The arteries are coiled.

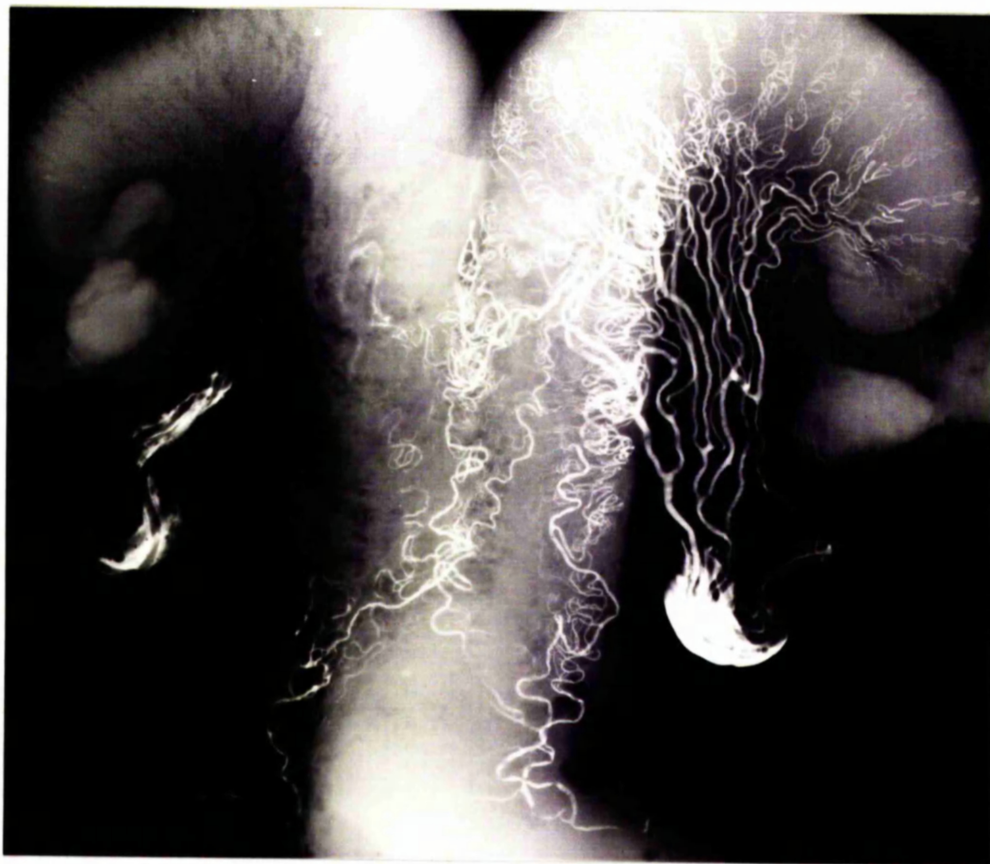


TABLE 2.1 INCIDENCE OF GROSS MACROSCOPICAL ABNORMALITIES AFFECTING THE OVIDUCTS OF 260 NON-PREGNANT BOVINE GENITAL TRACTS COLLECTED AT RANDOM AFTER SLAUGHTER

	Number of Tracts Examined	Number (%) Abnormalities					
		Adhesions		Hydrosalpinx		Cysts	
Cows	200	23	(11.5)	2	(1)	11	(5.5)
Heifers	60	0	(0)	0	(0)	2	(3.3)
Total	260	23	(8.8)	2	(0.8)	13	(5)

Plate 2.4 Bovine oviduct and ovary, showing gross adhesions enveloping the ovary and fimbria

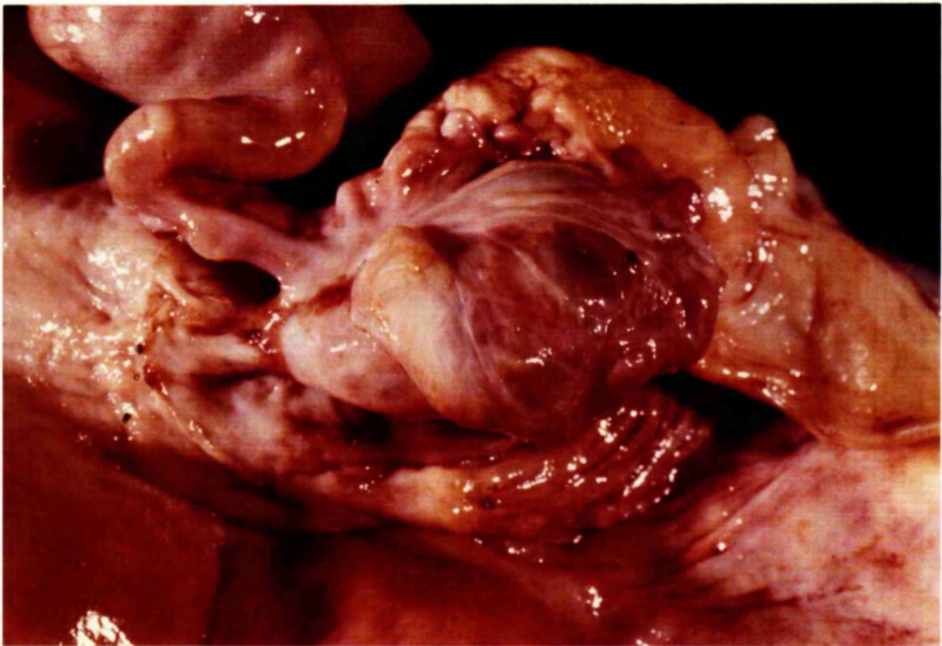


Plate 2 5 An example of an oviduct cyst, lying in the wall of the fimbria

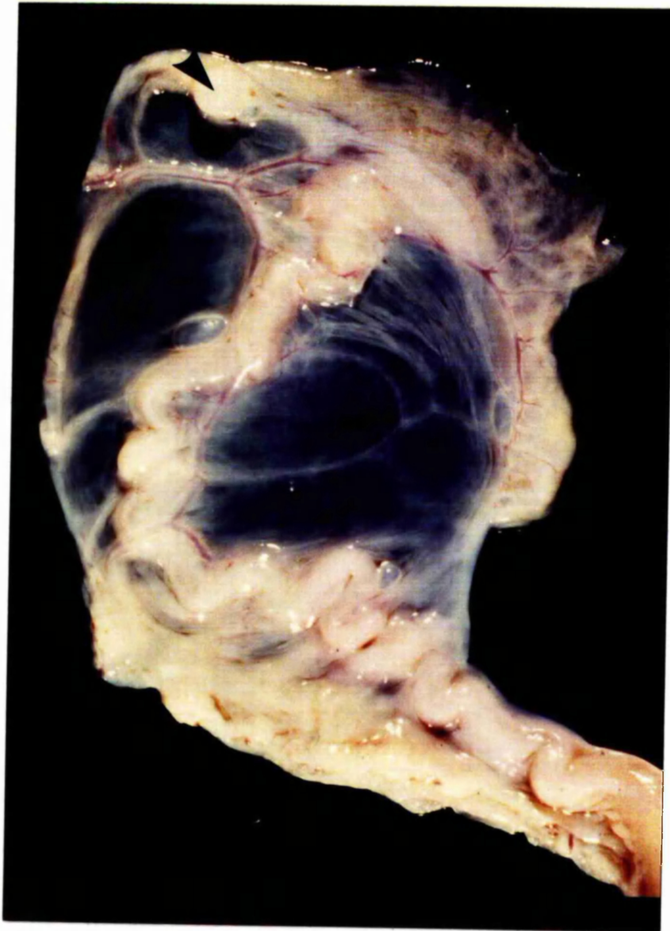
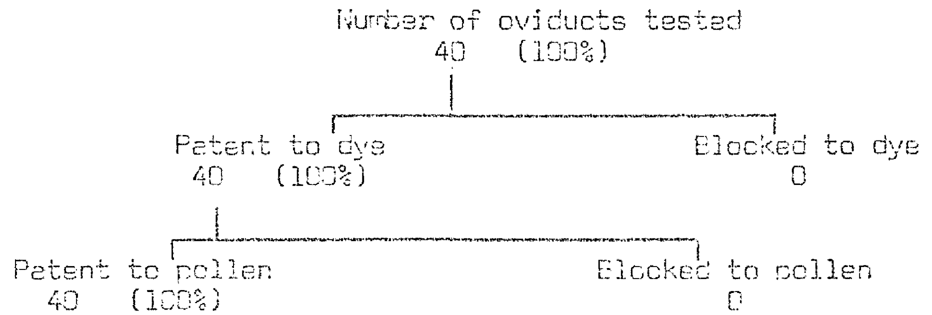
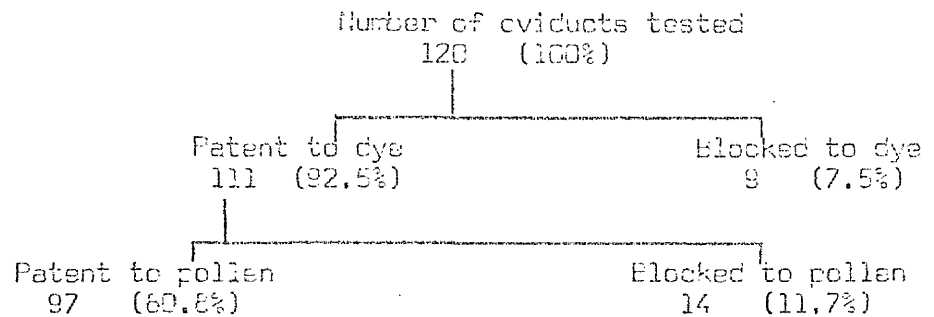


TABLE 2.2 RESULTS OF IN VITRO ASSESSMENT OF OVIDUCT PATENCY USING DYE, FOLLOWED BY A SUSPENSION OF POLLEN GRAINS, IN 60 COW TRACTS AND 20 HEIFER TRACTS

A. heifers

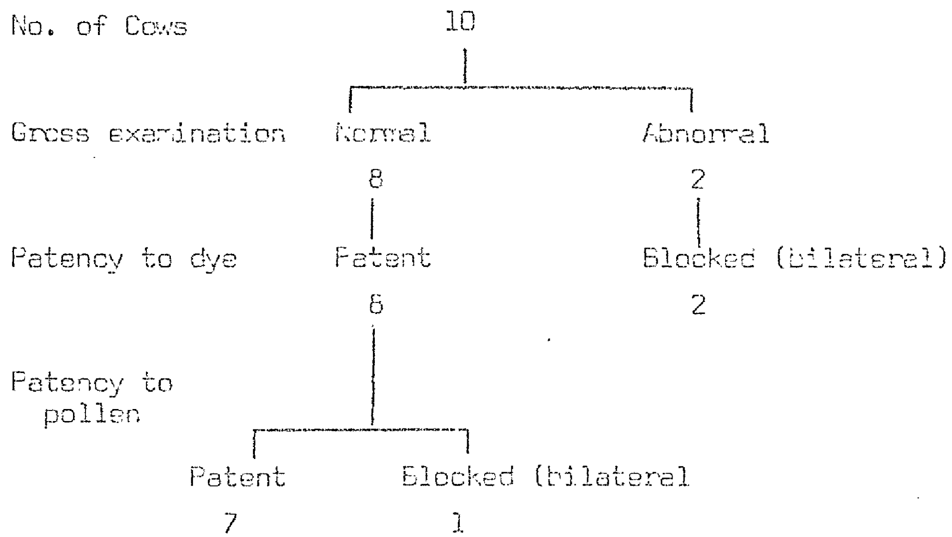


B. Cows



Note: The dye and pollen grain suspension were injected from the uterus to the fimbria in all samples.

TABLE 2.3 RESULTS OF ASSESSMENT OF OVIDUCT PATENCY USING DYE, AND A SUSPENSION OF POLLEN GRAINS IN A RANDOM SAMPLE OF 10 GENITAL TRACTS FROM REPEAT BREEDER COWS



dye followed by a suspension of pollen grains in 80 genital tracts. All heifer oviducts tested proved to be patent to the dye and to the pollen grains. 7.5% of the cow oviducts were blocked to the dye. It is interesting to note that 11.7% of the cow oviducts which had proved patent to the dye injection were blocked when tested with the pollen grain suspension.

Passage of dye from the fimbria to the uterus, and from the isthmus to the fimbriae was attempted but proved impossible to carry out in the majority of the oviducts tested. No results are shown.

The incidence of gross abnormalities affecting the oviducts in a random sample of 10 tracts from repeat breeder cows are shown in Table 2.3. Two tracts which were grossly abnormal were bilaterally blocked to the dye injection. One case which appeared normal on macroscopic examination proved to be bilaterally blocked when tested with the pollen grain suspension. In this small sample of repeat breeder cows, 30% have apparent oviduct abnormalities.

2.4 - DISCUSSION

There are several reports in the literature of the incidence of oviduct abnormalities affecting bovine female reproductive tracts Moberg, 1954; Dawson, 1956; Summers, 1974; Aldahash and David, 1977; Kothari, 1977; Duchateau and Whitmore, 1978). Much of the evidence is based on the examination of the reproductive tracts at slaughter. These authors report an incidence of oviduct abnormalities varying from 2.5% to 26%. From the slaughterhouse survey reported in this thesis the incidence of gross abnormalities was 18% in cows and 3.3% in heifers. Several different explanations for this wide range in

results recorded are possible. Some workers may have reported all types of abnormalities, with others noting only those which they considered would have an effect on the fertility of the animal. An example of this in studies reported here is that a common type of abnormality found on gross examination of the tract was the presence of paratubal cysts. Paratubal cysts are also frequently found in the human female. In the human, they are considered generally to be remnants of the mesonephric tubules or portions of the Muellarian ducts (Novak and Rubin, 1952; Monroe and Spector, 1962). Most workers consider them harmless. However, in 1964b, Dawson recorded that in a slaughterhouse survey of 518 cattle, 460 of which had been slaughtered for failure to breed, 12 had cystic structures surrounding the oviducts. In his cases, these cysts often involved the whole length of the oviduct and in his opinion were interfering with conception. However, as well as finding cysts on the oviducts, Dawson also found cysts present in both the ovaries and the endometrium of these same cases and he suggested that the aetiology of the cysts in this condition was endocrinological. The cysts in the tracts examined here were most commonly found at the fimbriated end of the oviduct and in the majority of cases did not infringe on the oviduct lumen as demonstrated by the passage of fluid, and fluid plus particles. There was only one exception where the cystic structure did surround the oviduct causing an obstruction and did not in this case allow fluid to be passed along the oviduct. In the different reports their appearance may or may not have been reported and so result in wide differences in recorded abnormalities in the different surveys.

Another aspect for consideration when viewing the wide range

of results reported is the time when the surveys were carried out. It appears that the earlier surveys, in general, report a higher incidence of abnormalities (Moberg, 1954; Dawson, 1958). Many changes in methods of treatment of problems associated with the reproductive tract have occurred during the past few years. For example, in the days before a pharmacological luteolytic agent was available the method most commonly used to shorten the inter-oestrous period was that of manual enucleation of the corpus luteum (Moberg, 1954; Roberts, 1957; Dawson, 1961). This technique is liable to lead to the formation of adhesions which may range in severity from several fine strands lying between the ovary and the bursa, to a mass of fibrous tissue completely surrounding ovary, bursa, oviduct and uterus (Moberg, 1954; Roberts, 1957). Such adhesions almost certainly have an effect on oviduct function. Nowadays manual enucleation of the corpus luteum is unnecessary as injectable luteolytic agents are available. This could explain the marked difference in the incidence of abnormalities reported in the literature between the times when enucleation was being used frequently and nowadays when it is hardly ever undertaken.

Another method of treatment which is no longer commonly practised is that of washing out the uterus with a solution of Lugol's iodine (Benesch and Wright, 1950). Workers have suggested that this treatment, if carried out on an animal where uterine infection is present, may have the effect of flushing the infective material from the uterus into the oviducts (Moberg, 1954; Kothari, 1977). However, one of these authors (Kothari, 1977) suggested that

in animals where no infection was present, it may have had a beneficial effect in that the flushing of the genital tract with fluid may have removed secretory material blocking the oviduct lumen or actually broken down adhesions present at the fimbria. It is difficult to say with any certainty whether the flushing of the uterus with Lugol's solution could result in either of the 2 conditions. However, when flushing the oviducts in the post-mortem specimens with dye, on a few occasions increased pressure was required to bring about the passage of the dye along the oviducts. This resulted in the separation of adhering fimbria and an immediate reduction in the pressure required. Such findings tend to suggest that where adhesions are the problem, carrying out irrigation of the uterus could be beneficial. If one considers the flushing technique as being the cause of infection gaining entry into the oviducts, then this could be another explanation why in the earlier years, when uterine irrigation was routinely carried out, there were apparently greater numbers of oviduct abnormalities found in cows.

Another form of infection which has been associated with abnormalities of the oviduct is tuberculosis. Tuberculosis was known to cause salpingitis in cattle (Rowson, 1942a, b; Moberg, 1954; Roberts, 1956). Tuberculosis has now been eradicated from almost all the cattle population in Britain. During the time it was relatively common in cattle it obviously resulted in increased recorded numbers of tracts with abnormalities of the oviducts and so is another reason for a variation with time of recording of the numbers of abnormalities found.

The incidence of oviduct adhesions in tracts from cows examined in this series was relatively high (11.5%) and of those with hydrosalpingitis, low (1%). These figures are lower than those reported by Kothari (1977) and greater than those of Aldahash and David (1977). The popularity of herd fertility control programmes in cattle with the need for more frequent palpation of the reproductive tract should perhaps be considered as a cause of abnormalities seen in the slaughterhouse surveys carried out during the past 7-10 years. Rough handling of the ovary at rectal palpation, particularly shortly after oestrus when a corpus haemorrhagicum is present in the ovary, may cause bleeding and subsequent adhesions (Coulthard, 1961). Where this was the case only 1 ovary would be affected as the lesions are produced by trauma as opposed to infection.

A method of determining whether the oviducts are patent in specimens after slaughter is of importance in proving whether the lesions present were likely to cause infertility. In the studies carried out, dye was injected from the uterine end of the oviduct to the fimbria. Attempts were made to inject the dye in the opposite direction, that is from the fimbria to the uterus. However, this technique was abandoned since great difficulty was encountered in all specimens tested. It has been suggested that this is due to the anatomy of the oviduct in that it is difficult to force a liquid from a broad tube (ampulla) into a very narrow one (isthmus) (Kothari, 1977). Also a valve effect may be produced by the angle adopted by the mucosal lining of the oviduct.

The results obtained from the dye infusion experiment

indicated that a relatively small number of the cow oviducts (7.5%) were totally occluded. Of these oviducts, only 4 had macroscopic lesions thought sufficient to affect fertility. The remainder appeared macroscopically normal. An attempt was made to determine histologically the site of occlusion in those normal-looking tracts. This proved unsuccessful. In 4 cases the dye appeared to have been stopped at some point along the isthmus, that is the narrow muscular area of the oviduct and it may be that the site of blockage was so small as to remain undetected unless the entire length of the isthmus was examined histologically. In the remaining oviduct, the dye was stopped at the fimbriated end by adhesions which had caused the mucosal layers of the infundibulum to stick together. This had not been seen on visual inspection. Just why this type of abnormality occurs is difficult to determine.

However, patency to fluid alone does not constitute normal function of the oviduct in the living animal. Patency such as to allow passage of the ovum is necessary. A suspension of pollen grains, similar in size to the ovum of the cow, were used to identify the oviducts unable to perform this requirement. In the studies described in this thesis, a number of cow oviducts which had proved patent when tested using an injection of dye were subsequently found to be abnormal to the extent that pollen grains of 80 μ in diameter were unable to pass freely from 1 end of the oviduct to the other. Since all heifer tracts permitted the passage of pollen grains, this inferred that an acquired lesion was present in these cow tracts, which prevented the movement of particles while allowing the passage of fluid alone. Two possible types of lesion could be considered as

causes for this occurrence. One is that strands of connective tissue are present in such an oviduct producing a meshwork in which the pollen grains are trapped but through which the dye solution can pass easily. It seems reasonable to suggest that this type of septal adhesion could be caused by rough handling of the oviduct during rectal palpation, producing bleeding into the oviduct lumen. On the other hand, a partial blockage could be produced by trapping of debris within the oviducts. A previous worker who used the injection of fluid to determine oviduct patency in vitro reported a slightly higher incidence compared to the results reported here and further suggested that, from histological studies carried out, even more oviducts were abnormally narrow such that the ovum would be unable to pass (Dawson, 1958).

Criticisms can be made of the use of slaughterhouse samples as a means of obtaining information regarding oviduct abnormalities. The oviduct, at post-mortem, is a very different structure compared to the organ in the living animal. In several of the slaughterhouse specimens, the UTJ was markedly flexed and appeared to be a barrier to the passage of the fluid. In several cases an increase in pressure was required to force the dye past. This may or may not be significant in the living animal.

Furthermore, all slaughterhouse surveys have the disadvantage that, as many dairy cows are culled for infertility, the incidence of abnormalities present in the abattoir may be higher than those found in the average dairy herd. However, the results reported here from examination of genital tracts of 10 cases with a known history of infertility indicate that oviduct lesions do play an important part in

the aetiology of infertility in repeat breeder cows, since a 30% incidence of oviduct disease of varying severity was found.

To summarise, these studies of slaughterhouse specimens have shown that the incidence of oviduct disease in the abattoir and in a small sample of repeat breeder cows is high. The use of an in vitro oviduct patency test using a suspension of particles comparable to the size of the ovum demonstrated that partial occlusion or narrowing of the oviduct can occur where no macroscopic abnormality is seen. A technique for assessment of oviduct patency in the living animal would give further information on the relative importance of oviduct disease in reproductive disorders in cattle.

CHAPTER 3

DETERMINATION OF OVIDUCT PATENCY IN VIVO USING PSP DYE

3.1.1 - INTRODUCTION - METHOD 1

Any investigation into the role that abnormalities of the oviduct may play in infertility in cattle must contain a method for assessing the normality of the oviducts. Although there are numerous reports in the literature describing oviduct patency tests in women, there is a limited amount of information on the application of similar techniques to cattle, and in particular, infertile cattle (Kothari, 1977; Ansari, 1979).

Essentially, the three methods described are: the use of air injected into the uterus (Hanley, 1953), starch grains injected on the surface of the ovary (Kessy and Noakes, 1979) or dye instilled into the uterus (Kothari et al, 1978; Kessy and Noakes, 1979). The first two methods have disadvantages. The introduction of gas even at low pressure into the bovine uterus has been shown to cause endometrial rupture in a large proportion of cases (Rowson, 1942a). With regard to the starch grain method, although some workers have reported accurate results (Kessy and Noakes, 1979), several others have questioned its reliability and suggested that the positive iodine reaction obtained could be due to the presence of starch in the swab used to wipe the vagina following the test rather than a response to the presence of starch grains (Johari and Sharma, 1964). There is also the question of the time required before a diagnosis is reached.

The use of a dye test appears more promising. Preliminary results indicated that this test is accurate in diagnosis of oviduct blockage, requires minimal equipment and appears to be a technically simple procedure (Kothari, 1977).

However workers in this field have used the test only on cows of known fertility or animals in which surgery was used to artificially disrupt the patency of the oviducts prior to carrying out the test. As yet no survey has been done which relates the incidence of oviduct abnormality in repeat breeder cows to that found using in vitro methods on slaughterhouse specimens.

Also, since the dye test was originally used as a test of kidney function, it is necessary to obtain some information on rates of the urinary excretion of PSP dye in cattle.

Therefore, the aims in using this test in repeat breeder cows were firstly, to attempt to validate the preliminary results obtained by others, and show that the technique was suitable in assessing a cow's fertility; secondly, to determine the incidence of oviduct abnormality in such a population of cows; and thirdly, by the use of intravenous and intraperitoneal injection of dye, and detecting its presence in the urine, acquire some knowledge of the rate of urinary excretion in the cow and its application to diagnosis of oviduct patency.

3.1.2 - MATERIALS AND METHODS

Animals

Cows of various breeds, ages and parity were used. At appropriate intervals the reproductive state of the majority of the animals was determined by behavioural observations and by rectal palpation of the reproductive tract. These animals were referred cases of infertility from veterinary practitioners. Animals were treated at all stages of the oestrous cycle.

Preparation of the PSP dye:

0.3 g of phenol red (pH range 6.8-8.4 B.D.H. Chemicals) and 4.2005 g of sodium bicarbonate anhydrous were dissolved in 1,000 ml of deionised water and the solution mixed by shaking. It was then filtered through a 0.45μ Millipore filter. In addition the dye was again sterilised by placing in universal bottles and autoclaving for 15 minutes at 15 lb/sq inch at 121°C in a portable steam steriliser (Thackeray).

Preparation of 1M sodium hydroxide (NaOH):

40 g of sodium hydroxide were dissolved in 1,000 ml of deionised water.

Equipment used in the dye test:

PSP dye solution

1M sodium hydroxide

Neilson catheter

Gibbon catheter (2 way 14 gauge, Franklins, High Wycombe)

20 ml syringes

Universal bottles

pH paper (range 6-8, BDH Chemicals).

Method for sterilisation of equipment:

Neilson catheters: These were packed in individual polythene bags which were sealed with temperature indicator tape and sterilised in a vertical steam steriliser (Thackeray).

Folytex catheters: These were packed as described above and sterilised using ethylene oxide at 55°C for 1 hour in a Victoria ethylene oxide steriliser Mark II.

Preparation of animals:

The test was carried out either with the animal haltered and restrained in a cattle crush or haltered in a stance in the byre. Her tail was tied to the halter to keep the perineal area clean and the perineum and vulva cleaned with a dilute solution of Savlon (chlorhexidine / cetrimide ICI Ltd., Macclesfield, Cheshire).

Conduct of the test:

A 14 gauge Gibbon catheter was inserted into the urethra, the cuff inflated, the bladder emptied and an initial urine sample taken. The catheter was left in situ.

A gloved hand was inserted into the rectum and the cervix grasped. The Neilson catheter was introduced into the vagina and manipulated through the cervix into the uterus. The Neilson catheter was maintained in the body of the uterus while the dye was instilled, using a 20 ml syringe.

The amount of dye instilled was that sufficient to cause palpable distension of the uterus and thus varied depending on the size of the uterus. The amount required was generally between 20 and 50 ml. Following the instillation of the dye, 10 ml of air was injected in order to ensure that no dye remained in the catheter. The catheter was then removed.

A second urine sample was taken 5 minutes after instillation of the dye and further samples were collected at 15, 30, 45, 60 and 90 minutes. The bladder was emptied at each sampling. The ph of the urine was tested, and a few drops of NaOH were added to any which were slightly acidic. The colour of the urine was assessed visually

for the red colour of the dye. Two positive samples were collected before stopping urine collection and if no dye had been detected by 90 minutes, the test was considered to be negative.

The assessment of normality or otherwise was based on conclusions of Kothari (1977) and the test repeated on 2 more occasions before a diagnosis of oviduct blockage was made.

Post-mortem assessment of oviduct patency:

Genital tracts from cows were examined for the presence of any gross abnormalities at varying intervals following dye tests. The horns of the uterus were cut open and the lumen checked for the presence of dye. The patency of the oviducts was then determined as previously described (Chapter 2) using dye solution followed by a pollen grain suspension in the case of apparently normal oviducts.

Intraperitoneal and intravenous injection of dye:

Animals: Four cows of different breeds and body weights were tested at 2 stages of the oestrous cycle. The body condition of each animal was assessed using the method outlined by Lowman, Scott and Somerville (1973).

Conduct of the test: Restraint of the animal and bladder catheterisation were as described previously.

20 ml of PSP dye was injected, using a 19 gauge, 1½ inch needle, into the jugular vein or intraperitoneally into the sublumbar fossa on the right side. Urine samples were drawn off continually from the bladder using a 20 ml syringe, a few drops of NaOH added, and the time noted at which the dye first appeared.

3.1.3 - RESULTS

Table 3.1.1 shows the detailed results of the dye test carried out on a total of 33 animals. The earliest time at which dye was seen in the urine following its instillation into the uterus was 30 minutes. The amount of dye required in order to cause uterine distension varied from 20 ml to 50 ml. In a high proportion of the cases, the test was unable to be completed due to leakage of dye from the cervical os. In a number of animals the urine was sampled at 35 minutes, usually due to difficulties in withdrawing urine.

A summary of the results contained in the previous table can be seen in Table 3.1.2. The number of animals diagnosed as having bilaterally blocked or unilaterally blocked oviducts is in excess of those diagnosed as having normal oviducts (39.4% of 36.4%). The number of inconclusive cases amounts to 24.2% of the total number of animals dye tested.

Results of application of the dye test compared to post mortem assessment of patency in 15 cows is shown in Table 3.1.3. In the 6 animals where the clinical test had indicated bilateral patency post mortem examination confirmed the diagnosis. However, of the 5 animals diagnosed as cases of bilateral blockage, 3 were found to have patent oviducts at post mortem (16, 22, 23). In the remaining 2 animals the clinical test results were confirmed.

