SOME ENDOCRINOLOGICAL STUDIES INTO PERIPARTURIENT REPRODUCTION IN THE COW

BY

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TABLE OF CONTENTS

FIGURES

TABLES

DIAGRAMS

PLATE

SUMMARY

This thesis describes a study of some hormonal, behavioural and gonadal changes during the peri- and post-parturient period of a group of suckled cows. The investigation was commenced just prior to calving, continued through the post-partum anoestrus period, and terminated when pregnancy was established.

To enable ovarian functional changes to be monitored radioimmunoassays were developed for progesterone, oestrogens and androgens. Validation of these assays for measurement of the steroids in peripheral plasma samples was by recognised reliability criteria. In addition, by applying the methods to samples from cows during the oestrous cycle comparison of the results with ovarian structural changes, and with the levels reported by other workers was possible.

At calving observations on the inter-relationship of progesterone, oestrogens, androgens and cortisol revealed alterations in the peripheral plasma levels of these hormones which gave information on possible sources of secretion, and suggested functional roles for these steroids in the process of birth.

Post-partum the peripheral plasma levels of progesterone, oestrogens, and androgens were related to the presence of follicles and corpora lutea in the ovaries, to the size and degree of turgidity of the uterine horns, and to behavioural changes typifying prooestrus and oestrus. Subdivision of the anoestrus period on the basis of endocrinological and gonadal structure changes, and comparison of silent, overt and false heats during the period of study elucidated some aspects of the aetiology of this period of altered reproductive function.

In animals in which pregnancy was established luteal function was compared to that observed earlier in the post-partum period and to dioestrus progesterone levels in cycling cows. The clinical significance of cystic ovarian structures recorded mainly during early pregnancy was studied.

The results of the thesis raised the question of the value of some of the accepted parameters to determine if oestrous cycles were occurring in cows. In addition the accuracy of endocrinological, behavioural and structural changes in the reproductive tract as a means of assessing the stage of the oestrous cycle were discussed.

CHAPTER ONE

GENERAL INTRODUCTION

It is now well recognised that in mammals reproduction demands the ability not simply to produce gametes but necessitates the occurrence of numerous events in different parts of the body. Not only must these many structural and functional changes take place, but they must be synchronised in many instances, both within and between individuals. Although the gametogenic aspects of reproduction had been elucidated by 1827, when Dumas showed that union of sperm and ovum led to the formation of a new individual, neither the regulation of events within the ovaries nor the influence of these organs on other parts of the body was appreciated.

Following studies such as those of Lataste (1887), Knauer (1896), Marshall (1903) and Bouin, Ancel and Villemin (1906) a functional relationship between the ovaries and the series of regular recurrent changes comprising the oestrous cycle had been proposed. Further work, notably by Stockard and Papanicolaou (1917) and Long and Evans (1922), confirmed the association between on the one hand, follicular development and the behavioural state of oestrus, and on the other, the presence of corpora lutea and proliferation of the uterine endometrium. Prior to this Loeb (1914) had provided the first major indication of a means by which the development of ovarian structures, and, therefore, in the light of later studies all the other changes of the oestrous cycle, could be regulated. By excising corpora lutea he was able to shorten the interval from one ovulation to the next thus reinforcing the concept of Marshall and Jolly (1905) that the cyclical development of follicles and corpora lutea basically depended on the presence or absence of the latter tissue. When present in the ovaries, the corpus luteum was

considered to exert a dominant effect which persisted until such time as the luteal tissue regressed at the end of its inherent life span when follicular development was re-initiated.

Heape (1905) questioned the concept of the ovaries themselves controlling the development of follicles and corpora lutea. Although circumstantial evidence of an extra gonadal influence on ovarian function had been suggested by the clinical observations of Lorain (1871) and the hypophysectomy experiments of Crowe, Cushing and Homaris (1909), it was not until 1921 that Evans and Long provided proof that there was indeed extra gonadal control of ovarian function. These early experiments of Evans and Long (1921) on anterior pituitary extracts were followed by the demonstrations by Zondek and Aschheim (1926) and Smith and Engle (1927) that injections of pituitary extracts to immature animals, or implantation of pituitary tissue to hypophysectomised hosts, could induce both follicular and luteal tissue development in the ovaries. Based on these observations these authors proposed that the pituitary, by release of specific substances, determined which structures developed in the ovaries.

Following the appreciation that ovarian structures had an endocrine role in control of events within the oestrous cycle, studies were directed towards identification of compounds that could fulfil the regulatory role of follicles and corpora lutea. These investigations led to the isolation of the first crystalline oestrogen, oestrone, by Doisy and Thayer (1929) and of progesterone (Corner and Allen, 1929). Efforts to confirm the concept of differing trophic substances from the pituitary controlling the development of ovarian structures led to

Fevold, Hisaw and Leonard (1931) isolating two biologically distinct fractions from crude anterior pituitary extracts. In test animals, one of these was found to produce follicular growth; the other if injected into animals with developed follicles led to ovulation and subsequent luteinisation.

A functional relationship between the endocrine products of the anterior pituitary and the ovaries was proposed by Moore and Price (1932). They suggested that not only did the pituitary gonadotrophic hormones regulate follicles and corpora lutea but that they themselves were in turn controlled by the secretions of the ovarian structure. Returning to the previously proposed concept of Loeb (1914) on the dominant effect of the corpus luteum during the oestrous cycle this was interpreted as being due to the secretion of a progestin inhibiting the release of the pituitary hormone required for ovulation. On the other hand a relationship between follicular development and the release of the hypophyseal ovulatory hormone was demonstrated by Lane (1935) who showed a marked reduction in pituitary ovulating activity following injections of oestrogens. By the mid 1930's therefore the outline of a system linking the transition from the follicular to the luteal phase was proposed. Pincus (1936) demonstrated that gonadotrophins were required for the meiotic division of the oocyte.

With the recognition that the pituitary was involved in the regulation of ovarian function attention turned towards possible mechanisms of control of the pituitary. As early as 1901 Frohlich had noticed that in some cases of defective morphological development of the genital tract lesions were present in the hypothalamic region

of the brain. The demonstration by Greep (1936) that the relative anatomical positions of the hypothalamus and anterior pituitary were essential to normal functioning of the latter tissue stimulated studies into the regulatory influence of the hypothalamus. Harris (1937) showed that electrical stimulation of the hypothalamus could lead to ovulation of the oestrous rabbit. It had previously been shown that ovulation in this species could be induced either by injections of gonadotrophins or of oestrogens. Taken together, therefore, these observations demonstrated the possibility of a functional connection between ovarian and hypophyseal activity, by way of the hypothalamus. Further evidence of the existence of the hypothalamic control concept was provided by the experiments of Dey, Fisher, Berry and Ranson (1940) who reported that if lesions were produced in the brain just caudal to the optic chiasma follicular growth occurred but ovulation did not take place.

Besides its probable involvement in the release of pituitary gonadotrophins the importance of the hypothalamic region to other aspects of the oestrous cycle became evident following the experiments of Dempsey and Rioch (1939) and Harris, Michael and Scott (1958). By sectioning the brain stem at different levels and by implantation of oestrogens to specific areas of the brain the importance of the upper mid brain and hypothalamus in bringing about the behavioural changes, referred to as oestrus, was revealed. Much of the work of the above authors and other contemporary studies has been reviewed by Sawyer (1960). It therefore appeared that the small hypothalamic region of the brain was involved not only in regulation of the

gametogenic aspects of reproduction, by virtue of the control exerted via the pituitary gland, but that it was also essential to the behavioural aspects.

Although the suggested sequential release of pituitary gonadotrophins as regulators of the formation of follicles and corpora lutea provided a possible explanation of the control of the oestrous cycle, doubts arose as to the validity of this concept. Experiments with increasingly purified pituitary extracts (Hisaw, Fevold and Greep, 1936) showed that although the pituitary follicle stimulating fraction could induce follicular growth a certain amount of luteinising fraction had to be administered at the same time if these follicles were to be capable of ovulating. Following further experiments with even purer preparations of follicle stimulating hormone (FSH) Greep, VanDyke and Chow (1942) postulated that normal follicular development required not only FSH but also luteinising hormone (LH) - the latter being a pre-requisite for oestrogen secretion. Therefore it appeared that the follicular phase of the cycle was not controlled exclusively by the release of a single gonadotrophin fraction.

Around the time the concept of a single hormone being responsible for follicular development was being questioned, the control of luteal function had become complicated by the possible involvement of a hormone called Prolactin. This substance, which had been partially purified from crude anterior pituitary extracts by Riddle and Bates in 1933, was clearly demonstrated to have luteotrophic properties in the rat by Astwood (1941) and Cutuly (1942). The identification of prolactin in the pituitaries of a range of animals

led to a general acceptance of its involvement in the regulation of luteal function. However, subsequent studies cast doubt on this suggestion in species other than rodents. In the cow, for example, Smith, McShan and Casida (1957) found that daily injections of prolactin were ineffective in prolonging the life of the corpus luteum. LH, but not prolactin, incubated with bovine corpora lutea slices led to significant progesterone production (Savard, Marsh and Rice, 1963). Armstrong and Hansel (1959) and Staples and Hansel (1961) showed that oxytocin inhibited both luteal growth and function in the cow. This inhibition could be overcome by LH but not by prolactin (Donaldson, Hansel and Van Vlek, 1965). In view of the possible synergistic effect of FSH and LH in producing normal follicular development, FSH was investigated as to its role in regulation of luteal function in the cow. Like prolactin, however, both by itself and in combination with other hormones FSH failed to demonstrate any luteotrophic activity.

Therefore, although much of the earlier work on regulation of the oestrous cycle was applicable to a wide range of animal species subsequent studies served to highlight species variability of the common pattern. A further example of this variance in relation to control of luteal function arose in the mid 1960's. Short (1964) demonstrated that in the sheep progesterone continued to be secreted in normal amounts following hypophysectomy. In contrast the studies of Donaldson et al. (1965) and Hansel and Seifert (1967) suggested that in the cow the corpus luteum required either several, or continued, LH stimuli for normal function. In the cow therefore LH alone appeared to regulate both growth of, and steroidogenesis by the corpus luteum.

