ENDOCRINE EFFECTS OF INTRAUTERINE MECHANICAL MANIPULATIONS AND OF SYSTEMIC ADMINISTRATION OF OXYTOCIN DURING VARIOUS STAGES OF THE BOVINE ESTROUS CYCLE

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LIST OF ABBREVIATIONS

PGF_{2a}	Prostaglandin F_{2alpha}
ng.	Nanograms
ml.	Milliters
C.L.	Corpus Luteum
L.H.	Luteinizing Hormone
H.C.G.	Human Chorionic gonadotrophin
Kg.	Kilograms
EDTA	Ethylene dinitro tetraacetic acid
Day 0	Day of heat
^{o}C	Degrees centigrade
min.	Minutes
S.Q.	Subcutaneous
mg.	milligrams
gm.	grams
PBSG	Phosphate Buffered Saline Gelatin
M.W.	Molecular weight
NaCl	Sodium Chloride
M.	Molar concentration
pH	Hydrogen ion concentration

c.p.m.	Counts per minute
T.C.T.	Total count tubes
N.S.B.	Non-specific bound
rT.C.T.	Recovery total count tubes
IX	Tritiated progesterone
B_o	Maximum binding
Prog.	Progesterone

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INTRODUCTION

Intrauterine manipulations involving cervical passage have been employed in artificial breeding techniques, artificial insemination, diagnostic techniques such as endometrial biopsy, and for therapeutic purposes as infusions of antibacterials e.g., sulfas, iodine solutions and antibiotics. The consequences of these intrusions on the estrous cycle have been studied in several species (Brimsfield and Hawk, 1968; Yamauchi et al., 1971; Nakahara et al., 1971; Seguin et al., 1974; Manns et al., 1975; Hurtgen et al., 1975; Kindahl et al., 1977) and in some cases have been reported to alter the endocrine profile and length of estrous cycles.

Similar alterations could be responsible for the adverse effects associated with nonsurgical ova transfer technique. A nonsurgical technique is a prerequisite for the development and use of ova transfer in large scale animal breeding but has so far resulted in unacceptably low pregnancy rates (Rowson and Moor, 1966; Rowson et al., 1969, 1972; Sugie et al., 1973). Artificial insemination, a breeding technique which has been in use for years,

makes it possible to increase the reproductive use of bulls. Recently development of surgical methods of embryo transfer (Rowson, Moor, Lawson, 1969) have opened the possibility of increasing the rate of reproduction of female cattle (Brand, 1977) by increasing twinning rates and faster genetic improvement. Thus, a genetically superior bull can be bred to a genetically valuable female (the donor) and the zygotes of this donor can then be transferred into recipients whose passive duty would then be to carry the live zygotes to term.

Since the first successful surgical ova transfer in a cow was performed by Willett et al. (1951), several attempts have been made to apply the surgical techniques under field conditions. Two methods which have been used so far are: 1. a midline approach to the uterus requiring the use of a general anesthetic, and 2. a flank approach requiring the use of a local anesthetic (Elsden, 1977). The need for extensive surgery facilities, use of anesthetic agents, limited use of donors and recipients due to repeated incisions and formation of adhesions are some of the drawbacks to the use of surgical techniques of ova transfer. Hence, to be of commercial significance, the development of nonsurgical methods which are atraumatic,

repeatable and applicable under field conditions, is highly desirable (Brand, 1977).

The average pregnancy rate in surgical transfers (60%) has been shown to be much higher than in nonsurgical transfers (30%) (Brand, 1977; Betteridge, 1977; Renard et al., 1977). Causes of this difference are not presently known. In nonsurgical ova transfer, a catheter is passed through the cervix and manipulated to the tip of the uterine horns at a stage of the estrous cycle varying from the third to the tenth day after estrus. Factors that have been suggested to be involved in the lowered pregnancy rates resulting from nonsurgical ova transfers include site of transfer, uterine infection and trauma, myometrial activity (Brand, 1977) possibly contributing to the ejection of the embryo (Harper, Bennett and Rowson, 1961), and depressed luteal function due to endocrine disturbance.

As a possible cause of embryo ejection, it has been shown that stimulation of the cervix of the cow causes the release of oxytocin, which increases uterine contractions (Rowson et al., 1964); and that injection of oxytocin induces uterine contractions at all stages of the estrous cycle (Vandemark and Hays, 1951, 1952; Hays and Vandemark, 1953a, b). Oxytocin induced release of prostaglandin in

isolated uteri from rats (Chan, 1974), ewes (Sharma and Fitzpatrick, 1974) and cows (Newcomb et al., 1977) has been demonstrated. Oxytocin and PGF have also been shown to cause increased uterine motility (Carsten, 1973) and luteal inhibition during a critical phase of the embryonic development. It could therefore be hypothesized that stimulation of the reproductive tract by the nonsurgical transfer method, and consequently a release of oxytocin from the pituitary gland (Harper et al., 1961) and a possible release of uterine PGF_{2a}, might be involved in the ejection of nonsurgically transferred ova or the resulting low pregnancy rates.

This study was therefore designed to determine: (a) if oxytocin and PGF_{2a} are released in response to mechanical manipulations of the cervix and uterus, (b) if there is any short or long term effect of these manipulations on the estrous cycle parameters as monitored by progesterone levels, and (c) to test the effect of exogenous oxytocin administration on PGF_{2a} release and estrous cycle length.

LITERATURE REVIEW

Earlier reports (Hays and Vandemark, 1953) suggested the release of an oxytocin-like substance following stimulation of the reproductive organs of the cow. Oxytocin, the hormone responsible for milk ejection (Gaines, 1915; Ely and Peterson, 1941), was released, after stimulation of the female genital tract as indicated by increased intramammary pressure (Hays and Vandemark, 1953). Moore and Nalbandov (1953) and Inskeep et al. (1962) observed a reduction of estrous cycle length of ewes when the uterus was distended with paraffin beads, and the former showed that this shortening effect was not seen when the section of the uterus which contained the bead was denervated. Hansel and Wagner (1960) reported that uterine dilatation and intrauterine infusion of seminal and preputial fluids (contaminated with bacteria) caused a large proportion of cows and heifers to have shortened estrous cycle lengths which were often 8-12 days. They suggested that the uterus played an important role in regulating estrous cycle length and luteal development in the bovine. That the hormone involved in distension effects is likely to be oxytocin was shown by Debackere et

al. (1961) using cross circulation techniques to show that vaginal distension in a ewe and massage of the seminal vesicles and ampulla of a ram often caused a sharp pressure rise in the udder of a connected ewe. Vaginal distension also causes milk ejection in ruminants via contractions of the myoepithelial cells of the udder (Debackere and Peters, 1960a).

It has also been shown that the uterine muscle of ruminants responds to posterior pituitary extracts _in vitro_ (Newton, 1934; Alexander, 1945; Asdell, de Alba and Roberts, 1945) and _in vivo_ (Van der Kaay, 1923; Benesch and Steinmetzer, 1931; Fitzpatrick, 1960). This ability of oxytocin to cause uterine contractility has been shown to depend on the stage of the cycle. Vandemark and Hays (1951) were able to show that stimulation of the cervix caused release of oxytocin which increased uterine contraction and that an injection of oxytocin induced uterine contractions at all stages of the estrous cycle. Normally, the activity of the uterus exhibits maximum rhythmic contraction at or near time of estrus (period of estrogen dominance) and minimal activity at mid cycle (period of progesterone dominance) in the bovine (Cupps and Asdell, 1944; Vandemark and Hays, 1951). This was also observed

by Bygdeman and Eliasson (1964) who used progesterone on isolated human myometrial strips, and in other species i.e., mouse (Kuriyama, 1961), rat (Marshall, 1959; Kuriyama and Csapo, 1961) and sheep (Bengtsson and Schofield, 1960). Daily injections of progesterone diminished the amount of oxytocin that appeared in jugular vein blood while estradiol elevated jugular oxytocin in normally cycling ewes when the vagina was distended with an air-filled balloon (Roberts and Share, 1969).

These observations have led several workers in the field of ova transfer to view oxytocin release and uterine contractions as a possible mechanism involved in the ejection of the fertilized ova soon after transfer. Utilizing resin spheres impregnated with radioactive gold to simulate 'artificial ova,' Harper, Bennett and Rowson (1961) were able to show that after 1-1/2 hours a large porportion of the spheres had been expelled from the uterus. The same results were obtained by these workers using actual ova. They also found that the number of expelled ova was higher around estrus than in the luteal phase. The latter observation has been confirmed by Lawson et al. (1975). Considering these results, the indication is given that with the beginning of diestrus (5-6 days after ovulation)

the increasing progesterone levels cause a relaxation of uterine musculature which makes nonsurgical transfer possible (Hahn and Hahn, 1976), but it is not clear if ova in later stages of development (morulae and blastocysts) show more resistance to the negative influences of the uterine environment than at earlier stages. Brand et al. (1976) have observed distinct electrical activity of the bovine myometrium 3 days before estrus which disappeared on day 4 or 5 of the next cycle. The cessation of spontaneous myometrial activity on days 4 to 5 coincides with an increase in serum progesterone concentrations above 1.5 to 2 ng/ml and could explain the improved pregnancy rate following both surgical and nonsurgical transfers at day 6 to 9 (Newcomb and Rowson, 1975; Lawson, Rowson, Moor and Tervit, 1975; Sreenan, 1975). Earlier observations (Cupps and Asdell, 1944) have indicated that proestrus and estrus are characterized by slow, even contractions of great amplitude while at metestrus, these become more rapid and tend to become feeble at diestrus. Thus the uterus may be more sensitive to oxytocin early in the cycle (period of estrogen dominance) than at diestrus (period of progesterone dominance). Whether oxytocin is the hormone involved in the expulsion of the eggs, is, however, not

clear.

The effects of uterine distension and other manipulations of the uterine tract on estrous cycle length, which may act via the release of oxytocin, have led several workers to investigate the effects of exogenously administered oxytocin on estrous cycle parameters in the bovine. Hansel and Wagner (1960) observed shortened estrous cycle lengths of 8-12 days in cows when oxytocin was administered during the first six days of the estrous cycle. These results have been repeated by Donaldson et al. (1964), Ginther et al. (1967), and Donaldson and Takken (1968). Concomitant injection of oxytocin and progesterone was able to sustain cycle length (Harms and Malven, 1969). However, they found that experiments in which C. L. were removed on Day 8 showed clearly that cycle length did not accurately reflect luteal inhibition in progesterone treated heifers. Other effects observed by Hansel and Wagner (1960) included cystic corpora lutea and very small corpora lutea with relatively few normal functional cells. This suggests that oxytocin may cause partial inhibition of C.L. development in contrast with the report of Staples and Hansel (1961) which suggested an all-or-none effect of oxytocin on C.L. function.

Studies of embryo survival in relation to luteal function (Staples and Hansel, 1961) have shown that the percentage of embryos recovered from oxytocin treated heifers is significantly reduced. These researchers also reported precocious ovulation without signs of estrus in some heifers treated with oxytocin. It thus becomes apparent that varied effects are obtainable when oxytocin is administered early in the cycle.

Another interesting observation was that of Mares and Casida (1963) who found that injections of oxytocin early in the cycle caused increased progesterone and pregnene--20β-ol-3-one content of the bovine C.L. However, these treated C.L. had a decreased ability to synthesize progesterone when incubated in vitro. This suggests that oxytocin could cause the release of a luteotropic factor that counteracts the luteolytic factor. This latter effect may be dose dependent as has been shown by Donaldson and Takken (1968) who observed that low dose units of oxytocin stimulated total progesterone content while high units caused a depression of total progesterone content of the ovary.

The mechanism by which exogenous oxytocin causes precocious estrus in cows and heifers has not been

elucidated. Donaldson et al. (1964) injected cows with oxytocin in combination with various gonadotropins. They found that bovine luteinizing hormone (L.H.) and human chorionic gonadotrophin (H.C.G.) overcame oxytocin inhibition on the C.L. Donaldson et al. (1964) and Labhsetwar et al. (1964) reported release of L.H. after Day 4 of the cycle. In other work, these workers (1964) observed that at least two periods of luteotrophic stimulation were needed for normal C.L. development, one at ovulation and another after Day 4 of the cycle. They thus postulated that part of the mechanism of luteal inhibition by oxytocin could involve depletion and/or depression of L.H.

Over the past decade, however, evidence has accumulated to indicate that the uterus has an influence on the corpus luteum and, in fact, is the source of a luteolytic mechanism that terminates the life of the corpus luteum. Removal of the uterus is associated with delayed regression of the corpus luteum (Wiltbank and Casida, 1956; Malven and Hansel, 1964; Melampy, 1968). It has been postulated that the uterus terminates the life of the C.L. at the end of a nonfertile estrous cycle by means of a uterine luteolytic mechanism which acts through two pathways: (a)

a systemic utero-pituitary-ovarian pathway involving a decrease in circulating levels of a pituitary luteotrophin, and (b) a local utero-ovarian pathway involving the transport of a uterine luteolysin from a uterine horn to the adjacent ovary (Ginther, 1968). Thus, the phenomenon of estrous cycle alterations following oxytocin injections may involve either or both of these pathways.

For oxytocin to be effective, the uterus must be present (Armstrong and Hansel, 1959; Anderson and Bowerman, 1963). In unilaterally hysterectomized heifers, oxytocin administration shortened the estrous cycle length only when the retained horn was adjacent to the ovary containing the C.L. (Anderson et al., 1965; Ginther et al,, 1967). This result indicates a local utero-ovarian mechanism and was not compatible with the theory that oxytocin affects corpus luteum function through the pituitary gland (Donaldson and Takken, 1968). Unilateral relationship via a uterine luteolysin has also been demonstrated by means of unilateral pregnancy in swine (du Buisson, 1961a); partial or unilateral hysterectomy in guinea pigs (Fischer, 1965); pseudopregnant rats (Anderson, Melampy and Chen, 1966); sheep (Moor and Rowson, 1966) and swine (du Buisson, 1961b) and unilateral uterine distension in cattle

(Ginther et al. 1966) and sheep (Ginther, Pope and Casida, 1965).