Table 3.1.4 shows the results of the dye test carried out at different stages of the oestrous cycle in a random sample of cows. In only 1 case (3) is there a difference in the time taken for the dye to appear when the test was repeated at a different stage of

TABLE 3.1.1 RESULTS OF DYE TEST CARRIED OUT IN 33 CASES OF VARIOUS BREEDS AND STAGES OF THE OESTROUS CYCLE

Cow Number	Breed	Stage of cycle	Amount of dye instilled (ml)	Time dye first appeared in urine (min)	Conclusion regarding oviduct patency
1	Charolais	Dioestrus	20	30	Normal
2	Jersey	Unknown	20	35	Normal
3	Jersey	Dioestrus	20	30	Normal
4	Jersey	Dioestrus	20	30	Normal
5	Ayrshire	Oestrus	30	35	Normal
6	Ayrshire	Unknown	40	30	Normal
7	Murray Grey	Oestrus	20	30	Normal
8	Simmental	Dioestrus	40	30	Normal
9	Shorthorn	Unknown	20	35	Normal
10	Friesian	Dioestrus	30	30	Normal
11	Friesian	Oestrus	30	35	Normal
12	Friesian	Unknown	20	30	Normal
13	Jersey	Oestrus	20	45	Abnormal
14	Ayrshire	Dioestrus	20	60	Abnormal
15	Ayrshire	Oestrus	20	-	Abnormal
16	Friesian	Dioestrus	20	-	Abnormal
17	Ayrshire	Dioestrus	20	-	Abnormal
18	Dexter	Dioestrus	20	-	Abnormal
19	British White	Metoestrus	20	-	Abnormal

TABLE 3.1.1 (contd)

Cow Number	Breed	Stage of cycle	Amount of dye instilled (ml)	Time dye first appeared in urine (min)	Conclusion regarding oviduct patency
20	Ayrshire	Oestrus	30	-	Abnormal
21	Ayrshire	Dioestrus	20	-	Abnormal
22	Friesian	Unknown	40	-	Abnormal
23	Friesian	Unknown	40	-	Abnormal
24	Charolais	Dioestrus	40	-	Abnormal
25	Charolais	Dioestrus	50	-	Abnormal
26	Charolais	Oestrus	40	-	Inconclusive
27	Charolais	Dioestrus	50	-	Inconclusive
28	Charolais	Metoestrus	30	-	Inconclusive
29	Charolais	Dioestrus	40	-	Inconclusive
30	Ayrshire	Unknown	20	-	Inconclusive
31	Ayrshire	Oestrus	20	-	Inconclusive
32	Shorthorn	Unknown	20	-	Inconclusive
33	Charolais	Dioestrus	30	-	Inconclusive

Note (1) - : No evidence of dye in urine at 90 minutes following its instillation.

(2) Tests were abandoned as inconclusive where dye escaped from the cervix and pooled in the anterior vagina following its instillation.

TABLE 3.1.2 SUMMARY OF RESULTS OF DYE TEST CARRIED OUT ON 33 COWS OF DIFFERENT BREEDS

Number of Cows	Bilateral patency		Unilateral blockage		Bilateral blockage		Inconclusive	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)
33	12	(36.4)	2	(6.1)	11	(33.3)	8	(24.2)

TABLE 3.1.3 RESULTS OF DYE TEST AND SUBSEQUENT POST MORTEM EXAMINATION IN 15 COWS OF VARIOUS BREEDS

Cow Number	Breed	Time dye first appeared in urine (mins)	Conclusion regarding oviduct patency	Post mortem assessment of patency	Comment
1	Charolais	30	Normal	Normal	Result confirmed
2	Jersey	35	Normal	Normal	Result confirmed
3	Jersey	30	Normal	Normal	Result confirmed
4	Jersey	30	Normal	Normal	Result confirmed
10	Friesian	30	Normal	Normal	Result confirmed
11	Friesian	35	Normal	Normal	Result confirmed
16	Friesian	-	Abnormal	Normal	False negative test
18	Dexter	-	Abnormal	Abnormal	Result confirmed
19	British White	-	Abnormal	Abnormal	Result confirmed
22	Friesian	-	Abnormal	Normal	False negative test
23	Friesian	-	Abnormal	Normal	False negative test
26	Charolais	-	Inconclusive	Normal	
27	Charolais	-	Inconclusive	Normal	
28	Charolais	-	Inconclusive	Normal	
32	Shorthorn	-	Inconclusive	Normal	

Note (1) - : No evidence of dye in urine at 90 minutes following its instillation.

(2) Test abandoned as inconclusive where dye escaped from the cervix following its instillation.

(3) Post mortem assessment carried out using dye followed by a pollen grain suspension as described previously.

TABLE 3.1.4 RESULTS OF THE DYE TEST CARRIED OUT AT DIFFERENT STAGES OF THE OESTROUS CYCLE IN A RANDOM SAMPLE OF COWS OF VARIOUS BREEDS

Cow Number	Breed	Amount of dye instilled (ml)	Time dye first appeared in urine (mins)				Conclusion regarding oviduct patency
			Dioestrus		Oestrus		
1	Charolais	20	30	(ii)	30	(i)	Normal
2	Jersey	20	35	(i)	35	(i)	Normal
3	Jersey	20	45	(i)	30	(ii)	Normal
4	Jersey	20	30	(ii)	30	(i)	Normal
13	Jersey	20	45	(i)	45	(ii)	Abnormal
18	Dexter	20	-	(ii)	-	(i)	Abnormal
19	British White	20	-	(i)	-	(ii)	Abnormal
27	Charolais	40	-	(ii)	-	(i)	Abnormal
24	Charolais	40	-	(ii)	-	(i)	Inconclusive
26	Charolais	50	-	(i)	-	(ii)	Inconclusive

Note (1) (i) indicates 1st dye test
(ii) indicates 2nd dye test.

(2) - : No evidence dye in urine at 90 minutes following its instillation.

(3) Cows 1, 2, 3, 4, 24, 26, 27 - on post mortem examination, both oviducts patent.
Cows 18, 19 - on post mortem examination, both oviducts blocked.
Cow 13 - not slaughtered.

the cycle. Note that the 2 cases in which the test was inconclusive were those tests which were carried out at 2 stages of the oestrous cycle with no difference in the result. In 1 case (27) a diagnosis of bilateral blockage made on tests conducted during dioestrus and oestrus was not confirmed at slaughter.

The results of intraperitoneal and intravenous injection of dye are seen in Table 3.1.5. After intravenous injection, dye was invariably seen in the urine by 5 minutes. In 2 animals (1 and 2) there was a marked delay in the time the dye appeared following the initial intraperitoneal injection. On repeating the injection dye invariably appeared within 7 minutes. It was assumed that in these 2 animals the first injection had not been into the peritoneal cavity. There is no apparent difference in the time taken for the dye to be absorbed from the peritoneal cavity between cows of different breed or body score, or at different stages of the oestrous cycle.

Plate 3.1.1 shows the cervical os and anterior vaginal area of 1 of the inconclusive cases where the dye pooled back into the anterior vagina. Photographs were taken immediately following instillation of the dye into the uterus. The dye can be seen pooling in the anterior vagina and around the external os of the cervix.

Plate 3.1.2 is the genital tract from 1 of the Charolais cases which was slaughtered 3 weeks after a dye test which gave a negative result. A large amount of dye is still present in the uterine horns, and the oviducts contain no dye. The oviducts were confirmed as bilaterally blocked.

TABLE 3.1.5 RESULTS OF INTRAPERITONEAL AND INTRAVENOUS INJECTION OF DYE IN 4 ANIMALS AT DIFFERENT STAGES OF THE OESTROUS CYCLE

Cow Number	Breed	Body score	Intraperitoneal		Intravenous	
			First appearance of dye in urine (mins)			
			Dioestrus	Oestrus	Dioestrus	Oestrus
1	Shorthorn	$2\frac{1}{2}$	-	7	$4\frac{1}{2}$	4
			6	7		
2	Friesian	2	-	-	5	5
			7	7		
3	Friesian	$2\frac{1}{2}$	6	6	4	$4\frac{1}{2}$
4	Ayrshire	2	6	$5\frac{1}{2}$	5	4

Note (1) 20 ml of dye was injected in all cases.

(2) Body scoring scheme was that outlined by Lowman, Scott and Somerville (1973).

(3) - : No evidence of dye in urine 15 minutes following intraperitoneal injection. Test repeated at later date.

Plate 3.1.1 The cervical os and anterior vagina of a cow immediately following instillation of the dye into the uterus. The dye is pooled in the anterior vagina and around the external os of the cervix.

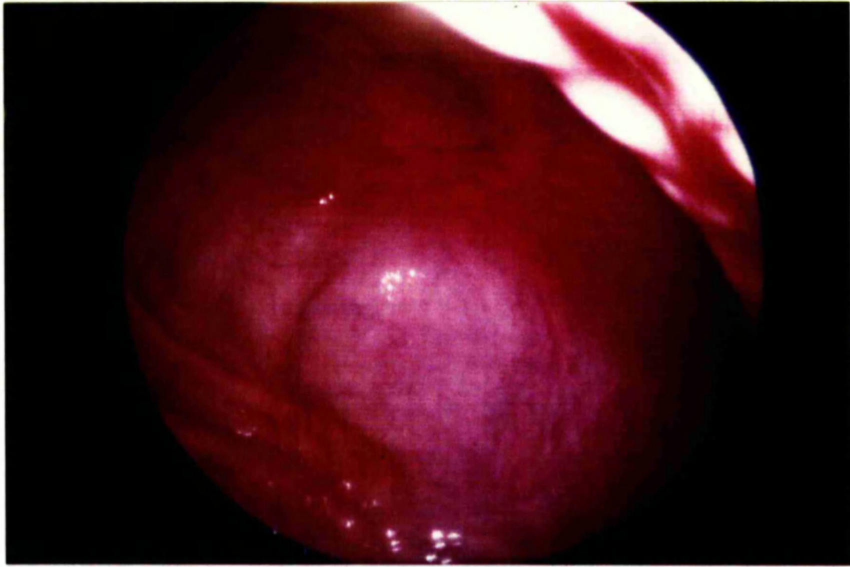
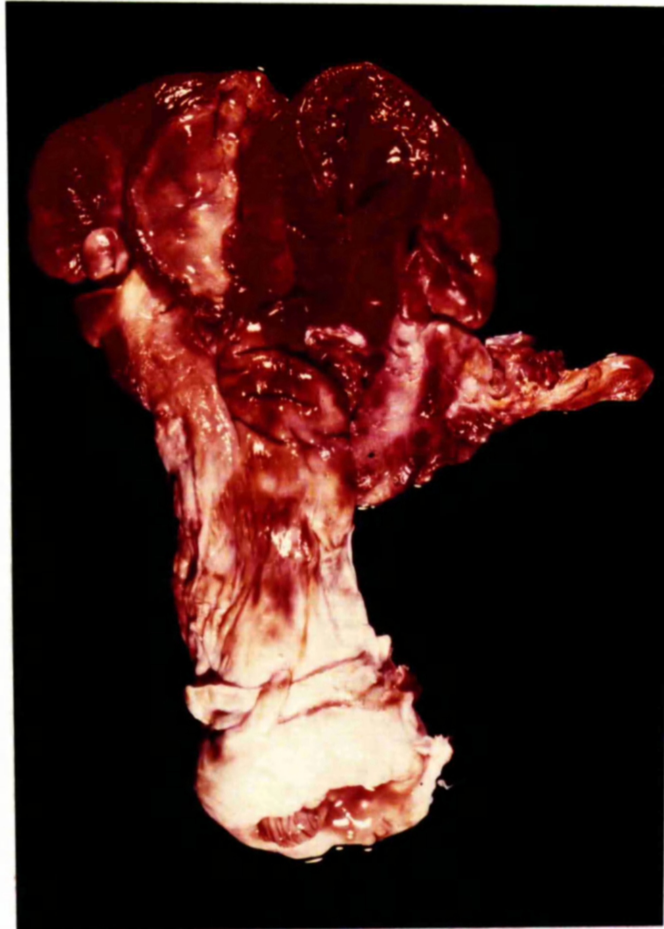


Plate 3.1.2 The reproductive tract from a cow slaughtered 3 weeks following a negative result to the dye test. Dye is pooled in the uterine horns.



3.1.4 - DISCUSSION

Using the dye test as described by Kothari et al (1978) initially various problems mainly of technique were encountered. These mainly consisted of difficulty in passing the catheter through the cervix in certain cows, and in catheterisation of the bladder. However these were quickly resolved with experience and subsequently a large number of dye tests were performed on cows of various breeds and at different stages of the oestrous cycle. In total 33 cows were tested, many of them on at least 3 occasions. The biggest problem encountered when carrying out this form of dye test was the large number of cases where the test had to be abandoned due to the escape of dye from the cervix, following its instillation into the uterine body. Normally the cervix in the cow is only dilated during oestrus (Arthur, 1975) and escape of dye from the uterus backwards into the vagina did occur in the several cases where the test was carried out with cows in oestrus. However it also happened in some pluriparous cows known to be in dioestrus by the circulating plasma progesterone levels and/or the presence of a palpable corpus luteum in one of the ovaries. Obviously this nullified the test since urine samples taken after this cervical backflow may have been contaminated. Kothari et al, 1978, made reference to such happenings in cows which they had tested and suggested that it could be overcome by packing the anterior vagina with sterile swabs. When this was attempted in the studies described here invariably the cow strained, forcing even greater amounts of dye through the cervix. Usually the expulsion of dye from the cervix occurred almost immediately after instillation, but on a few occasions a gradual seepage of dye from the cervix was seen

rendering the second or third samples of urine inconclusive even although the first sample had been clear. In those cows in dioestrus in which this backflow of dye occurred it would seem logical to suggest that they had an abnormality of the cervix itself. The uterus at dioestrus is under the influence of progesterone and is far more susceptible to infection than during oestrus (Rowson, Lamming and Fry, 1953; Hawk, Brinsfield, Turner, Whitmore and Norcross, 1964). A dilated cervix at this stage of the cycle may therefore be the means by which a subclinical uterine infection is established due to ascending infection from the vagina. A large percentage of the cows with this particular problem were repeat breeders. In only a few of those cows where backflow occurred did endoscopic examination of the cervical region give some indication as to the cause. The findings varied from a partial to a complete prolapse of the cervix backward into the vagina. However in the majority of animals no explanation was found on macroscopic examination. Obviously this is an area requiring further investigation.

In the remaining 25 animals in which no cervical backflow had occurred and the test was carried out to its conclusion, a total of 13 appeared to have oviduct abnormalities. This indicated an incidence of abnormal oviducts in this group of cows of over 50%. Even with the knowledge that these animals were all long standing cases of infertility this would seem an extremely high incidence of oviduct disease. However, in 3 animals (16, 22, 23) in which the clinical tests had indicated bilateral oviduct blockage on at least 3 occasions subsequent post mortem examination of the oviducts

revealed bilateral patency. In addition it was noted that no dye remained in the uterus of these 3 animals. This is in contrast to 2 other animals (18, 19) slaughtered following negative results where dye was seen to remain in the uterine lumen for as long as 3 weeks after the dye test. This indicates that endometrial absorption of dye proceeds very slowly, if at all, and that the dye in the 3 cases above did indeed pass through the oviducts into the peritoneal cavity. As only 5 of the animals giving a negative result were slaughtered the accuracy of a diagnosis of bilateral blockage in the remaining 6 must be suspect. There are several possible explanations for the occurrence of these false negative results.

False negative results of dye tests have been recorded by other workers but in their cases they occurred only when the test was carried out at oestrus (Kessy and Noakes, 1979). They suggested that this was due either to a tubal lock at the isthmoampullary junction or to increased oedema present around the uterotubal junction at oestrus producing a physical barrier to the passage of the fluid. There are two reasons to suggest that this was not the explanation in these studies. One, the tests were carried out at all stages of the oestrous cycle with the same result, and two, when the test was done in a number of other animals at oestrus, positive results were obtained.

There are several factors which may affect the movement of the dye through the oviduct. The amount of dye instilled into the uterus must be sufficient to cause uterine distension (Kothari, 1977). This in turn stimulates uterine contractions which force the dye past the uterotubal junction. In all the dye tests performed in these

studies sufficient dye was injected to cause uterine distension. During the test the dye must pass from an organ of a large diameter, that is the uterus, to one of a very small internal diameter, the oviduct. In order for this to occur rapidly enough for a sufficient dye concentration to build up in the urine to be detected visually, a certain amount of pressure is needed. This is brought about by the combination of uterine contractions stimulated by the presence of the dye, and the speed at which the dye is instilled into the uterus. In all cases, the dye was instilled rapidly until uterine distension had been achieved, so insufficient pressure and amount of dye is unlikely to be the explanation for these false negative results.

The presence of the dye in the urine is assessed visually and its detection depends on the amount of urine present in the bladder, the pH of the urine and the concentration of the dye being used. Obviously the fuller the bladder then the greater the dilution of the dye will be. In these studies the bladder was emptied before injecting the dye and at each urine sampling. Although it is difficult to ascertain if the bladder has been completely emptied most of the urine will be removed and the urine remaining should not be sufficient to mask the presence of the dye. PSP dye is visible only in an alkaline urine. The majority of cows have alkaline urine but in any case the pH was always tested and any samples which were slightly acidic were treated with sodium hydroxide. The concentration of dye used was standard for each test so this would be unlikely to be a cause of failure to detect any dye which was present. One other possible explanation for false negative

results could be a spasm of the uterotubal junction following the instillation of the dye into the uterus. This has been shown to occur quite often in human patients when similar techniques have been employed to assess oviduct patency giving a high number of false negative results (Hermann, Spadoni and Smith, 1969; Ansari, 1979) and is detected in the human by the use of hysterosalpingography. Hysterosalpingography has not yet been demonstrated in cattle. Thus it is difficult to pinpoint with any accuracy the reason for the occurrence of the false negative results obtained in these 3 animals.

In the work described by Kothari et al (1978), diagnosis of bilateral oviduct patency was made when dye was detected in the urine at 15-30 minutes following its instillation. In this study the earliest time at which dye was detected was 30 minutes. As already pointed out visual interpretation was the criterion used for the presence of the dye and this may be criticised as being too subjective. Certainly other workers have stressed the importance of the use of specialised equipment such as a colorimeter to pick up the slightest traces of dye in the urine and this may help to standardise the exact time at which the dye does appear (Kessy and Noakes, 1979). However it also takes the technique further out of reach of the practising clinician who may wish to use it as an on farm technique.

Kothari (1977) suggested that a delay in the appearance of the dye in the urine may indicate a unilateral blockage. However, while this may be so, it could equally well be due to a narrowing of both oviducts.

Once the dye has passed along the oviducts it is absorbed from the peritoneal cavity extremely rapidly. Studies in this thesis have shown that the rate of absorption is not affected by the body condition of the animal, and so any delay in the appearance of the dye in the urine is unlikely to be due to this. Also the stage of the oestrous cycle appears to have no effect on the speed at which the dye is absorbed through the peritoneum or excreted by the kidneys following intravenous injection. These results agree with those of other workers (Mixner and Anderson, 1958; Kothari et al, 1978; Kessy and Noakes, 1979).

Although this technique was accurate in a number of animals with bilateral oviduct blockage, it did produce some false negative results, was unable to be used in certain animals with cervical dilation and was not able to diagnose accurately cases of unilateral blockage. Therefore it could not be recommended as a diagnostic test, the results of which could determine the future of the animal.

3.2.1 - INTRODUCTION - METHOD 2

From the results obtained in the previous section it was clear that the dye test as described by Kothari et al (1978) had several disadvantages. A modified test was necessary which could overcome these, so making possible the accurate diagnosis of both unilateral and bilateral oviduct patency in all animals, regardless of the degree of dilation of the cervix.

Workers in the field of bovine embryo transfer looking for a more economical method of embryo recovery than surgical flushing of the tract, have developed a flexible, cuffed catheter for non-surgical use (Greve, Lehn-Jensen and Rasbech, 1977; Newcomb, 1980). The catheter is inserted along each uterine horn in turn, and the cuff inflated to prevent lose of the recovery medium. Another worker involved in this field had indicated that the use of a similar catheter could permit diagnosis of unilateral blockage with no problems of backflow of dye through the cervix (Coulthard, 1980).

Therefore it was decided to incorporate the use of such a catheter in carrying out dye tests as a means of diagnosis of oviduct patency.

3.2.2 - MATERIALS AND METHODS

Animals

Cows of various breeds and ages were used. In general these were animals referred by practising veterinary surgeons as cases of infertility. The reproductive state of each animal was determined by rectal palpation and any animals in which gross abnormalities were detected were excluded. The remaining animals were tested at all

stages of the oestrous cycle.

Preparation of the dye and 1M sodium hydroxide:

This was carried out as previously described.

Equipment used in the modified dye test:

PSP dye

1M sodium hydroxide (NaOH)

Stainless steel speculum (T. A. Saul, Boston, Lincs.)

Stainless steel introducer (T. A. Saul, Boston, Lincs.)

14 gauge 2-way Gibbon catheter (Franklins, High Wycombe)

60 ml syringe

20 ml syringes

Universal bottles

pH paper (range 6-8, BDH Chemicals)

Method for sterilisation of equipment:

The speculum and introducer were packed and sterilised as described for the Neilson catheter, and the Gibbon catheter as described for the Folytex catheter.

Preparation of the animals:

The cow to be examined was injected intramuscularly with 20 ml of hyosine-N-butylbiomide (Buscopan Composition: Boehringer Ingelheim) approximately 1 hour prior to the execution of the test. This was followed by an intramuscular injection of 1.5 ml acetylpromazine(ACP, C-Vet) approximately 40 minutes later. The cow was then haltered and placed in a cattle crush, which was raised 25 cm

from the floor at the anterior end. Immediately prior to performing the test, an epidural injection of 5-8 ml 2% lignocaine (Xylocaine, Astra Chemicals) was given. The tail was tied to the halter and the perineum was cleaned with a dilute solution of Savlon.

Conduct of the test:

A 14 gauge Folytex catheter was inserted into the urethra, the cuff inflated, the bladder emptied and an initial urine sample taken. The catheter was left in situ throughout the operation.

A notched stainless steel speculum (Plate 3.2.1) was lightly coated with lubricant (Lubrel, Dales Pharmaceuticals) and passed into the vagina as far as the cervix. The centrepiece was removed. An introducer (Plate 3.2.1) was passed along the speculum and thus reached the cervix without touching the vaginal mucosa. The outer-piece of the speculum was then removed leaving the introducer in situ.

The cervix was grasped per rectum, then, using rectocervical manipulation, the introducer was guided into the body of the uterus. One horn of the uterus was then gently eased back over the introducer until its tip lay approximately 5 cm into the horn. The centrepiece of the introducer was removed leaving its hollow outer tube in situ.

An assistant passed a 2 way Folytex catheter (Plate 3.2.1) through the introducer into the uterine horn and this was manipulated per rectum forward into the horn. During this phase, the uterus was pulled back over the catheter, rather than the catheter being pushed forward into the horn.

By rectal palpation of the tract the catheter tip was located within 5-7.5 cm of the anterior end of the uterine horn, and

Plate 3.2.1 Equipment used in the dye test (Method 2):

1. Speculum; 2. Introducer; 3. 2-way catheter;

4. PSP dye (universal bottle); 5. 20 ml syringe.

the retaining cuff inflated by injecting water. The degree of distension of the bulb required was assessed by rectal palpation. This ensured a seal on the horn, so preventing escape of dye. Sufficient dye was injected to cause a palpable distension of the anterior end of the uterine horn. The amount required varied from 10-50 ml depending on the size of the uterus and the position of the cuff of the catheter. After instillation of the dye, varying amounts of air were injected to displace the dye from the catheter. The equipment was left in place for 10 minutes. Afterwards the cuff was deflated and both the flexible catheter and the hollow outerpiece of the introducer were withdrawn. A urine sample was taken at 5 minute intervals and from then on until 30 minutes had elapsed since the instillation of the dye. The pH of the urine was tested using litmus paper (BDH), and a few drops of 1M NaOH added to any urine which was acidic. The colour of the urine was then assessed visually for the presence of the dye.

Two positive samples were collected before stopping urine collection and if there was no evidence of dye by 30 minutes, the test was considered to be negative. A final sample was taken at 60 minutes following dye instillation to confirm the negative result, and in all cases the test was repeated where delay or non-appearance of the dye in the urine had occurred. 24 hours were allowed to elapse before the test was repeated on the opposite horn.

3.2.3 - RESULTS

The results of the modified dye test carried out for the first time in a group of 38 cows are demonstrated in Table 3.2.1. The amount of dye instilled into the uterine horn varied from 15 ml

TABLE 3.2.1 RESULTS OF THE MODIFIED DYE TEST CARRIED OUT FOR THE FIRST TIME IN A GROUP OF 38 COWS OF VARIOUS BREEDS AND AT VARIOUS STAGES OF THE OESTROUS CYCLE

Cow Number	Breed	Stage of Cycle	Amount of dye instilled (ml)		First appearance dye in urine (min)		Conclusions regarding oviduct patency
			Left horn	Right horn	Left oviduct	Right oviduct	
1	Charolais	Dioestrus	30	30	20	20	Bilateral patency
2	Charolais	Dioestrus	35	30	15	20	Bilateral patency
3	Charolais	Oestrus	40	45	20	20	Bilateral patency
4	Charolais	Oestrus	40	40	15	15	Bilateral patency
5	Charolais	Oestrus	40	40	20	15	Bilateral patency
6	Ayrshire	Unknown	20	15	15	15	Bilateral patency
7	Friesian	Dioestrus	15	15	15	15	Bilateral patency
8	Friesian	Dioestrus	20	20	15	15	Bilateral patency
9	Friesian	Oestrus	20	20	15	15	Bilateral patency
10	Red Poll	Unknown	25	25	15	15	Bilateral patency
11	Charolais	Oestrus	40	35	20	15	Bilateral patency
12	Charolais	Dioestrus	40	40	15	15	Bilateral patency
13	Ayrshire	Unknown	15	15	15	15	Bilateral patency
14	Ayrshire	Unknown	20	15	15	15	Bilateral patency
15	Friesian	Dioestrus	20	20	15	20	Bilateral patency
16	Friesian	Dioestrus	20	20	20	15	Bilateral patency
17	Limousin	Oestrus	30	30	20	20	Bilateral patency
18	Simmental	Oestrus	40	45	20	20	Bilateral patency

TABLE 3.2.1 (contd)

Cow Number	Breed	Stage of Cycle	Amount of dye instilled (ml)		First appearance dye in urine (min)		Conclusions regarding oviduct patency
			Left horn	Right horn	Left oviduct	Right oviduct	
19	Friesian	Dioestrus	30	35	20	15	Bilateral patency
20	Friesian	Dioestrus	20	15	15	15	Bilateral patency
21	Simmental	Dioestrus	30	35	25	25	? Bilateral narrowing
22	Shorthorn	Unknown	20	15	30	30	? Bilateral narrowing
23	Charolais	Oestrus	40	50	30	30	? Bilateral narrowing
24	Charolais	Dioestrus	40	40	15	25	? Rt. oviduct narrowing
25	Ayrshire	Dioestrus	10	20	15	30	? Rt. oviduct narrowing
26	Charolais	Oestrus	40	35	30	20	? Lft. oviduct narrowing
27	Charolais	Dioestrus	50	50	25	15	? Lft. oviduct narrowing
28	Friesian	Dioestrus	20	25	-	-	Bilateral blockage
29	Friesian	Oestrus	30	25	-	-	Bilateral blockage
30	Charolais	Dioestrus	40	40	-	-	Bilateral blockage
31	Charolais	Dioestrus	40	40	-	15	Left oviduct blockage
32	Friesian	Oestrus	20	30	-	20	Left oviduct blockage
33	Ayrshire	Unknown	15	20	-	20	Left oviduct blockage
34	Charolais	Dioestrus	40	40	15	-	Right oviduct blockage
35	Friesian	Oestrus	20	30	15	-	Right oviduct blockage
36	Charolais	Dioestrus	40	40	20	-	Right oviduct blockage
37	Friesian	Oestrus	20	20	15	-	Right oviduct blockage
38	Friesian	Dioestrus	30	30	15	-	Right oviduct blockage

Note (1) - No evidence of dye in urine at 60 minutes following its instillation

to 50 ml. The earliest time at which dye was detected in the urine was 15 minutes following its instillation into the uterus. Of the 38 animals tested, 27 were diagnosed as having bilaterally patent oviducts, although it was noted that in 7 of these animals a slight delay occurred in the time taken for the dye to appear. The most common oviduct abnormality diagnosed was right oviduct blockage (5 cases) with 3 cases each of left oviduct blockage and bilateral blockage.

Table 3.2.2 summarises the results contained in Table 3.2.1. There were no inconclusive cases using this test, a result being obtained for every animal. Unilateral blockage appears to be almost 3 times as common as bilateral blockage in this group of cows (21.1% v 7.9%).

Table 3.2.3 shows the comparison between results obtained using the clinical test and subsequent post mortem examination of the oviducts. In every case post mortem confirmed the dye test result. It is interesting to note that in 2 animals (21 and 23) where the dye test had shown a delay in the time taken for the dye to appear in the urine, abnormalities sufficient to impair the progress of the pollen grains were found, as demonstrated by failure to pass the pollen grains along the oviducts.