Just as the concept of the consecutive independent release of two gonadotrophins in regulating the oestrous cycle was being questioned in the 1930's, so also was the idea that the ovarian steroids were secreted independently and produced specific unrelated effects. The suggestion that some of the changes ascribed solely to progesterone could not be produced without the prior actions of eostrogens was first made by Allen (1930). The validity of this hypothesis in relation to proliferative change in the uterine endometrium was demonstrated. The concept of oestrus as a function solely of the effect of oestrogen secretion was also questioned. In the ovariectomised guinea pig a more normal expression of oestrus resulted from the injection of oestrogens followed by progesterone than from oestrogens alone (Dempsey, Hertz and Young, 1936). Although this observation was confirmed in several other laboratory animals (Everett, 1948) in the cow Hammond and Day (1944) found that oestrogens alone could lead to a full mating response. However, Melampy, Emmerson, Rakes, Hanka and Eness (1957) considered that in this species small amounts of progesterone were also essential. This later suggestion was supported by the previous observation of Edger (1953) that pre-ovulatory follicular fluid in the cow contained progesterone.

In addition to the behavioural effects of oestrogens being complicated by the above studies the role of this hormone as a means of inducing ovulation through release of pituitary gonadotrophins was being questioned. In the rat Selye, Brown and Collip (1936) showed that injections of very small amounts of progesterone could facilitate ovulation and corpus luteum formation. Similar findings were reported

in the cow by Allegra and Pace (1950) and Hansel and Trimberger (1952). Along with the evidence on luteinisation of the theca interna and the progesterone content of pre-ovulatory follicles (Hansel et al., 1952; Edgar, 1953), these studies suggested that in the cow, as in rodents, progesterone may play a role in the normal process of ovulation and corpus luteum formation. However, in both the rat and cow, increasing the amount of progesterone injected was found to block ovulation in agreement with the observation that ovulation did not occur in the presence of a corpus luteum in the ovaries (Trimberger and Hansel, 1955). A situation had therefore been reached where progesterone appeared capable of both facilitating or inhibiting the release of LH depending on the dosage used, and that this hormone appeared in some instances to block, and in others to facilitate, the effects of oestrogens. In an attempt to explain the dual effect of progesterone in relation to LH release Kamakami and Sawyer (1959) and Sawyer, Kamakami and Kanematsu (1964) have proposed the existence of a hypothalamic threshold to gonadotrophin release which could be diminished by progesterone acting in the presence of oestrogens. Without oestrogens being present it was suggested that the effect of progesterone was to raise this arousal threshold and therefore inhibit LH release.

With the recognition of the regulatory influence of the hypothalamus on the pituitary efforts were directed towards isolation of substances from the former tissue that could influence hypophyseal function. Following the identification of a cortico-trophin-releasing factor by Saffron and Schally (1955) a substance which could bring about

a decline in pituitary LH was isolated (McCann, Taleisnik and Friedman, 1960; McCann, 1962; Chowers and McCann, 1965; Raimirez and Sawyer, 1965). In addition an FSH-releasing substance in hypothalamic extracts has been described (Igarashi and McCann, 1964). These substances were considered to be present in the nerve endings in the region of the hypophyseal portal blood vessels and to pass in the blood stream to the pituitary.

With the identification of the hypothalamic releasing factors further details of the system by which the recognised effects of gonadal steroids were linked to their regulatory influence on gonadotrophin release could be elucidated. Progesterone was found to decrease the LH-releasing factor (LHRF) content in the hypothalamus of the female rat (Minaguchi and Meites, 1966). This observation explained a means by which progesterone could exert its by now well recognised effect in blocking ovulation. Oestrogens were found to decrease the hypothalamic content of FSH-releasing factor (FSHRF) and produced ovarian atrophy (Mittler and Meites, 1966). This observation suggested the existence of a negative feedback system possibly operating to regulate follicular development. Oestrogens were also shown to be able to suppress plasma LH in ovariectomised rats - the decrease being prevented by administration of LHRF (McCann, 1962). As normal follicular development had previously been shown to require both FSH and LH the dual effectiveness of oestrogens in suppressing both of the releasing factors was in agreement with the feedback concept as a means of controlling follicular growth. However these latter results did not explain the mechanism whereby oestrogen administration could, in some

circumstances, bring about a discharge of pituitary LH leading to ovulation. One possible method by which this positive feedback could be achieved was proposed by Docke and Dormer (1965) and Piacsek and Meites (1966). These workers showed that a direct effect of oestrogens on the pituitary, not mediated through the hypothalamus, was possible and suggested that a pituitary sensitisation effect to LHRF must be involved in bringing about the pre-ovulatory LH release.

Combining the results of the above work with observations on the effects of experimental lesions in different parts of the hypothalamus Flerko (1966) proposed a scheme to account for the observed development of follicles and corpora lutea during the oestrous cycle. He suggested the existence of two centres regulating gonadotrophic-hormone-releasing factor (GNRF) release in the hypothalamus. He proposed the existence of an area responsible for the basal release of low levels of FSH and LH and a separate so called cyclic area which governed the release of the relatively large amount of LH required for ovulation. Gonadal steroids such as progesterone inhibited this cyclic centre thus blocking ovulation during dioestrous; however, basal LH and FSH secretion continued to occur. Thus, although ovulation was prevented, follicular development could take place and secretion of LH, if required as a luteotrophin, could continue. The pre-ovulatory positive feedback effect of oestrogens in triggering an LH discharge was achieved through stimulation of the cyclic centre by a critical concentration of these steroids.

Subsequent to the observations of Igarashi and McCann (1964) and Mittler and Meites (1966) doubts have been expressed as to the

existence of separate releasing factors for FSH and LH. The information available allows at least a possible series of events to be put together to explain the morphological and behavioural changes that take place during, and the mechanisms regulating, the oestrous cycle. This proposed pattern is summarised in diagrams 1 - 6.

1. Commencing with a situation where no corpus luteum is present in the ovaries. Due to the activity of the region of the hypothalamus, referred to as the basal centre, low levels of gonadotrophic hormone releasing factors are passed, via the portal vessels, to the anterior pituitary and bring about the release of low levels of gonadotrophins to the circulation. These low levels of FSH act to induce follicular growth in the ovaries and together with LH, these follicles start secretion of oestrogens. Oestrogens bring about changes in the tubular genital tract and stimulate nuclei in the hypothalamic and reticular areas of the brain to bring about the behavioural pattern referred to as pro-oestrus. In addition, by acting on the hypothalamus they serve, by negative feedback, to diminish the availability of large amounts of gonadotrophic hormone releasing factors.

2. With continued growth of the follicles the peripheral plasma oestrogen level rises until such time as a concentration occurs in the hypothalamic receptors which is sufficient to stimulate the cyclic centre. In this respect in species where pre-ovulatory luteinisation of follicles occurs progesterone may also serve to stimulate these hypothalamic nuclei. Stimulation of the cyclic centre leads to the release of a large amount of gonadotrophic hormone

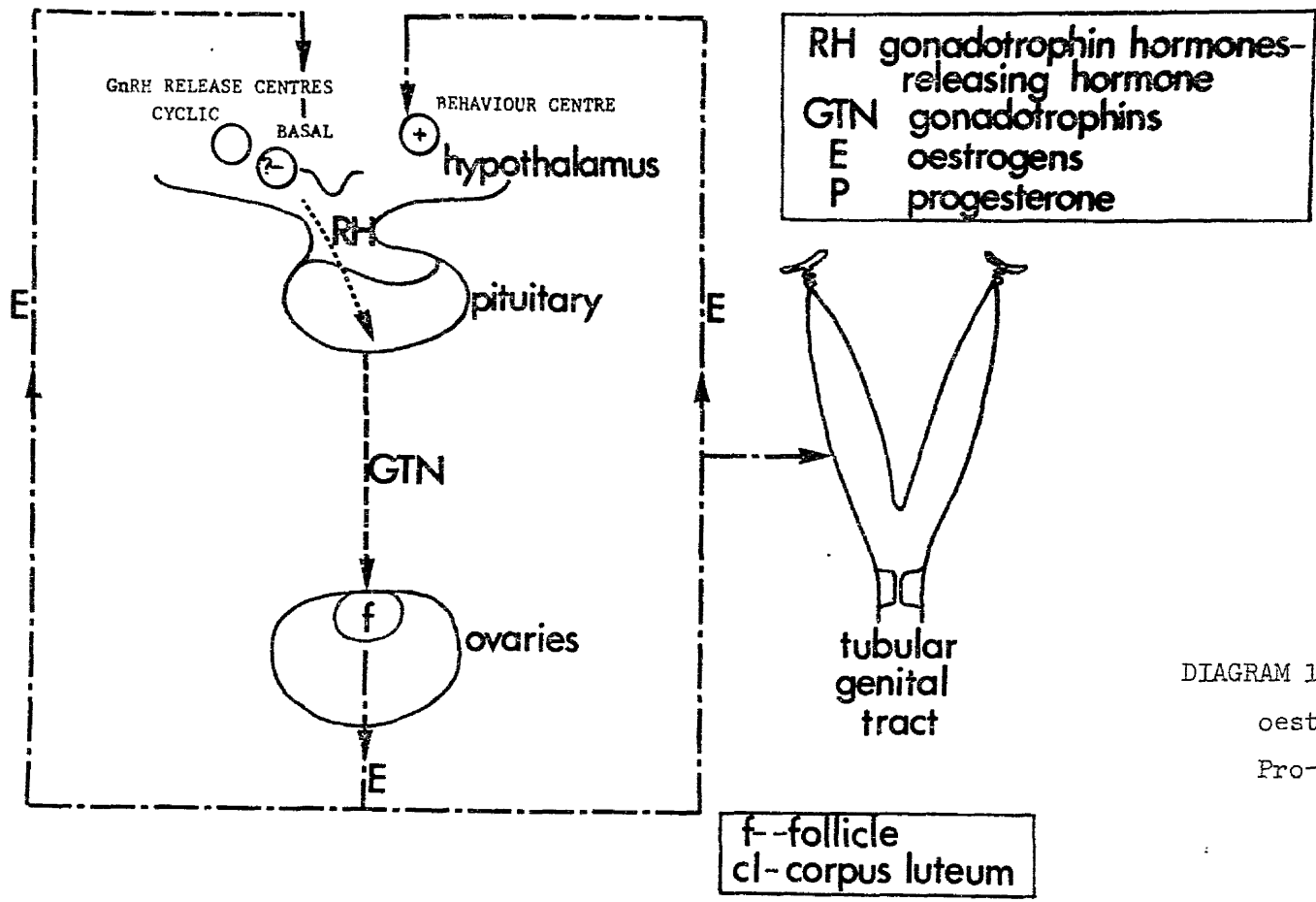


DIAGRAM 1. Control of the oestrous cycle. Pro-oestrus.