Recent work by Kindahl et al. (1977) may apply to the mechanism behind alteration of the estrous cycle with exogenous oxytocin. They showed that changes in the length of the estrous cycle after intrauterine infusions of iodine (very irritating to the endometrium, Seguin et al. 1974) were associated with release of prostaglandin F_{2a} (PGF_{2a}) and that the effect varied with the stage of the cycle. The effect of infusions of iodine on cycle length had previously been reported by Nakahara et al. (1967, 1971, 1975), Morrow et al. (1971), Grunert et al. (1973), Seguin et al. (1974), and Domeki et al. (1975). Infusions of nonirritating substances such as saline and some oil and aqueous-based antibiotic preparations do not cause altered cycles in cows (Oxender and Seguin, 1976).

It is thus possible that this mechanism may also be involved in expulsion of transferred ova. Whether the manipulations carried out during ova transfers are of the magnitude that could cause release of PGF_{2a} is not known. Exogenous administration of the compound (Rowson et al., 1972), as well as measurement of PGF_{2a} in the utero-ovarian vein (Nancarrow et al., 1973) or the uterine vein (Shemesh

and Hansel, 1975) indicate that the uterine luteolysin which could be involved is PGF_{2a}. Evidence for a preferential transfer of PGF_{2a} between the uterine vein and its adjacent ovarian artery has been reported (McCraken, 1971; Hixon and Hansel, 1974).

Prostaglandin F_{2a} can stimulate uterine smooth muscle contractions in many species including primates (Karim, 1968) and sheep (Liggins et al., 1973). Reports by Manns, Newcomb and Rowson (1975) did not show any release of PGF_{2a} into the uterine vein due to cervical stimulation. They have postulated that perhaps the bovine uterus does not synthesize and release PGF_{2a} into the uterine vein as a result of cervical and uterine stimulation or that PGF_{2a} may be synthesized in response to stimulations and yet not reflected in the uterine vein plasma levels, especially as it has been shown to be rapidly metabolized. Newcomb and Rowson (1976) found that there was a PGF_{2a} release after nonsurgical cervical and uterine manipulations on Day 3 of the cycle. The release was not conspicuous when the manipulations were done on Day 6. Whether the release was of the magnitude that it could cause uterine contractions was not reported.

Experiments by Oxenreider (1968) utilizing electrical stimulation to induce uterine contractions were ineffective in causing luteal regression in the beef heifer. He also found that anesthesia or severance of the lumbosacral spinal nerves did not prevent the induction of luteal regression by exogenous oxytocin, suggesting a local endocrine effect.

There, thus, seems to be a relationship between oxytocin and prostaglandin actions on the uterus and corpus luteum. The relationship has been well defined in sheep by Mitchell et al. (1975) who showed that oxytocin secretion occurred during second stage labor, vaginal distension and suckling, and influenced PGF levels in uterine venous blood. They postulated that a prostaglandin step may represent a bottleneck in myometrial activation through which myometrial stimulants must act. Flint et al. (1975) have also described a temporal relationship between changes in oxytocin and PGF_{2a} in response to vaginal distension in the ewe. The effect of exogenous oxytocin on the bovine estrous cycle (Hansel and Wagner, 1960; Donaldson et al., 1964; Anderson et al., 1965; Ginther et al., 1967; and Donaldson and Takken, 1968) presumably may have acted via the release of prostaglandins. PGF_{2a} release following

oxytocin injections in sheep as reported by Roberts and McCracken (1976), seems to support this view.

Oxytocin induced release of prostaglandin-like substances in isolated rat uterus has been demonstrated by Chan (1974) who suggested that oxytocin may cause uterine contractions via a prostaglandin activated mechanism. Vane and Williams (1973) showed that indomethacin, an inhibitor of PG biosynthesis, also inhibits the response of isolated rat uterus to oxytocin. Sharma and Fitzpatrick (1974) have demonstrated a release of prostaglandin F_{2a} in response to oxytocin treatment in anestrous ewes primed with estradiol, and that endometrial PGF_{2a} in sheep is affected by estrogens (Liggins, Grieves, Kendall and Knox, 1972) and progesterone (Wilson, Cenedella, Butcher and Inskeep, 1972).

Exactly how oxytocin causes this release of PGF is not known but earlier studies have shown that epinephrine inhibits uterine contractions caused by oxytocin (Vandemark and Hays, 1951). The estrous cycle shortening effect of oxytocin is also prevented by epinephrine (Armstrong and Hansel (1959), Black and Duby (1965), Auletta, Currie and Black (1972)). This work implies the involvement of adrenergic receptors in the bovine uterus and/or that oxytocin itself may act upon the hypothalamus and that

epinephrine blocks its action there as suggested by Black and Duby (1965).

Norepinephrine, an inhibitor of uterine contractions, has been shown to inhibit the oxytocin induced regression of the corpus luteum (Autletta et al., 1972). However, uterine contractions may not be a prerequisite for the release of PGF_{2a}. Roberts and McCracken (1976) found that the ewe's myometrium responded normally to oxytocin when the production of endogenous PGF_{2a} was suppressed with indomethacin and that contractile activity was not critical for oxytocin induced synthesis of PGF_{2a} in sheep. Mitchell et al., (1975) produced evidence to show that oxytocin induced contractions do not appear to "squeeze out" prostaglandins because PGF levels fell to basal, despite continued contractions and were even elevated before the onset of coordinated contractions in sheep suggesting that this effect may be metabolic.

MATERIALS AND METHODS

Animals and Blood Collection

All animals used were mature Holstein Friesian cows weighing approximately 500 kg, which had calved and lactated at least once. Experimental animals were followed through one cycle to establish normal cycle length prior to commencement of the experiment.

Blood was collected by jugular vein puncture into vacuum tubes containing EDTA[1]; the tubes were centrifuged immediately and plasma was removed and stored at -20°C until analyzed.

Experimental Design

Intrauterine Manipulations

Three cows were used and were each followed through a control and a subsequent treatment cycle. Blood samples were collected once each day on Days 0, 1, 2 of the cycle (Day 0 = Day of estrus). On Day 3, blood samples were drawn every hour for 3 hours starting at 8 A.M., then every

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[1] Vacutainer® System, Becton-Dickinson and Company, Rutherford, New Jersey.

20 minutes for 2 hours, and then every 5 minutes for 30 minutes. Manipulations were then done and blood samples collected every 5 minutes for 30 minutes, every 20 minutes for 2 hours, every hour for 3 hours, and every 3 hours until Day 6, when the same blood sampling and treatment scheme was repeated. Blood samples were then collected every 3 hours until the cows came in heat. The same bleeding schedule was carried out in the control cycle except that these cycles were devoid of manipulations.

Manipulation consisted of 'exaggerated' sham ova transfer techniques involving 2 minute vulvar cleaning with lukewarm water and a mild detergent; rectal palpation to determine the ovary bearing the corpus luteum; passage of a metal catheter through the cervix and into the tip of the uterine horn adjacent to the corpus luteum, and deposition of 5 ml. of sterile physiological saline into the same uterine horn. Total manipulation time was approximately 5 minutes.

Plasma samples collected were analyzed for oxytocin, 15-Keto-13, 14-dihydro-prostaglandin F_{2a} (a metabolite of prostaglandin F_{2a}) and progesterone.

Administration of Exogenous Oxytocin

Six cows were used in this experiment. Five of the cows were bled during the control cycle while sampling was carried out in all six cows during the treatment cycle following the control cycle. Blood was collected once each on Days 0 and 1. Starting at 6 A.M. on Day 2 through Day 6 of the cycle, blood was collected at 1 hour intervals for 2 hours, then every 10 minutes for 1 hour followed by subcutaneous injection of 230 International Units of Commercial Oxytocin[2] in the treatment cycle or an equal volume of physiological saline in the control cycle. After injection, blood was collected at 10 minute intervals for 1 hour, then at 30 minute intervals for another hour, then at 1 hour intervals for 4 hours and then at 3 hour intervals until the next day. Injections of 230 I.U. of oxytocin or saline were repeated at 6 P.M. of each day through Day 7 but the frequent blood sampling schedule was not carried out. After Day 6, blood samples were collected at 3 hour intervals until cows were observed in heat.

Plasma samples collected were analyzed for 15-Keto-

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[2]Vetocin [R] from Biological Specialties Division; The Mogul Corporation, Middleton, Wisconsin 53562.

13, 14-dihydro-prostaglandin F_{2a} (a metabolite of PGF_{2a}) and progesterone.

Heat Detection

Heat detection was achieved by using a teaser cow given 500 mg dihydro-testosterone propionate every 10-14 days as described by Kiser et al. (1977).

Radioimmunoassay Methods

Radioimmunoassay of Progesterone

Materials

Phosphate Buffered Saline--
Gelatin (PBSG)

Assay buffer was prepared by adding 1.0 gm of gelatin[2] to 1 liter phosphate buffer (0.1 M, pH 7.0, containing 0.14 M NaCl and 0.01% thimerosal). Phosphate buffer was prepared by adding 32.7 gm sodium phosphate dibasic heptahydrate (MW = 268), 10.8 gm sodium phosphate monobasic monohydrate (MW = 138), 0.2 gm thimerosal[3] and 18.0 gm sodium chloride (MW = 58) to a two liter volumetric flask and adding triple distilled water to a volume just under two liters. The mixture was stirred with a magnetic

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[3]Sigma Chemical Company, P.O. Box 14508; St. Louis, Missouri 63178.

stirrer until the salts were dissolved and pH adjusted to 7.0 $\pm$ 0.1. One gm/liter gelatin (0.1%) was then added and stirred while heating to dissolve the gelatin. The assay buffer was stored at 4°C for usually not more than two weeks.

Labeled Progesterone

Progesterone -1, 2, 6, 7 ^{3}H (Specific Activity = 0.25 Ci/mmole) was obtained from New England Nuclear Corporation[4] and stored at 4°C in 5 ml benzene.

To prepare the assay tracer ("IX"), an aliquot of the stock labeled progesterone was purified on a Sephadex LH-20 chromatography column as described by Carr et al. (1971), thus: 1.2 gm Sephadex LH-20 was weighed out into a glass vial and 10 ml of a benzene:methanol solution (85:15) added and allowed to swell overnight. A Whatman filter disk was placed at the bottom of the column and a few drops of the benzene:methanol solution added to wet the disk. The vial containing the Sephadex and solvents was shaken to form a slurry, poured into the column, allowed to run through and rinsed with 10 ml of the solvent. Meanwhile

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[4]New England Nuclear, Boston, MA. 02218.

0.5 ml of the stock labeled progesterone in benzene was dried down under air and taken up in .5 ml of a hexane: benzene:methanol solution (HBM, 85:5:4 v/v). Ten ml of the HBM solution was also added to the column to pack the Sephadex.

The stock progesterone dissolved in 0.5 ml HBM, was then applied to the column rinsing with another 0.5 ml HBM. 2 ml HBM was then applied to the column and allowed to drain. Then a 20 ml glass scintillation vial was placed under the column and 8 ml of HBM applied to the column. This fraction of the eluate (mls 3-10 after sample application) which contained the tritrated progesterone, was collected. This was dried down under air, taken up in 3 mls benzene, and stored at $4^{\circ}C$ for no longer than one month.

An appropriate fraction of this cleaned stock was dried under air, dissolved in PBSG, and diluted further to yield a concentration of 10,000 cpm (counts per min.)/ 0.1 ml.

Recovery tracer was prepared by diluting assay tracer to 1000 cpm/0.1 ml.

Antiserum

Antiserum S7#4, developed by Abraham et al., was obtained from Bar Harbor General Hospital, Torrance, California.[5] The lyophilized antiserum (.7 ml) was dissolved in 0.7 ml of PBSG and stored at -70°C in 0.1 ml aliquots.

Antibody dilution (avidity) curve. In order to titrate the antiserum, serial dilutions of 1:200, 1:300, 1:400, 1:600, 1:800, 1:1200 were made in assay buffer (PBSG), and 0.1 ml of each dilution in triplicate was added to tubes containing 10,000 cpm/0.1 ml of the assay tracer (IX) and 0.5 ml of assay buffer. Nonspecific bound tubes (NSB) containing 0.6 ml of assay buffer and 0.1 ml assay tracer, and total count tubes (TCT) containing 0.8 ml assay buffer and 0.1 ml assay tracer were also prepared in triplicate. Percent bound for each dilution was calculated by

$$\% \text{ Bound} = \frac{(\text{unknown cpm}) - \overline{\text{NSB}}}{\overline{\text{TCT}} - \overline{\text{NSB}}} \times 100$$

where $\overline{\text{NSB}}$ = average cpm of the three NSB tubes

$\overline{\text{TCT}}$ = average cpm of the three TCT tubes.

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[5]Harbor General Hospital, 1124 W. Carson St., Torrance, CA. 90502.

The 1:600 - 1:750 from the plotted curve gave % Bound close to 50% (Figure 1).

Standards

Progesterone for standards was obtained from Sigma Chemical Company.[6] The hormone was dissolved in ethanol to give a stock concentration of 1ug/ml. This was stored at -20°C. Aliquots of this were taken and diluted with PBSG to concentrations of 10 ng/ml buffer, 1 ng/ml buffer, and 0.1 ng/ml buffer to make high, medium and low standard stock solutions, respectively.