The results of a repeat dye test carried out in 10 animals showing delay in appearance of the dye in the urine at the first test are shown in Table 3.2.4. In 5 animals (22, 24, 25, 36, 37) a reduction in the time taken for the dye to appear has occurred in the second test. In 4 of the 5 animals post mortem examination of the oviducts has confirmed the results of the second test, with the remaining animal not slaughtered. In 2 animals (26 and 27), the

TABLE 3.2.2 SUMMARY OF RESULTS OBTAINED USING THE MODIFIED DYE TEST IN 38 COWS OF VARIOUS BREEDS

Number of Cows	Bilateral Patency		Unilateral Blockage		Bilateral Blockage		Inconclusive	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)
38	27	(71)	8	(21.1)	3	(7.9)	0	(0)

Note (1) Any cow showing a consistent delay in the appearance of the dye in the urine was nevertheless assigned to the bilateral patency group.

TABLE 3.2.3 RESULTS OF MODIFIED DYE TEST AND SUBSEQUENT POST MORTEM EXAMINATION IN 12 COWS OF VARIOUS BREEDS

Cow Number	Breed	First appearance dye in urine (mins)		Post mortem assessment of patency		Comment
		Left oviduct	Right oviduct	Left oviduct	Right oviduct	
28	Friesian	-	-	Blocked	Blocked	Result confirmed
30	Charolais	-	-	Blocked	Blocked	Result confirmed
34	Charolais	15	-	Patent	Blocked	Result confirmed
35	Friesian	15	-	Patent	Blocked	Result confirmed
31	Charolais	-	15	Blocked	Patent	Result confirmed
32	Friesian	-	20	Blocked	Patent	Result confirmed
21	Simmental	25	25	Narrowed	Narrowed	Result confirmed
23	Charolais	30	30	Hydrosalpinx	Hydrosalpinx	Result confirmed
4	Charolais	15	15	Patent	Patent	Result confirmed
6	Ayrshire	15	15	Patent	Patent	Result confirmed
1	Charolais	20	20	Patent	Patent	Result confirmed
7	Friesian	15	15	Patent	Patent	Result confirmed

Note (1) - : No evidence of dye in urine at 60 minutes following its instillation.

(2) Post mortem assessment of patency was carried out using a pollen grain suspension as described in Chapter 2.

TABLE 3.2.4 RESULTS OF REPEATING THE MODIFIED DYE TEST IN 10 ANIMALS WHICH SHOWED A DELAY IN THE APPEARANCE OF THE DYE IN THE URINE IN A PREVIOUS TEST

Cow Number	Breed	First appearance dye in urine (mins) (i)		First appearance dye in urine (mins) (ii)		Post mortem assessment of patency	
		Left oviduct	Right oviduct	Left oviduct	Right oviduct	Left oviduct	Right oviduct
21	Simmental	25	25	25	25	Narrowed	Narrowed
23	Charolais	30	30	30	30	Hydrosalpinx	Hydrosalpinx
22	Shorthorn	30	30	25	25	Patent	Patent
25	Ayrshire	15	30	15	20	Patent	Patent
37	Friesian	15	-	15	20	Patent	Patent
36	Charolais	20	-	20	20	Patent	Patent
35	Friesian	15	-	15	-	Patent	Blocked
24	Charolais	15	25	15	20	Not slaughtered	
26	Charolais	30	20	30	20	Not slaughtered	
27	Charolais	25	15	25	15	Not slaughtered	

Note (1) (i) indicates 1st dye test

(ii) indicates 2nd dye test

(2) - : No evidence dye in urine 60 minutes following its instillation.

(3) Post mortem assessment of patency carried out using a pollen grain suspension as described in Chapter 2.

delayed time was seen in the second test also, but unfortunately neither of these animals was slaughtered. It is interesting to note that both cows 21 and 22 showed delayed times in their second tests, but cow 21 was shown to have an abnormal narrowing of the oviducts at post-mortem, whereas cow 22 had apparently normal oviducts at post-mortem.

The genital tract of a cow slaughtered 3 months after a dye test giving a negative result is seen in Plate 3.2.2. The dye injected at post-mortem can be seen lying in the occluded oviducts, unable to pass beyond adhesions at the fimbria.

Plate 3.2.3 demonstrates bilateral hydrosalpingitis seen in 1 cow (23). This animal showed a delay in the appearance of the dye in the urine in repeated dye tests.

3.2.4 - DISCUSSION

The modified test using the cuffed catheter proved to have several definite advantages: firstly, as the cuff occluded the uterine horn dye backflow was prevented and the test could be used in any animal regardless of the degree of dilatation of the cervix or the stage of the cycle; secondly since the dye was instilled into one uterine horn only diagnosis of unilateral blockage was possible.

In cattle the degree of infertility resulting from unilateral oviduct abnormality depends on which oviduct is blocked. In the bovine more ovulations occur from the right ovary than

Plate 3 2.2 The reproductive tract from a cow slaughtered 3 months following a negative result to the dye test. Blue dye injected at post-mortem is lying in the occluded oviducts.

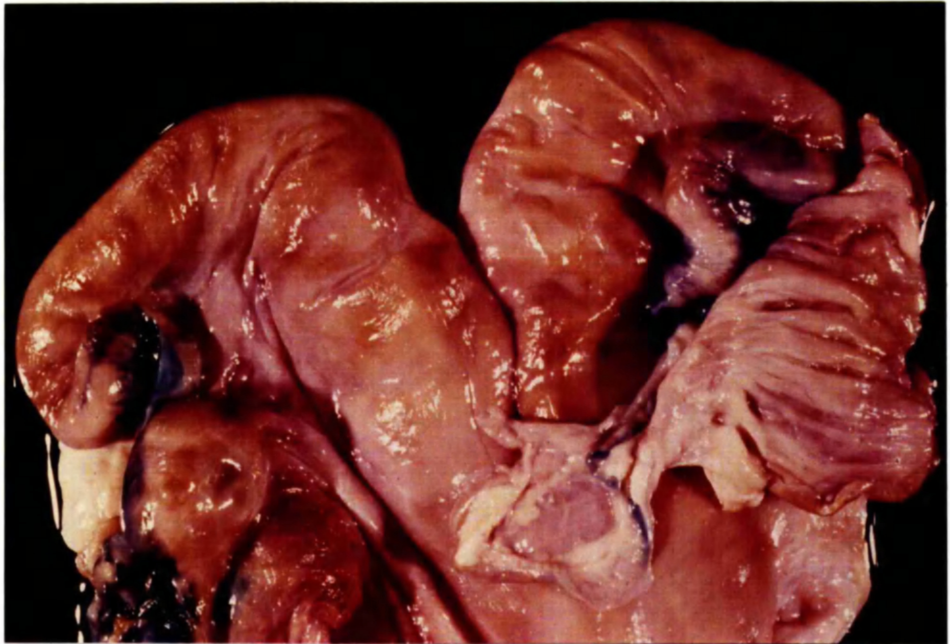
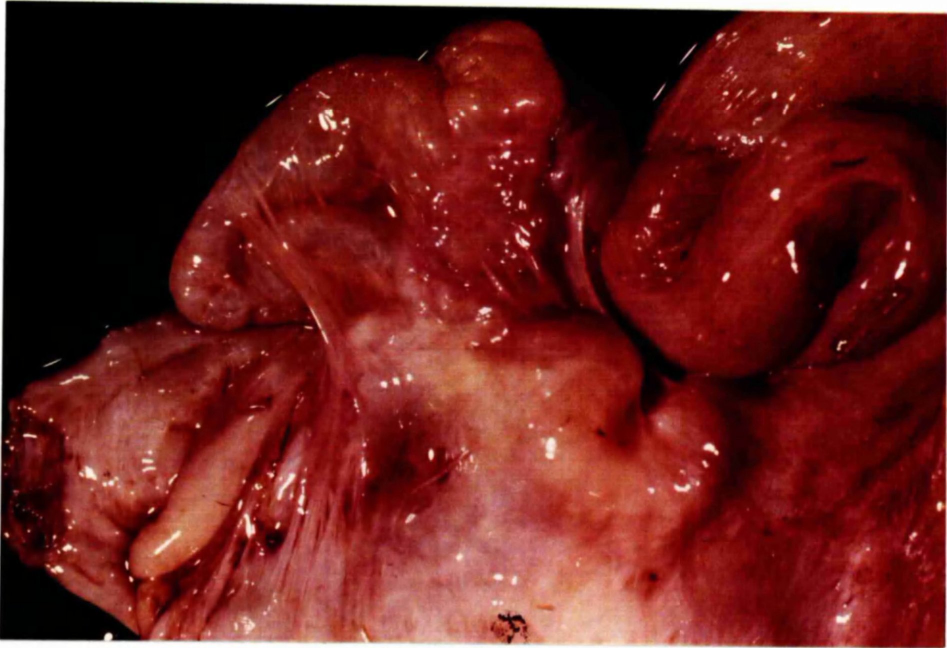


Plate 3.2.3 Hydrosalpinx, causing a fluid filled distension of the oviduct (Cow 23)



from the left (Al-dahash and David, 1977). Therefore where blockage of the right oviduct is diagnosed, fertility in such an animal is reduced by more than half. Treatment of an animal with unilateral blockage of the oviduct could be by either removing the ovary adjacent to the blocked oviduct, in which case ovulation should occur as often from the remaining ovary as when both ovaries are in situ, and under these conditions pregnancy would be much more certain since the corresponding oviduct is known to be patent. Or, rather than resorting to ovariectomy, determining by rectal palpation which ovary contains the corpus luteum and therefore was the source of the ovulation, then if ovulation had occurred from the ovary adjacent to the blocked oviduct, by using prostaglandins shorten the cycle. This should result in another ovulation which, this time, may be from the ovary adjacent to the normal side.

Another advantage of the modified method of the dye test is that the time taken for diagnosis is considerably shortened. Using the modified test the dye appears in the urine within 20-25 minutes where the oviduct is patent and a diagnosis of blockage can be made if no dye is present by this interval after instillation. This compares with more than 60 minutes in the original test for a negative result. Therefore this approach is more practical if the test is to be used by busy practitioners as an on farm technique.

However although this modified test is a much more accurate one, it does have the disadvantage of requiring a greater degree of manipulative skill, in that the flexible catheter must be positioned correctly in the uterine horn. In the cow, the uterine horn is curved

ventrally and therefore merely pushing the catheter horizontally causes it to impinge on the endometrium and cause damage. It was found with experience that one should ease the uterus backwards over the catheter rather than push the catheter forward. These manipulations are only possible if good anaesthesia is employed. The regime used here in which premedication with acetyl promazine and 'Buscopan' was used, followed by an epidural anaesthetic, produced excellent relaxation with the cow remaining on her feet. Under these circumstances the test was carried out relatively easily.

Another disadvantage of the test is that of the diameter of the stainless steel introducer which presented problems in animals with tightly constricted cervices. Indeed in 2 heifers it proved impossible to carry out this form of dye test even when a cervical dilator (T. A. Saul, Boston, Lincs.) was used to breach the cervical barrier.

The majority of animals tested using this method of dye test gave definite results which were confirmed, where possible, at post mortem. However, 1 cow (23) which at post mortem examination was found to have bilaterally blocked oviducts, gave a positive result to the dye test with dye appearing in her urine 30 minutes following its instillation. Although the slight delay in the appearance of the dye had aroused some suspicion of an abnormality being present, this was in effect a false positive result. At post mortem the oviduct blockage was seen to be caused by an acute bilateral hydrosalpingitis (Plate 3.2.3). Hydrosalpingitis causes a marked thinning of the oviduct wall and damage to the mucosal lining of the endosalpinx

(Benirschke, 1969). It would seem logical to suggest that in this case the dye may have passed through the damaged mucosa and so entered the blood stream without passing along the oviduct lumen. This explanation has also been proposed by workers in the human field, where this method of determining oviduct patency has been shown to produce false positive results when carried out on patients who have undergone tubal ligation operations (Hoffman, 1951; Williams and Gering, 1966). It is interesting to speculate as to the reason for the delay in the appearance of the dye in the urine of this cow. In the human patient only 1 urine sample is generally taken and this is done at 30 minutes following the instillation of the dye (Speck, 1948). In human cases of false positive results the dye is seen as clearly at this time as in cases where the oviducts are patent. However, the anatomy of the human oviduct is very different from that of the cow, being both broader and straighter (Francis, 1973). It may be that the surface area that is damaged and so able to absorb the dye is so reduced in the narrow oviduct of the cow that this caused a delay in the appearance of the dye. Two other explanations for a false positive result to the dye test are either the inadvertent injection of dye into the peritoneal cavity instead of into the uterus or blood in the urine being mistaken for dye. However, the sensation of the catheter puncturing the uterine wall is unmistakable and there would be no doubt if this had occurred. On the other hand, the presence of blood in the urine is obvious as, if the sample is left standing, the blood will sink to the bottom. Neither of these possibilities is applicable in this animal.

A delay in the time taken for the dye to appear bilaterally

occurred in 2 other animals (21, 22). Again, in the living animals, this delay aroused suspicion of some sort of abnormality present in the oviducts and this was confirmed in 1 case at post mortem (21). In this cow, although both oviducts were found to be patent to a fluid injection, the injection of the pollen grain suspension demonstrated a narrowing of both oviducts. However, when the remaining case which had shown a similar delay in the appearance of dye in the urine on several occasions was examined after slaughter, the oviducts were patent to both dye and pollen grains. Other workers have suggested that normal patency should be taken as the time taken for the urine to reach a 'cherry pink' colour and that a delay in formation of this colour rather than on the first appearance of the dye demonstrates a narrowing of the oviduct lumen (Coulthard, 1981). He based his confirmation of this method of diagnosis of narrowing on the subsequent failure to recover embryos following superovulation or the recovery of fewer embryos than expected from prior rectal palpation to determine the number of corpora lutea. However, as with the present studies, some cows in which a diagnosis of narrowing has been made, have apparently normal oviducts as shown by recovery of adequate numbers of embryos after superovulation.

Another possible explanation for the delay in dye appearance could be the differences in the rate of absorption of dye through the peritoneum in a thin animal compared to a fat animal. However it was found in these studies that when dye was injected directly into the peritoneum of cows of varying weights and body scores, no significant difference occurred in the time taken for the dye to reach

the bladder.

In 5 animals in which repeated dye tests were carried out a marked reduction in the time taken for the dye to appear occurred on the second and subsequent tests (22, 25, 37, 36, 24). Post mortem examination confirmed in 4 cases that the oviducts were patent (22, 25, 37, 36). It is hypothesised that this could be due to the removal of debris occluding the oviduct lumen or the breakdown of adhesions lying across the lumen by the pressure of the agent used for testing. The use of oviduct patency tests as a possible therapeutic treatment is well documented in the human field and has also been suggested by several workers in the veterinary field (Kothari, 1977; Ansari, 1979; Kessy and Noakes, 1979).

In these studies, several animals which had given negative results to the dye test, indicating abnormal oviducts, revealed adhesions at post mortem. These were easily broken down by the pressure needed to force dye through the oviducts. This could be the means by which the test works in certain cases to produce a patent tube where a blocked one existed previously. However, the main criticism in suggesting that the dye test has therapeutic value is that although the oviduct is made patent, this does not necessarily mean that it has returned to normal function. For example, an animal in which abnormality is present due to an acute infection is likely to have peritubal adhesions affecting the motility of the oviduct which may still cause problems although intralumenal adhesions have been broken down. In such an animal it would be probable that the oviducts were affected bilaterally and the prognosis would be guarded regardless of the outcome of repeated dye tests. The presence of salpingitis,

causing marked inflammation of the oviduct mucosa with subsequent reduction in the diameter of the oviduct lumen, will affect both transport of the gametes and the nutrition of the zygote if fertilisation has taken place, while still allowing the passage of fluid. However, the presence of septal adhesions around the fimbria, possibly produced by trauma due to rough handling during rectal palpation, may cause a reduction in fertility by their effect on the mobility of the infundibulum yet be broken down easily by fluid pressure with a return to normal oviduct function. Other workers have reported the use of surgery in an attempt to breakdown this type of adhesion and have produced pregnancies (Bowen, Elsden and Seidel, 1978; Noakes, 1979). It is suggested that if the past history of the animal under investigation reveals a long standing infection of the reproductive tract, then repeated dye tests will be of little benefit, whereas an animal with no history of uterine infection may benefit from repeated dye tests. This modified dye test certainly appeared to do no harm to the future fertility of the animal as several of the animals have since become pregnant following a succession of tests (2, 3, 8, 9, 12, 19).

To summarise, the dye test has been shown to be accurate in the diagnosis of unilateral and bilateral blockage in a number of cows. This is of importance where such a cow may be kept for some time with the incurrence of feeding and veterinary costs in an attempt to produce a pregnancy. A negative dye test on at least 3 occasions means that the cow can be regarded as having no further reproductive function. However, the main criticism of the dye test as a means of predicting future fertility is that it assesses the patency of the

oviducts to a fluid injection only and gives no information on the ability of the oviduct to transport and nourish the zygote. Therefore there will remain animals which although giving positive results to the dye test have oviduct abnormalities sufficient to prevent conception.

CHAPTER 4

USE OF SUPEROVULATION, EGG RECOVERY AND EMBRYO MORPHOLOGY IN THE ASSESSMENT OF OVIDUCT FUNCTION

4.1 - INTRODUCTION

In addition to patency, the oviduct in ensuring fertility must fulfil a number of other functions. In general these comprise the organ's ability to pick up the ovulated öocyte, transport ovum and sperm to the fertilisation site and the zygote to the uterus and maintain an appropriate internal environment. In monitoring oviduct function, a definitive test is therefore the establishment of a pregnancy. However, use of such a test for diagnostic purposes would be unpractical. Firstly, in the cow a considerable time must elapse between the time of mating and diagnosis of pregnancy; secondly, since a multiplicity of other factors are involved in an animal becoming pregnant, the finding of a negative result could be of limited significance as regards the oviduct. In addition the reliance on such an end point is associated with at least potential difficulties as animals for such investigations normally have a history of infertility.

As an alternative to the protracted method of establishing a pregnancy, the recovery of embryos, at an early state after mating, would provide information on oviduct function. The main disadvantage of using this method is that, under normal circumstances where a single ovulation takes place, only 1 oviduct could be tested. In addition there are practical difficulties in the recovery of a single embryo from the uterus of the cow (Sreenan, 1978; Shelton, Heath, Old and Turnbull, 1979). Both of these limitations could be overcome by simultaneously inducing multiple ovulations from both ovaries.

Several regimes for superovulation of the cow have been

described. These involve the administration of exogenous gonadotrophins from a range of sources in a variety of differing regimes (Foote and Onuma, 1970; Gordon, 1975). The materials used for producing a superovulatory response were originally pituitary extracts from sheep (Casida, Meyer, McShan and Wisnicky, 1943). In addition, the use of human menopausal gonadotrophin has been studied (Newcomb, 1980). However, the most commonly used methods involve the administration of a non-pituitary gonadotrophin - pregnant mare serum gonadotrophin (PMSG) (Foote and Onuma, 1970; Gordon, 1975). This latter gonadotrophin, which has mainly follicle stimulating hormone (FSH) properties has in some cases been used in combination with an additional ovulatory/ luteotrophic stimulus, both in the form of human chorionic gonadotrophin (HCG) and more recently, using the response of the cow to luteinising hormone releasing hormone (LHRH) (Scanlon, Sreenan and Gordon, 1968; Rowson, 1951; Newcomb, 1980).

The method of administration of these substances varies depending on the preparation. This ranges from single to multiple injections, administered in constant or increasing doses (Foote and Onuma, 1970; Gordon, 1975). In the earlier studies, the time of administration ranged throughout the oestrous cycle (Casida et al, 1943). However, it has been shown that better results are obtained when oestrus occurs within 4-5 days of administration of the preparation (Scanlon et al, 1968). This produces difficulties in predicting the ultimate time of oestrus. However, since 1974, the availability of luteolytic agents has removed some of the difficulties (Cooper and Furr, 1974; Elsden, Lewis, Cumming and Lawson, 1974; Leaver, Glencross and Pope, 1975).

It is difficult to compare the ovarian responses obtained by many of these studies as widely variable criteria were used to judge the efficacy of the treatments. For example, some workers assessed their results in terms of the increase in weight and volume of the ovary following treatment (Casida et al, 1943), others on the number of follicles present from which ova could be manually aspirated (Umbaugh, 1949), or the number of corpora lutea present as determined by laparotomy, rectal palpation or findings at slaughter (Henricks, Hill, Dickey and Lamond, 1973; Newcomb, Rowson and Trounson, 1976; Baker and Jillella, 1978; Hallford, Turman, Wetteman and Pope, 1979). Yet another measure of the response of the ovaries was the number of fertilised ova recovered from the tract (McGaugh and Olds, 1971; Newcomb, Christie and Rowson, 1976).

However, a consistent feature of this work is the wide variation in the response of individual animals to the treatments. This response may range from a failure of ovarian stimulation, to the shedding of large numbers of eggs (Hafez, Sugie and Gordon, 1963; Scanlon et al, 1968). In addition it is apparent that hyperstimulation of follicular development is not necessarily equated with ovulation (Folley and Malpress, 1944; Dowling, 1949; Elsden et al, 1974). In part these variations in response may be due to the lack of quantitative assay of the biological activity of the preparations used in earlier studies (Gordon, 1975). However, even with standardised preparations variation both amongst and within animals remains. The lack of predictability of response is a drawback in the commercial exploitation of these techniques as a prelude to embryo transfer (Hahn, Traub,

Agthe, Kohn and Lotthammer, 1976).

To permit superovulation and subsequent embryo recovery to be used in assessing oviduct function it would be necessary to have available an independent method for monitoring the ovarian response. This would be essential to confirm that a failure to recover eggs was not due to a lack of ovarian response.

Various techniques have been described to determine the success of superovulation regimes. These comprise direct visualisation of the ovaries, rectal palpation and assay of circulating gonadal steroids. By noting the presence of corpora lutea either at slaughter (Henricks et al, 1973), or after exposing the ovaries by laparotomy (Newcomb et al, 1976) an assessment of the superovulatory response can be obtained retrospectively. As a routine technique, however, this latter method, unless an integral part of embryo recovery is, although accurate, unpractical. In addition it carries the risk of introduction of infection and surgical trauma leading to ovarian and uterine adhesions (Baker and Jillella, 1978). A similar direct visualisation of the ovaries can be achieved by manipulating the ovary through an incision in the anterior vagina (Baker, 1968). Although employed in normal animals this method has not been used in the superovulated cow. Recently the use of a fibre optic laparoscope has been described. This relatively atraumatic technique has been reported to be highly accurate in the hands of an experienced operator (Wishart and Snowball, 1973). This latter technique has the potentially additional benefit of allowing sequential observations to be made of the ovaries. Rectal palpation of the ovaries has been commonly used in assessing the response to superovulation (Newcomb et al, 1978). However, comparison

of palpation and direct visualisation of the ovaries has highlighted potential inaccuracies, especially in the case of a marked response of the ovaries (Rowe, Delcompo, Eilts, French, Winch and Girnther, 1976). Also, the risks associated with manipulation of the ovaries around ovulation in the normal cow are liable to be compounded in the animal with hyperstimulated ovaries.

In general, following superovulation, increasing follicular numbers are associated with elevated plasma oestrogens in the period before ovulation (Saumande and Pelletier, 1975; Booth, Newcomb, Strange, Rowson and Sacher, 1975). Also, several workers have demonstrated that when there is an increase in the number of corpora lutea, this may be apparent from concurrently elevated levels of circulating progesterone (Henricks et al, 1973; Booth et al, 1975; Hallford et al, 1979). However, many conflicting results are found when attempts have been made to relate the levels of circulating gonadal steroids to the response of the ovaries at superovulation (Sreenan, Beehan and Gosling, 1978; Hallford et al, 1979).

Several techniques suitable for the recovery of eggs from the bovine genital tract have been described. Early workers, investigating either the incidence of early embryonic death in infertile animals, or the response of the ovaries to superovulatory drugs, slaughtered the animals at various times after mating (Casida et al, 1943; Tanabe and Casida, 1949). The genital tracts are then flushed with fluid while suspended on a bench and the eggs thus removed. This technique has the advantage of removing eggs from the uterus and the oviduct and so can be used within a short period of mating. Interest

in the use of embryo recovery as a research tool and commercially, stimulated research into methods for obtaining embryos from the living animal. Surgical techniques were developed, using a midline incision, with the animal under general anaesthesia (Rowson, Moor and Lawson, 1969). The uterus and oviducts are exteriorised and the flushing medium injected either from a puncture in the uterus to the oviduct fimbriae or in the opposite direction and collected. Again this technique can be used to obtain oviduct eggs, but repeated embryo recovery from the same animal introduces the danger of adhesions forming (Gordon, 1975). Also, the subsequent fertility of the cow could be adversely affected. The development of non-surgical techniques of embryo recovery constituted an advance in the field of commercial egg transfer. Various modifications have been used since the original work by Rowson and Dowling in 1949. Essentially the technique consists of flushing the uterine horns with media, via an apparatus inserted through the cervix. The washings are collected, either by a to and fro system using a 2-way bulbed catheter, or by continuous circulation using a 3-way bulbed catheter (Greve et al, 1977; Newcomb et al, 1978). Both rigid and flexible apparatus have been used (Baker and Jillella, 1978). This system has the advantage of being relatively atraumatic when used by an experienced operator and repeated flushings can be performed with no adverse effect (Seidel, Elsden, Nelson and Hasler, 1978). However, any eggs which remain in the oviduct will not be recovered.

Additional information on the normality of the oviduct may be gained by examination of eggs or embryos recovered from the uterus. Comparison of the stage of development of the embryo with that expected

from its estimated age may be useful. Although it is difficult in the cow to state precisely the time of ovulation and thus the exact time of egg cleavage, several workers have attempted to define this (Hamilton and Laing, 1946; Austin, 1961). Following fertilisation the first cleavage appears to occur approximately 20-24 hours after ovulation with subsequent cleavages every 19-24 hours. The time of entry into the uterus is 72-84 hours and therefore the embryo at this point is at the 8-16 cell stage. The sequence of events involved in embryonic development has been observed by noting the changes occurring in culture (Trounson, Willadsen and Rowson, 1976). Some changes resembling cleavage have, however, been shown in several species to occur in unfertilised eggs. These changes are associated with disorganisation and degenerative fragmentation of the eggs (Austin, 1961). Overall, these changes differ in certain features from those in fertile eggs and as such may be differentiated from them.

The growth of embryo transfer in cattle has prompted several workers to attempt to define what constitutes a 'normal' embryo. Various criteria have been used. For example, workers have scored embryos on the compactness, symmetry and density of the blastomeres at known stages of development. The validity of the scoring techniques has been assessed by subsequent transfer of the embryos to test cows (Church and Shea, 1976; Shea, Hines, Lightfoot, Ollis and Olson, 1976). As an alternative technique, morphological evaluation of cattle embryos has been related to their subsequent development in the rabbit oviduct (Boland, Crosby and Gordon, 1978). A different approach has been the measurement of the glucose consumption and growth of embryos in

culture systems (Renard, Menezo, Saumande and Heyman, 1978). It has been shown in the rabbit that expansion of the blastocyst is associated with an increase in glucose metabolism (Brinster, 1968).

Detailed changes in the morphology of the developing embryo have been described using phase contrast microscopy. From these studies a classification based on the degree of cellular organisation and the number of cells present was established. Embryos were divided into normal, those in the process of degeneration and those already degenerated (Linares and King, 1980). Subsequent studies using the transmission electron microscope confirmed these classifications with abnormal eggs appearing less well differentiated than normals (Plöen, Linares and Ekwall, 1980).

A means of examining the external structure of the embryos at a greater magnification may reveal functionally significant differences between viable and non-viable eggs. Recently techniques have been described for the examination of bovine blastocysts and developing mouse embryos by means of the scanning electron microscope (Flechon and Renard, 1978; Graham and Lehtonen, 1979; Reeve and Ziomek, 1981). Technical difficulties occur in the processing of eggs for scanning electron microscopy due to the small size of the egg. Reeve et al (1981) describe the use of a technique employing the adhesive qualities of poly-l-lysine, a polycationic substance which adsorbs strongly to solid surfaces. The cationic sites provide a firm attachment for the anionic sites on cell surfaces (Mazia, Schatten and Sale, 1975). Earlier techniques of preparation of tissues for scanning electron microscopy using freeze or air drying

caused artefacts. The use of a critical point drying method has been shown to preserve the overall size, shape and surface topography of embryonic tissues (Waterman, 1972). One major limitation of scanning electron microscopy is that only the surface of the sample can be examined. To overcome this limitation, workers involved in examination of mice embryos have removed the zona pellucida by means of the enzyme pronase (Reeve et al, 1981). A comparable technique is not applicable to the cow as the zona pellucida is resistant to pronase. Although the delicate technique of micromanipulation can be used to remove the zona from fresh cow eggs, this becomes more difficult with fixed eggs (Willadsen and Polge, 1981; Willadsen, 1980, 1981). The use of a reactive oxygen plasma has been described as a means of surface-etching kidney tissue for examination of the ultrastructure of the underlying cell organelles using the scanning electron microscope (Humphreys and Henk, 1979). The use of a similar method to remove successive layers of egg surface may provide more information on what constitutes a 'normal' egg.