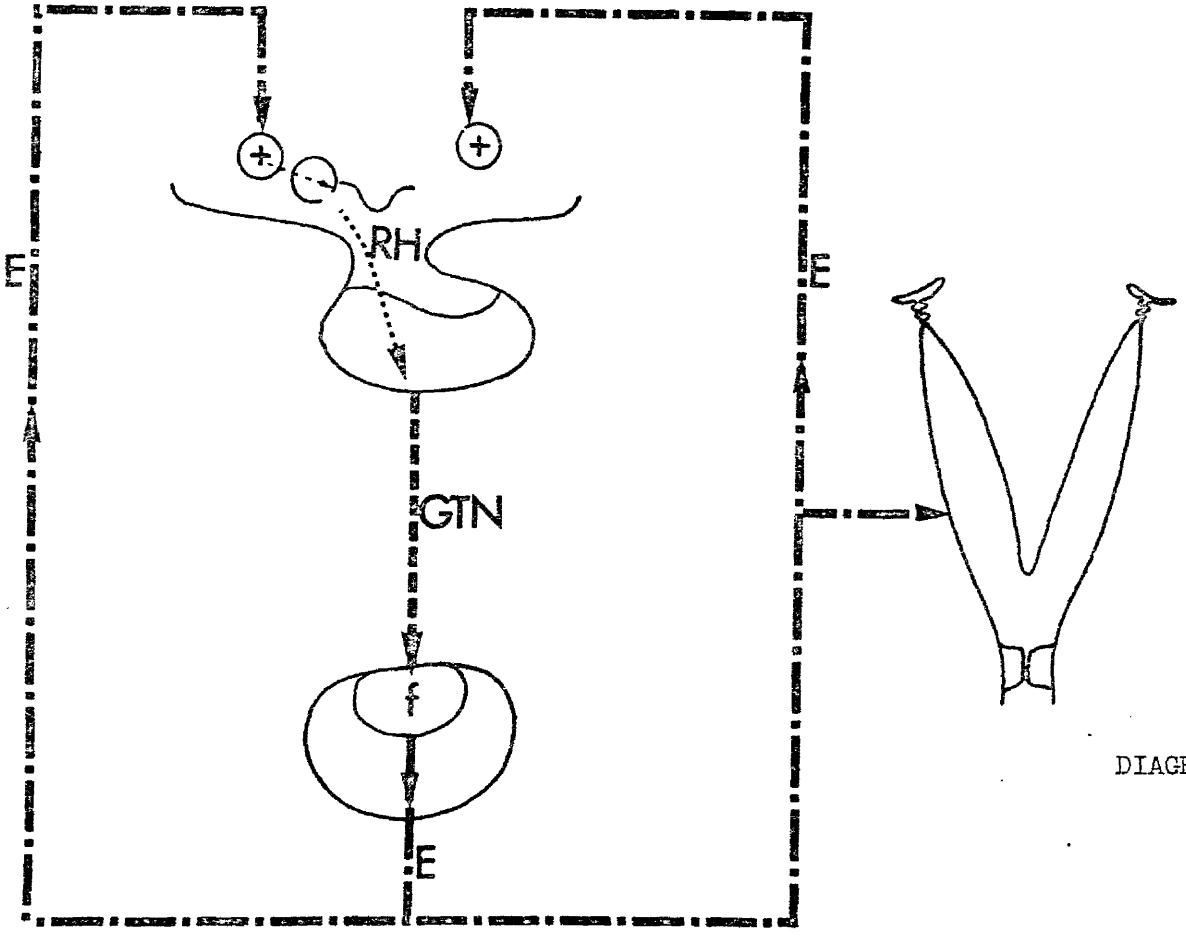
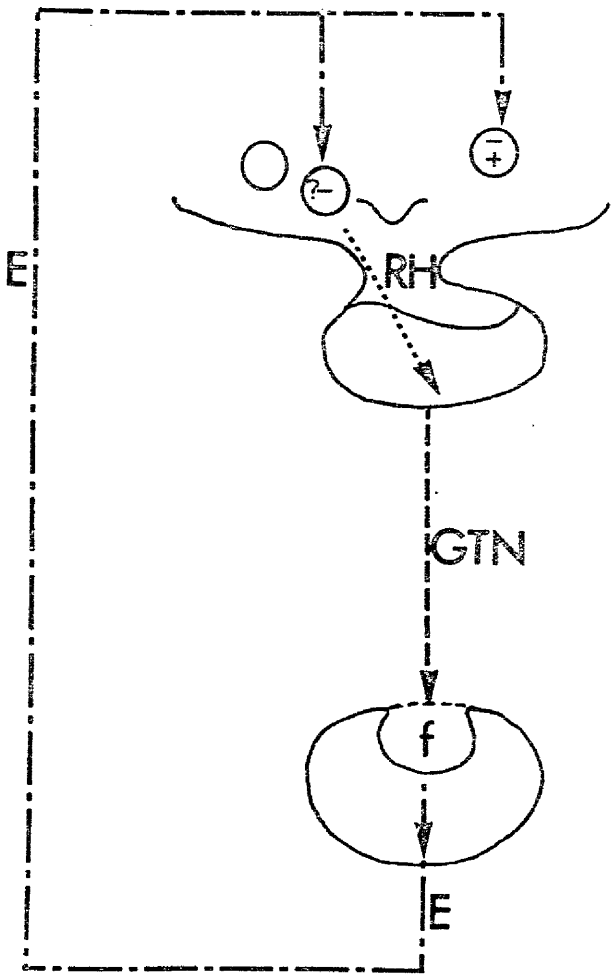


DIAGRAM 2. Control of the oestrous cycle. Oestrus.

releasing factors to the pituitary. Coupled with the possible sensitisation of the hypophyseal tissue to the releasing factors a large discharge of gonadotrophins occurs. Around the same time the level of oestrogens has also become sufficient to bring about the transformation from a behavioural pattern where the male is attracted to the female to one where superimposed on this she accepts his attentions. Pre-ovulatory progesterone secretion may also assist in bringing about this change. By employing a threshold to the positive feedback effects of oestrogens in both bringing about a large discharge of gonadotrophins and stimulating oestrus, synchronisation of the gametogenic and behavioural aspects of reproduction is achieved.

3. Following the large discharge of pituitary gonadotrophins the process of ovulation of one, or several follicles, is initiated and final maturation of the oocyte occurs. Due to the change induced in the follicle the secretion of oestrogens is diminished and oestrous behaviour terminated. In addition, the release of pituitary gonadotrophins at this time is low, possibly due to the concentration of oestrogens present in the circulation exerting a negative feedback on their release, but more probably due to the lack of available stored pituitary gonadotrophins following the massive pre-ovulatory discharge.

4. Following ovulation formation of a corpus luteum occurs. Progesterone from the corpus luteum acting on the oestrogen primed reproductive tract brings about the many changes characteristic of dioestrus. Although, in rodents, prolactin is required for steroidogenesis by the corpus luteum, in general, LH acts as the luteotrophin. In some species after an initial LH stimulus progesterone



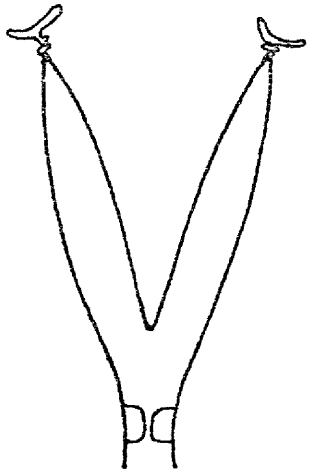


DIAGRAM 3. Control of the oestrous cycle. Ovulation.

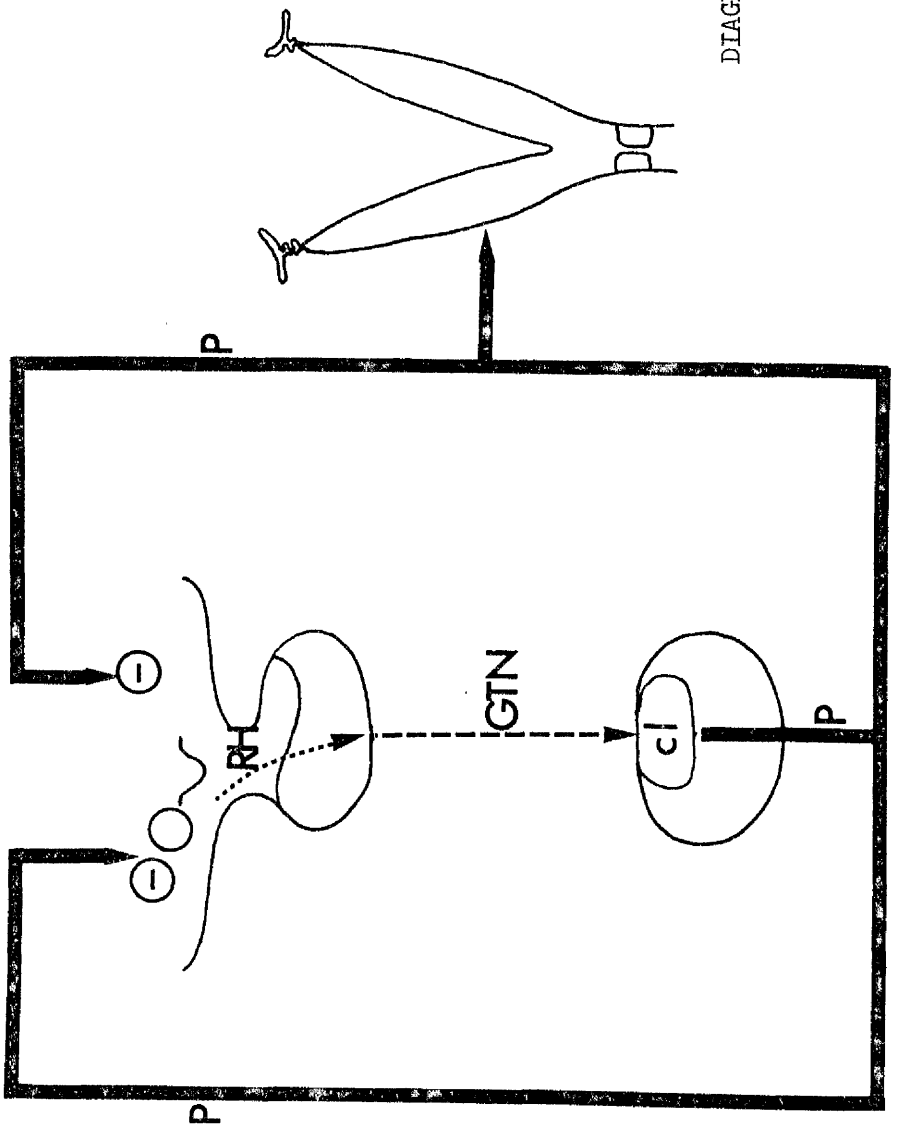


DIAGRAM 4. Control of the oestrous cycle.
Metoestrus/Dioestrus.

production continues for the life span of the corpus luteum but in others many or continual releases of LH are required. This situation is ensured in the latter group due to the fact that progesterone inhibits only the cyclic centre in the hypothalamus. Due to the continual activity of the unaffected basal centre gonadotrophins are released during dioestrus.