Dextran-Coated Charcoal (DCC)

A dextran-coated charcoal suspension was prepared using a concentration of 0.625% Norit A charcoal (Sigma Chem. Co.)[7] and 0.0625% Dextran T-70[8] in PBSG. The charcoal was cleaned by adding triple distilled water to a portion of activated charcoal in a beaker. This was stirred for 2 hours, the water decanted, and the charcoal dried in an oven overnight. To 0.625 gm of the cleaned charcoal and 0.0625 gm of Dextran T-70 in a flask, was added the

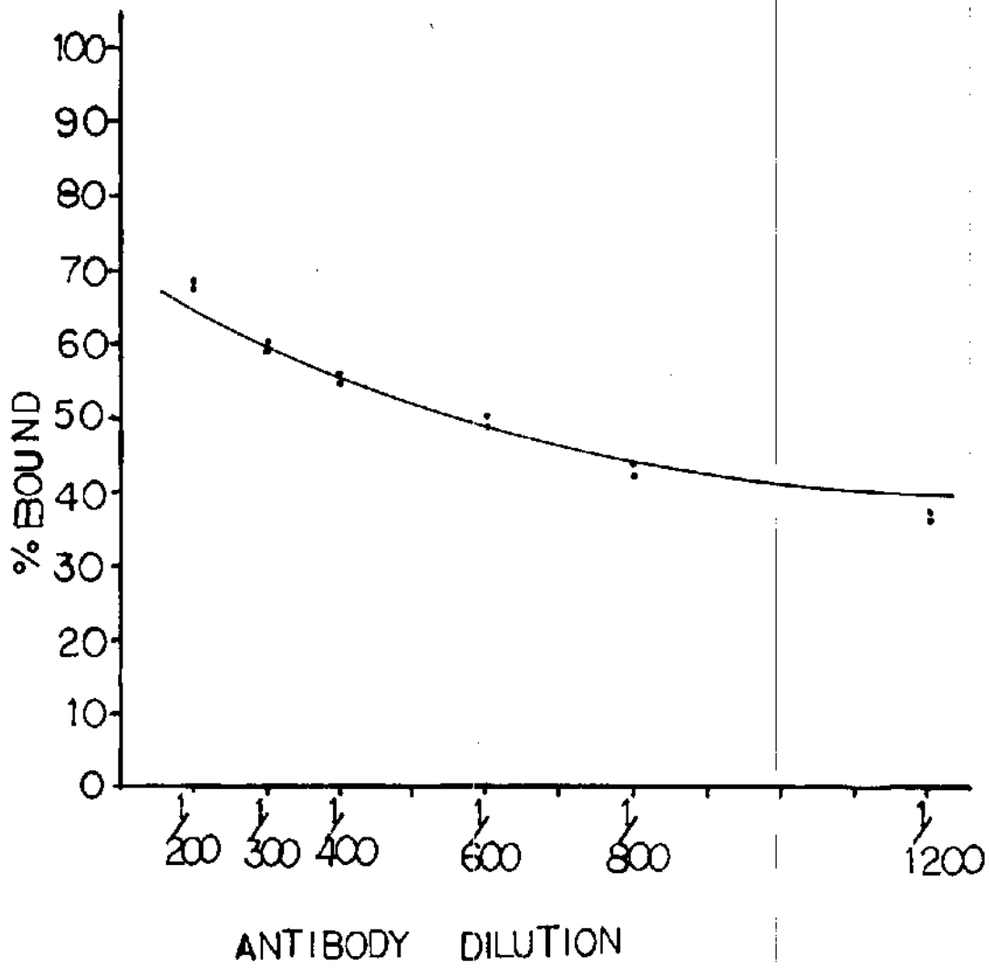
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[6]Sigma Chemical Co., St. Louis, MO. 63178.

[7]Sigma Chemical Co., St. Louis MO. 63178.

[8]Pharmacia Lab., Inc., Piscataway, N.J. 08854.

Figure 1. Avidity Curve for the S7#4 Anti-Progesterone Antibody


100
90
80
70
60
50
40
30
20
10
0
% BOUND
200
300
400
600
800
1200
ANTIBODY DILUTION

assay buffer up to the 100 ml mark and stored while stirring overnight at 4°C. This was prepared at least one day before use.

Scintillation Fluid

Scintillation fluid was prepared by adding 50 ml PPO-POPOP Concentrated Liquid Scintillator[9] and 198 ml paradioxane (Scintillation grade) to one 1 liter of toluene.[10]

Assay Procedure

Radioimmunoassay of plasma progesterone was carried out using a procedure similar to that described by Garza et al. (1975) using a specific antiserum to progesterone found by the authors to have minimal or no cross reaction with other steroids.

Extraction

One-tenth ml recovery tracer was added to each of three recovery total count vials (rTCT) and to 0.5 ml aliquots of each plasma sample in 15 x 125 mm pyrex test

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[9]Research Products International Corp., Elk Grove Village, IL. 60007.

[10]Fisher Scientific Co., N.J. 07410.

tubes. Contents of the tubes were mixed, and allowed to equilibrate at room temperature for 2 hours. Plasma steroids were extracted twice with 4 mls hexane (nonspectro),[11] mixing for 30 seconds and allowed to stand for a few minutes for the aqueous and hexane layers to separate. The aqueous (bottom) layer was frozen by placing tubes in a dry-ice:acetone bath, and the hexane layer then decanted into a glass vial. This was dried down in a warm water bath under a stream of filtered air,[12] rinsed down with 1 ml of hexane and dried down again. The dried extract was then dissolved in 1 ml of assay buffer, vortexed and stored at 4°C for a minimum of 4 hours. Two 0.2 ml aliquots of the extracted progesterone sample were counted to determine recovery of the tritiated tracer added prior to extraction. These were placed into 4 ml of scintillation fluid and each counted for 10 minutes (Beckman LS 330 Liquid Scintillation system)[13]

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[11]Burdick and Jackson Laboratories, Inc., Muskegon, MI. 49442.

[12]Multivap, Organomation Associates, Inc., Shrewsbury, ME. 01545.

[13]Beckman Instruments, Inc., Fullerton, CA. 92634.

$$\% \text{ Recovery} = \frac{(r_1 + r_2) - \overline{\text{Background counts}}}{\overline{\text{rTCT}} - \overline{\text{Background counts}}} \times \frac{5 \times 100}{1}$$

where $r_1 + r_2$ = average counts of the two 0.2 ml aliquots of the unknown

$\overline{\text{rTCT}}$ = average of the recovery total counts.

The mean recovery for all assays (n = 10) was $70 \pm 1\%$.

Assay Set-up

Two 0.2 ml aliquots of the extracted sample were pipetted into 12 x 75 mm disposable test tubes containing 0.3 ml PBSG. For the standard curve, 10, 25, 50, 100, 250, 500, and 1000 pg progesterone, each in 0.5 ml PBSG were added to tubes in triplicate. One-tenth ml of the diluted antiserum was added to each of the assay tubes, which were then vortexed. One-tenth ml of the assay tracer ("IX") was then added to each tube, which were vortexed, covered, and stored at 4°C for at least 2 hours (or overnight). After this period, 0.2 ml DCC (dextran-coated charcoal) was added to each tube. Contents of tubes were mixed, and allowed to stand at 4°C for 20 minutes, after which they were centrifuged at 4-5°C (10 minutes, x 1000 g). The supernatants were decanted into scintillation vials to

which 10 ml of the scintillation fluid had been added. Contents of the vials were mixed, and the vials were then placed in the scintillation counter.

Tubes containing no antiserum or unlabeled hormone, nonspecific binding tubes (NSB), and tubes containing antiserum but no unlabeled hormone, maximum bound (B_o) tubes were also run. These were treated in the same manner as tubes containing standards or unknowns. Triplicate total count tubes (TCT) were prepared by pipetting 0.1 ml of the IX directly into vials containing scintillation fluid. The average counts for the triplicate NSB tubes were subtracted from all other tubes in the assay. The B_o tubes (after subtraction of NSB) were used as a reference to which all other tubes were compared. The antibody bound radioactivity in the B_o tubes was taken as 100%, and tubes containing unlabeled hormone (standards or unknowns) were expressed as a percent of the B_o.

Calculations

The percent bound values obtained for standards were converted to logits (logit % bound = $\frac{\log \text{ \% bound}}{\text{100\% bound}}$). The logit transformation,

$$\text{logit } Y = \log_e \frac{Y}{100-Y} = 2.303 \log_{10} \frac{Y}{100-Y}$$

linearizes the curve between approximately 10% and 90% bound, and involves fairly simple computations and permits accurate estimation of the slope by either linear regression or graphical means (Johnston, 1977). The unknown hormone values were obtained using a computerized weighted regression analysis.

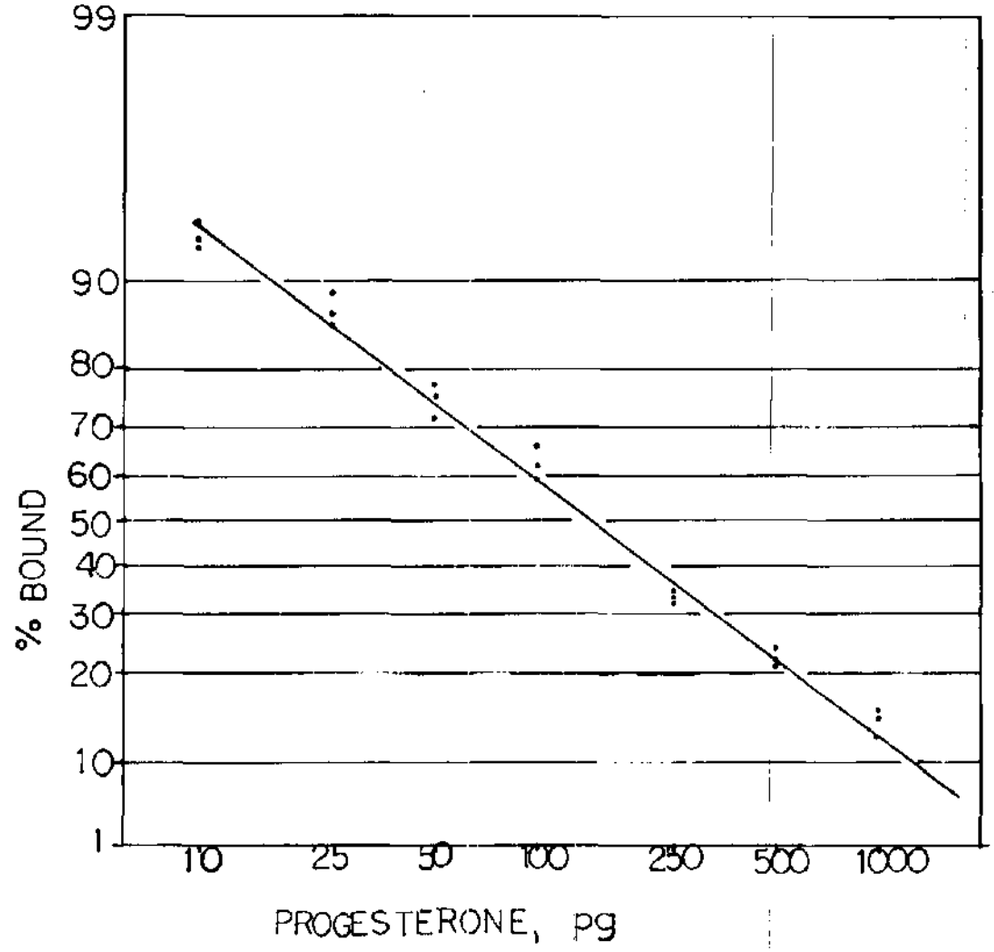
Percent of labeled hormone bound to antibody in the absence of unlabeled hormone (B_o tubes) was 47.98 $\pm$ 6.2% (n = 10). The slope of the standard curve was -1.080 $\pm$ 0.1200. Linear standard curves plotted on the logit - log scale using progesterone doses of 10 pg to 1000 pg ranged from 94.02% bound to 15.86%.

Assay Validation

Sensitivity

A mean plasma concentration of 0.2 ng/ml plasma in 10 assays was the minimum detectable amount that could be distinguished from zero. This value was calculated by subtracting the sum of 2 standard deviations from the maximum binding, extrapolating from the standard curves and then correcting for volume and recovery (Johnston, 1977).

Figure 2. Sample Standard Curve for a Radioimmunoassay of *Plasma Progesterone*


99
90
80
70
60
50
40
30
20
10
1
% BOUND
10
25
50
100
250
500
1000
PROGESTERONE, pg

Specificity

It has been shown, Garza et al. (1975), that selective solvent extraction with the nonpolar solvent hexane gave adequate specificity with the antiserum S7#4. Comparison between plasma progesterone levels obtained during a human menstrual cycle using S7#4 after hexane extraction only (Garza et al., 1975) versus using a nonspecific anti-P antiserum (Abrahams et al., 1971) with chromatographic purification prior to assay has further confirmed this (Garza et al., 1975). See Table 1 for characterization data on S7#4 progesterone antiserum (Garza et al., 1975). This table shows that S7#4 has minimal cross reactivity with other steroids.

Accuracy

This was determined by measuring known concentrations of untritiated progesterone at 10 ng, 7.5 ng, 5 ng, 2.5 ng, 1 ng and 0.5 ng added to 1 ml aliquots of the assay buffer. These were equilibrated overnight, then extracted, assayed and values corrected for procedural loss. Recoveries obtained are shown in Table 2 and Figure 3.