The recovery from the uterus of a normal egg at the expected time would be proof of oviduct function.

4.2.1 - SUPEROVULATION AND EGG RECOVERY

The use of superovulation and egg recovery to assess oviduct function entails the choice of firstly, a suitable method of superovulation; secondly, a technique for recovery of the eggs and thirdly, a way of monitoring the ovarian response to the superovulatory drug.

The drug chosen was PMSG since it is readily available as a standardised preparation, is easy to administer requiring a single injection and has been shown to produce a suitable superovulatory response in large numbers of cows (Gordon, 1975).
PMSG is commonly employed in superovulation regimes at a dosage of 3,000 iu (Scanlon et al, 1968; Booth et al, 1975; Newcomb, 1980). However, it may be that the optimum conditions for superovulation are not the most useful for testing oviduct function and the use of a differing dosage regime may be more appropriate. The choice of a non-surgical embryo recovery technique allows repetitive collection of embryos from the uterus. In the cow, the embryo enters the uterus at day 3 or 4 following mating (Hamilton and Laing, 1946). Workers in commercial embryo transfer generally recover these embryos at day 6 to 9, when they will readily transplant into another animal (Lawson, Rowson, Moor and Tervit, 1975). However, since these studies investigate oviduct function, embryo recovery was carried out at this time in some animals, but considerably earlier in others in order to recover eggs as soon as possible after their descent into the uterus. Monitoring the ovarian response comprised a combination of rectal palpation of the ovaries and assay of circulating gonadal steroids, with examination of the ovaries at slaughter checking the

accuracy of these techniques.

The object of this study is to assess if superovulation and embryo recovery is a valid method of assessing oviduct function in the cow. Studies directed towards selection of a superovulatory technique giving the required degree of hyperstimulation and the most appropriate time and method of recovering eggs will be done. The validity of the technique was confirmed by sequential monitoring of ovarian response prior to embryo recovery and subsequent slaughter.

4.2.2 - MATERIALS AND METHODS

Animals:

The cattle used were a mixture of breeds but mainly Ayrshires. All were lactating and with the exception of 1 animal were cows. Rectal palpation and behavioural observations were used to determine the normality of the reproductive tract prior to treatment, and only those animals with no gross genital abnormalities were used. The reproductive history of the cattle was, for the majority, unknown. The animals were kept in an outdoor cattle court, bedded on straw and fed on hay and concentrate. In general, cows were kept in groups of 4 or more. Artificial insemination was carried out by routine techniques using commercially available semen. Cows first observed in oestrus between 7.30 and 13.00 hours were inseminated during the afternoon of that day and again 24 hours later. Insemination of cows first observed in oestrus after 13.00 hours was delayed until the following morning and again repeated 24 hours later. One straw of semen was inseminated on each occasion. Weights and body condition of the cows were determined throughout the experiment.

Drugs:

Pregnant mare serum gonadotrophin (PMSG) (Folligon)	Intervet Lab. Ltd. Science Park, Milton Road, Cambridge.
Human chorionic gonadotrophin) (Chorulon)	Intervet Lab. Ltd.

The above preparations were stored at 4°C and reconstituted with the distilled water supplied by the manufacturer when required. The same batch number (2842) of Folligon was used throughout the experiment.

Dinoprost 5 mg/ml (Lutalyse)	Upjohn Ltd., Crawley, West Sussex.
Hyoscine N-butylbromide Dipyrone (Buscopan Composium)	Boehringer-Ingelheim Ltd., Bracknell, Berkshire.
Lignocaine 2% (Xylocaine)	Astra Chemicals
Acepromazine maleate (Acetylpromazine 10 mg/ml)	C-Vet Ltd., Bury St. Edmunds, Suffolk.
Oxytetracycline hydrochloride (Terramycin Q50 50 mg/ml)	Pfizer Ltd., Sandwich, Kent.

Superovulation regime:

The regime employed for superovulation is shown in the flow diagram. Day 0 was designated day of standing oestrus. PMSG and 'Dinoprost' were injected intramuscularly. 'Chorulon' was injected intravenously.

Day of oestrous cycle

Day 11 ——————— PMSG

Day 13 ——————— Dinoprost (25 mg)

Day 0 ——————— (HCG - 3,000 iu)*
AI

Day 1 ——————— AI

Day 4 + Day 5——— Embryo recovery**

Day 7 ——————— Embryo recovery**

* HCG was administered to a proportion of animals only.

** Embryo recovery was carried out at either of these times.

Embryo recovery:

Equipment

Stainless steel speculum (T.A. Saul, Lincs)

Stainless steel introducer (T.A. Saul, Lincs)

3-way, 14 gauge, 20 ml bulb, embryo collection catheter (Franklin & Sons, High Wycombe)

Catheter connectors - pale blue (T.A. Saul, Lincs)

Catheter stops - dark blue (T.A. Saul, Lincs)

Disposable syringes, 60 ml and 20 ml

100 ml glass boiling tubes

Glass egg collection cups (Camlab)

300-500 ml Dulbecco's phosphate buffered saline (PBS) (Oxoid Ltd.)

Dulbecco's PBS was made up by dissolving 5 tablets (Dulbecco A,

Oxoid Ltd.) in 500 ml of distilled water.

Details of the equipment used are shown in Plate 4.2.1.

Sterilisation of equipment

The stainless steel speculum and introducer, cuffed catheters and syringes were sterilised as described previously. The catheter connectors and stops were rinsed with distilled water and stored in surgical spirit.

The PBS fluid was sterilised using a portable steam steriliser (Thackeray type) at 121°C, 15 lb/sq in for 20 minutes. Immediately before use the fluid was warmed to 37°C in a water bath.

Preparation of the animal

The cow was premedicated using 20 ml Buscopan intramuscularly, and 1.5 ml acepromazine (10 mg/ml) into the tail vein. The rectum was evacuated and the perineal and tail area clipped and cleaned using a dilute solution of Savlon (Cetrimide/chlorhexidine I.C.I.). 5-8 ml Lignocaine (2%) was administered as an epidural anaesthetic. The animal was then placed in a crush raised approximately 25 cm at the front end and restrained by a halter. The tail was tied to the halter, for ease of working and finally the vulva and perineum were wiped with surgical spirit.

Method of recovery of eggs:

A metal speculum was inserted into the vagina as far as the cervix. The centre piece was removed. An introducer was then inserted through the speculum as far as the cervix and the speculum removed. By manipulation per rectum the introducer was passed through

Plate 4.2.1 Equipment used in embryo recovery:

1. Speculum; 2. Introducer; 3. 3-way catheter;

4. 60 ml syringe; 5. Catheter syringe;

6. Catheter connector; 7. Catheter stop.

the cervix. When the tip of the introducer reached beyond the biforcation of the uterus, and was lying in one of the uterine horns, the centrepiece was removed by an assistant while the cannula was held in position, through the rectum, by the operator. The assistant then fed the 3-way catheter along the cannula. The uterine horn at this stage was pulled upwards and backwards to facilitate passage of the catheter to within 5 cm of the uterotubal junction. The cuff of the catheter was inflated using a 20 ml syringe filled with Dulbecco's PBS, until it was considered to have occluded the uterine lumen.

Flushing of the uterine horn was begun, using consecutive 50 ml syringes filled with Dulbecco's PBS. The circulated fluid was collected into boiling tubes. As the first syringe of fluid was pushed through the catheter, the operator gently held the uterotubal junction in order to prevent the fluid flushing the oviduct. With subsequent flushings the uterus was lifted and massaged in order to create some turbulence inside the horns. When a steady flow of flushing fluid was achieved, the assistant commenced injection of the fluid in surges of 20 or 30 ml. After each surge an attempt was made by the operator to remove as much fluid as possible from the uterus by gentle compression and 'milking' of the uterine horn. Finally air was injected through the catheter to remove all fluid. Overall approximately 300 ml of fluid was injected, with a record kept for each cow of the volume injected/volume recovered.

The cuff was deflated and the 3-way catheter removed. The centrepiece of the introducer was replaced and the procedure repeated on the opposite horn, using a second sterile catheter.

Examination of fluid:

The boiling tubes containing the fluid were placed in a warm room and left for at least 30 minutes to allow any particles present to sediment. The gross appearance of the flushing fluid was noted, recording the presence of any blood and debris. The upper 60 ml was then removed using a length of plastic tubing attached to a 60 ml syringe. The remaining fluid was swirled in the tube, then decanted into glass egg collection cups. The cups were examined under the stereomicroscope and the number of eggs recovered was recorded. It was noted which eggs were fertilised, and the stage, by examining the number of cleavages and blastomeres which were present (Austin, 1961).

Monitoring response to superovulation:

1. Behaviour. All animals were marked using tail paint (Telltail:I.C.I) and observed at regular intervals from 7.30 am until 5.30 pm daily. The amount of paint removed from the tailhead was recorded. At times where an individual cow was expected in oestrus, an additional period of observation was carried out at 10.30-11.00 pm. Oestrus was considered to be when the cow stood to be mounted. Day of oestrus was designated day 0.

2. Rectal palpation of the reproductive tract. Rectal palpation was carried out on the day of PMSG administration and again on the day of flushing. This varied as to whether embryo recovery was carried out on day 4, 5 or 7 following oestrus. Rectal palpation was then continued on every second day until either the animal was slaughtered, or a second superovulation programme was commenced.

At rectal palpation, the tone of the uterus was recorded on a scale ranging from 0- (no tone) to 3- (highly toned). A drawing of the size and shape of the ovaries was made and the surface structures marked on the sketch. Structures were classified as follicles or corpora lutea following the description of Zemjanis (1970). However, any firm structure with no palpable ovulatory papillum was not regarded as a corpus luteum.

3. Peripheral plasma progesterone and oestrogen concentrations

Blood samples were taken from the animals daily between 11.00 a.m and 12.00 p.m. A 20 gauge, $1\frac{1}{2}$" vacutainer needle was inserted into the jugular vein and 20 ml of blood withdrawn into heparinised vacutainer tubes. The blood was centrifuged within 2 hours of collection and the plasma stored in glass vials at $-20^{\circ}C$ until required for analysis.

Plasma progesterone levels were determined from the day of PMSG injection, daily until the time of slaughter. In some animals plasma oestrogens were determined from 5 days prior to the day of oestrus, until 5 days following oestrus. Assays were performed using established radioimmunoassay methods (Abraham, Swerdloff, Tulchinsky and Odell, 1971; Hotchkiss, Atkinson and Knobil, 1971). The plasma sample volume used ranged from 50-200 µl for progesterone and 4 ml for oestrogens. For the most part extraction was done using pronalysis ether (May & Baker) but towards the end of the experiment petroleum spirit (40:60 : BDH) was used with no effect on results. The antibody used was Y29/6 for progesterone with major cross reactivities to 11α-hydroxy-progesterone (71.4%), deoxycorticosterone (11.1%) and 5β-pregnone-3,20-dione (8.8%), and for oestrogen 1802 with major cross reactivities 17β-oestradiol (100%), oestrone (0.7%), 17β-oestradiol-6-oxo (44%).

The sensitivity of the assay and the inter- and intra-assay coefficients of variation were 0.2 nm/l, 11.4%, 9.1% and 18.4 pm/l, 17.4%, 13.2% for progesterone and oestrogen respectively.

4. Post-mortem examination. The majority of the animals were slaughtered soon after their last superovulation treatment in order to examine the structures present on the ovaries. The day of slaughter varied between day 8 and 10 following embryo recovery on day 4, 5 or 7, to enable blood samples for gonadal steroid levels to be taken over a longer period, but while the structures on the ovary were still compatible with those present on the day of embryo recovery. Passage of dye or pollen grains following the techniques outlined in Chapter 2 was carried out on the tracts. Sections were taken from a number of ovarian structures. These were processed for histological examination using the fixation and staining techniques described previously (Chapter 2, page 29).

Using these techniques, the following studies were carried out to determine the number of eggs presented to the oviduct, and the numbers recovered from the uterus, following:

Experiment 1. A standard dose of PMSG.

Five cows were injected with 3,000 iu PMSG. Embryo recovery was carried out on day 7, and 3 of the animals were subsequently slaughtered between days 11 and 15.

Experiment 2. A single low dose of PMSG

Six cows were injected with 1,500 iu PMSG. Two of the animals were injected with 3,000 iu HCG at the time of the first A.I. Embryo recovery was carried out on days 4 and 5.

Experiment 3. Repeated low doses of PMSG

Four of the cows used in Experiment 2 were reinjected with a further 1,500 iu PMSG. The repeated injection was given 35-53 days after the first and followed the cows returning to oestrus spontaneously or following a double injection of prostaglandin. Two of the animals were injected with 3,000 iu HCG at the time of the first A.I. Embryo recovery was carried out on days 4 and 5. Two animals were slaughtered between days 8 and 10.

Experiment 4. Increasing doses of PMSG

Two of the cows used in Experiment 2 were reinjected with 3,000 iu PMSG. The second injection timing and subsequent treatment were as detailed for Experiment 3, but with neither cow receiving HCG.

4.2.3 - RESULTS

Experiment 1. Embryo recovery and ovarian response following a standard dose of PMSG:

All 5 cows were confirmed by rectal palpation as having a mature corpus luteum (CL) at the time of PMSG administration. Following prostaglandin (PG) injection all animals showed oestrus 3 days later. All of the cows were no longer in oestrus by day 4. The results of rectal palpation of the ovaries immediately prior to flushing (day 7) are detailed in Table 4.2.1. Marked variation occurred in the number of corpora lutea palpated. In cow 2 the left ovary contained a number of smooth, firm structures fulfilling neither the criteria for corpora lutea or follicles. With the exception of this animal, a similar response was found in individual ovaries within cows. Results of embryo recovery are also presented in Table 4.2.1. Overall, there

TABLE 4.2.1 RESPONSE OF 5 COWS TO ADMINISTRATION OF 3,000 iu PMSG

Cow	Ovarian Palpation		Embryos Recovered		In vitro Dye Passage		Slaughter Findings	
	Left Ovary	Right Ovary	Left Horn	Right Horn	Left Horn	Right Horn	Left Ovary	Right Ovary
1	1 CL 1 F	2 CL 1 F	1	0	Patent	Patent	1 CL 1 F	1 CL 2 F
2	8 ?	3 CL	0	0	Patent	Patent	2 CL 8 L 1 F 2 LF	3 CL
3	8 CL	7 CL	0	0	Patent	Patent	13 CL 2 F	8 CL 4 F
4	2 CL 1 F	4 CL 1 F	0	1	Patent	Patent	ND	ND
5	8 CL	7 CL	0	7	Blocked	Patent	ND	ND

Note:
CL Corpus luteum
F Follicle
L Luteinised structures
LF Luteinised follicles
? Not possible to identify structures
ND Not determined

were markedly fewer embryos recovered than was expected from the number of corpora lutea palpated. The egg recovered from cow 1 was infertile. All others were fertilised. Comparison of the ovarian structures found at slaughter, with those detected at rectal palpation, are shown for 3 animals (Table 4.2.1). Inaccuracies were apparent in the appreciation per rectum of both numbers and type of structures. The structures palpated in the left ovary of cow 2 ranged in size from 8-18 mm; were red or buff in colour; mainly soft in consistency; had a smooth surface and no evidence of an ovulatory papillum (Plate 4.2.2). On section, some contained a central lacuna, whereas others were solid throughout. On histological examination, lutein cells were identified (Plate 4.2.3). On the basis of gross appearance and histological examination, these were designated luteinised structures. Structures were also present which were smooth, thin-walled and contained follicular fluid. There was evidence of luteal tissue present on the follicular wall. These were designated luteinised follicles (Plate 4.2.2).

With the exception of the left oviduct of cow 5, all oviducts were patent to passage of dye (Table 4.2.1). Failure to recover embryos from 6 uterine horns was neither associated with a lack of ovarian response nor with blockage of the oviduct.

Peripheral plasma progesterone levels for these 5 animals, from day of PMSG administration to day 7, are illustrated in Fig. 4.2.1. In general, the administration of prostaglandin caused a marked reduction to basal levels. The subsequent pattern of plasma progesterone varied markedly amongst animals. In 2 cows (3, 5),

Plate 4.2.2 Left ovary of Cow 2, showing:

1. Corpus luteum; 2. Follicle; 3. Luteinised follicle;

4. Luteinised structure.

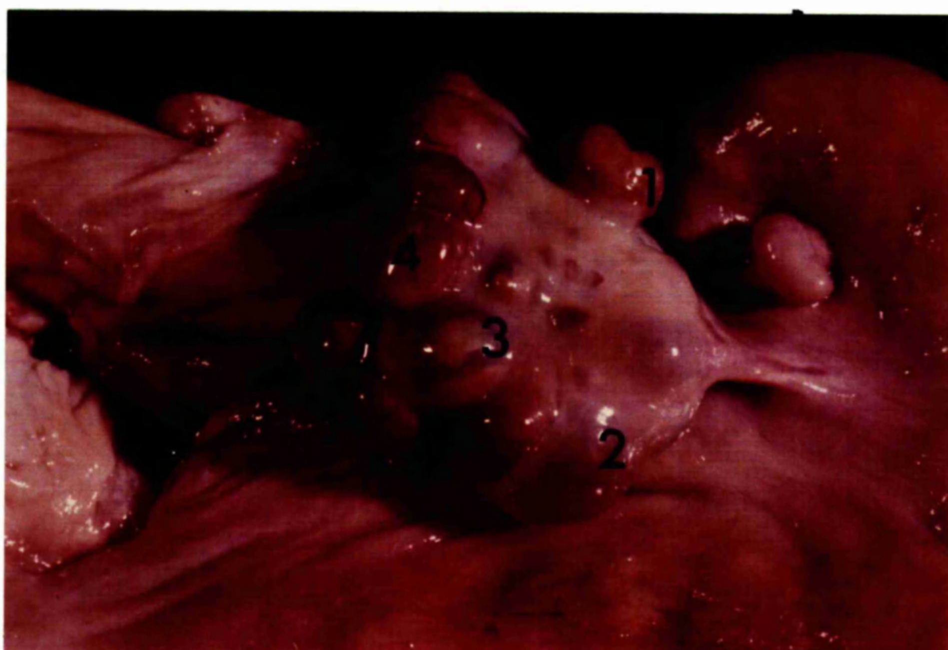


Plate 4.2.3 Section of luteinised structure from left ovary (Cow 2), showing lutein cells

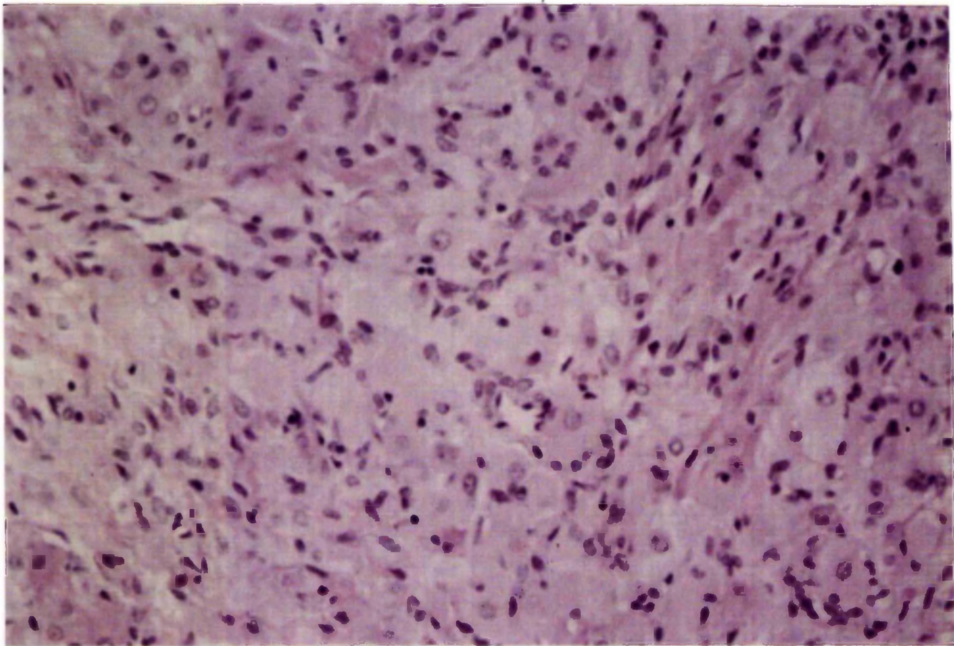
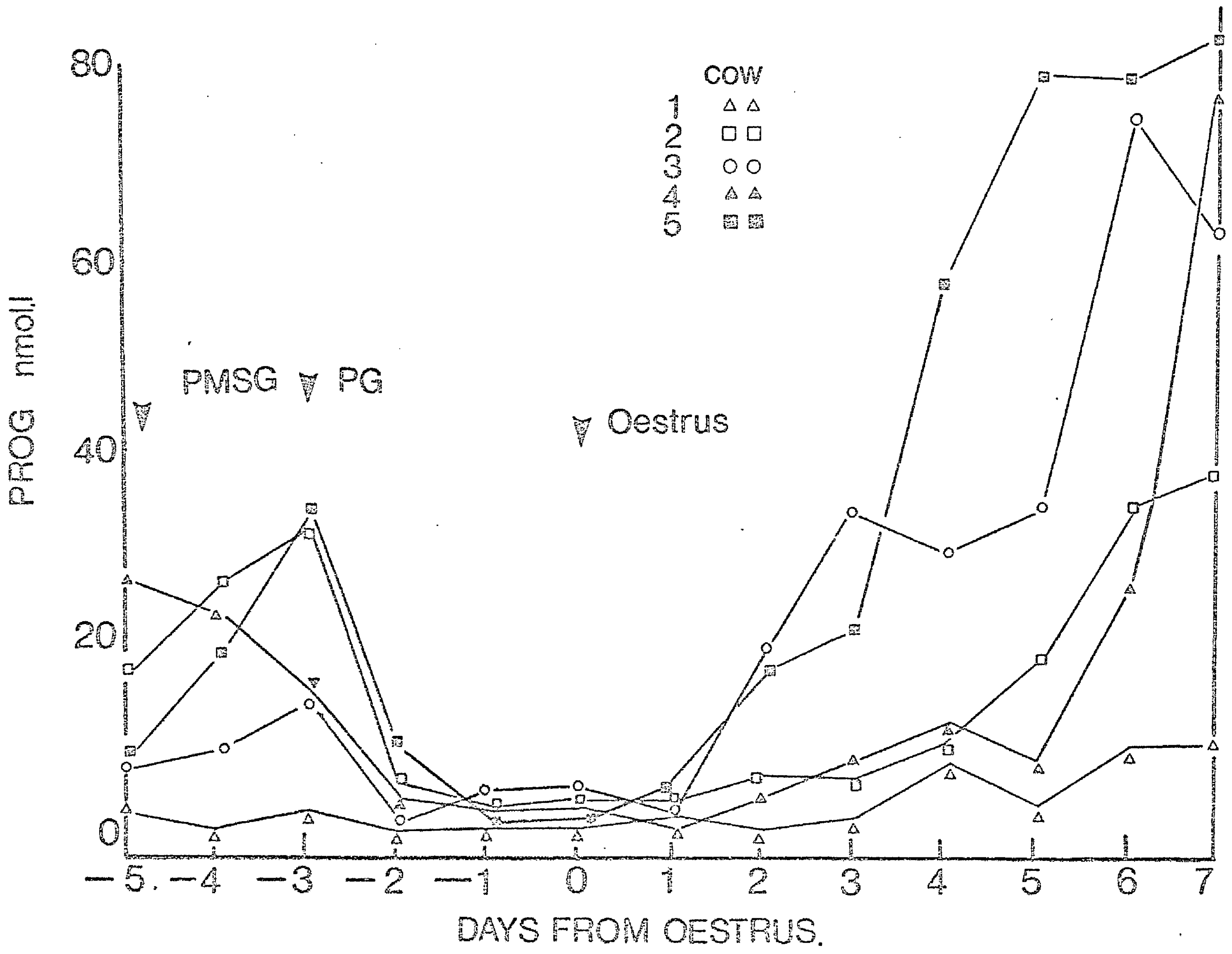


FIGURE 4.2.1 PLASMA PROGESTERONE PROFILES OF 5 COWS FOLLOWING ADMINISTRATION OF 3,000 iu PMSG



PROG nmol.l
80
60
40
20
0
COW
1
2
3
4
5
PMSG
PG
Oestrus
− 5. −4 −3 −2 −1 0 1 2 3 4 5 6 7
DAYS FROM OESTRUS.

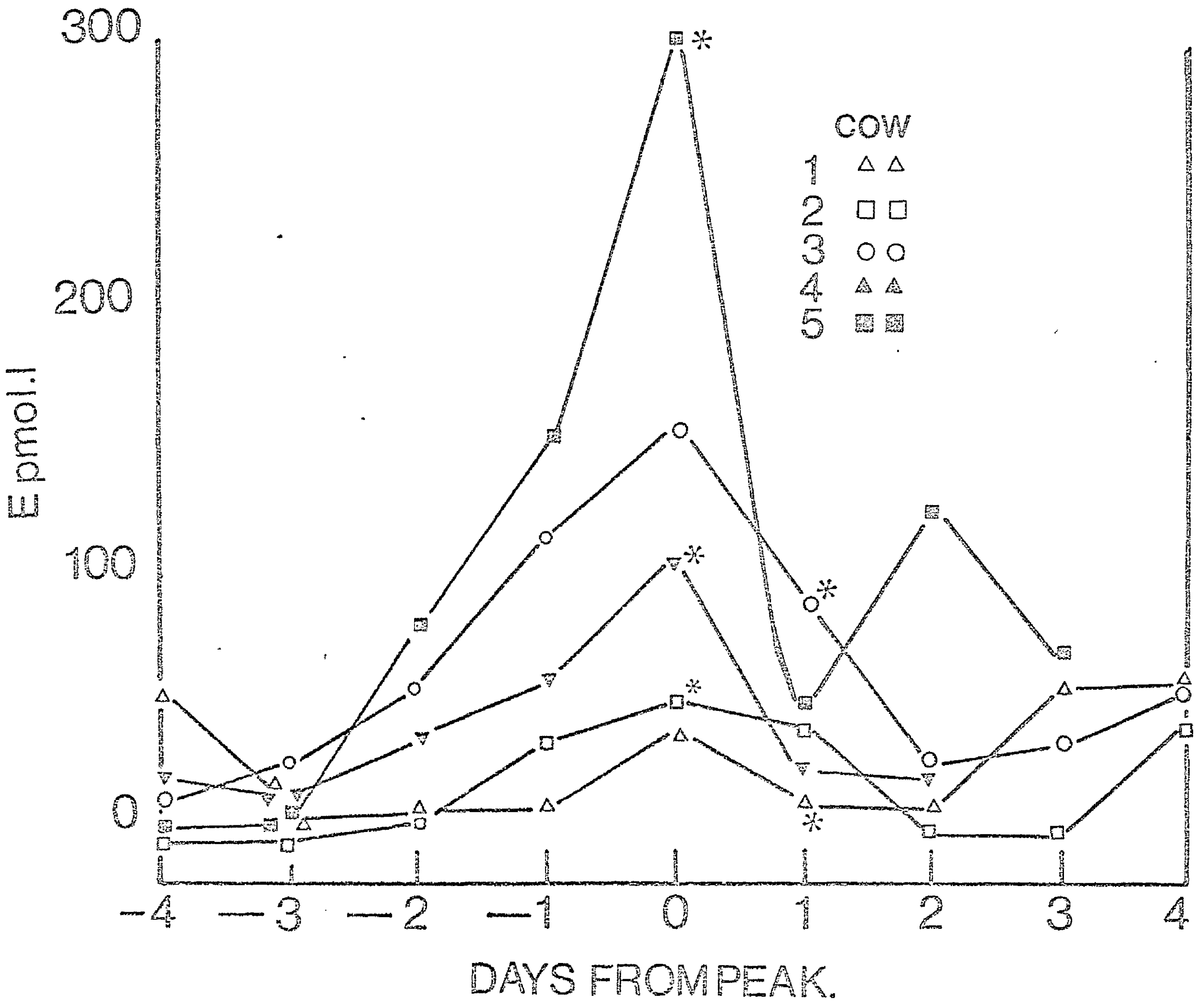
there was a rapid increase from day 2. A further 2 (2, 4) showed a more gradual and delayed increase. In 1 cow (1) plasma progesterone levels were already basal at the time of PMSG administration and showed no evidence of a consistently increasing pattern after oestrus.