5. Besides affecting luteal function these low levels of gonadotrophins are also able to bring about a degree of follicular development and oestrogen secretion during dioestrus. Due, however, to the secretion of progesterone these oestrogens are not capable of arousing the thresholds either to activate the cyclic centre or to initiate oestrous behaviour. Final follicular maturation and oestrous behaviour are therefore restricted to the period when a functional corpus luteum is absent from the ovaries. Throughout the period of dioestrus, due to the inhibitory effect of progesterone on the release of large quantities of gonadotrophins, pituitary stores of these hormones can be replenished.

The above description is all that is required to bring about one oestrous cycle. However, to account for the fact that the majority of animals are polyoestrus, an additional mechanism to allow the termination of one oestrous cycle and the commencement of the next is required. Obviously, due to the dominant effect of the corpus luteum in the cycle, termination of progesterone secretion by this structure is essential if follicular development leading to ovulation is to take place. Although it had been established that the corpus luteum in many species, such as the cow, does not function autonomously, it was

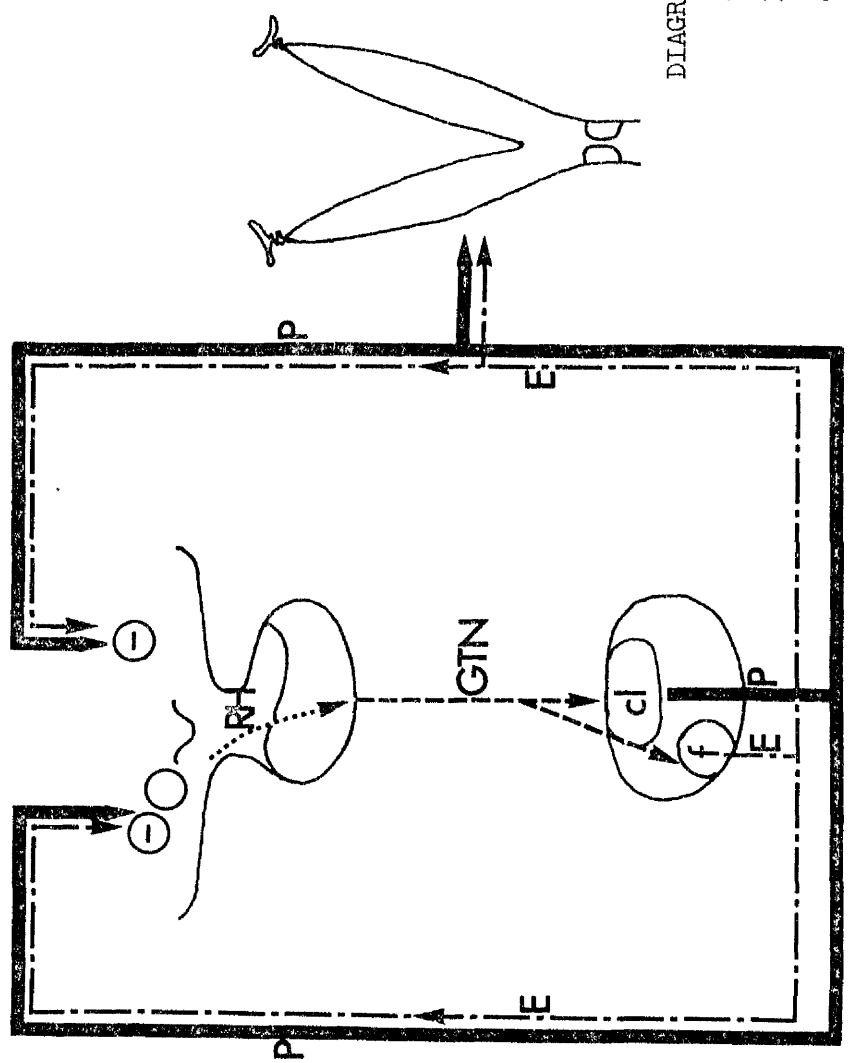


DIAGRAM 5. Control of the oestrous cycle. Follicular development during dioestrus.

not known whether it was simply withdrawal of trophic support, intervention of a lytic mechanism, or a combination of both, that naturally terminated dioestrus.

In 1923 Loeb, working with guinea pigs, noted that hysterectomy during dioestrus resulted in morphological and functional persistence of the corpora lutea beyond the expected time of regression. Further experiments demonstrated that this effect was related to the amount of uterine tissue removed rather than to any specific area of the uterus (Loeb, 1927). In the cow also, removal of the uterus was associated with prolongation of dioestrus (Wiltbank and Casida, 1956). These experiments, suggesting the existence of some substance of uterine origin which influenced the luteal life span, were extended to cover some of the previously proposed mechanisms influencing the corpus luteum. In cows the administration of either oestrogens, oxytocin or anti-LH serum, shown to be effective in intact animals as a means of bringing about luteal regression, were only partly effective in hysterectomised animals (Kaltenbach, Niswender, Zimmerman and Wiltbank, 1964; Wiltbank, 1966; Brunner, Donaldson and Hansel, 1969). Conversely, concurrent administration of LH could at least partly overcome oestrogen induced luteolysis in the hysterectomised cow, but was ineffective in the intact animal (Wiltbank, 1966). Further evidence that at least in the cow luteal regression occurred due to some substance other than the gonadotrophins was provided by the in vitro studies of Armstrong and Black (1966). They showed that luteal tissue removed 18 days after oestrus had lost its ability to respond to LH stimulation. This suggested that the corpus luteum had been acted on

by some factor prior to this time. Although evidence was accumulating to suggest the existence of a luteolytic substance of uterine origin, early attempts to isolate such a compound were unsuccessful (Malven and Hansel, 1965).

In spite of this, additional evidence implicating the uterus as the regulator of the duration of dioestrus was being presented. It was found that the uterus exerted a local, direct effect on the ovary of the same side and various routes by which the unidentified lytic substance acted were suggested (Fisher, 1965). The increasing weight of evidence suggesting the existence of a luteolysin of uterine origin has been reviewed by Ginther (1968), and Anderson, Bland and Melampy (1969). In the light of the proposed uterine luteolysin concept the continuance of luteal function in pregnant animals has been suggested as being due not to the release of an embryonic luteotrophin, as suggested by Nalbandov (1961), but due to the presence of the embryo inhibiting uterine luteolysin release (Moor and Rowson, 1966; Moor, 1968). In sheep, the substance prostaglandin F2α was found to fulfil many of the functions expected by the proposed uterine luteolysin (McCracken, Glew and Scaramuzzi, 1970). Evidence suggesting the possibility that this substance could reach the corpus luteum from the uterus by means of a counter current mechanism between the uterine vein and ~~ovarian~~ OVARIAN artery was presented (Baird and Land, 1973). Experimental luteal regression has been shown to be possible in a range of species, including the cow (Louis, Hafs and Morrow, 1972; Rowson, Tervit and Brand, 1972) using this compound.

In summary, the necessary termination of dioestrus to allow a non-pregnant animal to return to oestrus was at least tentatively shown to be achieved through release of a uterine luteolysin in response to absence of an embryo (see Diagram 6).

Although the concept of a reciprocal pituitary-gonadal relationship mediated through the hypothalamus provided, along with the uterine luteolysin, a mechanism for regulation of the oestrus cycle, it had long been appreciated that additional factors were involved. Studies such as those of Wallace (1907) and Loeb (1917), who demonstrated that sterility in non-seasonal breeders could be attributed to adverse climatic conditions and subnutrition, and Marshall (1908) who showed the effect of food intake of foetal numbers, highlighted the overriding influence of the environment on reproduction.

The role of the environment in relation to the occurrence of oestrous cycles in seasonal breeders also demonstrated that additional control could be superimposed on the mechanisms regulating events within the cycle. Marshall (1937) drew attention to this and suggested that these factors could exert their influence by way of the central nervous system. Neural, as opposed to endocrine, regulation of events within the oestrous cycle had previously been dismissed at an early stage of studies into the control mechanisms involved, e.g. (Knauer, 1896). However, as has been previously noted, during the 1950's and '60's the importance of the hypothalamic region of the brain in regulating both morphological development in the ovaries and the animal's reproductive behaviour had become apparent. This region of

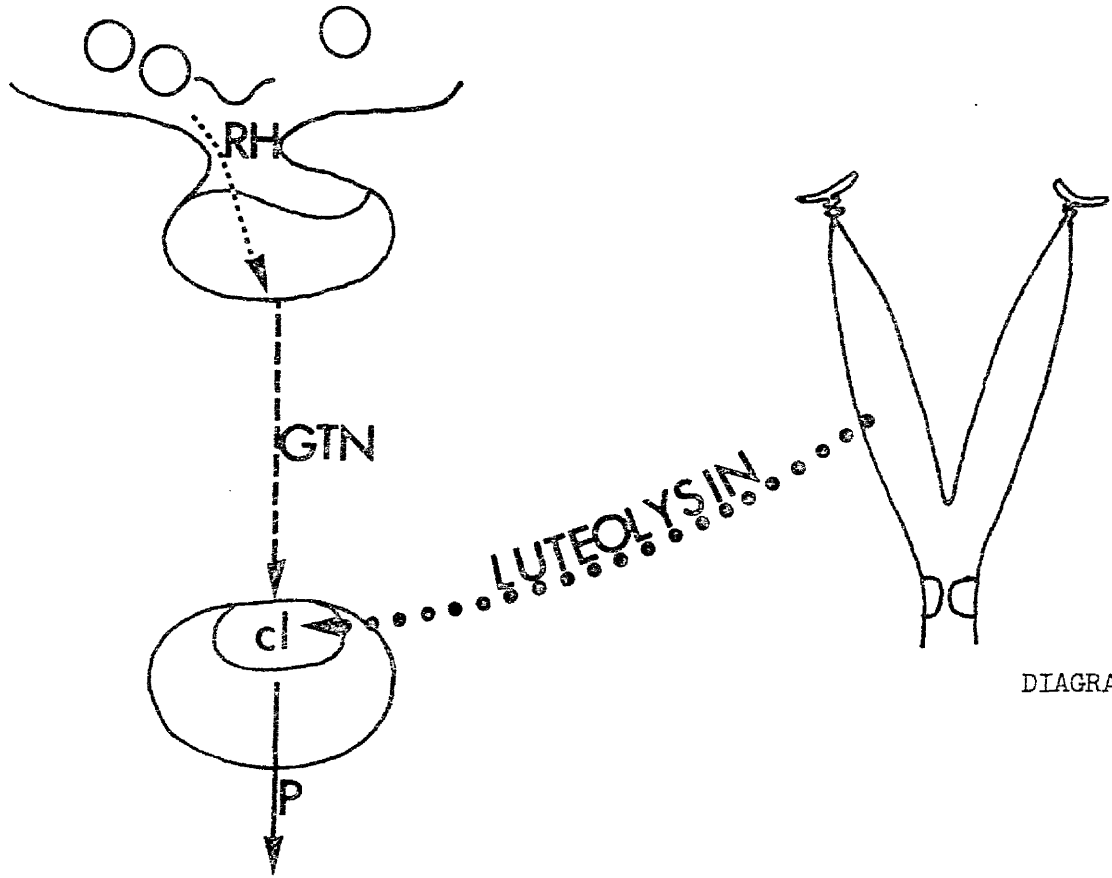


DIAGRAM 6. Control of the oestrous cycle. Termination of dioestrus.

the brain was found to receive numerous efferent pathways from other parts of the brain, the sense organs and the external genitalia. The convergence of these pathways, coupled with the previously noted control exerted by the hypothalamus on the oestrous cycle, provided a possible route whereby events in both the internal and external environment could influence reproduction. In many respects, therefore, as pointed out by Ganong (1966) who reviewed much of this work, the endocrine system in relation to reproduction can be regarded as an effector arm of the nervous system.