Blank Values

One-half ml aliquots of the assay buffer (PBSG) and

TABLE 1

CHARACTERIZATION DATA

Steroids	% Cross Reaction
Cholesterol	< 0.01
C_{21} Steroids	
Pregnenolone	0.8
Pregnenolone Sulfate	< 0.01
17-Hydroxypregnenolone	< 0.01
Progesterone	100
17-a-Hydroxyprogesterone	< 0.01
20-a-Dihydroprogesterone	0.3
Desoxycorticosterone	1.5
11-Desoxycortisol	< 0.01
Cortisol	< 0.01
Corticosterone	< 0.01
Aldosterone	< 0.01
C_{19} Steroids	
Androstenedione	< 0.01
Testosterone	< 0.01
Dihydrotestosterone	< 0.01
Dehydroepiandrosterone	< 0.01
Androsterone	< 0.01
Ethiocholanolone	< 0.01
C_{18} Steroids	
Estradiol-17 β	< 0.01
Estradiol-17-a	< 0.01
Estrone	< 0.01
Estriol	< 0.01

Method Reference: R. Garza et al., Anal. Let. 8: 1975 (March)

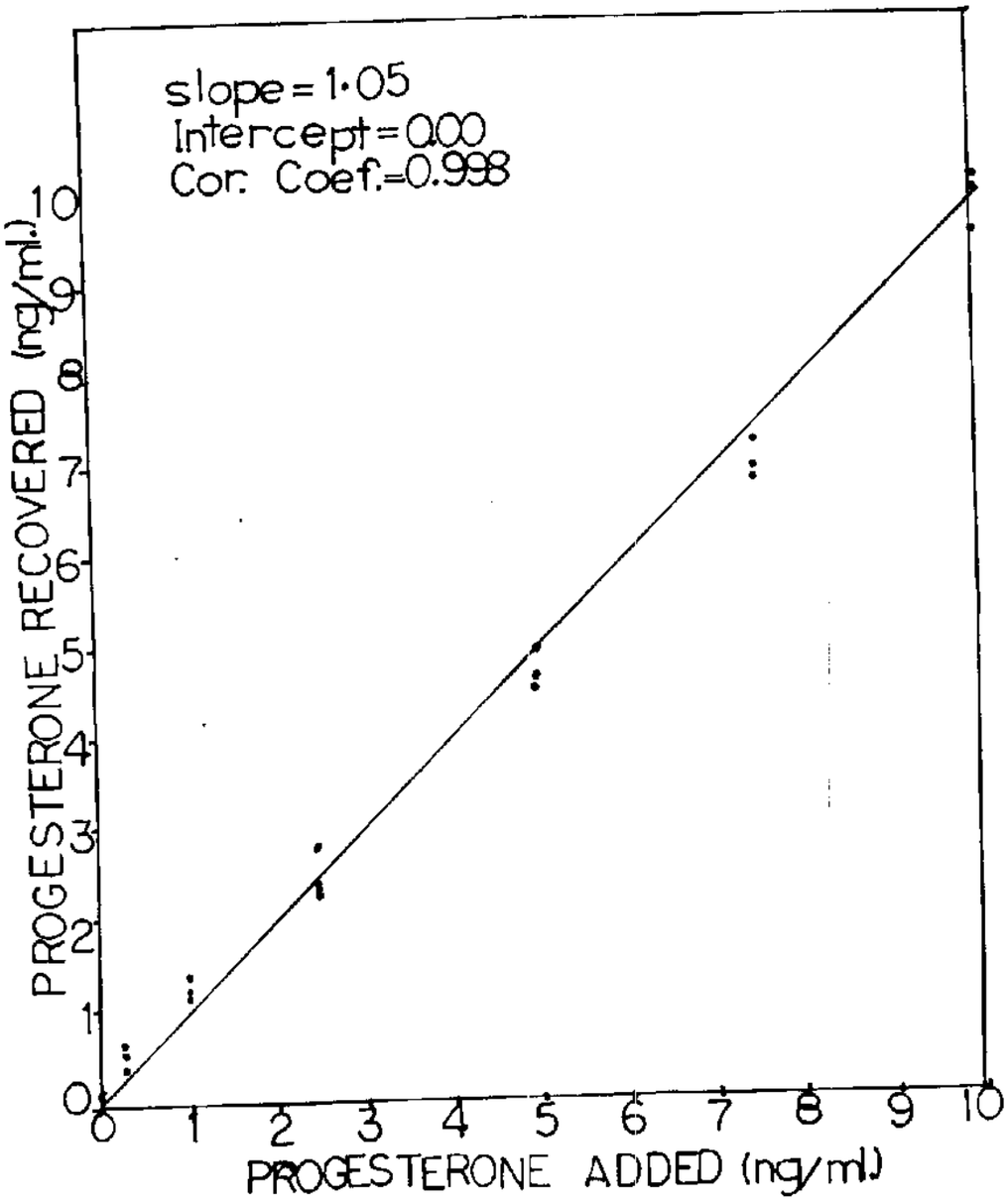
TABLE 2

RECOVERY OF NONTRITRATED PROGESTERONE ADDED TO ASSAY BUFFER

Progesterone Added (NG/ML)	Mean Progesterone[a] Measured (NG/ML)	S.D.	n	CV %
10.0	9.9	0.35	5	3.5
7.5	7.0	0.17	5	2.4
5.0	4.7	0.20	5	4.2
2.5	2.5	0.21	5	8.3
1.0	1.3	0.08	5	6.3
0.5	0.6	0.06	5	9.5
0.25	0.5	0.11	5	22.2

[a]Values are corrected for procedural loss.

Figure 3. Recovery of Nontritiated Progesterone Added to Assay Buffer


slope = 1·05
Intercept = 0.00
Cor. Coef. = 0.998
PROGESTERONE RECOVERED (ng/ml.)
PROGESTERONE ADDED (ng/ml.)
0
1
2
3
4
5
6
7
8
9
10

0.5 ml of bull serum were alsc extracted in triplicate and assayed like the unknowns within each assay. These gave nondetectable values for all assays (n = 10).

Precision

The precision of the assay is a measure of the variation between measured concentrations of progesterone in replicates of identical samples. This was determined using 10 frozen aliquots of serum from a diestrous mare in each assay to determine the inter-assay coefficients of variation. Within assay variation was determined by analyzing ten diestrous mare serum samples in a single assay (Table 3).

In order to minimize the effect of inter-assay variations, all plasma samples within each cow (both control and treatment cycle) were analyzed in one assay.

Evidence of Parallelism of Unknowns With The Standard Curve

This was obtained by assaying plasma samples from a diestrous cow, a diestrous mare and from a cow close to estrus. These samples were extracted, dried down and dissolved in 2 ml assay buffer. The extracted samples were then assayed 5 times each at volumes of 0.3 ml, 0.2 ml,

Table 3

COEFFICIENTS OF VARIATION WITHIN AND BETWEEN PROGESTERONE ASSAYS

	Intra-Assay	Inter-Assay
$\bar{x}$	5.7	5.7
S.D.	0.3	0.5
n	10	10
CV %	4.5	7.9

0.1 ml and 0.05 ml. Percent bound at each volume was plotted on a logit scale with the standard curve. The four lines were parallel, signifying that unknowns could be read with confidence over the length of the standard curve (Johnston, 1977). See Figure 4.

Radioimmunoassay of 15-Keto-13, 14-dihydro PGF_{2a}

15-Keto-13, 14-dihydro-PGF_{2a}, the main blood plasma metabolile of PGF_{2a}, was measured in bovine plasma by Drs. H. Kindahl and L.-E. Edgvist by a radioimmunoassay technique previously described (Granstrom E. and Kindahl H., 1976 and Kindahl et al., 1976a).

Radioimmunoassay of Oxytocin

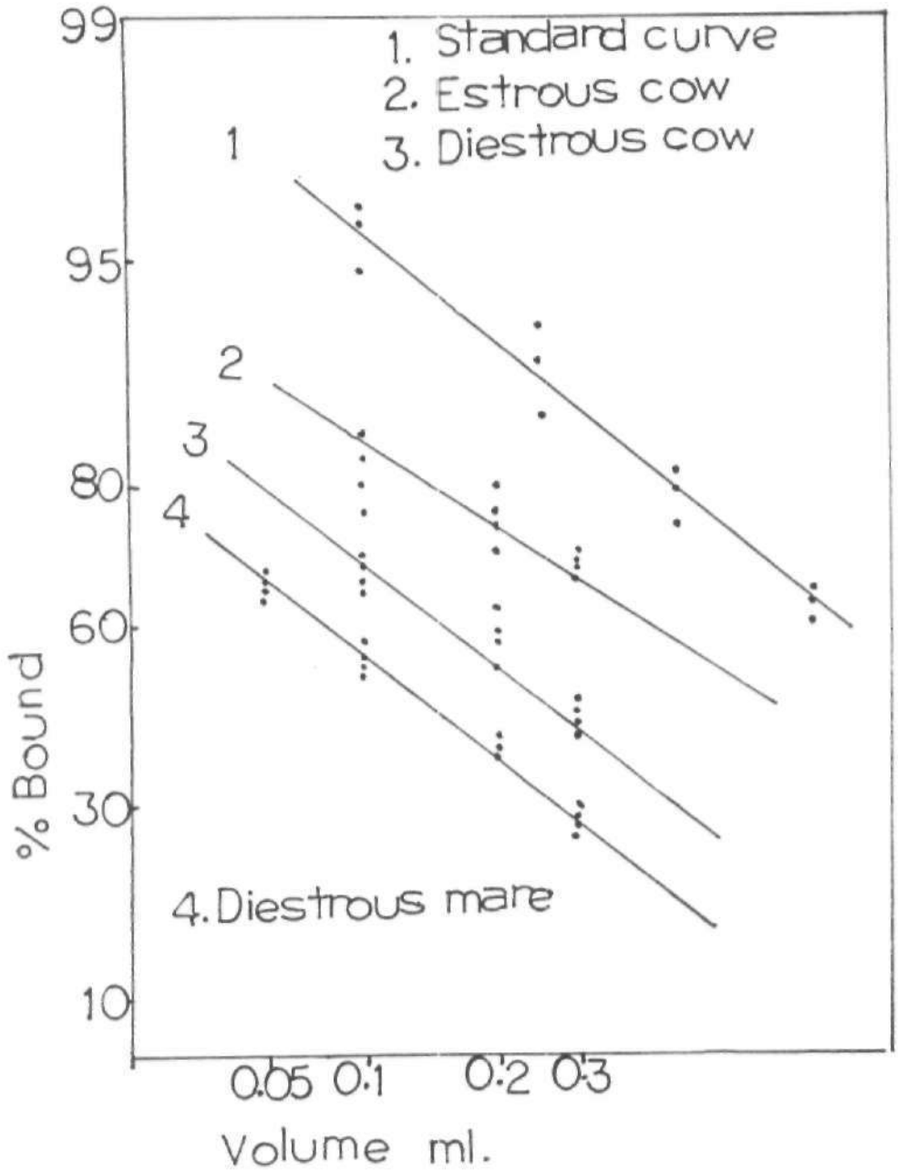
Oxytocin was measured in bovine plasma by Dr. P. Kumaresan at the Coney Island Hospital, Brooklyn, New York, by a radioimmunoassay technique previously described (Vascika et al., 1978; Kumaresan et al., 1978).

Statistical Analysis

Intrauterine Manipulations

Three way analyses of variance with individual contrasts were used to test the null hypotheses of no difference between control and treatment cycles, among the three

Figure 4. Evidence of Parallelism of Unknowns with the Standard Curve


99
95
80
60
30
10
% Bound
1. Standard curve
2. Estrous cow
3. Diestrous cow
1
2
3
4
4. Diestrous mare
0.05
0.1
0.2
0.3
Volume ml.

cows and among six time periods. Separate analyses of variance were used for prostaglandin metabolite, oxytocin and progesterone concentration values.

Differences among cows and time periods were tested by individual contrasts, and for interaction differences (e.g. cycles in different time periods), paired t-tests were used.

Oxytocin Administration

The same type of analyses were carried out except that five cows and ten time periods were considered.

RESULTS

Experiment 1 Intrauterine Mechanical Manipulations

Oxytocin

Mean plasma oxytocin concentrations in the control and treated cycles are shown in Table 4. Premanipulation levels on Day 3 were not different between control and experimental cycles. Oxytocin levels were higher ($p < 0.05$) after manipulations were performed on Day 3. On Day 6, premanipulation levels in the experimental cycles were higher than in the control cycles ($p < 0.01$) but manipulation on Day 6 caused a further increase in oxytocin. Oxytocin in the experimental cycles remained higher up to Day 14. The post-manipulation increase in oxytocin was greater ($p < 0.01$) after the Day 6 manipulation than after that on Day 3.

One cow had higher basal levels of oxytocin (approximately 300 pg/ml) than the other cows (approximately 150 pg/ml) throughout the trial.

PGF_{2a}

Data obtained with PGF_{2a}-metabolite showed that

TABLE 4

EFFECT OF CERVICAL AND INTRAUTERINE MECHANICAL MANIPULATIONS ON JUGULAR LEVELS OF OXYTOCIN AND PGF_{2a}-METABOLITE

Day of Cycle	Time Interval (Mins.)	Oxytocin (pg/ml)* Control Cycle		Oxytocin (pg/ml)* Exptal. Cycle		PGF_{2a}-Metabolite* (pg/ml) Control Cycle		PGF_{2a}-Metabolite* (pg/ml) Exptal. Cycle	
3	-330 to -120	175±107		167±50		97±13		72±13	
Permanipulation	-120 to - 30	161±108	a	175±80	a	110±19	d	74±18	e
	- 30 to 0	167±121		168±80		127±24		73± 9	
	0 to 30	172±122		173±91		117±27		86±25	
Postmanipulation	30 to 120	161±108	a	158±69	b	114±27	d	85±31	e
	120 to 330	175±117		190±90		106±36		83±28	
6	-330 to -120	167± 88		149±62		100±34		84±27	
Premanipulation	-120 to - 30	176± 87	a	191±91	b	100±27	d	84±20	e
	- 30 to 0	154± 80		182±72		98±40		79±16	
	0 to 30	163±100		194±88		109±44		81±11	
Postmanipulation	30 to 120	156± 88	a	183±86	c	97±32	d	88±20	e
	120 to 330	146± 90		199±107		100±30		90±21	

*Mean ± S.D.) No. of Cows = 3
No. of observations within each time interval = 6
abc and de Data with different superscripts are significantly different ($p < 0.05$)

manipulations did not cause release of PGF_{2a} either on Day 3 or 6 (Table 4). Mean PGF_{2a}-metabolite basal levels were higher ($p<0.01$) in control than in experimental cycles (Table 4).

Normal luteolytic patterns of release of PGF_{2a} were observed in all cycles around the time of estrus consistent with a fall in progesterone to basal levels.