The pattern of progesterone increase appeared related to the number of CL palpated in the ovaries, with the most marked increase taking place in cows with the greatest number of CL (3, 5). Plasma progesterone levels at day 7 were loosely related to the number of CL palpated in the ovaries, with the smallest number of CL associated with the lowest levels and the highest number with the greatest levels. In the 3 animals that were slaughtered (1, 2, 3), the direct relationship between CL numbers and plasma progesterone concentrations was maintained. In cow 2, with 8 luteinised structures present at slaughter, the plasma progesterone levels remained relatively low compared to cows 3, 4 and 5, but considerably higher than in the remaining animal.

Within individual animals, the peak plasma concentration of oestrogens was reached 4 to 5 days after PMSG administration. These peaks occurred either the day before or the day of oestrus. Peripheral plasma oestrogen levels for the individual animals are illustrated in Fig. 4.2.2. These results are presented as the levels for each day before and after the peak concentration. Oestrogens increased over a period of 1-3 days. A ten-fold difference was found in the peak levels amongst individual cows (5 v 1). The highest peak concentrations occurred in cows 3 and 5, in which the greatest number of CL were subsequently palpated. Conversely, cow 1, which according to results obtained at slaughter had only 2 CL, had the

FIGURE 4.2.2 PLASMA OESTROGEN PROFILES OF 5 COWS FOLLOWING ADMINISTRATION OF 3,000 iu PMSG. PMSG WAS ADMINISTERED AT DAY -5 OR -4.

* - Denotes the day of oestrus.


COW
1
2
3
4
5
E pmol.l
300
200
100
0
−4
−3
−2
−1
0
1
2
3
4
DAYS FROMPEAK.

lowest peak of plasma oestrogens. In all cases the peak concentrations were followed by a return to basal levels within 1-2 days.

Experiment 2. Embryo recovery and ovarian response following a single low dose of PMSG:

All 6 cows were confirmed by rectal palpation as having a mature CL at the time of PMSG administration. With the exception of cow 8, all animals showed oestrous behaviour 2 days following prostaglandin injection. Cow 8 showed no signs of oestrus at any time and was not inseminated. The results of rectal palpation of the ovaries immediately prior to the first flushing (day 4) are shown in Table 4.2.2. There was a wide variation amongst cows in the response to the same dose of PMSG. However, within cows, similar responses were found in each ovary. Cow 11 apparently failed to respond to the drug. On rectal palpation, both ovaries of cow 10 contained an undefinable number of fluid filled structures. Overall ovarian dimensions were 6 x 4 x 3 cm and 7.5 x 5 x 3 cm. These ovaries were considered to contain either large numbers of follicles or cysts. Among other cows, the numbers of CL palpated varied from 18 in cow 8 to 5 in cow 6. In the majority of cases, fewer embryos were recovered than CL palpated. Eight infertile eggs were recovered from cow 8. All other embryos collected were fertilised and ranged from the 4-cell to the 16-cell stage. Reflushing on day 5 yielded no further embryos.

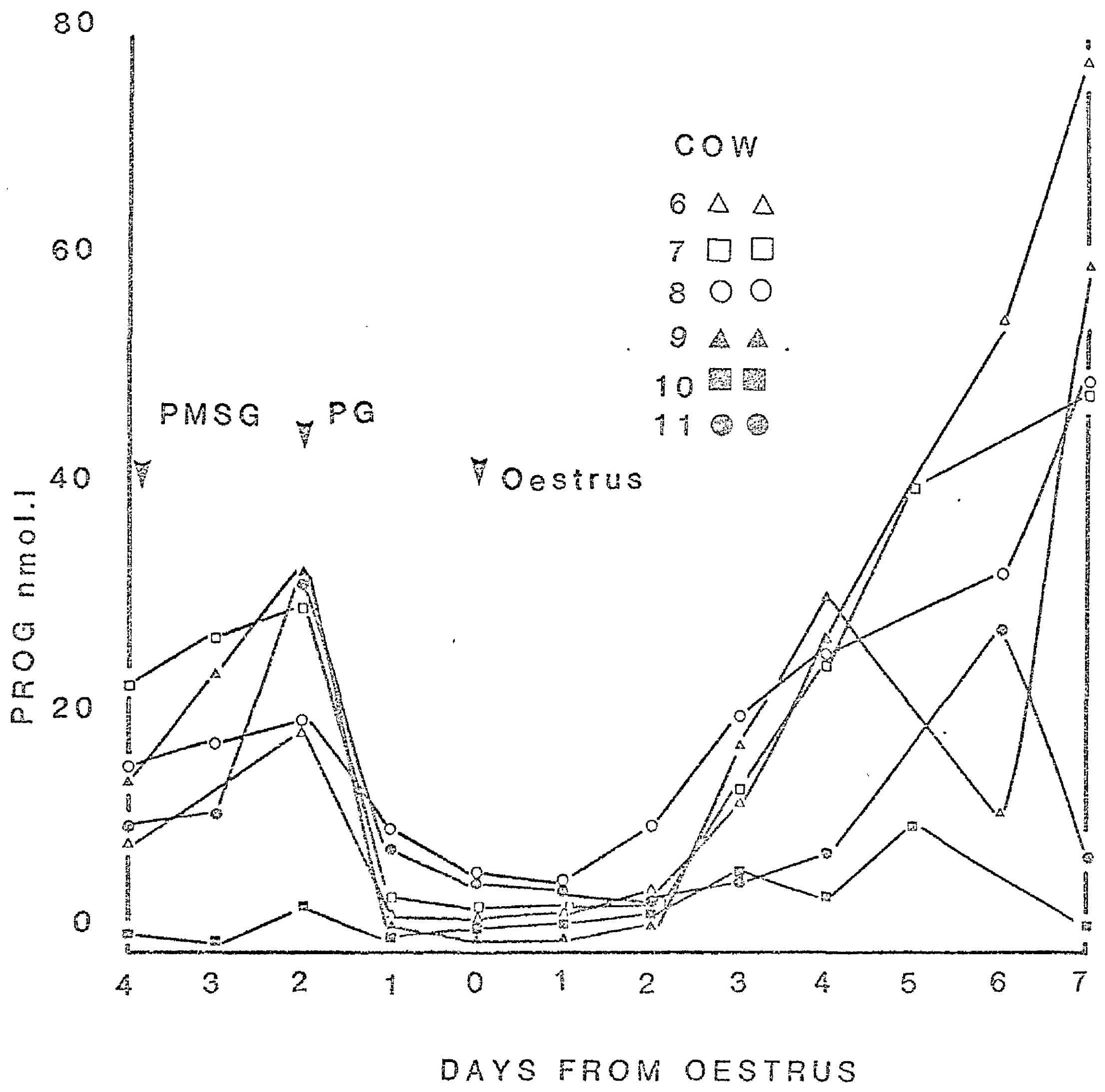
Peripheral plasma progesterone levels for these animals, from day of PMSG administration to day 7, are illustrated in Fig. 4.2.3. In general, the administration of prostaglandin caused a marked reduction to basal levels. Thereafter levels remained low throughout oestrus.

TABLE 4.2.2 RESPONSE OF 4 COWS TO ADMINISTRATION OF 1,500 iu PMSG AND OF 2 COWS TO 1,500 iu PMSG AND 3,000 iu HCG

Cow	HCG (3,000 iu) Administration	Ovarian Palpation		Embryos Recovered Day 4		Embryos Recovered Day 5	
		Left Ovary	Right Ovary	Left Horn	Right Horn	Left Horn	Right Horn
6	+	2 CL	3 CL 2 F	0	0	0	0
7	+	4 CL 1 F	5 CL 2 F	5	1	0	0
8	-	9 CL	9 CL	8	0	0	0
9	-	4 CL	4 CL	0	0	0	0
10	-	Multiple F/cysts	Multiple F/cysts	0	0	0	0
11	-	1 CL 1 F	NPS	0	0	0	0

Note: CL Corpus luteum F Follicle NPS No palpable structures

FIGURE 4.2.3 PLASMA PROGESTERONE PROFILES OF 6 COWS FOLLOWING ADMINISTRATION OF 1,500 iu PMSG


PMSG
PG
Oestrus
COW
6
7
8
9
10
11
PROG nmol.l
80
60
40
20
0
4
3
2
1
0
1
2
3
4
5
6
7
DAYS FROM OESTRUS

Marked differences amongst animals occurred in the time and pattern of increase. Cow 10 did not show any consistent pattern of increase throughout the period of study. In the other animals, the time progesterone started to increase from oestrus varied from day 2 to day 5. Dividing the cows into 2 groups - those with a minimal response (10, 11) and those that responded well (6, 7, 8, 9), cows in the second group showed the most consistent rate of increase. The cows with the maximum progesterone levels at day 7 were the ones in which the greatest numbers of CL were palpated (6, 7, 8, 9). However, amongst animals with high progesterone concentrations at day 7, there was no clear relationship between CL numbers and plasma progesterone levels.

The administration of HCG to 2 animals (6, 7) was neither associated with an increase in the number of CL nor a characteristic alteration in the pattern of plasma progesterone.

Experiment 3. Embryo recovery and ovarian response following repeated low doses of PMSG:

All 4 cows were confirmed by rectal palpation as having a mature CL at the time of the second PMSG administration. Following prostaglandin injection at day 13, 3 animals were observed in oestrus 2 days later (6, 7, 9). Cow 8 showed no signs of standing oestrus at this time and was not inseminated. The results of rectal palpation of the ovaries immediately prior to the second flushing (day 4) are shown in Table 4.2.3. Amongst cows, differences were apparent in the numbers of CL detected, ranging from 10 CL in cow 9, to 2 CL in cow 8. In all animals, a similar response was found in individual ovaries

TABLE 4.2.3 RESPONSE OF 4 COWS TO 2 SUCCESSIVE INJECTIONS OF 1,500 iu PMSG, WITH AND WITHOUT HCG ADMINISTRATION

Cow	HCG Administration	Ovarian Palpation		Embryos Recovered Day 4		Embryos Recovered Day 5		Slaughter Findings	
		Left Ovary	Right Ovary	LH	RH	LH	RH	Left Ovary	Right Ovary
6	+	1 CL 2 F	1 CL 4 F	1	0	0	0	-	-
		(2 CL)	(3 CL 2 F)	(0	0)	(0	0)		
7	+	5 CL	4 CL	3	0	0	0	-	-
		(4 CL 1 F)	(5 CL 2 F)	(5	1)	(0	0)		
8	-	3 CL 4 F	2 CL 4 F	0	0	0	0	1 CL 4F	5 F
		(9 CL)	(9 CL)	(8	0)	(0	0)		
9	-	5 CL	5 CL	2	6	0	0	6 CL 2 F	6 CL 2 F 2 LF
		(4 CL)	(4 CL)	(0	0)	(0	0)		

Note: All oviducts were patent to fluid and pollen at slaughter

Results of previous administration of PMSG in brackets

CL Corpus luteum

F Follicel

LF Luteinised follicle

within cows. Results of embryo recovery are also detailed in Table 4.2.3. In the majority of cases, fewer embryos were collected than CL palpated. All embryos recovered were fertilised and ranged from the 4-cell to the 16-cell stage. Reflushing on day 5 yielded no further embryos. Comparison of the ovarian structures found at slaughter with those detected at rectal palpation are shown for 2 animals (Table 4.2.3). The remaining 2 animals (6, 7) were slaughtered 2 and 3 months later and so comparisons are not possible. In cows 8 and 9, inaccuracies were apparent in the appreciation per rectum of both numbers and types of structures. From observation of the ovarian structures at slaughter, cow 8 apparently responded with only 1 ovulation to the second administration of PMSG.

All oviducts were patent to passage of dye and to a suspension of pollen grains (Table 4.2.3). Failure to recover embryos from 7 uterine horns was neither associated with a lack of ovarian response nor with blockage of the oviduct.

Results of ovarian palpation of structures present following the first administration of PMSG are shown in brackets (Table 4.2.3). With the exception of cow 8, there is a similar response to the first and second treatments. Cow 8 shows a marked reduction in the response to the second PMSG injection compared to the first (1 CL v 18 CL).

Peripheral plasma progesterone levels for cows 6, 7, 8 and 9, from the time of first PMSG administration to day 7 following the second flushing, are shown individually in Figs. 4.2.4, 4.2.5, 4.2.6 and 4.2.7 respectively. The results of the first treatment have been given previously (Fig. 4.2.3). Prostaglandin, given as a

FIGURE 4.2.4 PLASMA PROGESTERONE PROFILES OF COW 6 FOLLOWING REPEATED LOW DOSES OF PMSG

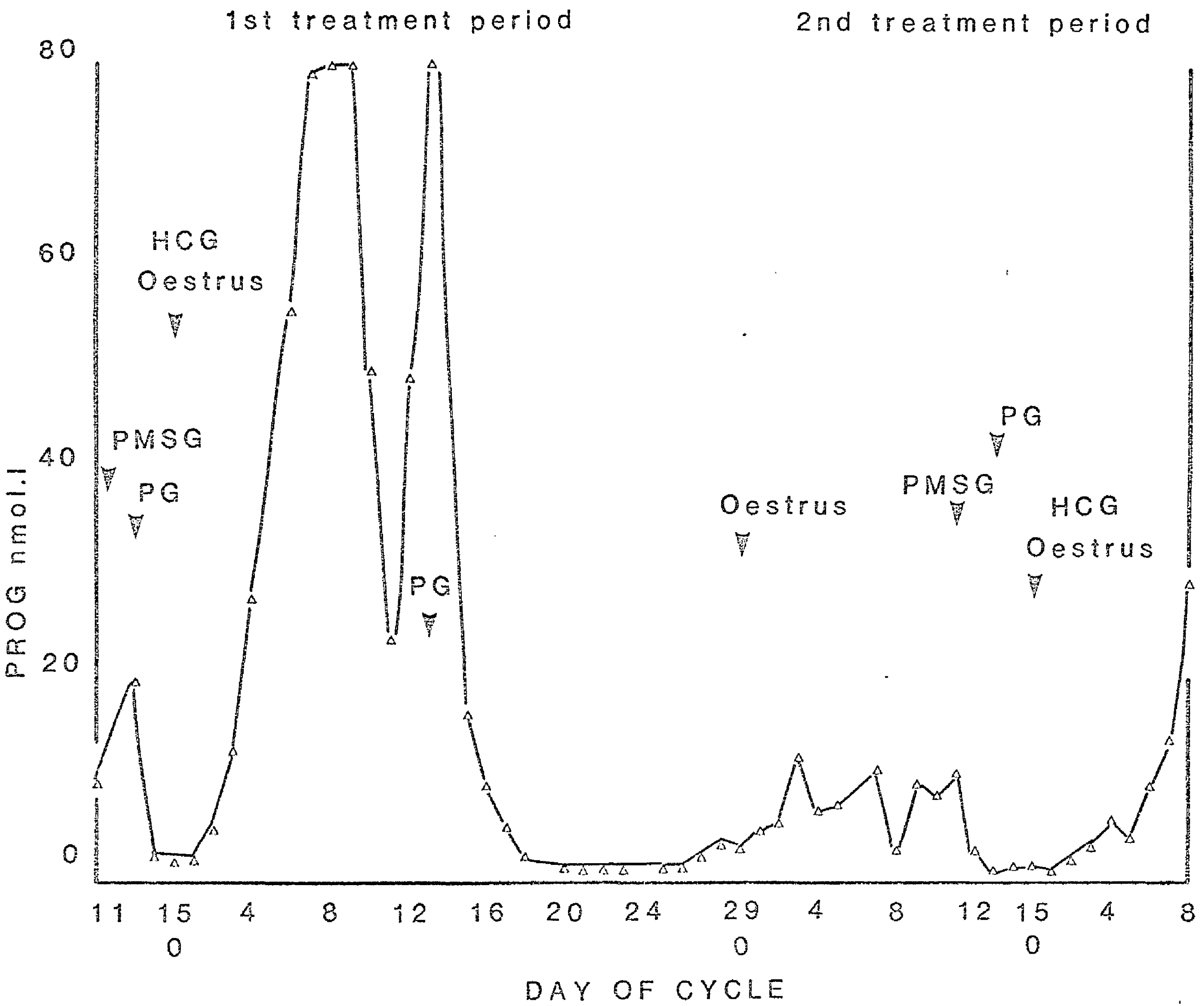

1st treatment period
2nd treatment period
HCG
Oestrus
PMSG
PG
PG
Oestrus
PMSG
PG
HCG
Oestrus
PROG nmol.l
80
60
40
20
0
11
15
0
4
8
12
16
20
24
29
0
4
8
12
15
0
4
8
DAY OF CYCLE

FIGURE 4.2.5 PLASMA PROGESTERONE PROFILES OF COW 7 FOLLOWING REPEATED LOW DOSES OF PMSG

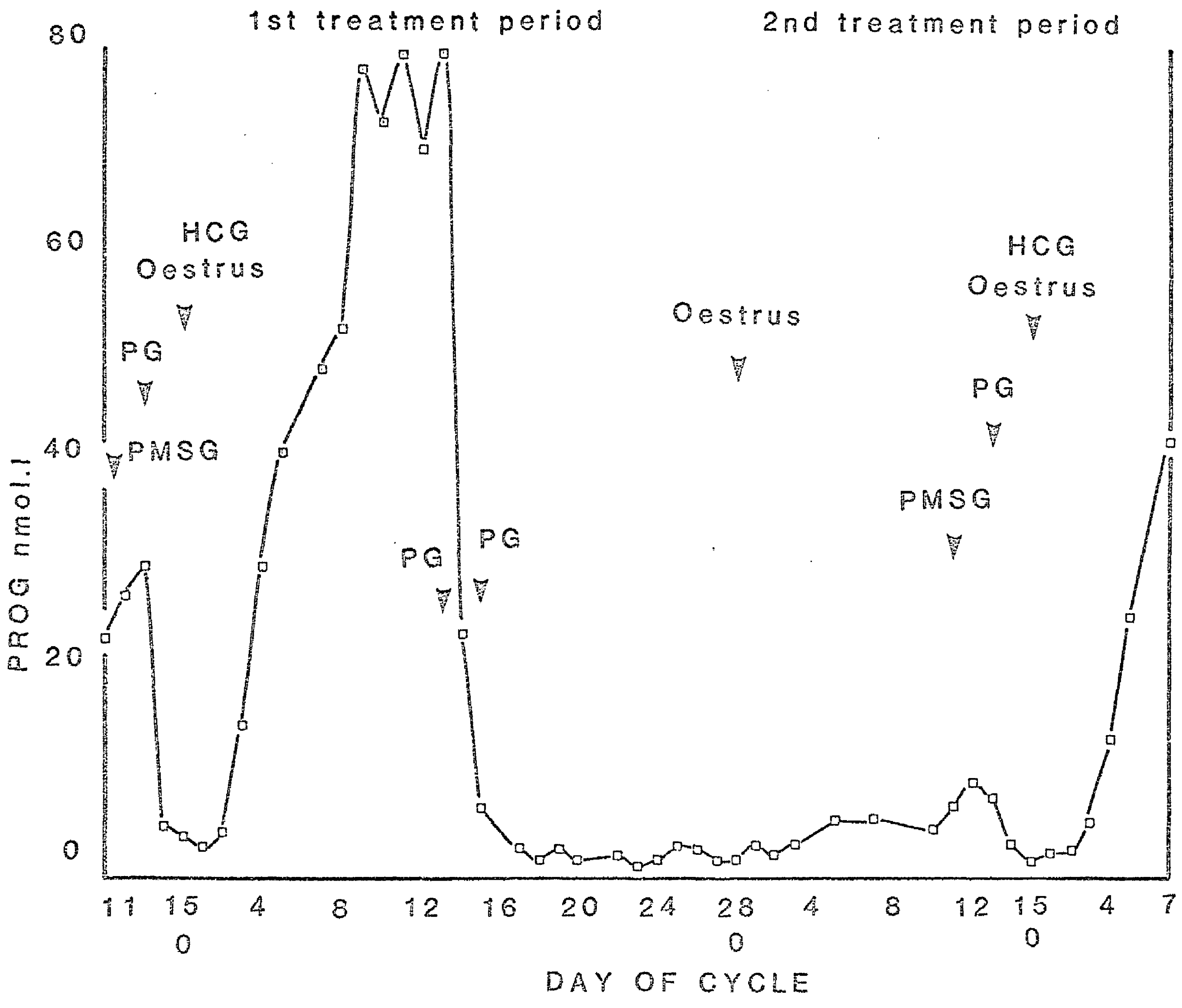

1st treatment period
2nd treatment period
PROG nmol.l
80
60
40
20
0
HCG
Oestrus
PG
PMSG
PG
PG
Oestrus
HCG
Oestrus
PG
PMSG
11
15
0
4
8
12
16
20
24
28
0
4
8
12
15
0
4
7
DAY OF CYCLE

FIGURE 4.2.6 PLASMA PROGESTERONE PROFILES OF COW 8 FOLLOWING REPEATED LOW DOSES OF PMSG.

* - THIS DAY DESIGNATED AS DAY TO ALLOW COMPARISON WITH THE OTHER COWS IN THE GROUP, ALTHOUGH THE COW WAS NOT OBSERVED IN OESTRUS AT THIS TIME.

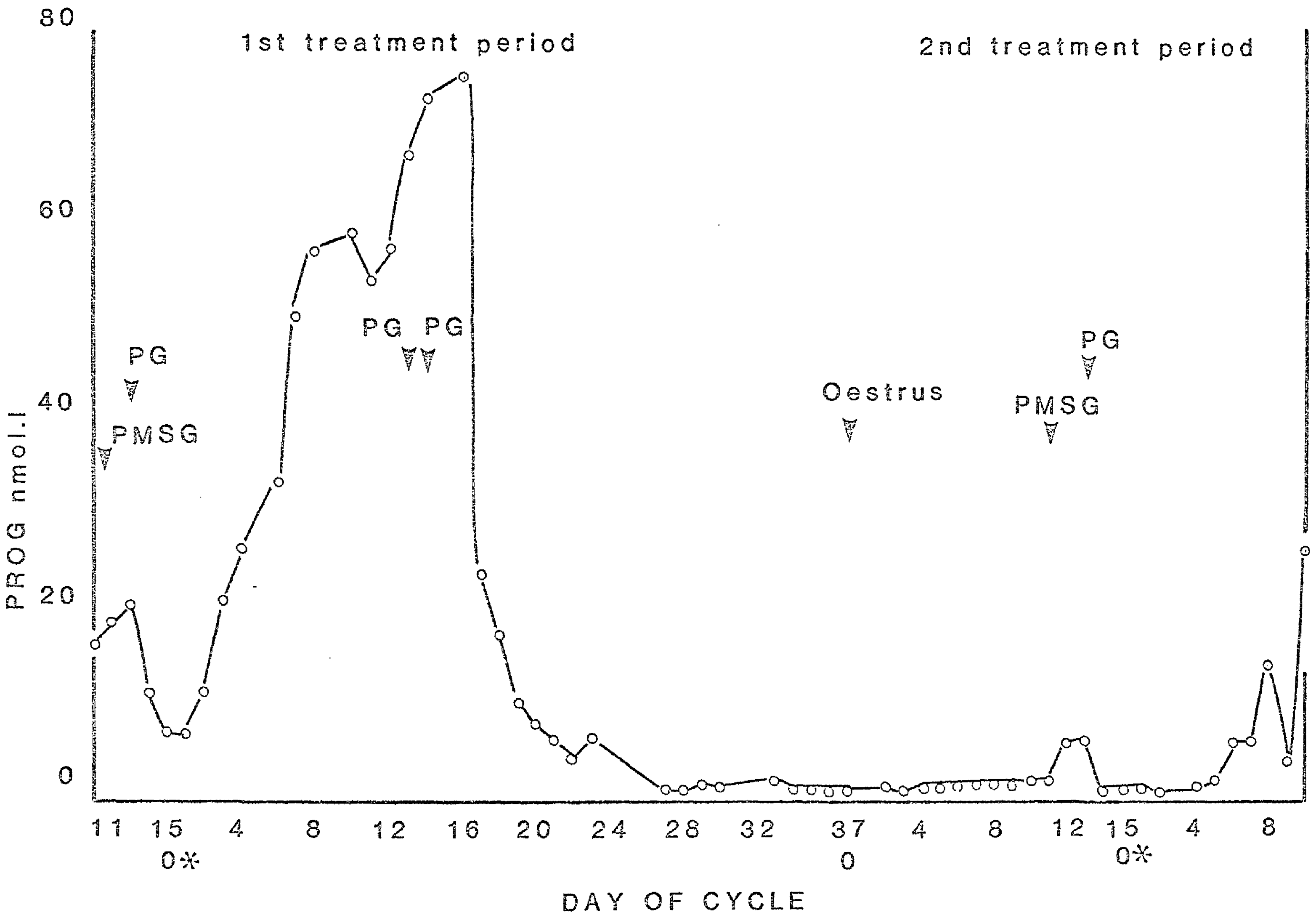
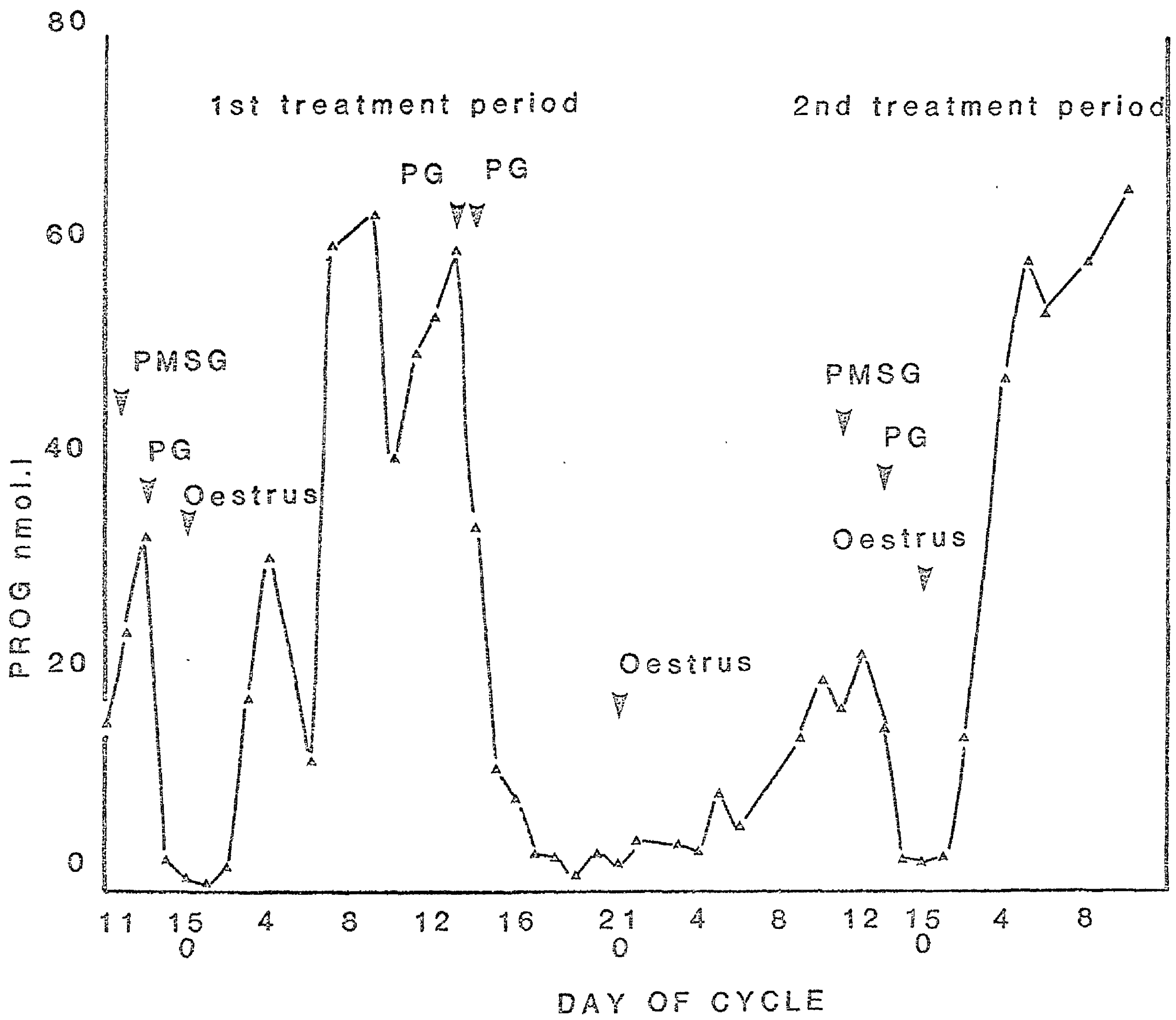

1st treatment period
2nd treatment period
PG
PMSG
PG PG
Oestrus
PG
PMSG
PROG nmol.l
80
60
40
20
0
11 15 4 8 12 16 20 24 28 32 37 4 8 12 15 4 8
0* 0 0*
DAY OF CYCLE

FIGURE 4.2.7 PLASMA PROGESTERONE PROFILES OF COW 9 FOLLOWING REPEATED LOW DOSES OF PMSG


1st treatment period
2nd treatment period
PMSG
PG
Oestrus
PG
PG
Oestrus
PMSG
PG
Oestrus
80
60
40
20
0
PROG nmol.l
11
15
0
4
8
12
16
21
0
4
8
12
15
0
4
8
DAY OF CYCLE

double dose in 3 animals (7, 8, 9) and as a single dose in the remaining cow (6), at day 13 or 14 following the first flushing, in all cases caused a marked decrease in plasma progesterone levels. This was not reflected in the occurrence of oestrous behaviour, until day 29 in cow 6, day 28 in cow 7, day 37 in cow 8 and day 21 in cow 9. For the second treatment period, PMSG was administered at day 11 following this oestrus, followed by PG 2 days later. In 3 cows (8, 9, 6) progesterone levels were already decreasing prior to administration of PG. In the remaining cow (7) the administration of PG caused a marked reduction to basal levels. Thereafter, levels remained low throughout oestrus. Marked differences amongst animals occurred in the time and pattern of increase of progesterone. The time progesterone started to increase varied from day 2 to day 5. There was no clear relationship between CL numbers and plasma progesterone levels at day 7.