It is therefore apparent that although the systems regulating events within the oestrous cycle may be potentially capable of functioning, physiological and pathological factors can act to both totally abolish, or modify the efficiency of, reproductive productivity. Physiological absence of oestrous cycles, and therefore the ability to reproduce, occurs prior to puberty, during senescence, outwith the breeding season in some animals, and for a variable period of time following parturition. Many factors such as genotype (Cole and Casiday, 1947), age and bodyweight (Reynolds, De Royen and High, 1963) and the time of year (McDonald, 1969) have been found to influence the occurrence of puberty. In spite of extensive studies into this aspect of anoestrous and of the effects of a similar range of parameters on the onset of the breeding season the means by which oestrous cycles are initiated at a specific time are not known. By injections of gonadotrophins the ovaries of pre-pubertal animals, such as calves, have been shown to be capable of responding by developing follicles (Marden, 1953). Studies in lambs have demonstrated the capacity of

the prepubertal hypophysis to synthesise LH, and the potential functional ability of hypothalamic GnRF release to be activated (Land, Thimonier and Pelletier, 1970). These and similar studies in seasonal breeders, have, in general, failed to implicate a deficiency of any of the hormones known to be involved in regulation of the oestrous cycle as the reason for the state of anoestrus.

Even in adult non-seasonal breeders a recurrent state of physiological anoestrous occurs, as mentioned above, after parturition. In the cow, due to the economic necessity of ensuring a return to the pregnant state within a specific period of time, a great deal of effort has been expended into observations on structural changes in the reproductive tract, environmental factors influencing the duration of this phase, and its prolongation when various pathological conditions are present. However, in spite of this accumulation of information on the post-partum period the underlying factors governing the observed deviations from normal cyclical ovarian activity and reproductive behaviour have not been elucidated.

It is well established that the presence of a hormone gives no guarantee that it will produce an effect at a target organ. Studies of endocrine mechanisms therefore involved the determination of the occurrence of hormones reaching the site of action on the one hand, and on the other the responsiveness of the target organs to the presence of the trophic hormones. With the previously referred to observations on the control mechanisms regulating the normal oestrous cycle a basis exists for examining the post-partum period when these mechanisms apparently are non-operational. An obvious integral part of a study

of this nature is the determination, not only of the development of ovarian structures, but also of more relevance, the hormones they are secreting and the amounts reaching the target organs. Obviously, only when this information is available and the results, for the period of defective reproductive functioning, compared with those from normal cycling animals, will elucidation of at least part of the aetiology of the state of post-partum anoestrus be possible.

The work in this thesis involves an intensive investigation into some endocrinological aspects of reproductive function in periparturient cows. By terminating the study only after pregnancy had been re-established the complete period encompassing not only the re-initiation of oestrous cycles but the return of the complete reproductive system to normality will be considered.

CHAPTER TWO

THE ESTIMATION OF PERIPHERAL PLASMA LEVELS OF PROGESTERONE, OESTROGENS, ANDROGENS AND CORTISOL IN THE COW

2.1. Introduction

The relatively recent development of immunoassay techniques using radioactive tracers and either naturally occurring binding proteins or specific antisera has enabled investigations to be carried out into the levels of certain gonadal steroids in the peripheral blood of a range of animal species. Using these methods the peripheral plasma progesterone and oestrogen levels have been reported in the cow (Donaldson, Bassett and Thorburn, 1970; Smith, Edgerton, Hafs and Convey, 1973; Glencross, Munro, Senior and Pope, 1973). General agreement exists as to the overall pattern of progesterone and oestrogen levels during the oestrous cycle of the cow. Differences are apparent however between studies, in the actual levels found, and in the timing of transient peaks and declines within the cycle. The peripheral plasma levels of either total or specific androgens during the oestrous cycle in the cow have not been reported although radioimmunoassay techniques for estimation of these hormones in the females of other species have been described (e.g. Niswender and Midgley, 1970).

It is generally accepted that any assay applied to the estimation of hormone levels must satisfy certain reliability criteria. Borth (1957) defined reliability as encompassing estimates of the specificity, sensitivity, accuracy and precision of the method. Abraham (1974) has reviewed the application of these reliability criteria to radioimmunoassays of steroid hormones in biological fluids and throughout this thesis these recommendations for calculating

reliability have been adopted. The various components of assay reliability have been defined as follows:

Specificity implies the determination of one chemical entity in the assay to the exclusion of others. In a steroid immunoassay it represents the non-specific interference from other steroids extracted from plasma along with the required hormone, the concentrations of these other steroids present, and their affinities for the antibody.

Sensitivity is taken as the smallest single result which with some assurance can be distinguished from zero. It defines the detection limit of a steroid immunoassay when this is applied to the measurement of steroid levels in biological fluids.

Accuracy expresses the nearness by which a given analytical result approaches the true result.

Precision describes the within and between assay variables when a plasma sample is repeatedly estimated in the system. In addition to these considerations practicability must be taken into account.

Practicability is judged by the speed, cost, and skill required to perform the assay and is subjective.

In this thesis radioimmunoassay techniques for the estimation of peripheral plasma levels of progesterone, oestrogens and androgens in the cow will be described. In addition, as the studies into reproductive function in the cow necessitated an indication of

adrenocortical function, an existing technique, validated for cortisol estimation in the human (Mattingly, 1962), will be described in relation to the cow. Besides presenting information on the specificity, accuracy, precision and sensitivity of the assays, the techniques will be compared with the currently used methods of other workers. Finally by measuring the circulating levels of hormones in a group of cows during the oestrous cycle the relationship between the results in this thesis and those reported in similar studies will be established.

2.2. Determination of plasma progesterone levels in the cow

Following the demonstration by Murphy (1967) that cortisol binding globulin could be used as the basis of a progesterone assay, modifications of this technique have been applied to the estimation of progesterone in the peripheral blood of the cow. Both dog (Swanson, Hafs and Morrow, 1972) and human (Shemesh, Lindner and Ayalon, 1971) globulins have been used in these competitive protein binding (CPB) assays. The levels of progesterone in the peripheral blood of cows before puberty (Donaldson et al., 1970; Swanson et al., 1972), during the oestrous cycle (Donaldson et al., 1970; Shemesh et al., 1971; Henricks, Dickey, Hill and Johnston, 1972; Garverick, Erb, Niswender and Callahan, 1971; Christensen, Hopwood and Wiltbank, 1974) and during pregnancy (Donaldson et al., 1970; Hunter, Erb, Randel, Garverick, Callahan and Harrington, 1970; Henricks et al., 1972; Shemesh, Ayalon and Lindner, 1973; Arije, Wiltbank and Hopwood, 1974) have been described.

To a certain extent the more recent availability of antisera directed towards progesterone derivatives coupled to carrier proteins, and the advantages of specificity and sensitivity conferred by these, has resulted in the CPB methods being superseded by radioimmunoassay (RIA) systems. The circulating levels of progesterone during the oestrous cycle and pregnancy in the cow have been reported using RIA techniques (Glencross et al., 1973; Dobson and Dean, 1974).

2.2.1. Materials

Glass distilled water and ethanol (Burroughs) was used throughout.

1. Phosphate buffered saline pH 7.0 (PBS)

Sodium chloride AR (B.D.H., Poole, England)	40 g
Thiomersal (Sigma, London)	0.5 g
NaH_2PO_4 $2H_2O$ (B.D.H., Poole) 0.5 M	34 ml
Na_2HPO_4 (B.D.H., Poole, England) 0.5 M	68 ml
Water to	5000 ml

2. Phosphate buffered saline 0.1% gelatin (PBS gel)

Gelatin (B.D.H., Poole, England)	1 g
PBS	1 l

3. Progesterone (Sigma, London)

10 ng/ml and 1.0 ng/ml solutions in ethanol were prepared. The required amount of steroid was obtained, unless

otherwise stated, by evaporation of the ethanol under a stream of air at 40°C.

4. (1, 2, 6, 7 (n)-^{3}H)Progesterone (Radiochemical Centre, Amersham, England)

This had a specific activity of 80 - 110 Ci/mmol. A stock solution was prepared by diluting 250 µCi in 100 ml ethanol. The required amount of tritiated steroid was obtained, unless otherwise stated, by evaporating off the ethanol as above and reconstituting in PBS.

5. Diethyl ether Pronalys (May & Baker Ltd., Dagenham, England)

6. Charcoal Norit A (Sigma, London)

Before use this was washed several times in methanol to remove impurities and fines. Excess methanol was removed by rotary evaporation.

7. Dextran T-70 (Pharmacia, Uppsala, Sweden)

Stock solutions of progesterone and tritiated progesterone were stored at -15°C. All other liquid reagents were kept at 4°C.

8. Scintillation fluid ELS 93 (Koch-Light Laboratories Ltd., Colnbrook, England)

9. Extraction tubes Quickfit 10/19 tubes (Jobling, Stone, England) modified to have pointed bases were used for the extraction of samples.