Progesterone

There were no differences between mean progesterone levels in the control and experimental cycles up to Day 14 (Figure 5). However, two of the three manipulated cows (Nos. 1 and 2) had 2-3 day shorter experimental cycles than control cycles. Progesterone profiles between Day 14 and estrus in the control and experimental cycles for each cow are shown in Figures 6, 7, and 8.

Experiment 2 Exogenous Oxytocin Administration

In order to analyze data concerning injections of oxytocin given twice each day for 5 days, the duration of the injections was divided into ten periods for each cow as follows:

Figure 5 Mean Progesterone in Control and Experimental Cycles After Manipulations.

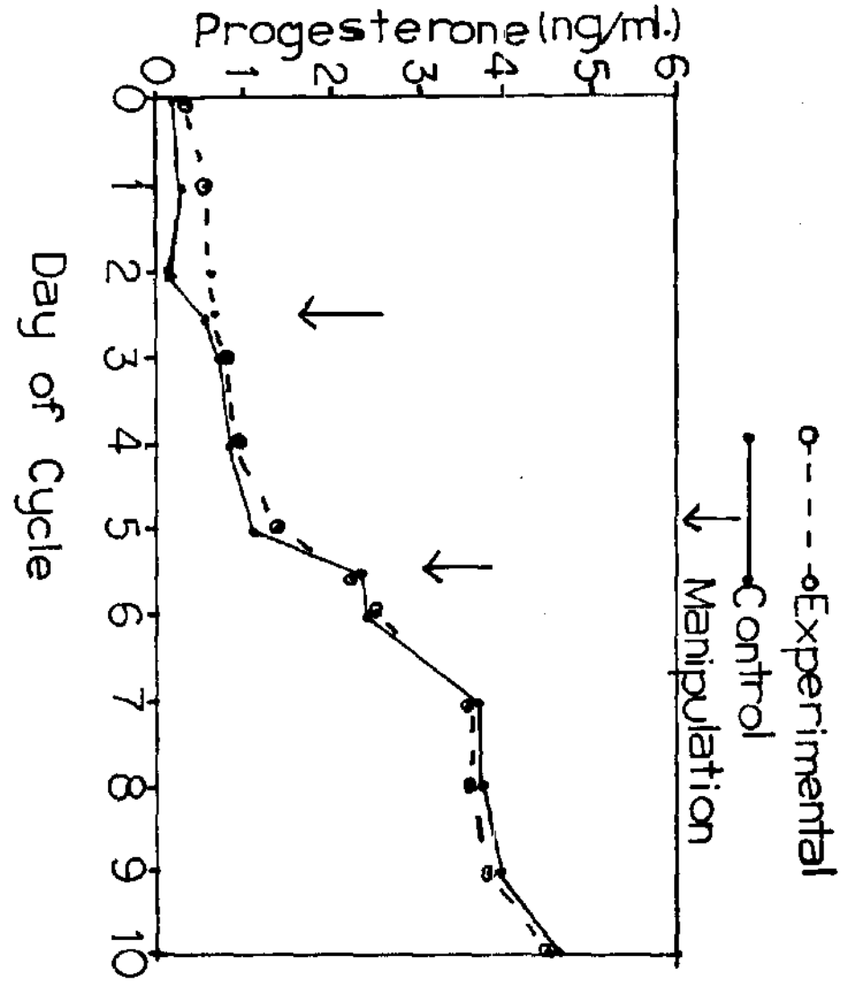

Progesterone (ng/ml.)
0
1
2
3
4
5
6
Day of Cycle
0
1
2
3
4
5
6
7
8
9
10
Experimental
Control
Manipulation

Figure 6 *Progesterone* Profile Between Day 14 and Estrus in Control and Experimental Cycles After Manipulation, Cow No. 1.

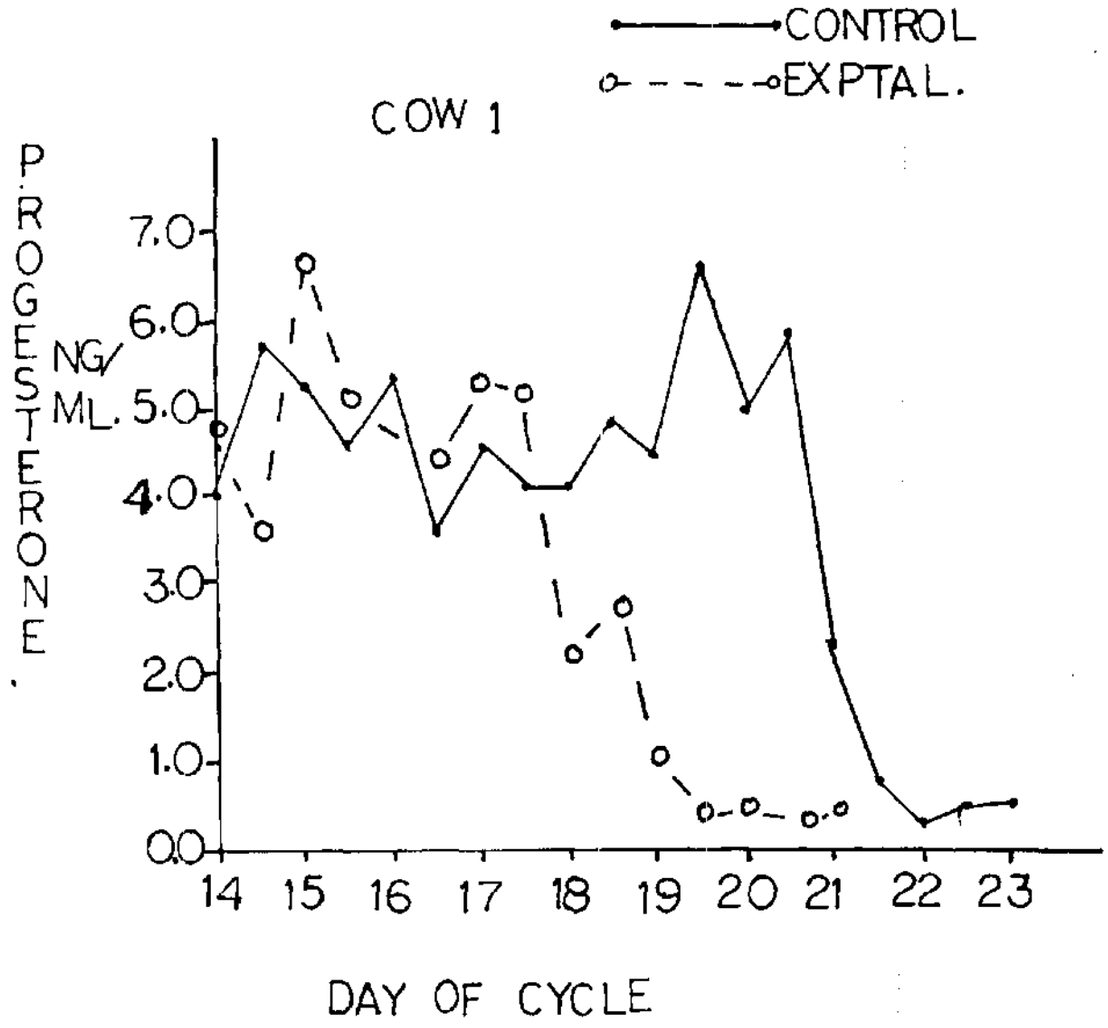

CONTROL
EXPTAL.
COW 1
PROGESTERONE
NG/ML.
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
14 15 16 17 18 19 20 21 22 23
DAY OF CYCLE

Figure 7 Progesterone Profile Between Day 14 and Estrus in Control and Experimental Cycles After Manipulation, Cow No. 2.

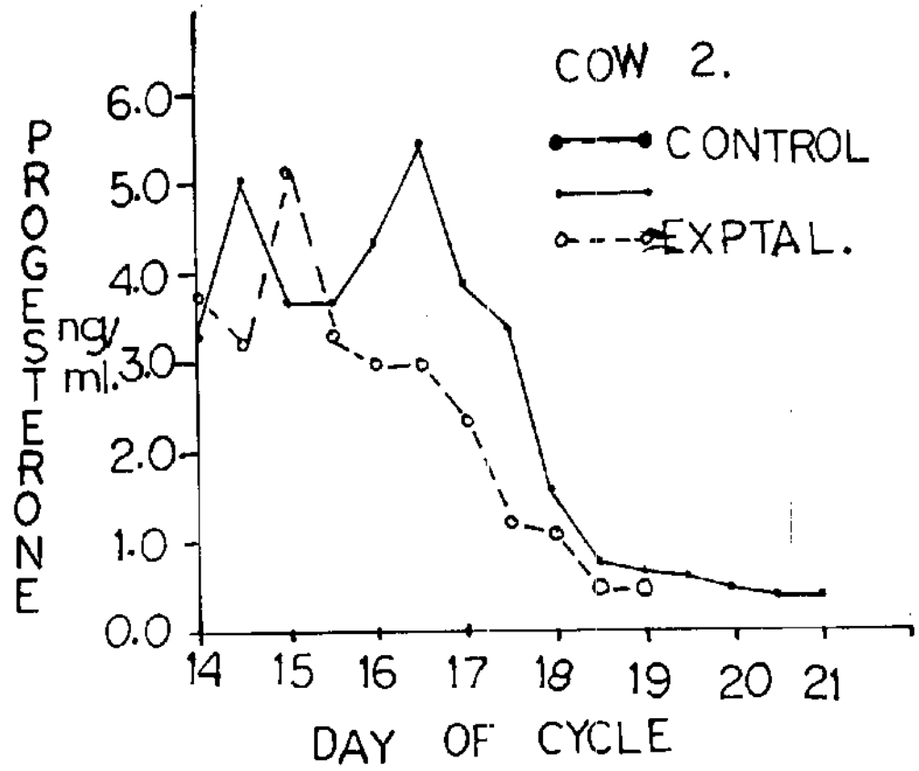
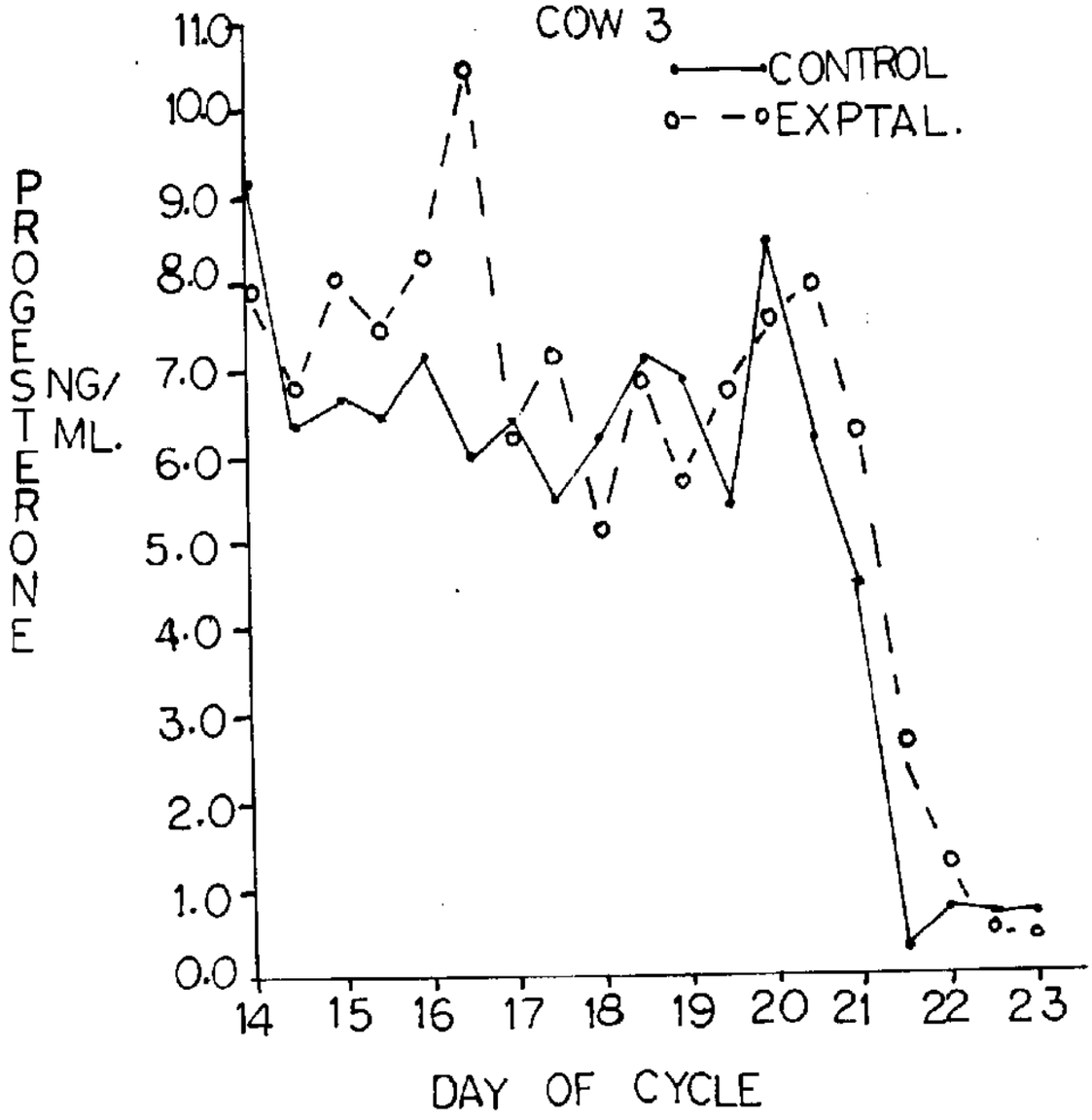

COW 2.
CONTROL
EXPTAL.
PROGESTERONE
ng/ml.
6.0
5.0
4.0
3.0
2.0
1.0
0.0
14
15
16
17
18
19
20
21
DAY OF CYCLE

Figure 8 Progesterone Profile Between Day 14 and Estrus in Control and Experimental Cycles After Manipulations, Cow No. 3.