The administration of HCG to 2 cows (6, 7) was neither associated with an increase in the number of CL nor a characteristic alteration in the pattern of plasma progesterone.

Experiment 4. Embryo recovery and ovarian response following increasing doses of PMSG:

Both cows were confirmed by rectal palpation as having a mature CL at the time of the second PMSG injection. Following PG injection at day 13, both cows were observed in oestrus 2 days later. The results of rectal palpation of the ovaries immediately prior to the second flushing (day 4) are shown in Table 4.2.4. The findings in the 2 cows are markedly different. The ovaries of cow 10 apparently contained a number of fluid filled multiple follicles

TABLE 4.2.4 RESPONSE OF 2 COWS TO 3,000 iu OF PMSG FOLLOWING A PREVIOUS DOSE OF 1,500 iu PMSG

Cow	Ovarian Palpation		Embryos Recovered				Slaughter Findings	
			Day 4		Day 5			
	Left Ovary	Right Ovary	LH	RH	LH	RH	Left Ovary	Right Ovary
10	Multiple F/cysts	Multiple F/cysts	0	0	0	0	Multiple F/cysts	Multiple F/cysts
	(Multiple F/cysts)	(Multiple F/cysts)	(0	0)	(0	0)		
11	2 CL 2 F	1 CL 2 F	0	0	2	0	5 CL 10 F 1LF	6 CL 11 F
	(1 CL 1 F)	(NPS)	(0	0)	(0	0)		

Note: All oviducts were patent to fluid and pollen at slaughter

Results of previous administration (1,500 iu) PMSG in brackets

CL Corpus luteum

F Follicle

LF Luteinised follicle

NPS No palpable structures

as described previously (Experiment 3). Cow 11 had 3 CL present on the ovaries. A similar response was found in the individual ovaries of each cow. Results of embryo recovery are also detailed in Table 4.2.4. No embryos were recovered from either cow at day 4, with 2 infertile eggs collected from cow 11 at the repeat flushing on day 5. Comparison of the ovarian structures found at slaughter with those detected at rectal palpation are also detailed in Table 4.2.4. The structures observed on the ovaries of cow 10 were as expected from rectal palpation (Plate 4.2.4). In cow 11, marked inaccuracies were apparent in the appreciation per rectum of both numbers and types of structures. The luteinised follicle found on the left ovary was similar to that described in Experiment 1, cow 2.

All oviducts were patent to passage of dye and to a suspension of pollen grains (Table 4.2.4). A failure to recover the expected number of embryos from cow 11 was not associated with either a lack of ovarian response or with blockage of the oviduct.

Results of ovarian palpation of structures present following the first administration of PMSG are shown in brackets (Table 4.2.4). The increased dosage produced a similar response in cow 10. A marked increase in the response to the increased dose occurred in cow 11.

Peripheral plasma progesterone levels for cows 10 and 11, from the time of first PMSG administration to day 7 following the second flushing, are shown individually in Figs. 4.2.8 and 4.2.9 respectively. The results of the first treatment have also been included (Fig. 4.2.3). The double injection of FG administered to

Plate 4.2.4 Left ovary of Cow 10, showing multiple follicles/ cysts

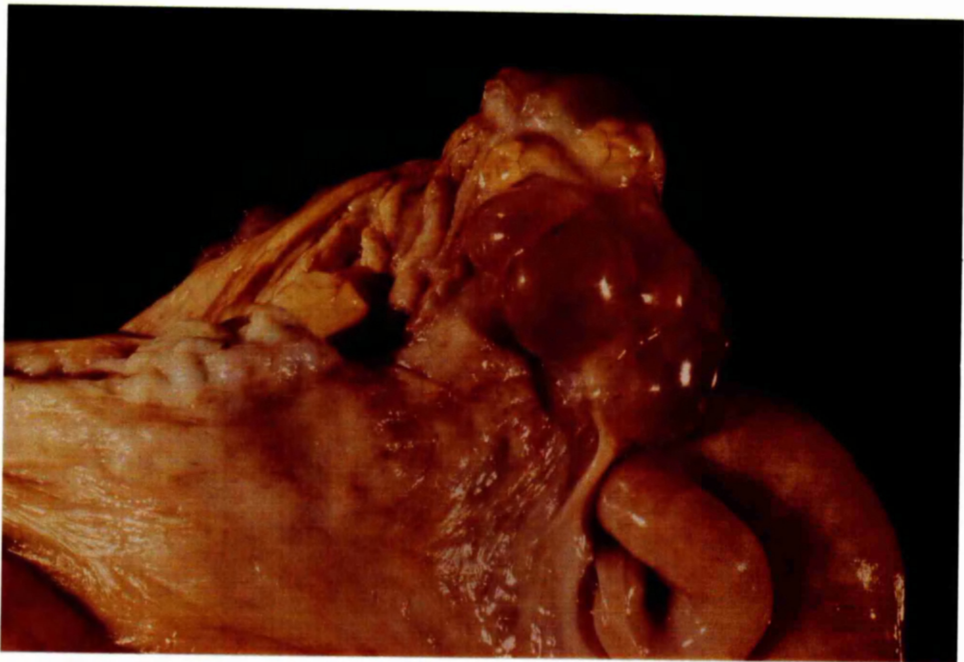
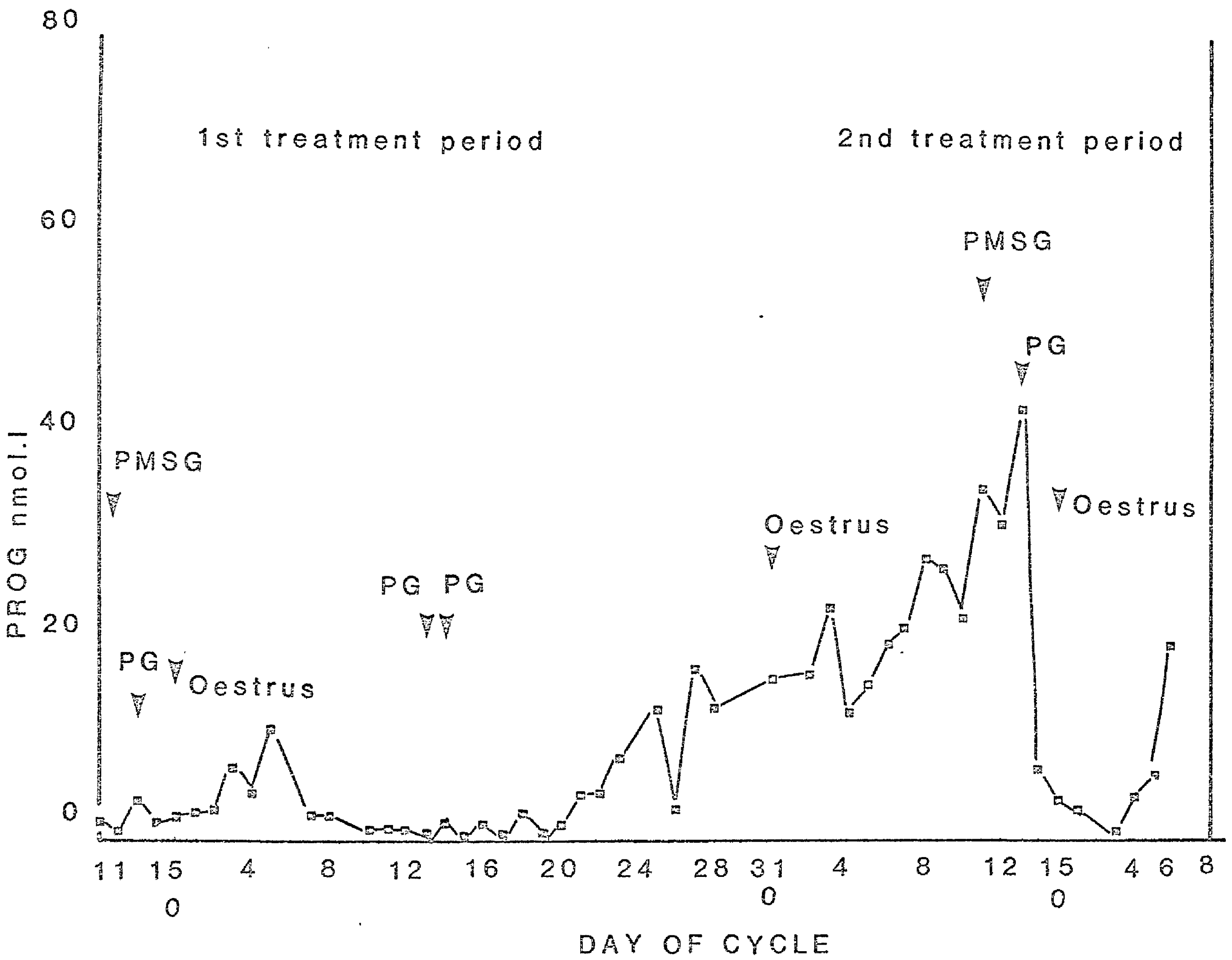
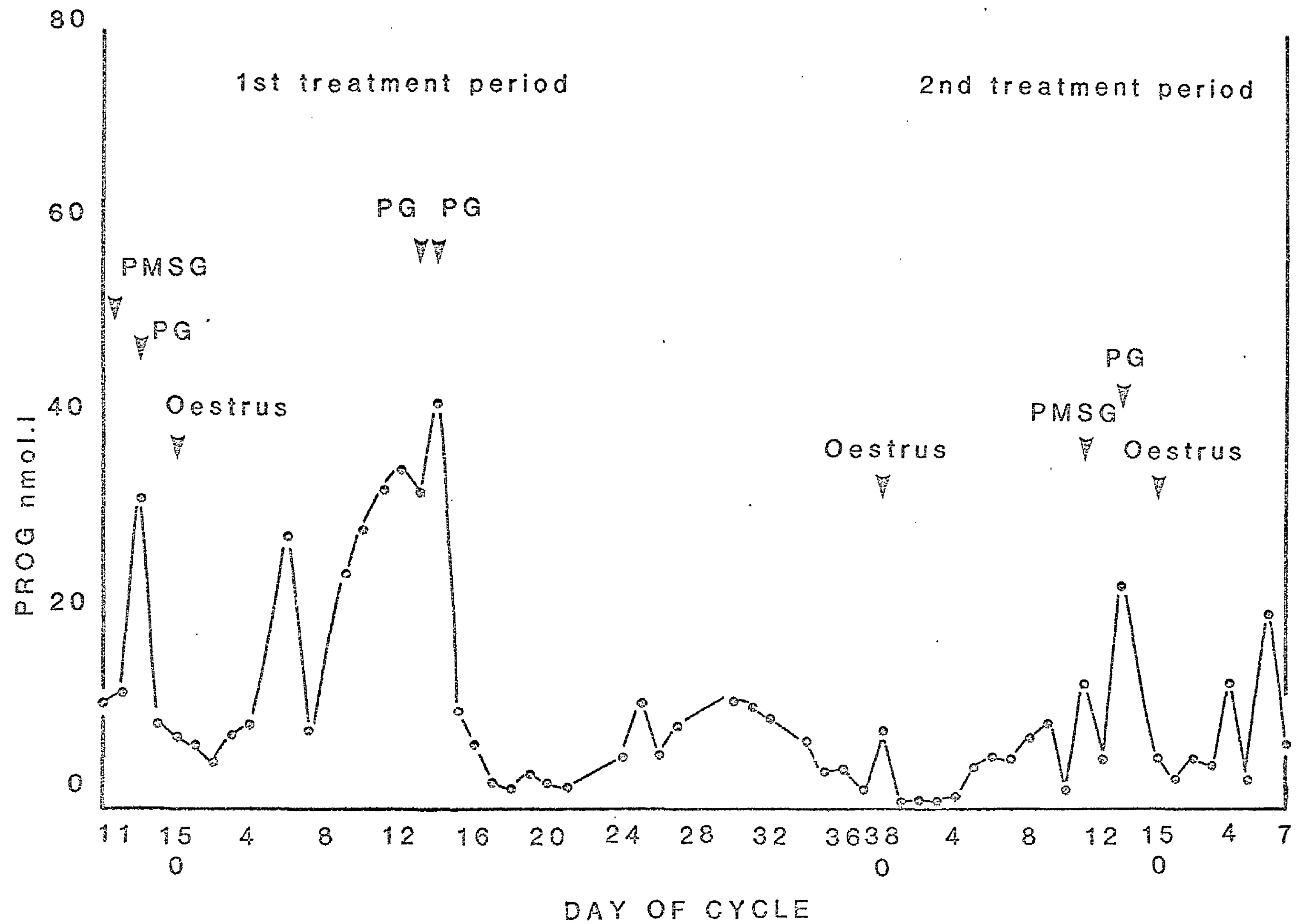


FIGURE 4.2.6 PLASMA PROGESTERONE PROFILE OF COW 10 FOLLOWING INCREASING DOSES OF PMSG



1st treatment period
2nd treatment period
PMSG
PG
Oestrus
PG PG
Oestrus
PMSG
PG
Oestrus
PROG nmol.l
80
60
40
20
0
11 15 4 8 12 16 20 24 28 31 4 8 12 15 4 6 8
0
0
0
DAY OF CYCLE

FIGURE 4.2.9 PLASMA PROGESTERONE PROFILE OF COW 11 FOLLOWING INCREASING DOSES OF PMSG


1st treatment period
2nd treatment period
PMSG
PG
Oestrus
PG PG
Oestrus
PG
PMSG
Oestrus
PROG nmol.l
80
60
40
20
0
11 15 4 8 12 16 20 24 28 32 3638 4 8 12 15 4 7
0
0
0
DAY OF CYCLE

cow 10 on day 13/14 had no effect as progesterone levels were already basal. Oestrous behaviour was not observed until day 31, at which point progesterone levels were high and increasing. The double dose of PG given to cow 11 caused a marked reduction in progesterone levels. This was not reflected in the occurrence of oestrous behaviour until day 38. During the second treatment period, PMSG was administered to both cows at day 11 following this oestrus, followed by PG 2 days later. The administration of PG caused a marked reduction in progesterone levels in both cows. Levels then remained low through oestrus. Progesterone started to increase at day 4 in cow 10 and at day 2 in cow 11. Although ovarian structures in both cows differed markedly, progesterone levels at day 6 were similar.

The breeds, weights and body scores of the 11 animals used in the study are shown in Table 4.2.5.

Criteria used to estimate the efficiency of the flushing technique are recorded in Table 4.2.6. In all cases the tip of the catheter was placed within 5.5 cm of the uterotubal junction. The presence of debris in the flushing fluid may be associated with a failure to recover embryos in 5 cows (3, 4, 6, 7, 8). Substantial loss of fluid (less than 85% recovered) has occurred in 3 cows (4, 7, 11).

TABLE 4.2.5 BREED, STATUS, WEIGHT AND BODY SCORE OF 11 ANIMALS SUBJECTED TO SUPEROVULATION TREATMENT

Cow	Breed	Status	Weight (kg)	Body Score
1	Ayrshire	Cow	460	2
2	Friesian	Cow	551	$2\frac{1}{2}$
3	Friesian	Heifer	465	$2\frac{1}{2}$
4	Shorthorn	Cow	351	$2\frac{1}{2}$
5	Friesian	Cow	530	2
6	Ayrshire	Cow	542	$2\frac{1}{2}$
7	Ayrshire	Cow	417	2
8	Ayrshire	Cow	467	$2\frac{1}{2}$
9	Ayrshire	Cow	399	2
10	Ayrshire	Cow	476	2
11	Ayrshire	Cow	406	2

Note - Body scoring scheme was that outlined by Lowman et al (1973)

TABLE 4.2.6 DETAILS OF METHODS USED TO ESTIMATE THE EFFICIENCY OF THE FLUSHING TECHNIQUE

Cow No.	Treatment Period	Day of Flush	Placement Catheter Tip		Fluid Recovered %		Presence Debris/Blood		Embryos Recovered	
			Left	Right	Left	Right	Left	Right	Left	Right
1	1	7	3	3	85	90	-	-	1	0
2	1	7	3	2.5	95	95	-	-	0	0
3	1	7	5	4.5	95	90	***	***	0	0
4	1	7	5	6	90	75	*	**	0	1
5	1	7	3	3	99	99	-	-	0	7
6	1	4	5	4	90	90	***	***	0	0
		5	5	5	90	90	***	***	0	0
	2	4	3	3	95	95	-	-	1	0
		5	3	4	90	95	-	-	0	0
7	1	4	5	5.5	95	95	*	*	5	1
		5	5	5.5	90	85	*	**	0	0
	2	4	4	5	90	50	-	-	3	0
		5	5	5	90	90	-	-	0	0
8	1	4	3	3	95	95	-	-	8	0
		5	3	3	90	90	-	**	0	0
	2	4	3	3	95	95	-	-	0	0
		5	3	3	95	95	-	-	0	0

TABLE 4.2.6 (Contd.)

Cow No.	Treatment Period	Day of Flush	Placement Catheter Tip		Fluid Recovered %		Presence Debris/Blood		Embryos Recovered	
			Left	Right	Left	Right	Left	Right	Left	Right
9	1	4	4	3	95	98	*	-	0	0
		5	4	4	90	90	-	-	0	0
	2	4	3	5	95	90	-	-	2	6
		5	3	5	90	90	-	-	0	0
10	1	4	4	4.5	99	99	-	-	0	0
		5	4	4	95	99	-	-	0	0
	2	4	4	4	90	90	-	-	0	0
		5	5	4	95	90	-	-	0	0
11	1	4	4	5	99	95	-	-	0	0
		5	5	5	95	95	-	-	0	0
	2	4	5	4	50	95	-	-	0	0
		5	4	4	95	95	-	-	2	0

Note (1) Placement of catheter measured from its tip to UTJ. (cm)

(2) Fluid recovered given as the percentage of the amount instilled.

(3) - Fluid clear; *** Debris and blood; ** Debris; * Traces of debris.

4.2.4 - DISCUSSION

Although in vivo tests exist to monitor oviduct patency assessment of oviduct function necessitates information on its ability to allow ovum fertilisation and to transport the embryo to the uterus. Information on this latter aspect could be obtained by the collection and examination of early embryos from the uterus. Recovery techniques have been documented as a means of providing embryos for subsequent transfer into donor animals. Embryo transfer has been employed to increase the number of progeny from genetically superior females; to increase the rate of twinning in beef cattle; as a means of rapidly altering breed composition on farms and as a method of obtaining calves from certain infertile cows (Gordon, 1975; Newcomb, Rowson and Trounson, 1978; Betteridge, 1980). Embryo transfer techniques require a good superovulatory response from the treated cows in order to produce the maximum number of viable embryos. In contrast to the maximal response required in these programmes, the testing of oviduct function requires a more limited response. Both techniques, however, necessitate a regime that ensures that a predictable number of ovulations takes place from the ovaries.

Using embryo recovery as the criterion for oviduct patency, in this current study, only 10 of 22 oviducts were normal. At slaughter 21 of these 22 oviducts were patent to dye or pollen (Table 4.2.7). These apparently incompatible results may be due to two factors. Firstly, some animals may have failed to respond to the superovulatory drug with no embryos having been shed from the ovaries. Secondly, in cows whose ovaries have responded, other

TABLE 4.2.7. RESULTS OF OVIDUCT FUNCTION TEST CARRIED OUT IN 11 ANIMALS

Cow No.	Age	Dose PMSG (iu)	Response Both Ovaries	Embryos Recovered		In Vitro Oviduct Patency to Dye or Pollen	
				Left Horn	Right Horn	Left	Right
1	Old	3,000	Yes	Yes	No	Patent	Patent
2	Old	3,000	Yes	No	No	Patent	Patent
3	Young	3,000	Yes	No	No	Patent	Patent
4	Old	3,000	Yes	No	Yes	Patent	Patent
5	Young	3,000	Yes	No	Yes	Blocked	Patent
6	Old	1,500 (1)	Yes	Yes	No	Patent	Patent
		1,500 (2)	Yes				
7	Old	1,500 (1)	Yes	Yes	Yes	Patent	Patent
		1,500 (2)	Yes				
8	Old	1,500 (1)	Yes	Yes	No	Patent	Patent
		1,500 (2)	No				
9	Young	1,500 (1)	Yes	Yes	Yes	Patent	Patent
		1,500 (2)	Yes				
10	Old	1,500 (1)	No	No	No	Patent	Patent
		3,000 (2)	No				
11	Young	1,500 (1)	No	No	Yes	Patent	Patent
		3,000 (2)	Yes				

Note (1) (2) - Refer to 1st and 2nd PMSG treatments.

Young - aged 6 years or less; Old - over 6 years.

factors may have prevented embryos being recovered from the uterus.

During the normal cycle of the cow, ovulation is associated with formation of a corpus luteum (CL). Formation of an additional CL may be taken as indicative of a response to a superovulatory regime. Using this criterion, 4 out of 17 PMSG injections failed to produce a response. Amongst animals that did respond marked variation was found in the number of CL. In addition, there appeared no consistent relationship between the amounts of PMSG injected and the superovulatory response. This is in contrast to the findings of workers using larger groups of animals, who have recorded a linear dose/response relationship to PMSG (McGaugh and Olds, 1971; Moore, 1975). The breed, age, stage of lactation and nutritional status of the cow have been suggested as influencing the response to PMSG (Gordon, 1975; Saumande, Chupin, Mariana, Ortevant and Mauleon, 1978). To minimise these effects the majority of animals used in the study were non-lactating Ayrshire cows. However, both their ages and body condition were different and their previous reproductive history were unknown. No apparent relationship was observed between the age of the cows and their response to PMSG (Table 4.2.7). The nutritional status of the animals was assessed by their body condition (Table 4.2.5). No cow was in poor condition. In addition, efforts were made to ensure they received an adequate diet throughout the period of study. Although a seasonal effect of response to PMSG has been suggested, this was not apparent in this study where animals were injected at various times of the year (Gordon, 1975; Saumande et al, 1978). Controversy

exists on the effect of different batches of PMSG on the ovarian response (Gordon, 1975). This could not have contributed to the results obtained in the present study as the same batch of PMSG was used throughout.

Besides these factors, the ovarian structures present at the time of PMSG injections and the interval between treatment and the onset of oestrus appears to affect the ovarian response (Scanlon et al, 1968; Saumande et al, 1978). Previous studies have established that PMSG given at day 9-12, followed by prostaglandin (PG) 2 days later to terminate the cycle, gives a more consistent response than when the drug is administered either earlier or later in the cycle (Sreenan and Gosling, 1977). The variable responses noted in the cows in this study all followed PMSG injection at this recommended time. Such a regime applied to an animal with a normal oestrous cycle length means that the cow is injected with PMSG when a mature CL, capable of responding to the subsequent PG treatment, is present. This ensures that oestrus will follow at the optimum 4-5 days after PMSG (Scanlon et al, 1968). Three animals (1; 10, treatment 1; 8, treatment 2) in the current investigation, although considered to have a mature CL on their ovaries, both by rectal palpation and interval from oestrus, did not have concurrently high concentrations of progesterone at the time of PMSG injection. Using the previously defined criterion of a superovulatory response, 2 of these animals did not respond to the PMSG (10, 8). It is likely that PMSG was administered in 2 of the animals (10, treatment 1; 1) at or shortly after functional regression of the CL. A CL may still be palpable at this time, as

functional luteolysis precedes rectally palpable regression of this structure (Watson and Munro, 1980). This in turn would mean that these cows had abnormally short oestrous cycles. Some short cycles have been associated with uterine infection (Hafez, 1974).
It may be relevant that 1 of the cows (1) previously had a vulvar discharge several months before this study commenced, but appeared clinically normal at the time of PMSG injection. However, short cycles are recorded in normal cows (Asdell, de Alba and Roberts, 1949). At subsequent slaughter of these animals, no evidence of gross uterine pathology was observed. The lack of response in these 2 animals was not associated with a prolonged PMSG-oestrus interval. In the third animal (8, treatment 2), although a CL was also palpable at the time of PMSG injection, this was preceded by a prolonged period with no evidence of a functional CL having been present. PMSG has previously been shown to produce inconsistent results in initiating cycling in anoestrous animals (Asdell, 1949). It is possible that in this animal, the administration of PMSG served to reinstate ovarian cycles, and as such should be considered independently from the PMSG responses of the other cycling cows.

The use of human menopausal gonadotrophin (HMG) as an alternative superovulatory drug to PMSG has been studied. However, the variability found with PMSG was also observed with this drug. Some advantage associated with its use, and reflecting different cow responses, was that some animals which had failed to respond to PMSG were successfully superovulated with HMG (Newcomb, 1980).

Pituitary follicle stimulating hormone (FSH) of equine

origin has also been used with apparently less variation in response than that previously attributed to PMSG (Elsden, Nelson and Seidel, 1978; Seidel, Elsden, Nelson and Bowen, 1978). However, a practical disadvantage of both FSH and HMG is that, unlike PMSG, they must be administered as multiple injections (Avery, Fahning and Graham, 1962; Bellows, Anderson and Short, 1969; Newcomb, 1980). This may ultimately, however, prove to be of benefit compared to PMSG which once injected (reflecting its half-life) continues in an uncontrolled manner to exert its effect (Schams, Menzer, Schallenberger, Hoffman, Hahn and Hahn, 1978).

In two of the instances of failure of response to PMSG (cow 10, treatments 1 and 2), no CL formed after treatment and the ovaries were found to contain multiple follicles/cysts. This indicates that although follicles were stimulated to develop in this animal, ovulation did not take place. Other workers have observed that stimulation of follicular growth in response to PMSG administration may not be followed by ovulation (Folley and Malpress, 1944; Hafez et al, 1963; Elsden et al, 1974). Such structures may reflect a lack of the normal stimulus for follicles to ovulate, or failure of the responsiveness of the follicles. LH is the ovulatory hormone in the cow and is normally released as a preovulatory surge 24 hours prior to ovulation (Hansel and Snook, 1970; Chenault et al, 1975; Schams et al, 1977).

Several workers have estimated LH levels in superovulated cows (Henricks and Lamond, 1972; Henricks et al, 1973; Hallford et al, 1975; Saumande and Pelletier, 1975; Saumande, 1978; Schams

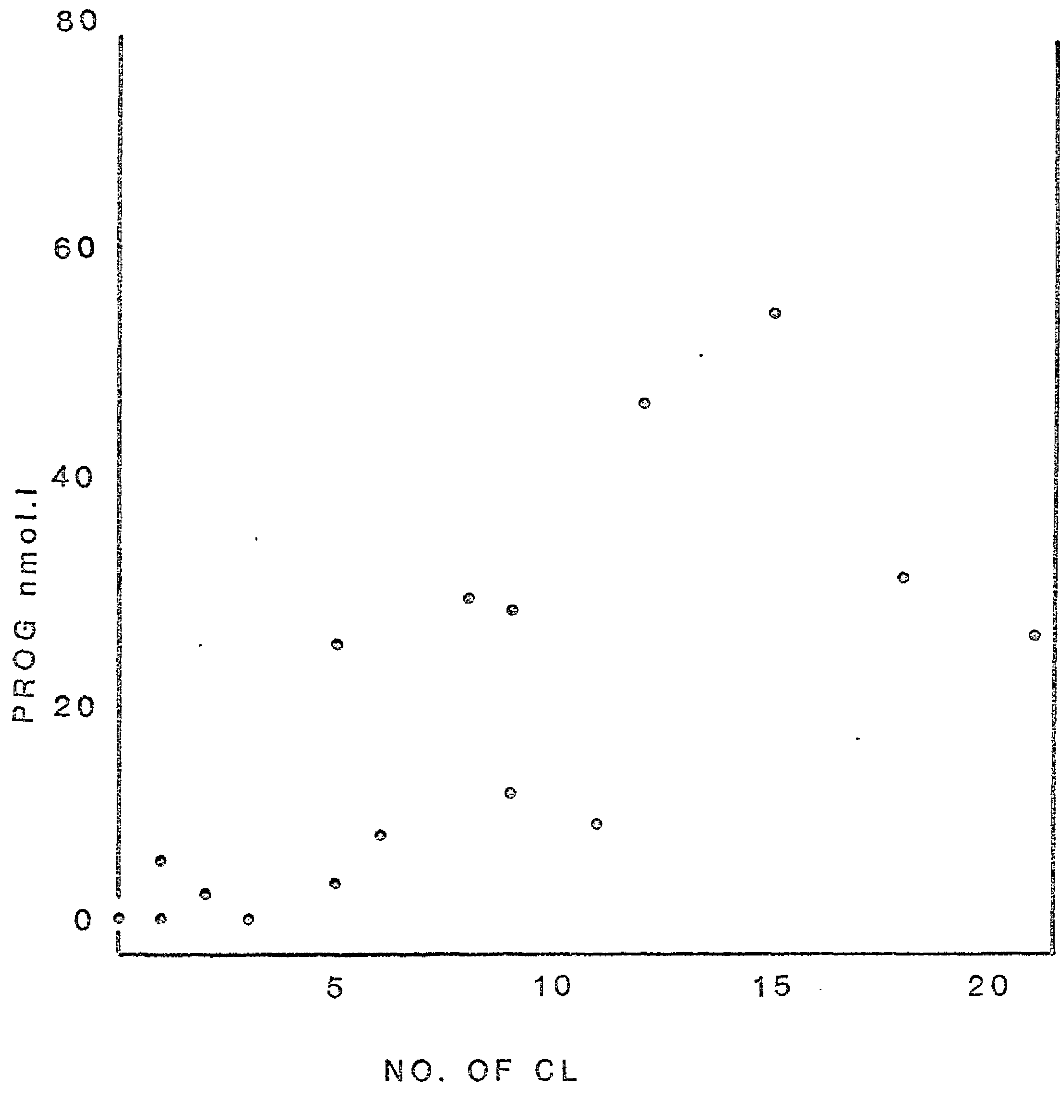
et al, 1978). Both no effect, and earlier, greater LH peaks have been observed. These reports on the magnitude of the LH peak would explain the lack of additional ovulatory response following LH-RH or HCG (Moore, 1975; Newcomb, 1980). However, the possibility of abnormal LH release cannot be ruled out in this particular animal, and it may be that LH-RH, shown to be effective in treating cystic ovarian disease in cattle, may be appropriate in such cows which consistently form these structures (Cantley, Garverick, Bierschwal, Martin and Youngquist, 1974).