10. Assay tubes. Disposable 13.5 x 100 mm rimless glass test tubes (Beckton and Dickinson, Wembley, London).

2.2.2. Methods

a. Preparation of anti-progesterone serum

Immunisation. Using the method of Niswender and Midgley (1970) a hemisuccinyl derivative of 11α-hydroxyprogesterone was prepared through position C-11. By employing the mixed anhydride reaction of Lieberman, Erlanger, Beiser and Agate (1959) the hydroxy-progesterone 11-hemisuccinate was linked to bovine serum albumin. A 1.0 mg/ml solution of the steroid-protein conjugate was dissolved in normal saline and 2.5 ml of this solution homogenised with an equal volume of Freunds complete adjuvant (Difco, Detroit, Michigan, U.S.A.). The mixture was then injected into an adult ewe distributing the total volume between two intramuscular sites (in the region of the popliteal lymph nodes) and six subcutaneous sites along the back. The injections were repeated at 2 - 3 week intervals. The serum used in the assay system was obtained after a total of 6 sets of injections had been given.

Characterisation of the antiserum

Characterisation of the antiserum involved a series of experiments designed to establish the suitability of the potential antibodies in the proposed radioimmunoassay, and the optimal conditions for their use. In these investigations the behaviour of the antibodies

was studied by incorporating the relevant steroid carrying a radioactive label to the system. To monitor the reactions therefore necessitated the availability of a means whereby the antibody bound steroid could be separated, and distinguished by virtue of its label, from the excess free steroid. Following the recommendations of Jiang and Ryan (1969), dextran coated charcoal was employed to adsorb the free steroid, thus leaving the antibody bound fraction in solution.

Determination of the concentration of dextran coated charcoal.

The concentration of dextran coated charcoal which would, if required, be capable of adsorbing the total quantity of labelled steroid was established as follows:

To a range of assay tubes, each containing approximately 10,000 c.p.m. tritiated progesterone in 200 µl PBS gelatin suspensions of charcoal ranging from 0.5 g to 0.03125 g/100 ml PBS, with dextran T-70 present at 10% of the charcoal concentration, were added in 1 ml aliquots at 4°C. After a brief thorough mixing the tubes were left at 4°C for 10 minutes. Following this the tubes were centrifuged (10 minutes at 4°C, 2,500 g) and the supernatants decanted to plastic scintillation vials. 10 ml scintillator fluid were added to each vial and after mixing the c.p.m. determined. The total quantity of tritiated steroid in each tube prior to charcoal addition was established by substituting 1 ml PBS for the charcoal-dextran solution and treating these tubes as above.

Results

The results are illustrated in Table 1. It is apparent that 0.125 g charcoal/100 ml PBS containing 0.0125 g dextran T-70 was capable of adsorbing in excess of 90% of the tritiated progesterone present. No marked increase in absorption occurred with higher concentrations of charcoal. The 0.125 g suspension was therefore employed in subsequent experiments.

Antibody titre

The antiprogesterone titre of the antiserum was determined by the method of Abraham (1969). Various dilutions of the serum were incubated with a fixed mass of tritiated progesterone, and the antibody dilution that bound 50% of the added steroid determined.

Serial dilutions of antiserum ranging from 1:500 to 1:16,000 were prepared in PBS gelatin. 100 µl of each dilution were pipetted into assay tubes in duplicate. To give an indication of the amount of tritiated progesterone used in the test, a further two tubes containing 100 µl PBS gelatin in place of antibody were included. 100 µl of progesterone-H^3 in PBS, containing approximately 12,000 c.p.m. were added to each tube and the contents thoroughly agitated. Incubation of the tubes was carried out at $4^{\circ}C$ for 18 hours. At the end of this time the antibody bound steroid was separated from the free steroid by the addition of dextran coated charcoal solution. 1.0 ml of the charcoal suspension at $4^{\circ}C$ was added to all tubes, with the exception of the two tubes with no antibody which received 1.0 ml PBS. Addition

TABLE 1 ADSORPTION OF TRITIATED PROGESTERONE BY DEXTRAN COATED CHARCOAL

Charcoal concentration G/100 ml	Average initial c.p.m.	Average final c.p.m.	Average % c.p.m. adsorbed
0.5	7692	59	99.2
0.126	"	56	99.3
0.0625	"	66	99.1
0.03125	"	101	98.7

of charcoal was carried out within a total time of 5 min. After mixing, the tubes were left at 4°C for 10 min. Separation of the supernatant was as described above.

Results

The antibody bound radioactivity in the presence of increasing dilutions of the antiserum is presented in Fig 1. It was apparent from these results that the dilution of antibody binding 50% of the c.p.m. tritiated progesterone was 1:6,000.

Displacement of labelled progesterone from the antibody

The antibody dilution at which maximum displacement of labelled progesterone occurred, when a fixed mass of progesterone was present, was determined. The amount of progesterone used was chosen as representing the approximate mid point of the anticipated working range of the assay.

At the same time as the antibody titre tubes were set up, an identical series of antiserum dilutions were added to duplicate tubes each containing 0.5 ng progesterone. The addition of labelled hormone, the period of incubation, and the charcoal separation were carried out at the same time, and in the same manner, as the antibody titre tubes.

Results

The antibody bound c.p.m. for each antiserum dilution are presented in Fig 2. It was apparent from these results that maximum