COW 3
CONTROL
EXPTAL.
PROGESTERONE NG/ ML.
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
14
15
16
17
18
19
20
21
22
23
DAY OF CYCLE

Period 1 (Pretreatment)	Day 1 to 9 A.M. of Day 2
Period 2	9 A.M. of Day 2 to 6 P.M. of Day 2
Period 3	6 P.M. of Day 2 to 9 A.M. of Day 3
Period 4	9 A.M. of Day 3 to 6 P.M. of Day 3
Period 5	6 P.M. of Day 3 to 9 A.M. of Day 4
Period 6	9 A.M. of Day 4 to 6 P.M. of Day 4
Period 7	6 P.M. of Day 4 to 9 A.M. of Day 5
Period 8	9 A.M. of Day 5 to 6 P.M. of Day 5
Period 9	6 P.M. of Day 5 to 9 A.M. of Day 6
Period 10	9 A.M. of Day 6 to 6 P.M. of Day 6

PGF_{2a} Response to Oxytocin

Prostaglandin F_{2a}-metabolite levels after oxytocin injections are shown on Tables 5 and 6. The values shown indicate that oxytocin injections caused an acute release of PGF_{2a} with the greatest response following the first oxytocin injection at 9 A.M. on Day 2. In general, decreasing PGF_{2a} responses were observed with each subsequent oxytocin injection. The overall mean prostaglandin F_{2a}-metabolite levels were higher ($p < 0.01$) throughout the duration of injections in the oxytocin treated cycles than in the saline treated cycles.

TABLE 5

THE MEAN ± S.E.M. LEVELS OF $PGF_{2\alpha}$-METABOLITE (PG/ML) BEFORE AND AFTER OXYTOCIN ADMINISTRATION BY PERIODS

Day of Cycle		2		3		4		5		6	
Time Periods		1 Pretreatment	2	3	4	5	6	7	8	9	10
Cows	Treatment		9AM 6PM	9AM 6PM		9AM 6PM		9AM 6PM		9AM 6PM	
4	Saline	70±2.7	69.0± 2.1	74.7± 1.9	69.9± 2.1	62.1± 2.9	71.1± 2.8	75.1± 2.7	73.4± 2.7	71.9± 3.3	79.5± 2.5
	Oxytocin	114.0±5.0	206.0± 15.1	122.0± 10.5	131.2± 6.5	104.6± 6.1	104.7± 5.4	126.9± 3.3	121.7± 5.0	129.1± 3.6	126.8± 4.4
5	Saline	84.5±1.9	77.8± 2.6	81.1± 3.2	75.3± 2.4	63.6± 6.4	90.4± 7.0	91.2± 7.0	87.2± 4.7	54.8± 4.1	86.3± 2.7
	Oxytocin	85.4±3.4	132.3± 12.4	108.2± 9.1	129.3± 7.9	91.8± 5.2	113.9± 7.2	101.3± 4.2	122.5± 9.2	90.0± 6.0	95.1± 7.8
6	Saline	57.8±5.5	64.6± 4.2	49.3± 1.8	54.3± 2.5	58.4± 4.4	57.9± 3.8	49.3± 2.2	57.4± 2.5	50.3± 2.4	62.3± 3.9
	Oxytocin	49.0±1.9	76.8± 8.2	40.7± 9.7	51.1± 7.7	36.7± 1.6	46.2± 2.0	43.1± 1.6	45.1± 1.6	33.1± 1.7	63.9± 11.8
7	Saline	51.6±2.2	65.3± 2.4	48.6± 1.5	59.0± 2.5	50.7± 2.1	71.8± 5.7	76.0± 3.6	79.7± 3.6	55.8± 8.8	42.4± 1.7
	Oxytocin	49.7±1.0	87.5± 8.3	41.5± 2.7	48.5± 3.7	33.8± 3.3	48.0± 3.9	40.3± 2.9	41.2± 2.4	39.7± 1.8	49.1± 2.6
8	Saline	73.4±3.3	84.4± 3.0	65.8± 1.7	67.1± 3.3	66.7± 2.1	68.2± 1.5	61.1± 1.3	61.8± 1.8	57.2± 2.0	54.1± 1.1
	Oxytocin	79.3±3.0	72.4± 2.4	95.7± 6.9	93.0± 4.0	97.2± 4.4	87.9± 3.6	95.6± 5.3	111.9± 5.7	112.2± 6.8	110.4± 4.9
9	Oxytocin	55.4±3.1	62.1± 2.8	66.5± 2.7	69.3± 2.5	60.1± 2.5	68.6± 1.9	49.4± 2.3	55.8± 1.4	54.8± 1.7	55.7± 2.2
Averages ±S.D.	Saline	67.5±5.8	72.2± 3.8	63.9± 6.6	65.1± 3.8	60.3± 2.7	71.9± 5.3	71.9± 5.5	71.9± 5.5	58.0± 3.7	64.9± 8.1
	Oxytocin	72.1±10.5	106.2± 22.3	79.1± 14.2	87.1± 15.1	70.7± 12.8	78.2± 11.7	76.1± 14.9	83.0± 16.1	76.5± 16.3	83.5± 13.0

No. of Cows = 6

No. of observations per period = 13

TABLE 6

THE MEAN ± S.E.M. LEVELS OF 15 KETO, 13, 14-DIHYDRO $PGF_{2\alpha}$ (PG/ML) BEFORE AND AFTER S.Q. INJECTION OF 230 UNITS OF OXYTOCIN OR PHYSIOLOGICAL SALINE

Day of Cycle	Treatment	Pretreatment Levels	Time From Oxytocin/Saline Injection (Mins.) 0-30	30-60	60-90	90-120	120-180	180-240	240-300	300-360	Pretreatment	Post P.M. Treatment Time from Oxytocin Injection (Mins.) 0-180	180-360	360-540
2	Saline	67±5.9	70.2± 4.6	74.2± 6.0	68.2± 5.4	79.4± 4.5	67.6± 7.6	72.6± 3.9	72± 6.6	73.8± 5.7				
	Oxytocin	75.7±12.1	98.1± 25.1	122.9± 24.8	161.8± 34.1	139.6± 33.0	143.8± 34.6	145.2± 43.9	93.8± 12.4	107.5± 20.0	67.6± 16.5	134.5± 37.0	91.2± 21.0	84.8± 25.0
3	Saline	63.9±6.6	64.6± 5.3	67.0± 3.1	70.4± 4.3	69.2± 8.0	64.0± 5.9	66.2± 4.5	61.8± 4.4	66.4± 6.2				
	Oxytocin	75.3±16.9	93.5± 19.3	87.6± 20.5	103.8± 22.6	115.2± 23.4	102.6± 21.2	98.8± 14.8	84.4± 13.8	91.6± 16.3	60.0± 16.5	83.0± 19.2	81.0± 17.6	104.8± 22.8
4	Saline	59.8±2.8	60.3± 4.4	76.2± 7.7	78.6± 5.4	83.2± 14.0	75.4± 10.8	71.0± 5.8	71.4± 9.5	73.6± 4.9				
	Oxytocin	68.7±14.3	77.2± 14.4	87.0± 17.2	90.3± 18.7	75.2± 9.5	73.4± 9.9	76.8± 15.4	73.6± 12.7	89.2± 19.5	74.8± 16.4	80.6± 17.4	73.2± 12.8	96.3± 19.3
5	Saline	70.4±7.2	77.9± 5.9	67.8± 6.6	78.2± 8.8	66.4± 5.1	71.6± 7.0	74.4± 6.3	67.8± 2.1	66.8± 5.7				
	Oxytocin	82.1±17.6	88.9± 20.6	79.4± 15.2	82.2± 16.1	103.2± 25.8	86.0± 16.4	94.2± 22.6	92.2± 20.8	101.2± 24.6	86.6± 22.0	84.8± 21.4	101.6± 25.1	79.3± 22.7
6	Saline	57.4±3.6	61.7± 7.6	63.2± 8.9	62.4± 6.8	63.2± 6.0	64.8± 12.3	71.6± 9.1	68.6± 9.7	73.6± 9.2				
	Oxytocin	63.4±16.8	81.2± 18.6	88.1± 18.8	102.0± 16.6	111.6± 20.8	104.4± 15.3	84.4± 10.8	82.0± 14.5	89.0± 23.4	76.4± 16.9	87.4± 14.2	72.0± 13.4	77.0± 10.4
Averages	Saline	63.7±2.4	66.9± 3.2	69.7± 2.4	71.6± 3.1	72.3± 3.8	68.7± 2.1	71.1± 1.4	68.3± 1.8	70.8± 1.7				
	Oxytocin	73.0±3.2	85.8± 3.6	93.0± 7.6	108.0± 14.0	109.0± 10.4	102.0± 11.9	99.9± 12.0	85.2± 3.7	95.7± 3.7	73.1± 4.5	94.1± 10.2	83.8± 5.6	88.4± 5.3

No of cows = 6

Estrous Cycle Length, Progesterone, PGF_{2a} and Clinical Findings

The response of the cows to the oxytocin injections varied considerably in relation to the estrous cycle length, the interaction between progesterone and PGF_{2a}-metabolite levels; and clinical findings. Progesterone through Day 8 of the control and experimental cycles are shown for each cow on Table 7. The mean progesterone levels in the control and experimental cycles up to A.M. of Day 6, did not differ (Figure 9). As from P.M. of Day 6, progesterone levels had begun to differ significantly and was higher in the saline treated cycles as thus:

P.M. of Day 6 $p = 0.05$

Day 7 $p = 0.01$

Day 8 $p = 0.003$ (Figure 9)

Because of the variation in response among the cows to the oxytocin injections, the six cows have been subdivided for the subsequent discussion of these parameters.

a. Cows with shortened estrous cycles:

Oxytocin injections shortened the interestrous intervals for two cows (Nos. 4 and 6) to 10 and 12 days. These two cows had elevated levels of PGF_{2a}-metabolite

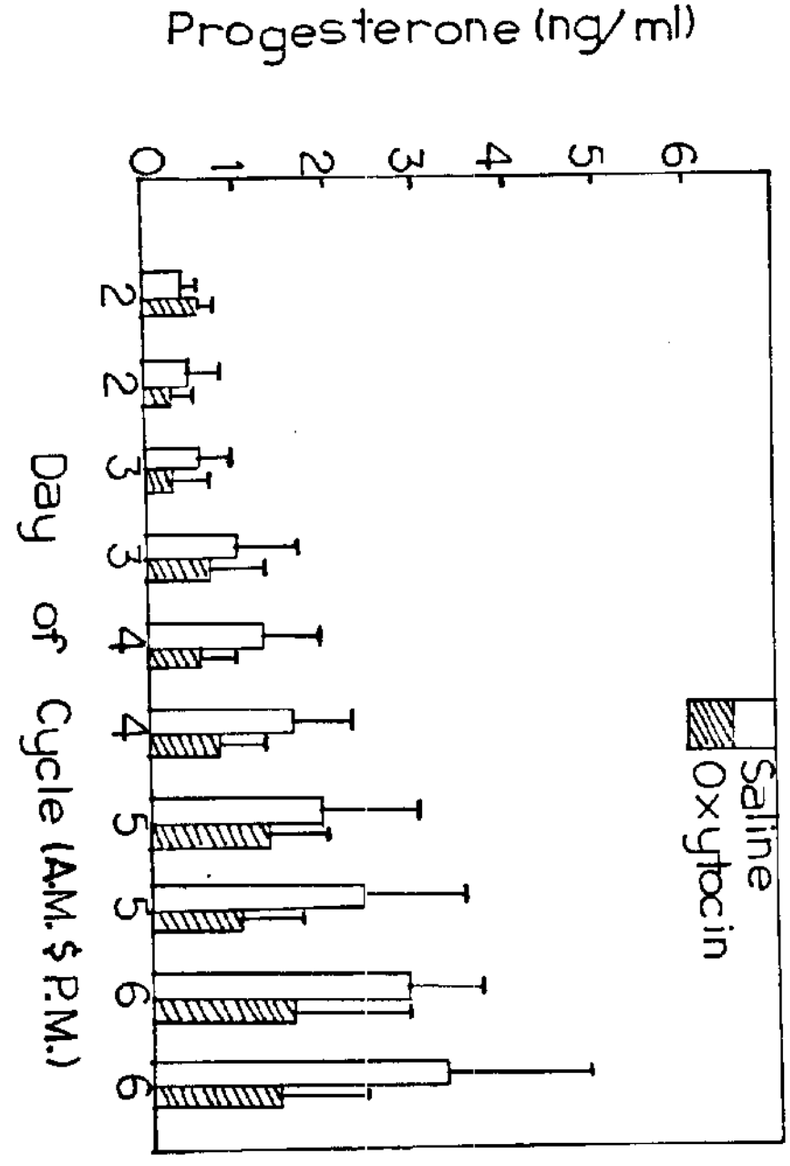
TABLE 7

PROGESTERONE (ng/ml) LEVELS IN OXYTOCIN AND SALINE TREATED COWS UP TO DAY 8 OF THE ESTROUS CYCLE

Day of Cycle			2		3		4		5		6		7	8	Estrous Cycle Length (Days)
Time Periods			1	2	3	4	5	6	7	8	9	10			
			9AM	6 PM	9 AM	6PM	9AM	6PM	9AM	6PM	9AM	6PM			
Cows		Treatment													
1	4	Saline	0.3	0.2	0.3	0.3	0.7	1.1	1.2	1.6	1.7	2.2	3.4	4.7	20
		Oxy.	0.2	0.2	0.3	0.6	1.0	1.6	1.5	1.7	3.7	3.0	2.2	1.5	10
2	5	Saline	0.4	0.8	1.0	1.7	1.7	2.5	3.0	3.3	2.8	5.0	7.3	4.2	22
		Oxy.	0.3	0.2	0.3	1.7	0.2	0.2	1.9	0.3	0.5	0.5	2.1	2.0	23
3	6	Saline	0.5	0.4	0.3	0.3	0.6	0.7	0.5	1.0	2.6	2.7	5.2	4.1	20
		Oxy.	0.5	0.6	1.1	0.7	1.1	0.9	1.3	1.4	1.5	1.4	1.6	0.3	12
4	7	Saline	0.5	0.4	0.9	1.5	1.9	1.9	2.8	2.4	3.4	3.4	4.2	3.5	21
		Oxy.	0.3	N.D.*	N.D.	N.D.	0.3	0.7	1.0	0.6	1.3	1.0	1.4	2.3	Cystic
5	8	Saline	0.5	1.0	0.9	1.2	1.6	2.1	2.0	3.5	3.8	3.5	2.8	4.7	22
		Oxy.	0.6	0.4	0.3	0.5	0.5	0.4	0.6	1.1	1.1	1.4	1.8	2.8	20
6	9	Oxy.	0.5	0.3	N.D.	N.D.	N.D.	0.3	N.D.	N.D.	N.D.	N.D.	0.6	0.5	Cystic
Aver-		Saline	0.4	0.6	0.7	1.0	1.3	1.7	1.9	2.4	2.9	3.4	4.6	4.2	
ages			±0.1	±0.3	±0.3	±0.7	±0.6	±0.7	±1.1	±1.1	±0.8	±1.1	±1.8	±0.5	
± S.D.		Oxytocin	0.4	0.3	0.3	0.6	0.5	0.7	1.1	0.9	1.4	1.2	1.6	1.6	
			±0.2	±0.2	±0.4	±0.6	±0.4	±0.5	±0.7	±0.7	±1.3	±1.0	±0.6	±1.0	

No. of cows = 6
No. of observations per period = 2
*N.D. = Non-detectable or Zero
Cystic = Corpus Luteum cyst
S.D. = Standard Deviation

Figure 9 Mean Progesterone Levels Between Day 2-6 in Saline and Oxytocin Treated Cows.