The alternative mechanism to explain the presence of these follicles/cysts, that is lack of responsiveness, may account for the luteinised structures observed in cow 2. In this latter case, since CL were also present in the ovaries, this strongly suggests that a normal LH surge has occurred. The presence of luteinisation, but without the occurrence of an ovulatory papillum, suggested that these structures were not CL. However, Zemjanis (1970) states that some CL do not have an ovulatory papillum. Other workers have recorded CL as structures with 'dented heads' (Folley and Malpress, 1944). The structures, described as luteinised structures, in cow 2 had neither ovulatory papilla or 'dented heads'. In addition their size range was different to that recorded in normal CL (Zemjanis, 1970). However, the levels of progesterone present in this cow cannot be used to differentiate these structures from CL, since the correlation between CL numbers and progesterone levels was found not to be significant (Fig. 4.2.10).

Based on circumstantial evidence, it has been stated that ovulation can occur over a period of 8 days in superovulated animals

FIGURE 4.2.10 THE CORRELATION BETWEEN PLASMA PROGESTERONE (PROG) LEVELS AT DAY 4, AND THE NUMBER OF CORPORA LUTEA (CL) PRESENT IN THE OVARIES.

CORRELATION COEFFICIENT - 0.728. NOT SIGNIFICANT.



PROG nmol.l
80
60
40
20
0
5
10
15
20
NO. OF CL

(Hafez et al, 1963). Using fibre optic laparoscopy, a spread of approximately 30 hours has been proposed (Newcomb, 1976). It is possible that this spread of ovulation may contribute to the type of structures present in cow 2. An LH surge, in the presence of mature follicles, may produce ovulation. Large numbers of developing follicles, which produce sufficient oestrogens to trigger an LH surge, may fail to respond to the luteotrophic stimulus and persist as multiple follicles/cysts. It is possible in some instances that follicles intermediate between these 2 groups may undergo luteinisation without ovulation. It may be that all of these can occur within the same animal, with the LH surge having been triggered by one or more normal mature follicles. In this case, any follicles that continue to develop after the LH surge, possibly due to the continued action of PMSG, would not be capable of producing a further LH peak. This occurs due to the fact that the bovine pituitary requires a period of time to resynthesise sufficient LH (Hansel and Snook, 1970). It may be that to ensure ovulation of these 'slow developing' follicles an LH substitute should be administered but that this should be given after the endogenous LH surge has occurred. In addition, as PMSG contains LH activity, this could contribute to the presence of these luteinised structures.

In any technique of ovarian stimulation, a prerequisite is a means to assess the success of the treatment. This was especially important in the current investigation in eliminating a lack of ovarian response as a reason for failure to recover eggs. A commonly employed technique is the detection of CL in the ovaries either by

rectal palpation or by measurement of progesterone in blood or milk (Dobson, Midmer and Fitzpatrick, 1975).

In the current study, although rectal palpation of CL provided adequate information on whether an individual ovary had responded, there were quantitative differences between the numbers of CL palpated and the numbers observed at slaughter. The differences generally comprised an underestimation of the numbers of CL. Dawson (1975) reported errors in the identification of individual CL in even the single-ovulating animal. Other workers have commented on the difficulty in handling large ovaries per rectum (Rowe et al, 1976). These errors are, therefore, likely to be compounded in the super-ovulated animal.

Although some workers report that it is possible to identify the corpus haemorrhagicum, most workers consider that the CL is just palpable at day 4-5 (Hancock, 1962). In this study, the difficulties in identifying structures, and the underestimation compared to later slaughter findings, may have been compounded by the fact that assessment was being made at a relatively early stage. Should early embryo recovery be employed as a test of oviduct function, the use of rectal palpation to assess ovarian response may be associated with potential error.

Progesterone levels have been shown to be markedly increased in the superovulated cow compared to untreated controls (Booth et al, 1975; Sreenan and Gosling, 1977). In general, in the current study, such a pattern of increase was observed. However, although generally large numbers of CL were associated with increased progesterone levels (Fig.4.2.10) considerable overlap occurred at a range of ovarian

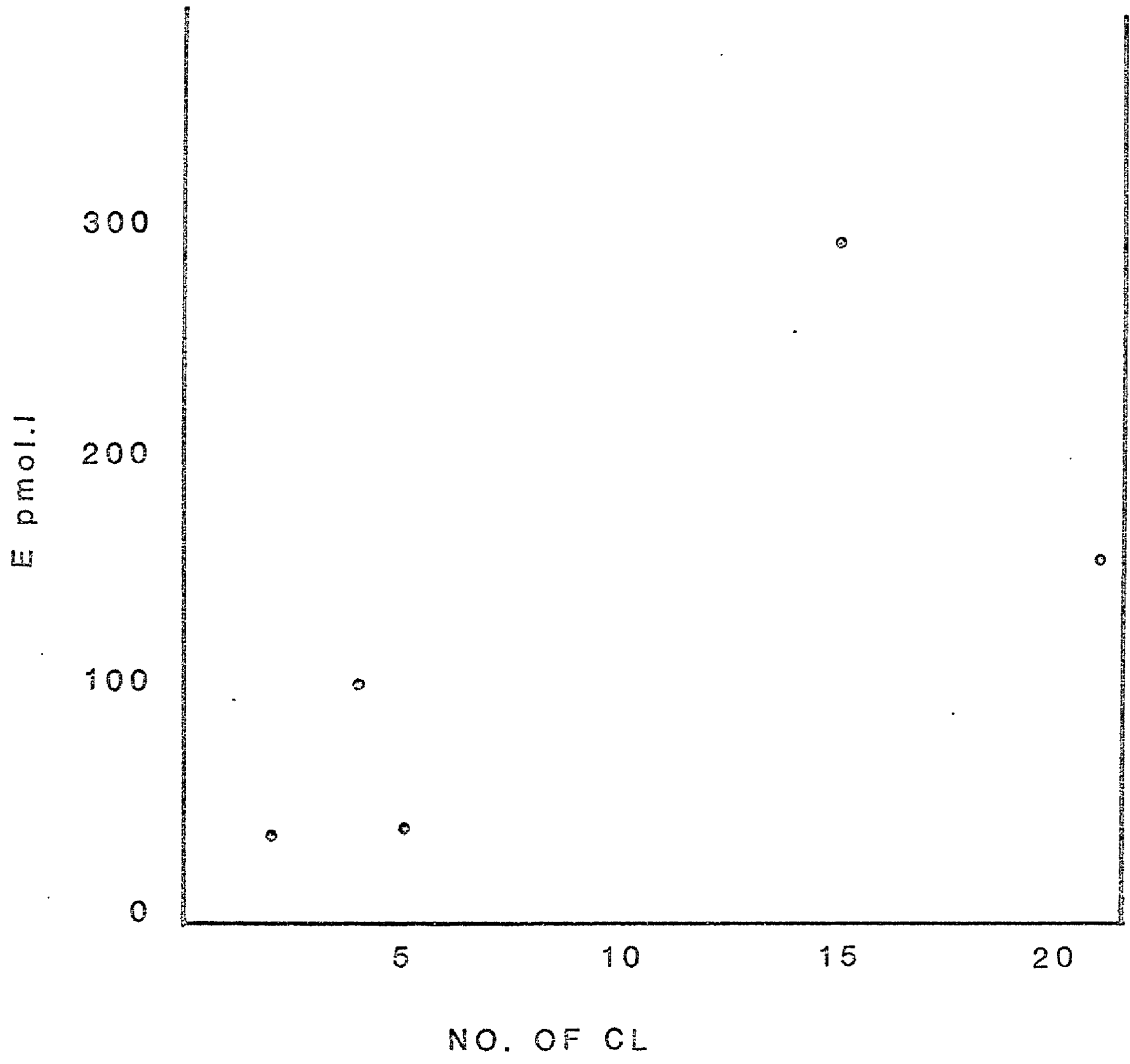
responses all considered appropriate to testing oviduct function. In addition, it is not possible, using plasma progesterone assay, to determine whether an appropriate response has taken place in both ovaries. A further disadvantage of this technique compared to rectal palpation is that due to the delay in processing samples, results will be retrospective. In this study, overall progesterone levels at day 4 or 5 could not be used to indicate accurately CL numbers. Although other workers have shown a significant correlation using larger numbers of animals, this has little practical benefit when dealing with individual cows (Sreenan et al, 1978).

Higher levels of oestrogens have been reported in superovulated cows compared to normal cows (Glencross et al, 1973; Henricks et al, 1973; Booth et al, 1975; Sreenan et al, 1978). In this study, although only a small number of animals were sampled, peak oestrogen levels showed a relationship to total numbers of CL (Fig. 4.2.11). Other workers have found a relationship between peak oestrogen levels and the numbers of CL subsequently found in the ovaries (Saumande and Pelletier, 1975; Hallford et al, 1975). It may be that in individual animals, as seen from the limited results in the current study, only a broad indication of the ovarian response can be obtained. However, should subsequent work establish a high correlation between numbers of CL and plasma oestrogen profiles, this would be useful in predicting the ultimate ovarian response, and therefore the number of embryos that should be recovered.

Direct visualisation of the ovaries by fibre optic laparoscopy has been used to determine ovarian structures, with accurate

FIGURE 4.2.11 THE CORRELATION BETWEEN PEAK PLASMA OESTROGEN (E) LEVELS AND THE NUMBER OF CORPORA LUTEA (CL) PRESENT IN THE OVARIES.

CORRELATION COEFFICIENT - 0.726. NOT SIGNIFICANT.


E pmol.l
300
200
100
0
5
10
15
20
NO. OF CL

results in normal and superovulated cows (Wishart and Snowball, 1973; Saumande et al, 1978). However, laparoscopy has also been shown to reduce the interval from PMSG administration to oestrus, and as such may reduce the number of follicles which are mature enough to ovulate (Saumande et al, 1978).

In any work of this nature, it would be most useful if the response of an individual cow to PMSG was predictable. The high level of repeatability found in the small number of animals in this investigation suggests the feasibility of such techniques. Other workers have reported similar consistent results with the same dose, where the interval between treatments has been short (Scanlon, 1972; Coulthard, 1978). Extension of this interval leads to increased variability. An obvious extension of such techniques to increase the response to superovulation is to assess the response to an initial low dose of PMSG prior to the superovulatory treatment. Where larger numbers of animals have been treated with individual amounts of PMSG, a linear response between the production of ova and dose has been reported (McGaugh and Olds, 1971). Within the individual cow (11) treated in this study, such an increased responsiveness to a larger dose was also apparent. It has been suggested that repeated administration of PMSG causes a reduction in response due to antibody production (Miller, Larsen, Nancarrow and Cox (1975). This would limit the application of techniques to predict ovarian response. However, antibody production to PMSG has been shown not to occur in the cow (Schams et al, 1978).

In several of the animals injected with PMSG, behavioural

oestrus occurred under high levels of progesterone. It is well established that oestrus can occur both in pregnant animals and in some cycling cows where progesterone is transiently increased at this time (Williamson et al, 1972). The reasons for this apparent absence of a progesterone block to oestrous behaviour are not known.

Accurate control of superovulation requires the ability to terminate the luteal phase of the cycle in a predictable fashion. This is normally carried out using natural or synthetic PG. In the normal cycling cow, PG administration to a cow with a mature CL leads to behavioural oestrus in 2-3 days. In the superovulated animals in this study, in most cases the injection of PG after the first PMSG treatment period was not followed by behavioural oestrus. However, in these animals, progesterone levels markedly decreased indicating that luteolysis had occurred. Following a suggestion from the manufacturers, the dose of PG administered at this time was greatly in excess of that which is required in the normal cow. Apparent lack of oestrous behaviour after PG treatment in superovulated cows has been noted by other workers (Coulthard, 1981). Such an occurrence may be due to lack of observation of behavioural oestrus. This would seem unlikely in the current study as other heat periods in these same cows were, in general, detected. Alternatively lack of observation of oestrus could have arisen due to the occurrence of silent oestrus. No explanation can be offered as to why these particular ovulatory periods should have been associated with lack of normal behavioural signs of it.

In cows that responded to PMSG, as mentioned previously

failure to recover eggs may be due to several factors. The technique of non-surgical embryo recovery in the cow requires manipulative dexterity and experience. Initial difficulties were encountered in the correct positioning of the catheter in the uterine horn and this may have contributed to a reduction in the embryo recovery rate in some of the animals. Table 4.2.6 summarises the efficiency of the flushing technique using the criteria of placement of the catheter, clarity of the recovered fluid and amount of fluid recovered. The placement of the catheter has been shown to affect significantly the embryo recovery rate (Newcomb et al, 1978). In this study, recovery was carried out at day 4 in some animals, when the embryos are high in the uterine horn, and thus the correct positioning of the catheter may be even more important. Due to interference with identification of eggs, debris present in the fluid may have contributed to a lower recovery rate in 5 animals (3, 4, 6, 7, 8). Loss of recovery fluid occurred in 3 cows (4, 7. 11) and in these animals may be associated with a failure to recover the expected number of embryos.

Another reason for low recovery rates may be the early timing of flushing. Although in general the cow embryo enters the uterus between days 3 and 4, this may be altered in the superovulated animal (Hamilton and Laing, 1946). Administration of oestrogens has been shown to affect ovum transport in other species. Both acceleration of the time of entry of the embryo into the uterus and a 'tubal locking' leading to a delay in entry into the uterus have been described (Burdick and Pincus, 1935; Greenwald, 1961). The increased

levels of oestrogens in the superovulated cow may, if embryo transport is delayed, affect the embryo recovery rate.

One other factor which may be relevant to the timing is the handling of the tract at such an early stage of the cycle. It has been suggested that rectal palpation of the cow genital tract, shortly after oestrus, may lead to contractions, which could cause rapid transport of eggs through the uterus (Newcomb, 1980). In the current study, it may be that embryos not recovered at day 4 were still present in the oviduct, and that the manipulations used caused their rapid transport through the uterus. Repeated flushing at day 5 would then fail to recover these embryos. This remains a possibility in some of the cows, although the finding of 2 eggs at day 5 in cow 11 suggests that this did not occur at least in this animal.

Finally some aspects of the anatomy of the oviduct itself may be a factor in the low recovery rates. The large size of ovaries containing many CL may cause difficulties in the capability of the fimbriae to pick up all the eggs in some cows. This has also been suggested by other workers (Hafez et al, 1963). However, other workers have reported high recovery rates in animals with more than 24 ovulations, so it appears that the fimbriae are capable of harvesting large numbers of eggs in a number of cows (Scanlon et al, 1968). The normality of the oviducts was assessed at slaughter by the injection of dye or pollen grains. However, the pollen grain used is 80 μ in diameter which is still smaller than the bovine egg. Therefore oviducts permitting the passage of pollen grains may still have septal adhesions or narrowing sufficient to prevent passage of the egg. The pollen grain suspension test may underestimate the

number of oviducts with abnormal patency.

Some points regarding the embryos recovered are of interest. The fertilised eggs recovered at day 4 from cows 6, 7 and 9 ranged from the 4-cell to the 16-cell stage. This may be due either to ovulation occurring over a long period of time, or acceleration of egg transport into the uterus, or a combination of both. Acceleration of egg transport may be associated with the high oestrogen levels and their slow decline after oestrus, present in the superovulated animal. Recently workers have shown that the administration of progesterone causes a more rapid descent of the embryo into the uterus (Crisman, McDonald and Thompson, 1980). It has been suggested that premature entry into the superovulated uterus has a deleterious effect on eggs (Newcomb, Rowson and Trounson, 1976). The apparent structural normality of all the day 4 fertilised eggs, as assessed by light microscopy, may be attributed to the short time spent in the uterine environment. This tends to suggest that the increased levels of circulating gonadal steroids, which may affect the oviduct environment, appear to have no deleterious effect on the eggs, prior to entry into the uterus. However, the technique of light microscopy only provides limited data on the detailed structure of the embryo. It may be that the use of the embryo as an indicator of oviduct normality would be benefited by more refined techniques for examination of its morphology.

4.3.1 - EXAMINATION OF EGGS BY LIGHT AND SCANNING ELECTRON MICROSCOPY

The structural development of the bovine egg has been well described (Hamilton and Laing, 1946). However controversy still exists as to the degree of abnormality which will cause the egg to be non-viable. Studies were undertaken to investigate a means of determining further the morphological characteristics of the bovine embryo.

The eggs collected were examined by the established light microscope methods, identifying the cell stages and looking for symmetry and compactness of the blastomeres (Church and Shea, 1976). The potential application of techniques of scanning electron microscopy were investigated using a modification of the method described by Reeve and Ziomek, 1981. In addition, exposure of the eggs to a reactive oxygen plasma, prior to scanning electron microscopy, as a means of examining the inner layers of the embryo was undertaken.

4.3.2 - MATERIALS AND METHODS

The eggs and embryos used were those collected from the superovulated cows described in the previous section. Additional material was kindly supplied by Mr. R. Coulthard, Woodhall Spa, from a variety of superovulated cows.

Preparation of eggs for light microscopy:

The morphology of the eggs recovered from the cows was recorded. Criteria determining fertilisation and stage of development was as described by Hamilton and Laing, 1946, and Austin, 1961.

Preparation of eggs for scanning electron microscopy:

Reagents

1. Stock buffer. 0.2M sodium cacodylate, pH 7.2.

This was prepared as follows: 21.4 g of sodium cacodylate (BDH) was dissolved in 250 ml distilled water, to produce 0.4M sodium cacodylate. 50 ml of this solution was added to 8 ml 0.2M hydrochloric acid and made up to a volume of 100 ml, using distilled water. The stock buffer was stored at 4°C and used within one month of preparation.

2. Working buffer. 0.1M sodium cacodylate, pH 7.2

This was prepared as follows: 25 ml of the stock buffer (adjusted to pH 7.2, using 1M hydrochloric acid if necessary) was made up to a volume of 50 ml, using distilled water. The working buffer was stored at 4°C and kept for a maximum of one month.

3. Fixative. 4% glutaraldehyde in working buffer.

This was prepared as follows: 16 ml of 25% glutaraldehyde (Agar Aids) was made up to 100 ml, using 0.1M sodium cacodylate buffer.

4. Poly-l-lysine solution.

A 0.1% solution of poly-l-lysine (Type 1-β hydrobromide P1886, Sigma) was prepared in distilled water. The solution was used immediately and any excess remaining was discarded.

5. Acetone.

This was diluted to the required concentration, using distilled water.

Equipment

2 ml autoanalyser cups (Sarstedt)

6 mm diameter round glass cover slips (Macfarlane Robson)

Glass egg collecting dishes (Camlab)

Critical point dryer (Polaron, Watford)

Aluminium stubs (Agar Aids)

Sputter coater (Emscope)

Scanning electron microscope (Philips SEM 500)

Plasma chemistry unit (Plasma prep. 100, Nanotech).

Fixation

The embryos were transferred from the flushing medium to the fixative and left for 1-2 hours. They were then stored in the working buffer in 2 ml autoanalyser cups until processing for the scanning electron microscope was carried out.

Attachment of eggs to cover slips

Round glass cover slips were scored on one side with a diamond pencil, washed in ethanol and dried in an oven at 37°C. A drop of freshly prepared poly-l-lysine solution was placed on to the scored surface of each cover slip, using a Pasteur pipette. The cover slips were left for 15 minutes at room temperature. The excess poly-l-lysine was then removed without allowing the cover slip to dry out. The cover slip was then washed 3-4 times with working buffer. A drop of buffer was left on the cover slip, which was transferred to the bottom of a glass egg collecting cup, with the buffer coated side uppermost. Three or four eggs were transferred to each cover slip, using a pipette which had previously been pulled over a flame to a diameter of approximately 200 microns. Care was taken to pipette as little fluid as possible with the eggs. Adhesion of eggs was checked by gently lifting and shaking the egg collecting cup.

Generally the eggs stuck immediately to the cover slip. The collecting cup was then flooded with working buffer.

Dehydration and examination

The buffer was removed almost completely from the collecting cups. Dehydration was performed in graded concentrations of acetone (30-50-70-90%) for 5-10 minutes each, followed by three changes with 100% acetone for 10 minutes each. The cover slips covered with 100% acetone were transferred to the critical point dryer and the carbon dioxide critical point was quickly obtained. The eggs remained in the dryer for approximately one hour. In cases where the zona pellucida was to be examined, the critical point dried eggs, on the cover slips, were stuck on to aluminium stubs, by means of a conductor silver paint. They were coated with a thin layer of gold in a sputter coater and examined under the scanning electron microscope at an acceleration voltage of 15 kV.

Assessment of morphology was carried out by viewing the eggs on a television monitor. In addition, to record detailed observations, photographs were taken at magnifications varying from 640 to 5,000.

In cases where the zona pellucida was required to be removed, the critical point dried eggs, on the cover slips, were placed on a watchglass. This was placed in a plasma chemistry unit at 100w, with a gas flow (oxygen) of 20 cc/min for 30 seconds. The cover slips were then placed on stubs, gold coated and examined under the scanning electron microscope as described above.

4.3.3 - RESULTS

Plate 4.3.1 illustrates an unfertilised egg (cow 8, day 4) under the light microscope. No sign of cleavage is apparent. For comparison, Plate 4.3.2 demonstrates three fertilised eggs (cow 9, day 4) under the light microscope. A 4-cell and two 8-cell stages are shown. The blastomeres are compact and symmetrical, with no degenerative changes visible.

A scanning electron micrograph (SEM), showing the surface of the zona pellucida of an infertile egg is shown in Plate 4.3.3. At this magnification, folds on the surface are visible. However, a large amount of extraneous material is also present on the surface. The same egg can be seen at a higher magnification in Plate 4.3.4. In this micrograph the folds or 'rugae' present on the zona pellucida are clearly visible.

Plate 4.3.5 is an SEM of a blastocyst. The overall shape of the egg is markedly distorted. However, it is still apparent that the embryo is larger than the unfertilised egg shown in Plate 4.3.3. Also the surface of the zona pellucida appears more amorphous in structure. Plate 4.3.6 illustrates the same blastocyst at a greater magnification. In this micrograph, the zona pellucida surface is not arranged in folds, as with the unfertilised egg (Plate 4.3.3), but has a rougher, more irregular appearance.

Plate 4.3.7 shows an infertile egg, under the scanning electron microscope. The egg was subjected to a reactive oxygen plasma for 30 seconds. The top half of the zona pellucida surface is arranged in folds, similar to the unetched egg shown in Plate 4.3.3. The bottom half is much smoother in appearance.