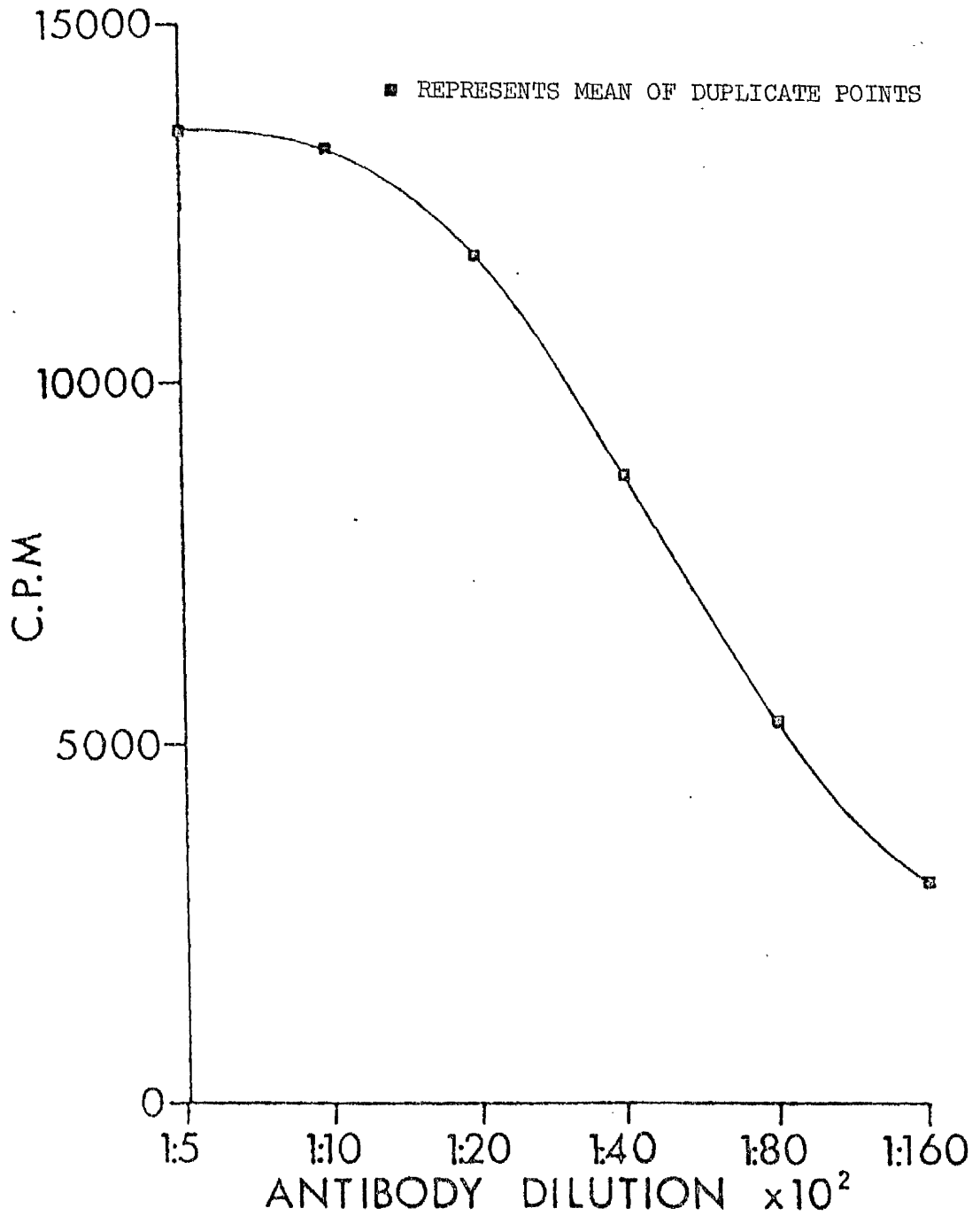


FIG 1 ANTI-PROGESTERONE SERUM DILUTION CURVE

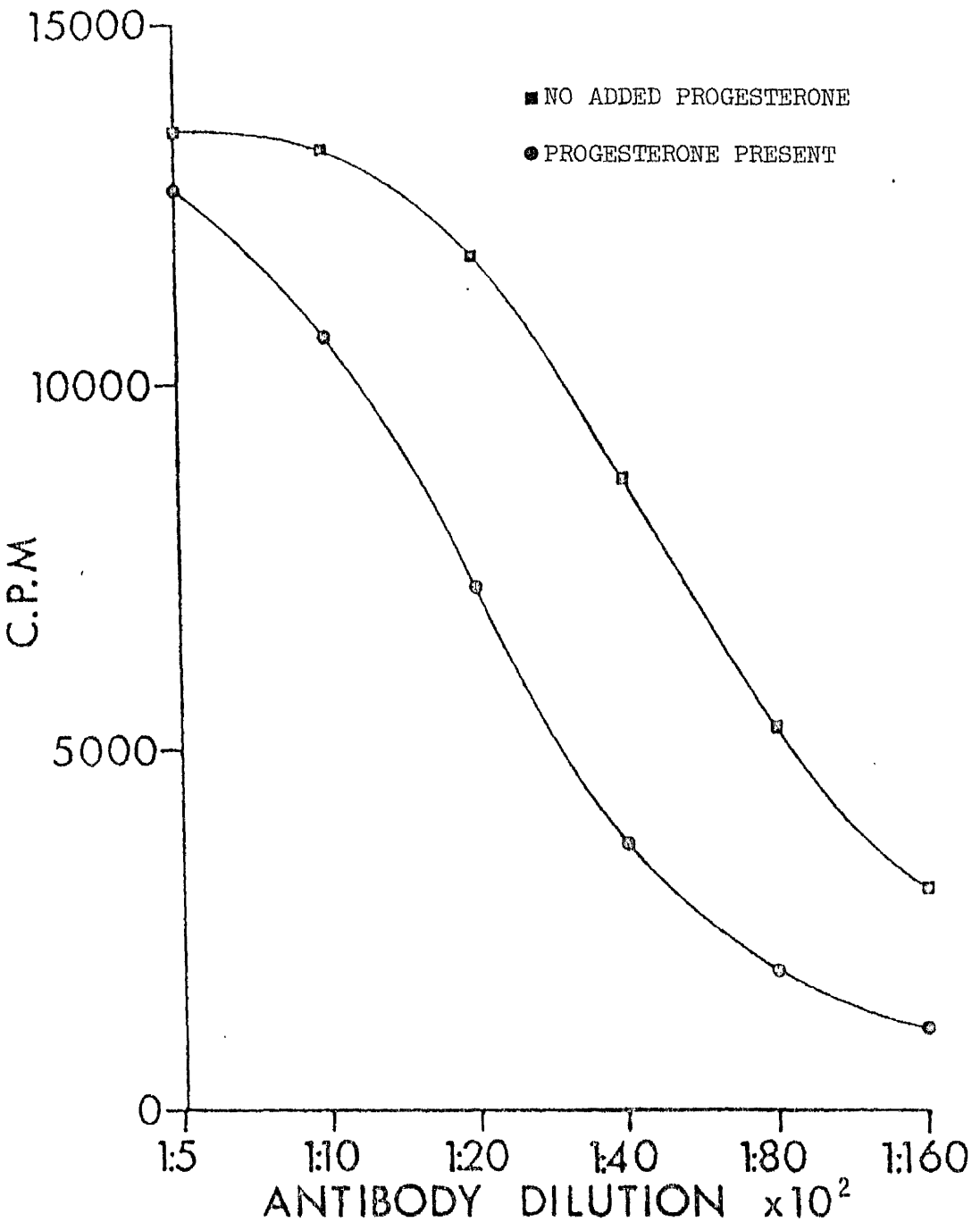


FIG 2 DISPLACEMENT OF TRITIATED PROGESTERONE FROM VARIOUS ANTIBODY DILUTIONS BY THE PRESENCE OF UNLABELLED PROGESTERONE

displacement of the labelled hormone between the tubes with no added progesterone (antibody titre tubes) and those with 0.5 ng progesterone occurred at an antibody dilution of 1:4,700. Besides indicating that the labelled steroid could be displaced from the antibody by progesterone, due to the magnitude of the displacement the results suggested the possibility that the above antiserum in a dilution of 1:4700 - 1:6000 could be used in an assay where the 0.5 ng progesterone level lay around the mid point of the values to be measured.

The effect of time on the binding of tritiated steroid by the charcoal

The stability of the charcoal absorption step was determined by adding charcoal suspensions to a series of tubes containing anti-progesterone serum and progesterone-H^3. Twenty assay tubes containing 100 µl 1:6000 dilution of antibodies in PBS gelatin and 100 µl progesterone-H^3 in PBS had 1000µl charcoal-dextran T-70 added to each. Separation of the supernatants was carried out immediately - 0 time - and at 5, 10, 15 and 20 minutes after charcoal addition. A further three assay tubes containing 100 µl PBS in place of the antibody had 1000µl PBS added to them at the separation stage. These tubes gave an estimate of the total amount of tritiated steroid used.

Results

The results are presented in Fig 3. Based on these observations on the change in supernatant counts with time, 10 min was allowed to elapse in all subsequent experiments between charcoal addition and separation of the supernatant.

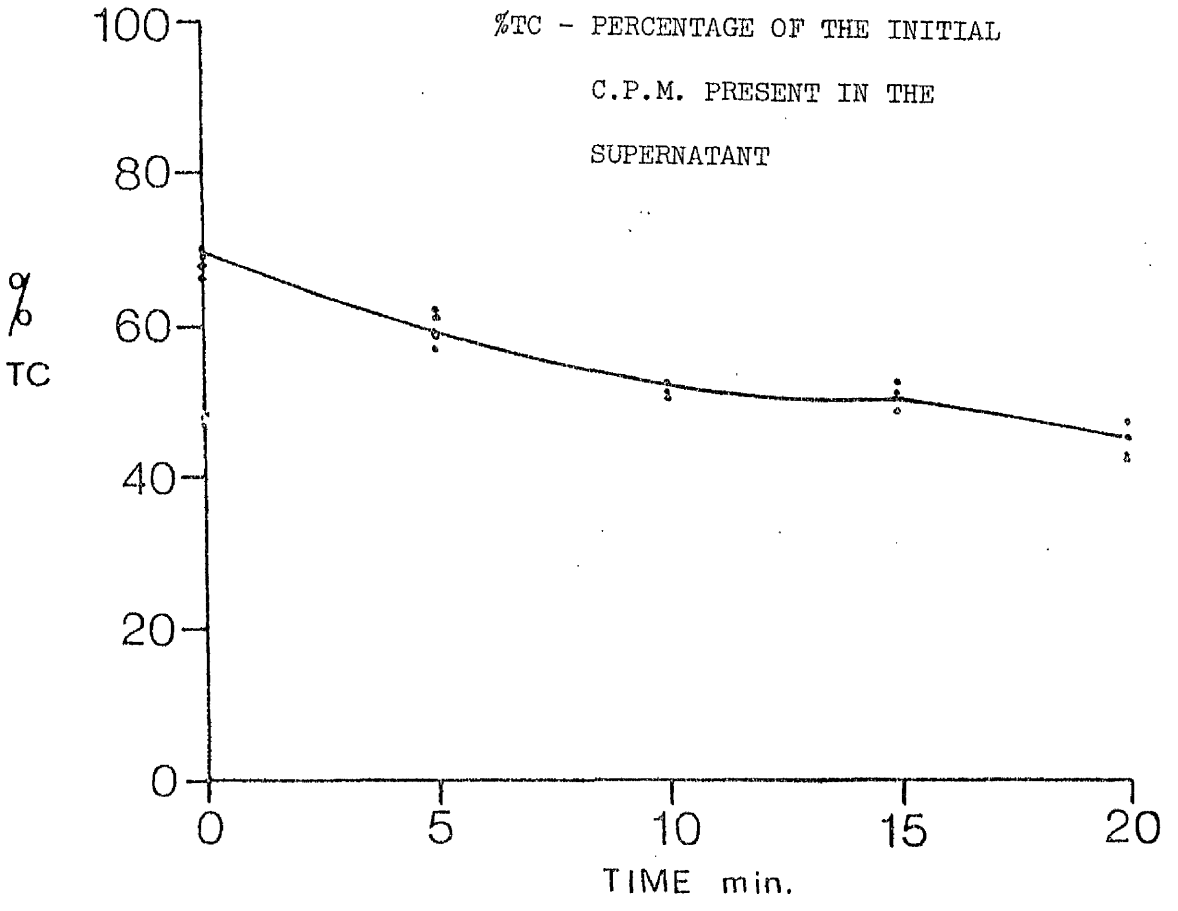


FIG 3 EFFECT OF TIME ON THE BINDING OF LABELLED PROGESTERONE BY DEXTRAN COATED CHARCOAL

(b) Determination of the standard curve

The potential range of the assay was established by determining the displacement of tritiated steroid by increasing amounts of progesterone. The antiserum dilution that bound approximately 50% of the labelled steroid, and which gave maximum displacement with 0.5 ng progesterone was used.

From the 1.0 ng/ml and 10.0 ng/ml progesterone solutions in ethanol 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 ng of hormone were pipetted to assay tubes, in duplicate, and the alcohol removed by evaporation under a stream of air at 40°C. 100 µl of a 1:6,000 dilution of the antibody were then added to each tube and thoroughly agitated. 100 µl dilute antiserum were also dispensed to three empty tubes which were used to determine the amount of tritiated steroid bound in the absence of any competing hormone-bound count tubes (BC). To all tubes 100 µl progesterone-H^3, containing approximately 12,000 c.p.m. were added. An equal volume of tritiated steroid was pipetted to a further two tubes where the antibody solution had been substituted by 100 µl PBS gelatin. These two tubes were incorporated to give an estimate of the total amount of tritiated steroid used - total count tubes - TC. All tubes were thoroughly agitated and then incubated at 4°C for 18 hours. Separation of bound and free hormone was as described above. The degree of displacement of the tritiated steroid was determined by expressing the counts obtained at each point of the standard curve as a percentage of the counts in the BC tubes ($\frac{BT}{BC}$ %).

Results

Fig 4 illustrates a typical standard curve. The wide range of progesterone that produced a displacement of approximately 80% ($\frac{BT}{BC}$ %) confirmed the suitability of the antiserum, in a dilution of 1:6,000 as the basis of a radioimmunoassay for this steroid.

(c) Specificity of the antiserum

The specificity of the antiserum was tested by cross reaction studies. Using an antibody dilution of 1:6,000 the percentage cross reactions, compared to 100% for progesterone, of a range of steroids was determined. The cross reaction of potential interfering steroids was assessed by comparison of the displacement of tritiated progesterone from the antibody by the various steroids. The displacement relative to progesterone, which was assigned a value of 100%, was recorded. The cross reactivity data of the antiserum Y 29/6 was provided by Dr. I. Coutts.

Results

The cross reactivities of the range of steroids tested are presented in Table 2. It was apparent that significant cross reaction existed with 11α hydroxyprogesterone. However the lack of significant cross reaction with the majority of other steroids tested suggested the suitability of the antiserum in a specific progesterone radio-immunoassay system provided certain steroids, such as deoxycorticosterone, could be excluded from competing in the antibody incubation reaction.