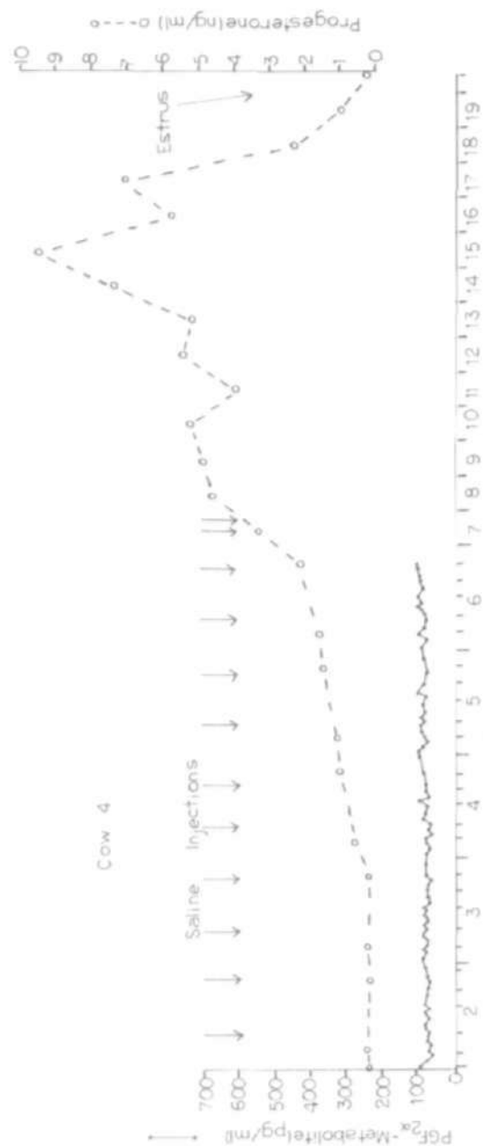

Progesterone (ng/ml)
0
1
2
3
4
5
6
Saline
Oxytocin
Day of Cycle (A.M. & P.M.)
2
2
3
3
4
4
5
5
6
6

after oxytocin injections on Days 2 and 3 but little response on Days 4, 5 and 6. However, a typical luteolytic pattern of release of PGF_{2a}-metabolite was observed in both cows starting around Day 7. This was followed by a decline in progesterone with subsequent expression of estrus between Days 10-12 as opposed to the interestrous interval of 20 days in the control cycles (Figure 10). This figure is for cow No. 4 but is representative of the responses for both cows 4 and 6. In these 2 cows, progesterone levels up to Day 6 in the saline and oxytocin treated cycles, did not differ. Cow No. 6 was slaughtered 10 days after precocious estrus which was 14 days after the last injection of oxytocin and a mature corpus luteum was found further confirming the occurrence of precocious estrus prior to slaughter. A corpus luteum cyst was also found on the other ovary (Figure 13).

b. Cows with normal estrous cycle lengths

Two cows (Nos. 5 and 8) responded to the oxytocin injections with releases of PGF_{2a} on Days 2 through 6 with the greatest response occurring on Day 2 (Figure 11). After the last oxytocin injection on Day 7, no further releases of PGF_{2a} were observed until luteolysis around Day

Figure 10 PGF_{2a}-Metaboiite and Progesterone in Cow No.4 with Shortened Estrous Cycle After Oxytocin Administration.


Progesterone (ng/ml) o---o
Estrus
Saline Injections
Cow 4
Day of Cycle
PGF2α-Metabolite (pg/ml)

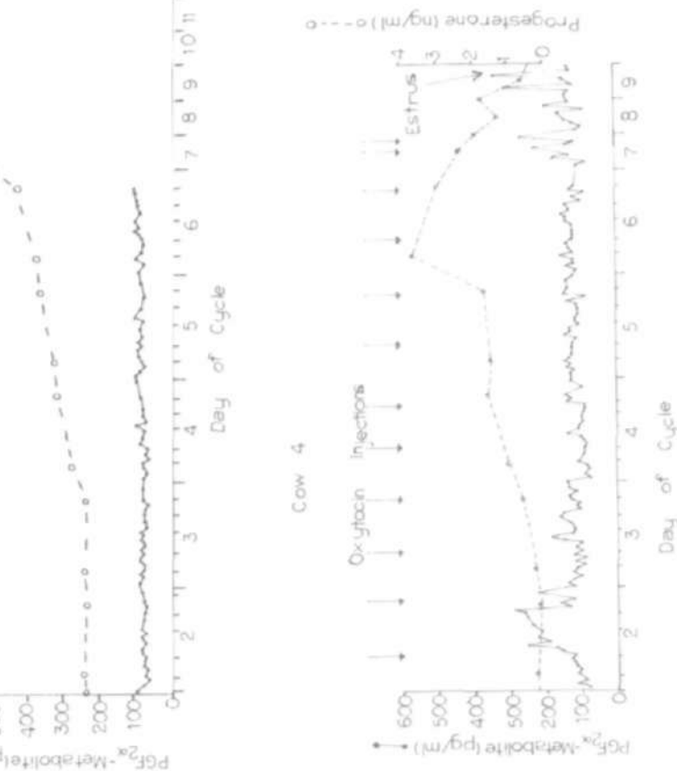
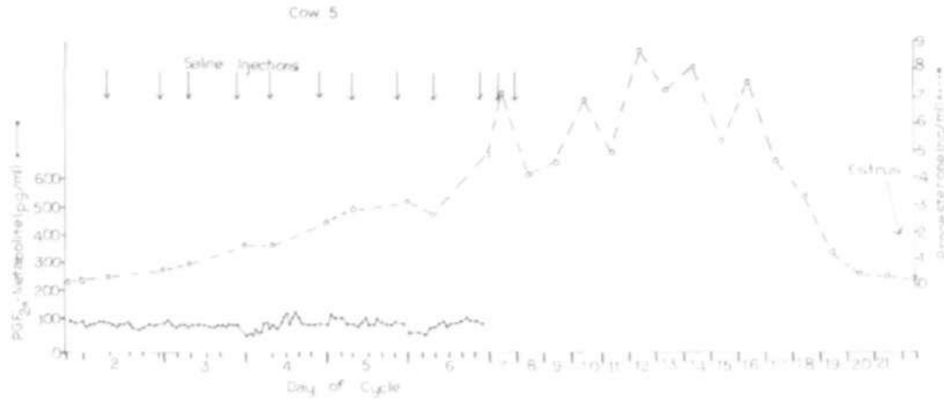
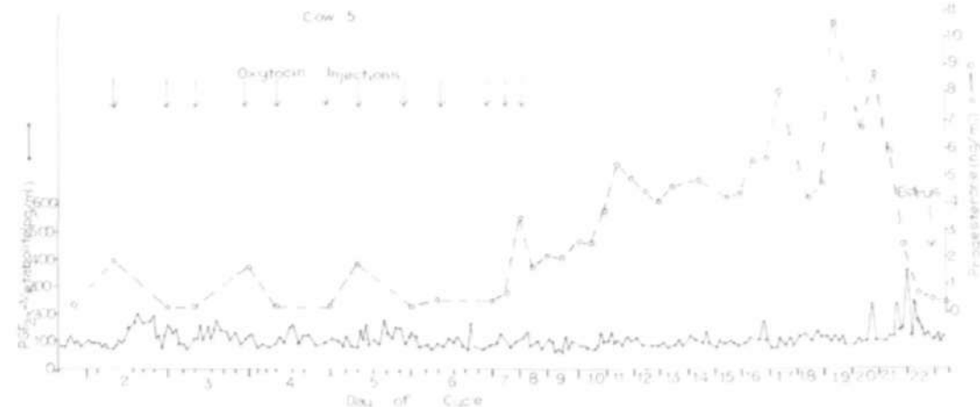

Progesterone (ng/ml) o---o
Estrus
Oxytocin Injections
Cow 4
Day of Cycle
PGF2α-Metabolite (pg/ml)

Figure 11: PGF_{2a} Release, Depressed Progesterone Secretion without Estrous Cycle Alteration in an Oxytocin Treated Cow, Cow No. 5.


Cow 5
Saline Injections
Estrus
Day of Cycle


Cow 5
Oxytocin Injections
Day of Cycle

18. Subsequent expression of estrus occurred on or about Day 22. The interestrous intervals in the saline and oxytocin treated cycles in these cows were the same. However, progesterone levels in the oxytocin treated cycles were lower than in the control cycles. An irregular progesterone pattern was observed up to Day 6, rising occasionally up to about 2 ng/ml and falling to non-detectable levels until the oxytocin injections were stopped. After the last oxytocin injection on Day 7, progesterone recovered to normal levels until estrus on Day 22. Palpation findings revealed depressed C.L. development and formation of multiple follicles (which disappeared without evidence of ovulation) during the oxytocin injections. Reddening of the vulva and external os of the cervix, clear mucous discharge and occasional acceptance of the teaser were also common occurrences in these cows during the oxytocin treatment. These endocrine changes are illustrated in Figure 11. This figure is for cow No. 5 but is representative of the responses for both cows.

c. Cows which became cystic

Two cows (Nos. 7 and 9) were diagnosed cystic after the oxytocin injections. Slight elevations of PGF_{2a}-

metabolite after oxytocin injection was observed on Day 2 without any response on Days 3, 4, 5 and 6 even with continued injections of oxytocin. The progesterone patterns indicated a complete suppression of progesterone synthesis and/or secretion throughout the duration of the oxytocin injections and reaching high levels when the injections were stopped. The cows were diagnosed cystic (corpus luteum cyst) on palpation and confirmed at post mortem on Day 25 (Figure 13).

Throughout the duration of the oxytocin injections, these cows would occasionally stand to be mounted by the teaser but no external signs of estrus were observed. The endocrine changes for cow 7 are illustrated in Figure 12 but this figure is representative of the responses for both cows.

Figure 12 PGF_{2a}, Luteal Inhibition and Subsequent Cystic C.L. Formation in an Oxytocin Treated Cow, Cow No. 7.

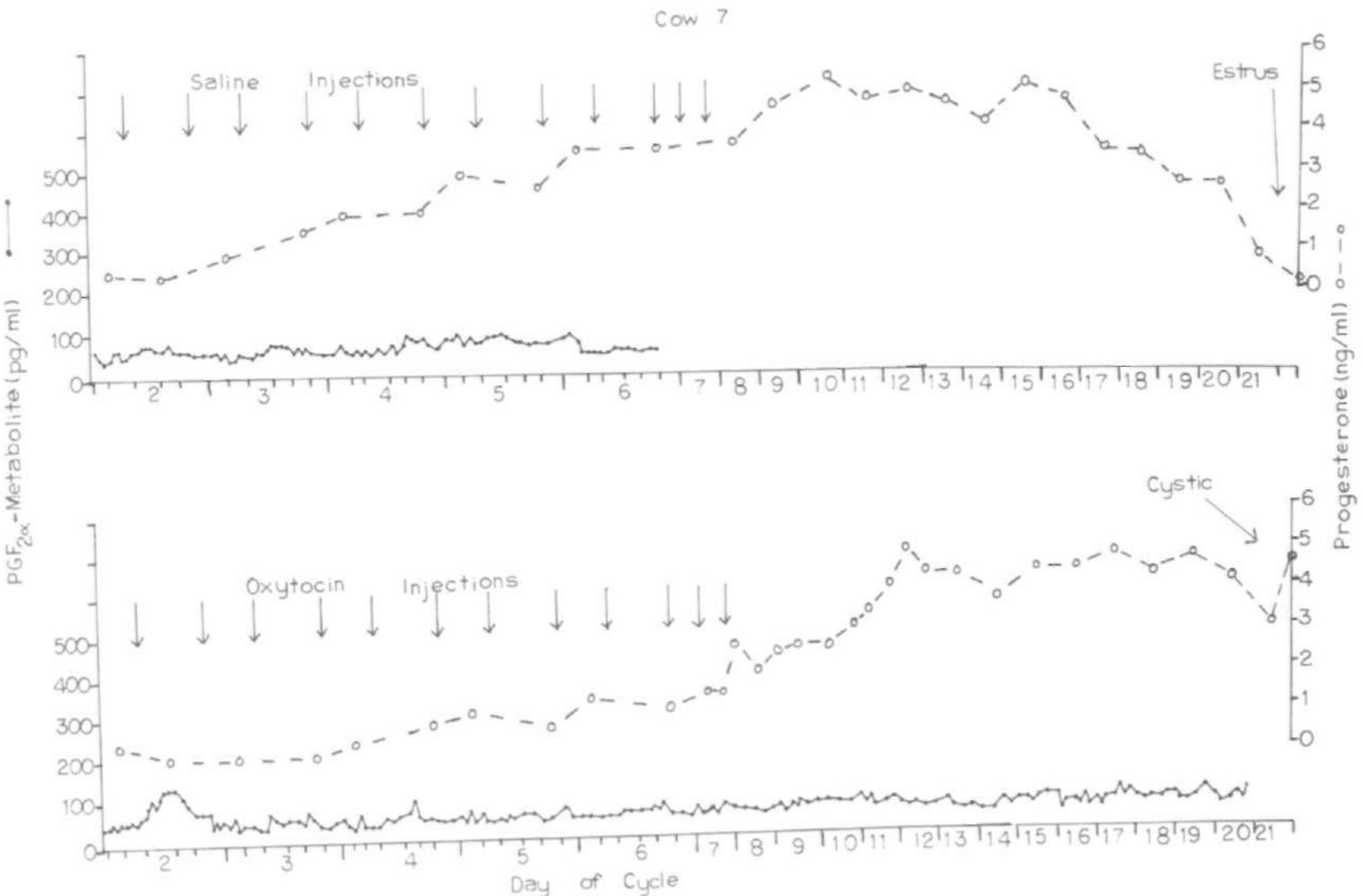

Cow 7
Saline Injections
Estrus
Oxytocin Injections
Cystic
PGF2α-Metabolite (pg/ml)
Progesterone (ng/ml)
Day of Cycle