Plate 4.3.1 Light micrograph of an infertile egg recovered from Cow 8

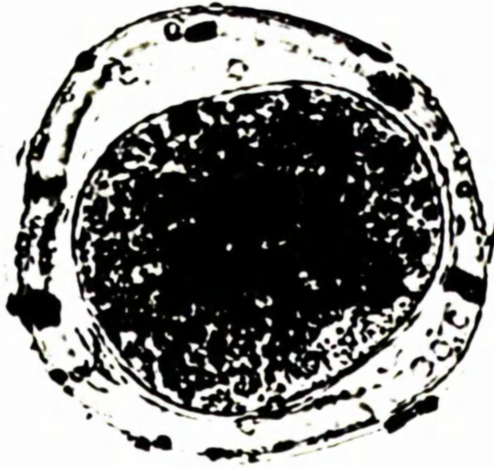


Plate 4.3.2 Light micrograph of 4-cell and two 8-cell eggs recovered from Cow 9

Plate 4.3.3 SEM Infertile egg. x 640

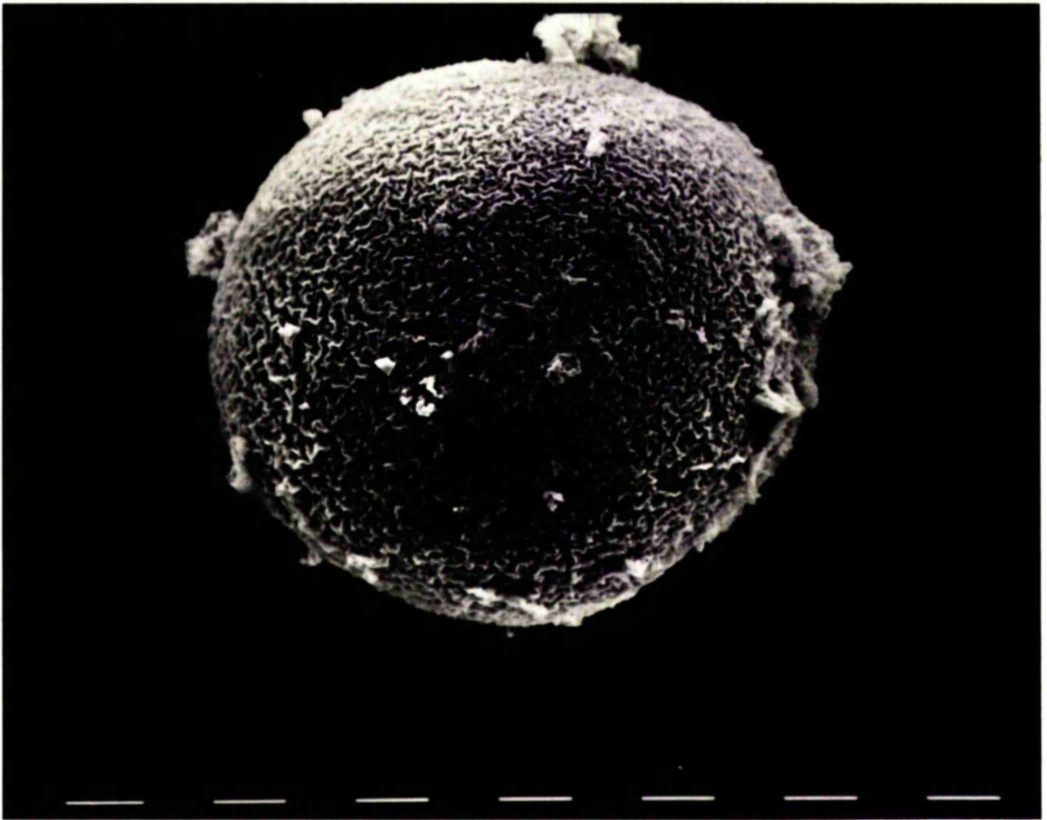


Plate 4.3.4 SEM Infertile egg. × 2,500

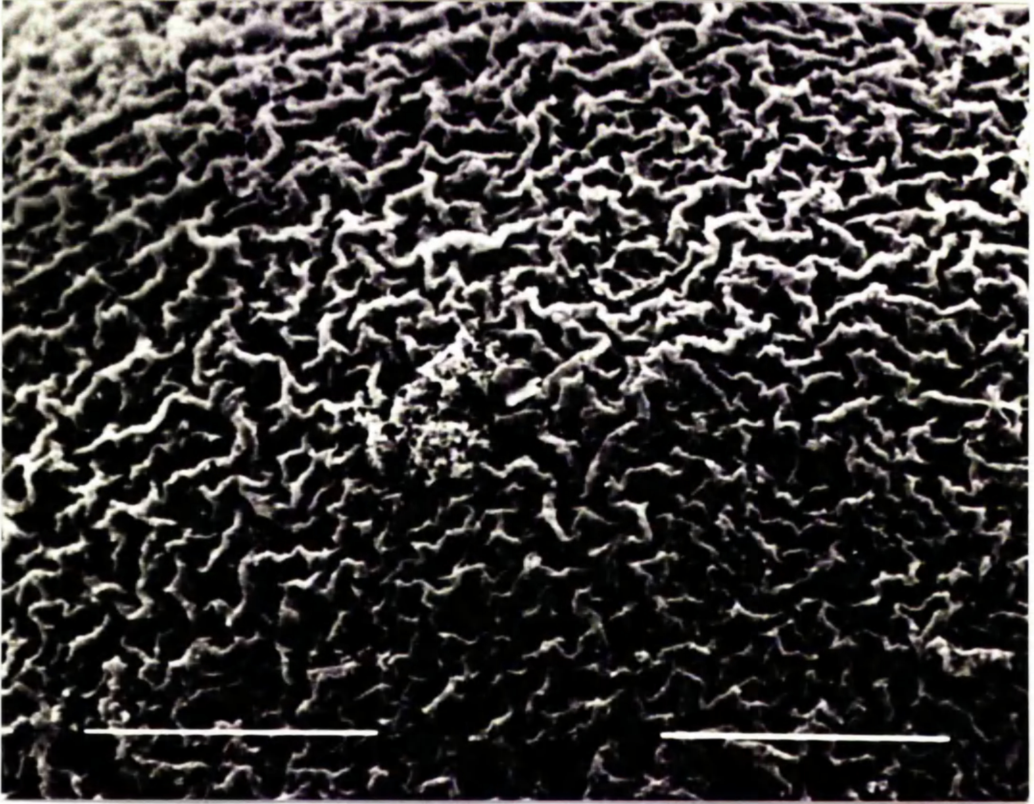


Plate 4.3.5 SEM Blastocyst. x 640

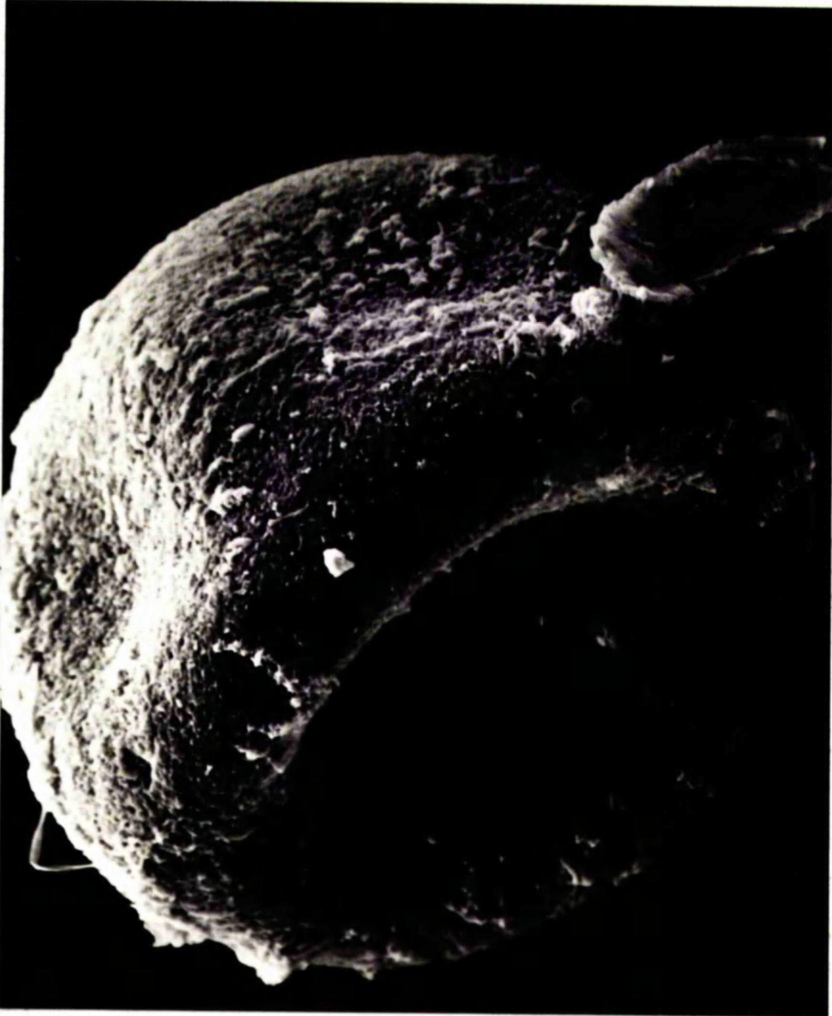


Plate 4.3.6 SEM Blastocyst. x 1,250

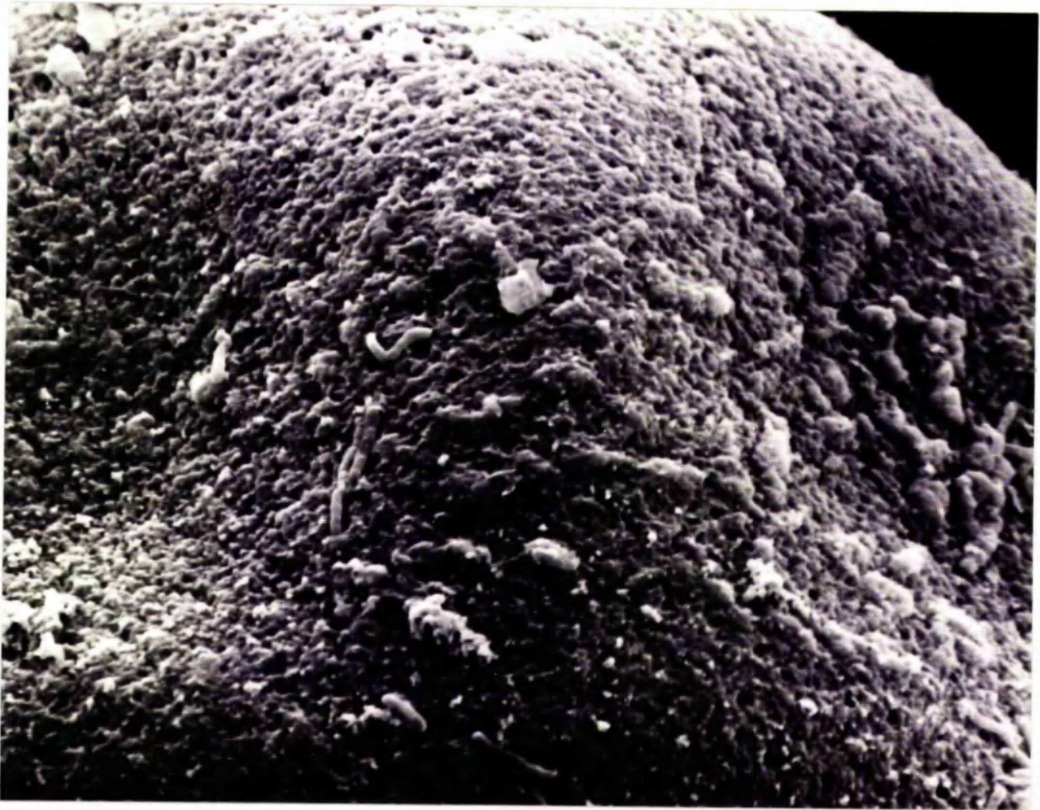


Plate 4.3.7 SEM Infertile egg, plasma etched for 30 seconds.

× 640

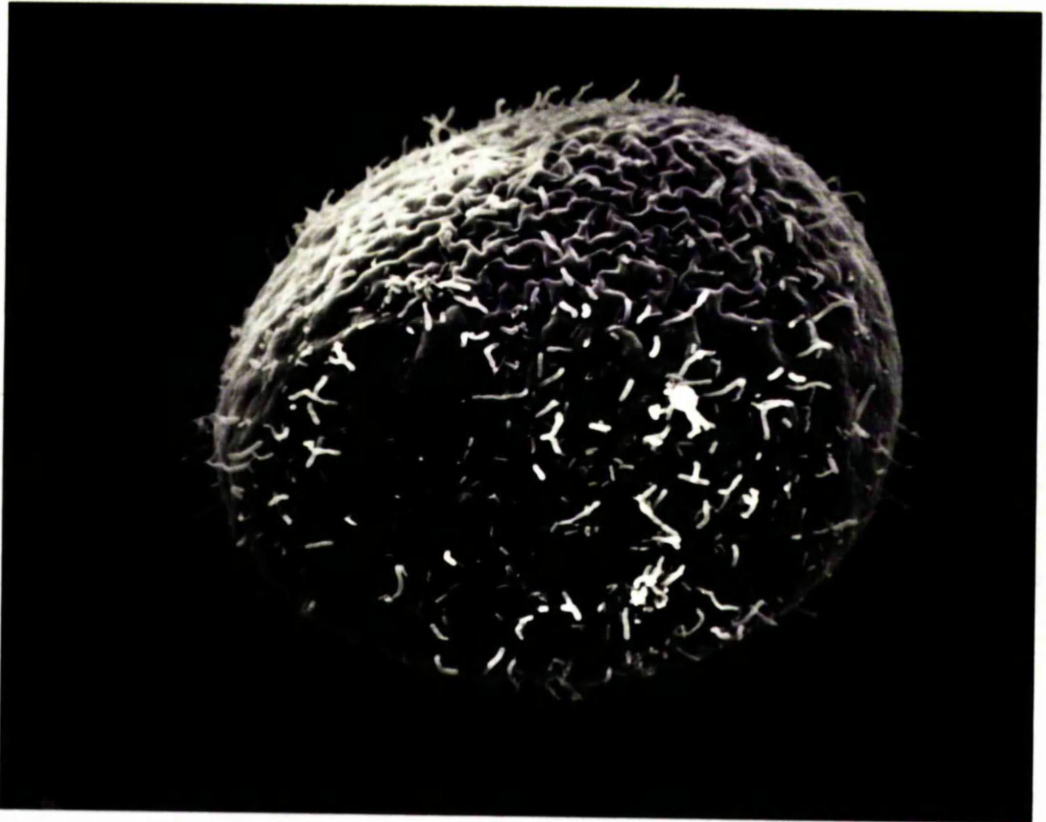
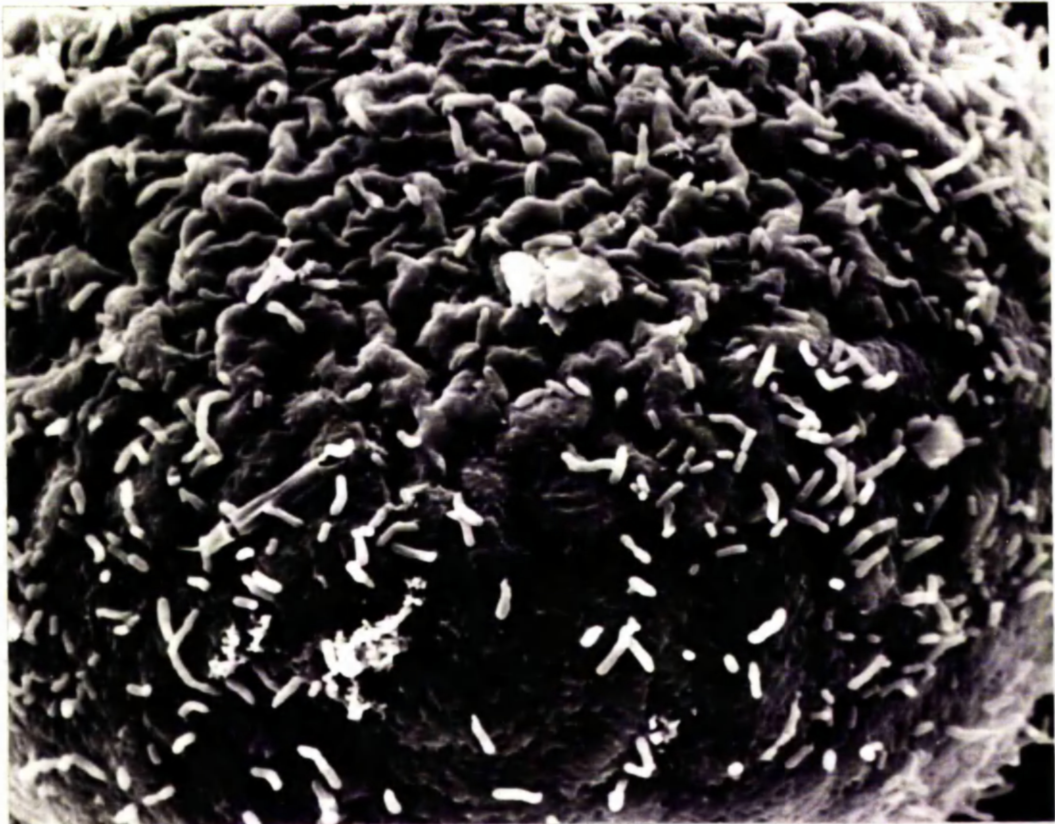


Plate 4.3.8 SEM Infertile egg, plasma etched for 30 seconds.

x 1,250



Additional material is present on the surface of the egg, white structures which appear evenly distributed over the zona surface. At a greater magnification, these structures are plainly bacteria (Plate 4.3.8). Both bacilli and cocci are apparent. In this micrograph, the change in surface appearance from the top of the egg to the bottom of the egg is clearer.

4.3.4 - DISCUSSION

None of the various techniques available for detailed study of morphological changes occurring in the bovine egg during development can be used to define precisely what constitutes a viable embryo. At present, the only way to do this is to transfer the embryo to a recipient uterus and monitor its further development until pregnancy is well established. The use of embryo recovery as a test of oviduct function requires that the embryos collected can be identified as normal or abnormal. The studies described in this section have attempted to examine the bovine egg using both the light and the scanning electron microscope (SEM).

Initially various technical problems were encountered. In the light microscope studies, these were mainly due to inexperience in handling and transferring the embryos. However, the use of a Pasteur pipette, pulled over a flame to a diameter just greater than that of the egg, and controlled by mouth suction, gave excellent results. This was particularly important during the SEM work, since the eggs were transferred into drops of fluid on tiny cover slips and any excess fluid pipetted resulted in their being washed away. The adhesive properties of poly-l-lysine were a major advantage, since subsequent processing of the embryos could then be carried out while they were firmly stuck to glass cover slips.

In this study examination under the light microscope was used to determine whether or not the eggs had been fertilised. Evidence of fertilisation was taken as cleavage producing compact symmetrical blastomeres, with no evidence of degeneration inside the zona pellucida. However, it is acknowledged that the fragmentation

which occurs in infertile eggs may superficially resemble cleavage (Austin, 1961; Church and Shea, 1976). This is of particular relevance during the early stages of development when the dividing blastomeres may be unequal in size, and easily mistakenly identified as fragmenting eggs instead of fertile eggs. It has been suggested that where any confusion exists special staining techniques can be used to identify the nuclei of the blastomeres, and also the penetrating sperm head (Bedford, 1971). In this study the eggs were collected at day 4, 5, or 7. At this time many of the eggs collected were at the 8 or 16-cell stage. Under the light microscope there was no sign of degenerative changes in any of the eggs examined in this thesis (Plates 4.3.1 and 4.3.2).

The use ofthe SEM to examine the detailed surface of the zona pellucida has produced several interesting observations. The zona surface of the infertile egg shown in Plates 4.3.3 and 4.3.4 appears much folded with regular rugae. It is difficult to be certain if this configeration has been produced by the fixatives used in processing the egg for the SEM. However, since the overall topography of the egg, that is, its shape and size appears normal, this would seem unlikely. It may be that the presence of these rugae in vivo help spermatozoa penetration at fertilisation by guiding the sperm heads into the bases of the folds. The micrograph of the blastocyst (Plates 4.3.5 and 4.3.6) shows a different appearance of the zona surface. No regular configerations are visible, but the surface is rough and appears porous. This appearance is similar to the observations of Flechon and Renard (1978) of the zona pellucida of the cow blastocyst. The blastocyst in Plate 4.3.5 can be seen to

be relatively larger, compared to the infertile egg in Plate 4.3.3. It seems logical to suggest that the zona pellucida has expanded and thus the rugae present in the early stages have been flattened out. However, it is difficult to be certain with this sample, since marked distortion occurred during fixation and it must be borne in mind that any subsequent observations may be due to this. In Plates 4.3.3 and 4.3.5 contaminants can be seen on the surface of the eggs. These findings agree with those of Flechon and Renard (1978) who suggested that the zona surface was viscous and adhesive, so allowing extraneous material to stick. The use of a Millipore filter to remove any debris from the fixative solutions immediately prior to their use could prove helpful in overcoming this problem.

The use of a reactive oxygen plasma as a means of removing the surface layers of the egg was also investigated. A limited amount of material was subjected to this technique. Plates 4.3.7 and 4.3.8 demonstrate that a change has occurred on the surface of the infertile egg. The oxygen plasma has apparently 'etched' the folds from the lower area of the egg, leaving a very much smoother surface. However, a longer period of 'etching' appears to be necessary before the zona pellucida is completely removed. Two modifications of this technique may be useful for future use. Firstly, the use of a carbon coating over the egg would permit sequential observations under the scanning electron microscope during the etching procedure. Since the reactive oxygen plasma produces a total ashing effect on carbon particles, the egg could be recoated prior to re-examination (Humphreys and Henk, 1979). Secondly, the zona pellucida could be removed by microman-

pulation from the fresh eggs which could then be fixed (Willadsen, 1980). Examination under the SEM after carbon coating, would reveal the surface of the blastomeres. Subsequent treatment with a reactive oxygen plasma could be accurately timed to allow identification of cell organelles (Humphreys and Henk, 1979).

An interesting observation has been the presence of a number of types of bacteria on the surface of the infertile egg in Plates 4.3.7 and 4.3.8. Other workers have noticed that certain bacteria (Escherichia coli) can grow in the fixative used in these studies. These same workers routinely Millipore filter all fixatives before use (Ziomek, 1981). However, in these studies, the bacteria present at the egg surface are a variety of different types, both cocci and various sizes of bacilli being evident. Also other smaller particles are evident. Identification of these bacteria was not attempted in these preliminary srudies. However, these smaller particles could be virions or mycoplasma. The fixative used in these studies was not Millipore filtered prior to use. However, not all of the eggs stored in the same fixative had bacteria adhering to their surfaces. Another source of contamination could have been the plasma chemistry unit. This also seems unlikely since several eggs were placed in the unit and only a proportion demonstrated bacterial contamination. Another possible explanation of this contamination could be that bacteria may have been present in the oviduct or uterus of the cow. This is impossible to ascertain without further controlled studies. Direct surgical collection of eggs from the oviduct using aseptic techniques would be one method of attempting to determine whether or not this was the case. Non-surgical collection is a procedure where

it is difficult to ensure complete sterility. To offset this, workers use sterile recovery medium with added broad spectrum antibiotics. The apparent adhesive properties of the bovine zona pellucida and ease with which bacteria stick to this surface reaffirms the necessity for this. The finding of bacteria associated with some of the eggs examined during these studies is an area worthy of further investigation.

These preliminary studies have shown that more detailed information on the morphology of the embryo may be gained by use of the scanning electron microscope. The procedure used for fixation and processing was relatively simple to perform and not time consuming. However, many more samples need to be examined before the significance of the observations can be established. More information on the ability of the embryo to continue development may be gained by observation of the cell organelles. A reactive oxygen plasma would appear a suitable method of progressively etching the surface of the embryo, such that the cell organelles become visible. These techniques, although at the research level initially, may ultimately provide information for workers involved both with infertile cows, and in commercial embryo transfer work. The current controversy over the morphology of viable eggs re-emphasizes the need for continued research in this field.

GENERAL DISCUSSION

GENERAL DISCUSSION

Passage of the ovum from the ovary to the site of fertilisation, and from there of the zygote to the uterus is an extremely involved process. It involves morphological, physiological and endocrinological changes. These changes occur within the oviduct itself and also in the brain and other regions of the reproductive tract. Therefore a test for clinical evaluation of the oviduct in infertile cows should, if possible, test the normality of these changes which are necessary if pregnancy is to be achieved.

Certain aspects of the procedure can be investigated by testing the patency of the oviducts. The form of PSP dye test eventually devised from studies carried out in this thesis, appears to be accurate in the diagnosis of unilaterally or bilaterally blocked oviducts. This knowledge is of value as a means of reducing the time cows with blocked oviducts are kept, so decreasing the cost of feeding, and further veterinary investigation. The equipment necessary to carry out this dye test is easy to obtain, and with practice, the procedure should be within the capabilities of most large animal veterinary surgeons. The diagnosis of occlusion involves time since a series of urine samples over a period of 60 minutes must be collected. However, a number of animals can be tested on one farm at one visit. Nevertheless the dye test only indicates that the oviducts are fully or partially patent to a fluid injection.

The work carried out on slaughterhouse samples using a suspension of pollen grains suggests that a significant number of

animals which give positive results to a dye test may not in fact have oviducts sufficiently patent to allow passage of the bovine ovum. Other workers have shown that septal adhesions may exist, sufficient to prevent the passage of the ovum, while allowing the passage of fluid (Dawson, 1958, 1964a). Diagnosis of the degree of narrowing is not possible in the living animal using pollen grains in a similar manner to the dye. However, the theory behind the starch grain test where grains of varying diameter are injected around the ovary and subsequently collected by vaginal flushings, may have a place in further studies. Workers involved with this test have noticed that generally only starch grains of a small diameter are recovered from the anterior vagina (Kessy and Noakes, 1979). This may be associated with degradation of the grains in their passage down the reproductive tract, rather than with a narrowing or partial occlusion of the oviduct. An alternative technique may be the use of a pollen grain suspension in a similar manner since it is less likely that the pollen grains would undergo reduction in size. However, difficulties would be encountered in the detection of the pollen grains in the vaginal washings. This could be overcome by staining the grains, for example, with PSP dye. This test would provide information on the ability of the fimbria to harvest the pollen grains, of the oviduct to transport particles to the uterus and on the subsequent passage of the grains to the anterior vagina. It may be possible to use the well-established techniques of non-surgical egg recovery to collect the pollen grains directly from the uterus, if difficulties were encountered in their collection from the vagina.

However, a major advantage of the dye test is that in a number of animals it may remove debris and break down adhesions present in the oviduct. Therefore the routine use of the dye test in infertile cows as a therapeutic treatment, as well as a diagnostic aid, may be of benefit.

Another aspect which requires further investigation is the occurrence of cervical backflow of dye which occurred in several animals, when dye was instilled into the uterine body without the use of an inflated cuff. Whether this is of any significance is difficult to say since some workers claim that a proportion of pluriparous cows have a degree of dilation of the cervix even in dioestrus (Arthur, 1975). However, the possibility of infection gaining entry to the uterus by this means may result in reduction in fertility in such animals.

Cervical backflow of dye has also been recorded by other workers, using the dye test as described by Kothari et al (1978) when the test was carried out at oestrus, producing a high incidence of false negative results (Kessy and Noakes, 1979). This is in disagreement with the findings in this thesis and of others (Kothari et al, 1978). Oestrus is the desirable time to carry out this test, both in terms of the degree of dilation of the cervix and the ability of the uterus to withstand infection. Further studies directed towards elucidating this problem could involve the use of superovulated animals, since some of the changes occurring around oestrus in the normal cow tend to be increased in superovulated animals. Kessy and Noakes (1979) suggested that their false negative results

might have been due to increased oedema present around the uterotubal junction, or to a 'tubal locking' effect within the oviduct itself. These changes precipitated by increased oestrogen levels should be compounded in the superovulated animal. If this was the case, negative results to the dye test would occur in all superovulated cows tested at oestrus.

The precise role of the uterotubal junction (UTJ) in the cow remains to be determined. Studies carried out in this thesis suggested that a proportion of the pollen grains, in the slaughterhouse samples, were being stopped at the UTJ, and could only be pushed forward by marked increases in pressure. It is dangerous to infer changes observed in post-mortem specimens with what may be happening in the living animal. However, the use of betamimetic drugs, in vivo, specifically to cause uterine relaxation may be a means of elucidating the degree of constriction exerted at the UTJ to substances passing from the uterus into the oviduct.

An important aspect to be considered when PSP dye is used to assess oviduct patency is that no information is available as to whether or not the dye is excreted in the milk of lactating cows. Since a high proportion of repeat breeders are dairy cows, the PSP dye test would be required in lactating animals (Boyd and Reed, 1961). If it were found necessary to withhold milk from cows under test, due to contamination with dye, this would greatly increase the cost and inconvenience of carrying out the procedure. This aspect requires further investigation.

The degree of patency, whether to fluid or particles, is only

one component of the oviduct's role in the establishment of a pregnancy. In addition, the environment of the oviduct is involved both in capacitation of sperm prior to fertilisation and in the development of the zygote before it enters the uterus. These studies have investigated superovulation and egg recovery as a means of monitoring oviduct function. The finding of a fertilised egg in the uterus indicates that growth of the follicle, ovulation, transport of sperm, transport of ovum, fertilisation, and passage of the zygote to the uterus have all proceeded. The eggs that are produced, however, must be normal. The use of superovulatory drugs to produce multiple ovulations has advantages and disadvantages. It is easier to collect eggs from the uterus where there are a large number of eggs present, as opposed to only one. In addition, both oviducts can be tested at one treatment period. However, the variation in response to superovulatory drugs may be the limiting factor in using this technique. Some workers have recorded that overstimulation of the ovarian response appears to produce an increase in the number of abnormal eggs produced (Boland et al, 1978). This may be due to the markedly increased levels of gonadal steroids produced by the superovulatory response. These studies were a preliminary attempt to find a dose regime, and timing of egg collection suitable for the assessment of oviduct function. Ideally what is required is an ovarian response producing enough eggs to enable at least a proportion of them to be collected, but not so many that the morphology of the eggs could be affected by the superovulation treatment. In the biological situation, this is extremely difficult to manipulate. The results of this small study showed a

marked variation in response among cows to the same dose of PMSG. Future studies using other drugs such as HMG or FSH require to be undertaken in an attempt to find more consistent results.

Having established a regime likely to produce a reasonable increase in the number of eggs produced, examination of the eggs must be undertaken to determine that such conditions can produce normal eggs. One method which could be tried is to maintain the eggs in a suitable culture medium and monitor their development (Trounson et al, 1976). One effect of this technique which has been noticed by workers in the field of commercial embryo transfer, is that a 'cremated' embryo will frequently have an improved, more symmetrical appearance after several hours in culture (Coulthard, 1981). Another method is by the use of light microscopic aids to detect any obvious morphological abnormality (Church and Shea, 1976; Linares and King, 1980). These techniques have the disadvantage that many eggs which develop normally and appear morphologically normal, may fail to establish a pregnancy when transplanted into a bovine uterus. Conversely, many eggs discarded as abnormal may have gone on to maintain a pregnancy after transfer.

The studies undertaken were a preliminary investigation of the morphology of bovine eggs using the scanning electron microscope. Further work to clarify the normal appearance of certain features on the egg surface is necessary. A regime where eggs which appeared morphologically similar under the light microscope were subjected either to scanning electron microscopy or to embryo transfer and the results compared, could be of benefit. The use of a reactive oxygen

plasma to remove progressively the surface layers of the egg, eventually revealing cell organelles such as the mitachondria, may reveal ultrastructural differences in viable and non-viable eggs. Day 12-16 embryos have been shown to develop into normal foetuses after biopsy (Hare, Singh, Betteridge, Eaglesome, Randall and Mitchell, 1978). Use of a similar technique may enable a section of the embryo to be processed and examined by the scanning electron microscope and reactive oxygen plasma, with the remaining material used for embryo transfer.

An interesting aspect of the scanning electron microscope work has been the finding of various types of bacteria adhering to the egg surface in some cases. The significance of this is not known until further controlled experiments are carried out. However, the possibility remains that the presence of these bacteria may be a result of infection occurring in the oviduct or the uterus of the donor animal. The implications of this in either causing damage to the egg, and so impeding fertilisation, or to the transfer of infection to the recipient, are grave. In commercial egg transfer, the recovery medium contains antibiotics which may act on the bacteria prior to transplantation of the egg into a recipient. However, the presence of smaller organisms on the egg surface, which may or may not be virions or mycoplasma, could be important. Workers have recorded a higher incidence of mycoplasma present in the oviducts of infertile cows as compared to normal cows (Hoare, 1969). In the human field, some mycoplasma have been shown to have a marked detrimental effect on the motility of the oviductal cilia (Mardh, Weström, von Mecklenburg and Hammar, 1976). Antibiotics are not effective against viruses, and the type of anti-

biotic commonly added to recovery media (penicillin/streptomycin) is not effective against mycoplasma. As stated previously, further controlled experiments are necessary to clarify the significance of these findings.

These studies have highlighted the many areas concerning the role of the oviduct in infertile cows, which require further investigation. The function of the UTJ, the aetiology of conditions which may produce narrowing or partial occlusion of the oviducts, and the examination of the eggs recovered from apparently unproductive cows, are all areas for further study resulting in an increase of our knowledge of infertility in cattle.

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