Figure 13 Cystic C.L. in Oxytocin Treated Cows

A. Cow No. 6
B. Cow No. 9

A. Cow No. 6



B. Cow No. 9



DISCUSSION

The higher concentrations of oxytocin found after manipulations confirm earlier reports of an oxytocin release as a result of genital stimulation. The increase in oxytocin is in agreement with the studies of Vandemark and Hays (1952) who showed that oxytocin is released at the time of natural mating and during artificial insemination due to the manipulations of the reproductive organs (Vandemark and Hays, 1951). Increased oxytocin levels in the manipulated cycles on Day 3 could have been aggravated by the manipulations on Day 6 to cause the sustained increase found up to Day 14. The release of oxytocin could thus be responsible for the ejection of transferred ova.

Clitoral massage during artificial insemination has been found to increase pregnancy rate in cattle (Randel et al, 1975), possibly due to oxytocin release which may enhance sperm transport. The manipulations involved in A.I. are, however, not as severe as those employed in ova transfer, and pipettes are not usually manipulated into the tip of the uterus. Whether the release of oxytocin observed in the present study is strong enough to cause uterine contractions, is not known. Spontaneous myometrial activity which started three days before estrus, culminated at estrus and disappeared on Day 4 or Day 5 after estrus was observed by Brand et al

(1976) and appears to follow the estrogen pattern of the cycle. These authors postulated that spontaneous myometrial activity before Day 5 could be responsible for the low pregnancy rates when non-surgical embryo transfers are carried out early in the cycle (Days 3-5). In effect, even if oxytocin were higher after manipulation on Day 6 than on Day 3, there may be a more pronounced effect of oxytocin on the uterus after the manipulations on Day 3. Day 3 of the estrous cycle has been shown (Bygdeman and Eliasson, 1964) to be characterized by high estrogen levels while Day 6 (early diestrus) is characterized by higher progesterone level, both of which play a part in uterine responsiveness to oxytocin (Roberts and Share, 1969). Brinsfield and Hawk (1968) observed that motility *in vitro* was greatest in strips of myometrium from estrous ewes, intermediate in strips from overiectomized ewes and least in strips from luteal phase ewes. The uterine contraction stimulating effect of estrogen as opposed to the relaxing effect of progesterone, may interact with the oxytocin levels to cause ejection of the embryo. Brand et al. (1976) observed that manipulation of the genital tract during non-surgical embryo transfer after Day 5 did not elicit uterine contractions which resulted in embryo expulsion. Seidel et al. (1975) showed that introduction of sterile pipette through the cervix of inseminated cows did not depress fertility. An already existing state of the uterus, however, may be exaggerated by the higher oxytocin

levels in the manipulated cycle. Cupps and Asdell (1944) have shown that the uterus is characterized by slow and feeble contractions at diestrus while at metestrus, the contractions tend to be more rapid. Higher levels of oxytocin were anticipated in the present study, but the five minute interval bleeding could have been too wide to pick up the initial release.

The pharmacological dose of oxytocin (230 international units) injected into cows in the second experiment was expected to be higher than the levels of oxytocin (physiological concentration) released due to manipulations. The results obtained with exogenous oxytocin injections, are, however, indications of some possible effects of plasma oxytocin levels. In support of this, Burns et al. (1978) has indicated that plasma oxytocin levels may be associated with ovarian function in mares. The mechanism by which this occurs, however, still remains obscure, but may be related to PGF_{2a}.

The absence of PGF_{2a} release after manipulations was not surprising especially as similar results have been observed by Manns et al. (1975). In their study, PGF_{2a} in the uterine vein was measured, and not the PGF_{2a}-metabolite. It was thought that measurement of PGF_{2a}-metabolite levels in the present study would be more adequate since the parent compound has a half-life of about 30 seconds and the metabolite, a half-life of 10 minutes. The observations of a lack of

PGF_{2a} release after manipulations in this study do not necessarily contradict the findings of Newcomb et al. (1977) who observed a greater release of PGF_{2a} in heifers following manipulations on Day 3. It is possible that the uterus of the matured cow may be less likely to respond to manipulations by a release of PGF_{2a} than heifers. Release of PGF_{2a} following manipulations may also depend on the severity of the manipulations. Manipulations severe enough to cause traumatic damage to uterine endometrium may lead to a release of PGF_{2a}.

The sequence of elevations in oxytocin and PGF_{2a} following manipulations, as observed in ewes was not seen. Flint et al. (1975) were able to show an endogenous rise of oxytocin after vaginal distension in pregnant and puerperal ewes which were rapidly followed by elevated PGF levels. In sheep, a release of PGF_{2a} has been observed (Roberts et al, 1975) following mechanical stimulation. These workers found that the release occurred only very early and very late in the cycle, and that it depended on the steroid hormone status of the animal. They also observed elevations in peripheral blood levels of oxytocin after mechanical manipulation of the uterus.

The higher PGF_{2a}-metabolite levels found in the control cycles in the manipulation experiment may have been due to a sub-clinical infection or may be associated with post estral bleeding (Kindal et al., 1977). Roberts et al. (1975), were able to elicit an increase in PGF_{2a} by in-

jecting endotoxin into the uterine arterial supply of the sheep.

Whether an endogenous release of oxytocin can be followed by PGF_{2a} release in the bovine is not known. However, the fact that exogenously injected oxytocin was able to cause PGF_{2a} release would indicate that this may be possible. Elevations in PGF_{2a}-metabolite after oxytocin injection on Day 2 which declined on Days 3,4,5,6 are fairly consistent with the findings of Newcomb et al. (1977). However, these workers observed a greater release of PGF_{2a} on Day 3 in heifers. It is possible that mature cows may respond quicker to the effects of oxytocin injection by a release of PGF_{2a}. An observation has been made by Roberts et al. (1975) in sheep that release of PGF_{2a} depends on the hormone steroid status. They showed that estrogens early and late in the cycle can elicit PGF_{2a} release by the uterus. The lowered PGF_{2a} response to oxytocin injection on Days 4, 5 and 6 may indicate that the uterus is insensitive to the administered oxytocin on those days. Days 4, 5 and 6 are marked by a decline in peripheral blood estrogen levels. PGF_{2a} release on Day 2 may not be physiologically important since the C.L. is largely undeveloped on that day.

The mechanism by which exogenous oxytocin causes release of PGF_{2a} is not known. It has been shown that uterine contractions may not be a prerequisite for oxytocin induced synthesis and/or release of PGF_{2a} (Roberts and

McCracken, 1976). These workers found that the myometrium responded normally to oxytocin when the production of endogenous PGF_{2a} was suppressed with indomethecin and that contractile activity was not critical for oxytocin induced synthesis of PGF_{2a} in sheep. The duration of uterine contractions after oxytocin administration was not determined in this experiment. Mitchell et al. (1975) suggested that the mechanism by which oxytocin induces PGF_{2a} synthesis and release may be metabolic in origin. Roberts et al. (1975) have also suggested that PGF_{2a} may be released in response to oxytocin to carry out important physiological functions such as altering uterine blood flow or changing cervical tone. It is possible that the physiological relationship of PGF_{2a} to other hormones culminating in luteolysis during normal estrous cycle, could also be involved here, so that a closer examination of the estrogen pattern during oxytocin administration is indicated. In anestrus ewes, Sharma and Fitzpatrick (1974) found that the amount of PGF_{2a} released appeared to depend more on the priming dose of estrogen. In ovariectomized ewes, Hays and Vandemark (1953) showed that estrogens caused a marked increase in response to oxytocin, whereas progesterone greatly reduced the response.

The mechanism behind the luteolytic release of PGF_{2a} prior to precocious estrus in cows with shortened estrous cycle is also unclear. However, an examination of the figures representing the responses due to oxytocin

injection reveals a steady rise in progesterone even during the oxytocin injections in cows with shortened estrous cycle. This suggests that the uterus requires a minimum exposure time to progesterone before it can elicit PGF_{2a} release.

Decreased interetrous interval, cystic C.L. and normal cycles found after oxytocin administration are consistent with those of Hansel and Wagner (1960). In the manipulation experiment, the two to three day shorter interestrous intervals observed in the manipulated cycles are however within normal limits of estrous cycle length. This experiment also failed to produce an evidence of luteal inhibition due to manipulation. However, a temporary suppression of C.L. function in some cows is also suggested by the observation of non-detectable levels of progesterone in Cow 9 (Figure 12) during the oxytocin injections. Progesterone began to rise after the oxytocin injections were stopped, followed by formation of a cyst. Luteal inhibition has been shown to be a common feature of oxytocin treated cows (Armstrong and Hansel, 1959; Hansel and Wagner, 1960; Auletta et al, 1972). In our studies, mean progesterone was found to be significantly lower in oxytocin treated cows and are consistent with the findings of Auletta, Currie and Black (1972) who found a significant decrease in oxytocin treated cows on Day 7. Milvae and Hansel (1978) found significant progesterone decrease only after Day 8.

The level of inhibition may depend on the amount of circulating luteinizing hormone. Donaldson et al. (1964), suggested that the cow requires at least two periods of luteotrophic stimulation for normal corpus luteum development: One at ovulation and another after Day 4 of the cycle. Whether this is true for most or all cows has not been proven, but it could be postulated that this may be related to the different responses observed. Thus in cows with higher levels of circulating LH, there could be some sort of competition with PGF_{2a} or oxytocin for receptor sites on the C.L. Whether the lack of C.L. development is due to the oxytocin injections, is not known. The indication of depressed progesterone levels in some of the cows even without PGF_{2a} release on Days 4, 5, and 6 and the development of cysts would suggest that oxytocin could affect the cycle by mechanisms other than the release of PGF_{2a}.

The double dose of oxytocin injected was necessitated from the observations of Donaldson et al (1968) and Donaldson (1969) that a much higher dose was required to alter the estrous cycle of mature cows. Preliminary studies carried out by injecting 150 units of oxytocin once a day between Days 2 to 6 in two cows did not cause release of PGF_{2a}. No alteration of the estrous cycle was observed, and the clinical signs observed when 230 units of oxytocin were injected were not found.

SUMMARY AND CONCLUSIONS

Intrauterine mechanical manipulations were carried out in three cows. Plasma samples were collected at frequent intervals before and after manipulations. Plasma samples were also collected in control cycles devoid of manipulations. The plasma samples collected were analyzed by radioimmunoassay methods for oxytocin, 15-Keto-13, 14 dihydro PGF_{2a} and progesterone. The results showed that oxytocin was released due to cervical and uterine mechanical manipulations. There was no release of PGF_{2a} due to the manipulations. Intrauterine manipulations did not cause either luteal inhibition or estrous cycle shortening as have been observed in the mare. It is concluded that in the cow, physical damage to the endometrium may be required to cause PGF_{2a} release following manipulations..

This would indicate that oxytocin release rather than PGF_{2a} may be responsible for the ejection of ova after nonsurgical transfer in the bovine. Higher basal oxytocin levels (300 pg/ml) in one cow than the others (150 pg/ml) may indicate that some cows could be more susceptible to ejection than others.

In order to determine the possible effects of oxytocin on PGF_{2a} response and estrous cycle, oxytocin (230 International units) was injected subcutaneously into six mature

cows twice a day on Days 2 through 7 of the cycle (Day 0= estrus). Plasma samples were collected at frequent intervals before and after oxytocin injections and were analyzed for 15-Keto-13, 14 dihydro PGF_{2a} and progesterone by radioimmunoassay.

Oxytocin injections caused a release of PGF_{2a} with the greatest response occurring after the first injection at 9:00 A.M. on Day 2 of the cycle. The PGF_{2a} response then declined up to Day 6 even with continued injections of oxytocin. The ultimate effects of injected oxytocin varied among the cows. Injected oxytocin shortened the interestrous intervals of two cows to 10-12 days. Luteolytic release of PGF_{2a} was observed prior to precocious estrus in both cows. Depressed progesterone, lack of luteal tissue and cystic corpora lutea were found in two cows. Following oxytocin injections, two cows showed depressed progesterone during oxytocin injections without any alteration of estrous cycle length. What triggered the luteolytic release of PGF_{2a} prior to precocious estrus could not be determined.

The effects of exogenous oxytocin (230 international units) on PGF_{2a} response may not necessarily reflect the effects of endogenous levels of oxytocin (one international unit) in the bovine.

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