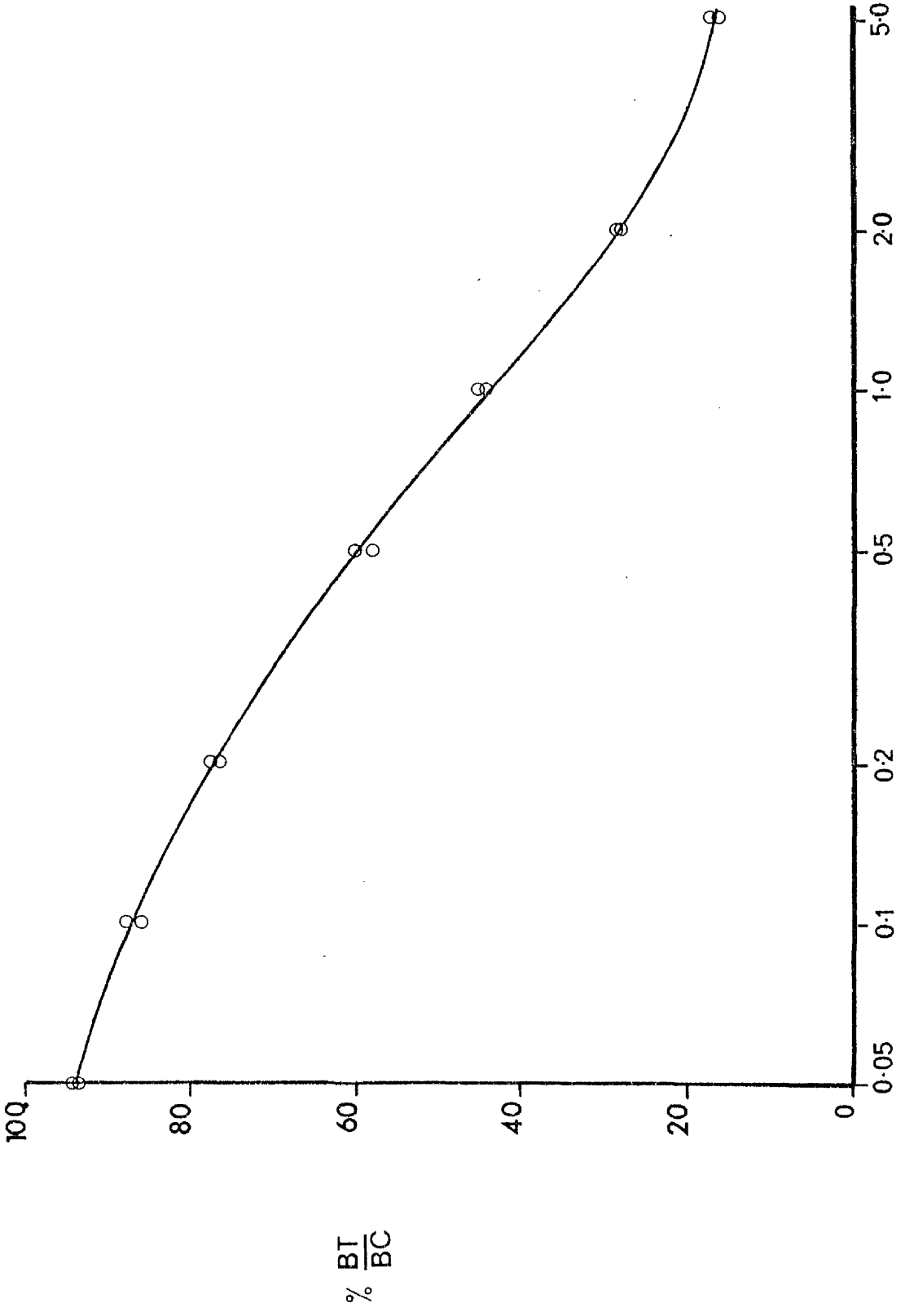


FIG 4 PROGESTERONE STANDARD CURVE

TABLE 2 CROSS REACTION OF VARIOUS STEROIDS WITH ANTI-PROGESTERONE SERUM Y29/6

Steroid	% Cross Reaction
Progesterone	100
11α hydroxyprogesterone	71.4
17α "	0.15
20α "	1.8
20β "	< 0.2
5β pregnane, 3,20-dione	8.8
Testosterone	0.2
Androstenedione	0.2
11β hydroxyandrostenedione	0.2
17-Oestradiol	< 0.2
Oestrone	0.2
Oestriol	< 0.2
Deoxycorticosterone	11.1
Cortisol	< 0.2

(d) Extraction of progesterone from plasma

The efficiency of diethyl ether as a means of extracting progesterone from cow plasma was assessed.

100 µl progesterone H^3 in ethanol, containing approximately 15,000 c.p.m., were dried in extraction tubes (modified Q & Q 10/19). The actual c.p.m. used were determined by adding 100 µl of progesterone H^3 to two counter vials, adding 1.0 ml PBS and 10 ml scintillator to each and counting. 500 µl plasma, from an ovariectomised cow, were added to each extraction tube and after mixing the tubes were allowed to stand at room temperature for 30 minutes. 2.5 ml diethyl ether at $4^{\circ}C$ were then added and the tubes rotated end over end for 10 minutes. At the end of this time the tubes were centrifuged to separate the phases. Following freezing of the lower (plasma) layer at $-15^{\circ}C$ the ether layer was decanted to counter vials. After evaporating the ether to dryness the vials were counted following the addition of 1.0 ml PBS and 10 ml scintillator. The contents of further tubes were re-extracted by repeating the mixing and separating step with an additional 2.5 ml ether, following removal of the first aliquot. In this case both ether extracts were dried in the same counter vial. By comparison of the recovered counts with the total counts present at the start, the extraction efficiency was determined.

Results

The results are presented in Table 3. It was apparent that 2.5 ml diethyl ether efficiently extracted progesterone from cow plasma. Only a slight extraction efficiency advantage against a marked increase in effort could be achieved by double extraction.

TABLE 3 : EXTRACTION OF PROGESTERONE FROM PLASMA WITH DIETHYL ETHER

Average Initial c.p.m.	Recovered c.p.m. 1 extraction	Extraction efficiency %	Recovered c.p.m. 2 extractions	Extraction efficiency %
15311	11885	78	12612	82
	11466	75	12173	80
	11800	77	12688	83
	11569	76	12556	82

Average extraction efficiency - single 77%

- double 82%

(e) Assay technique

Based in part on the previous experiments, the following reagents were used for the routine assay of plasma progesterone. Unless otherwise stated, the reagents were as described before.

1. Phosphate buffered saline pH 7.0 (PBS).

2. Phosphate buffered saline 0.1% gelatin.

3. Progesterone

   50, 20, 10, 5, 2, 1 and 0.5 ng/ml solutions were prepared in ethanol. Routinely 100 µl of these solutions were dried in tubes to give the required amount of hormone. The solutions were stored at -15°C.

4. (1, 2, 6, 7(n)^{3}H) Progesterone

   600 µl of the stock solution were dried in a tube. 7.5 ml of PBS were then added and the contents thoroughly mixed. The stock solution was stored at -15°C, and the working dilution prepared fresh for each assay.

5. Antiprogesterone serum

   A 1:100 dilution of the antiserum in PBS 0.1% gelatin was stored in small aliquots at -15°C. The working antibody dilution of 1:6,000 was prepared, in PBS 0.1% gelatin, from the stock for each assay.

6. Diethyl ether.

7. Dextran coated charcoal

Charcoal	0.125 g
Dextran T-70	0.0125 g
PBS to	100 ml

8. Liquid scintillator.

9. Blank plasma

Plasma from an ovariectomised cow was aliquoted and stored at $-15^{\circ}C$.

Assay principle

The assay consisted of:

i. Extraction of progesterone from plasma.

ii. Incubation of the extracted hormone from samples with antibody and tritiated progesterone. Simultaneous incubation of known quantities of progesterone with the antibody and labelled hormone.

iii. Separation of antibody 'bound' from 'free' progesterone.

iv. Comparison of the displacement of labelled progesterone from the antibody by the unknown quantities of progesterone with the displacement produced by standard quantities of the hormone. This gave the concentration of hormone in the unknown sample tubes.

v. Plasma with known quantities of progesterone present were treated as unknown samples in the assay. The apparent quantities of progesterone in these tubes gave an estimate of the recovery of progesterone in the assay.

vi. The hormone content of the unknown sample tubes were corrected for the recovery of progesterone in the assay, and the results expressed as ng/ml.

The assay was carried out as shown in the flow diagram - Diagram 7. In all cases where progesterone was added to tubes it was the first reagent to be dispensed. No other reagents were added until the solvent alcohol had been removed by evaporation. After adding the plasma to the extracted standard tubes (S8 - S10) they were allowed to stand at room temperature for approximately 30 minutes to allow the added hormone to be bound to plasma proteins.

Routinely 500 µl plasma samples were assayed but this volume was reduced if a prior assay had shown this to be necessary to allow the expected value to fall within the working range of the standard curve. In the event of modification of the sample volume the BL PBS gelatin, BLD plasma, and S8 - S10 plasma volumes were similarly altered. Within an assay these volumes were kept constant.

Calculation of results

The concentration of progesterone in plasma samples was determined either by a computer programme or manually. The computer programme, developed by Rodbard and Lewald (1970) presented results

DIAGRAM 7 FLOW DIAGRAM PROGESTERONE ASSAY

EXTRACTION

Set up the following in extraction tubes (Q & Q 10/19)

	No. of Tubes	PBS Gelatin	Blank Plasma	Progesterone	Sample Plasma
Ether Blank (BL)	2	500 µl	-	-	-
Plasma Blank (BLD)	3	-	500 µl	-	-
Extracted Stds (-S8)	2	-	500 µl	0.2 ng	-
(-S9)	2	-	500 µl	0.5 ng	-
(-S10)	2	-	500 µl	1.0 ng	-
Samples (A, B etc.)	Max. 30	-	-	-	500 µl

Add 2.5 ml diethyl ether to each tube

Mix for 10 min

Centrifuge and freeze lower layer

Pour ether extracts to assay tubes (Disposable test tubes)

and evaporate solvent

BL, BLD, S8, S9, S10, and samples in assay tubes.

DIAGRAM 7 CONTINUED FLOW DIAGRAM PROGESTERONE ASSAY

INCUBATION

Set up the additional assay tubes below*, along with the assay tubes above, as follows:

	No. of Tubes	Progesterone	PBS Gelatin	Antibody	Progesterone H^3
*Total Counts (TC)	2	-	100 µl	-	100 µl
*Bound Counts (BC)	3	-	-	100 µl	100 µl
*Standards S1	2	0.05 ng	-	100 µl	100 µl
" S2	2	0.1 "	-	100 µl	100 µl
" S3	2	0.2 "	-	100 µl	100 µl
" S4	2	0.5 "	-	100 µl	100 µl
" S5	2	1.0 "	-	100 µl	100 µl
" S6	2	2.0 "	-	100 µl	100 µl
" S7	2	5.0 "	-	100 µl	100 µl
Extracted Stds. S8	2	-	-	100 µl	100 µl
" " S9	2	-	-	100 µl	100 µl
" " S10	2	-	-	100 µl	100 µl
Blank Plasma - extracted (BLD)	3	-	-	100 µl	100 µl
Ether Blank - extracted (BL)	2	-	-	100 µl	100 µl
Samples - extracted (A, B etc.)		-	-	100 µl	100 µl

Mix between each addition
Incubate for 18 hours at 4°C.

DIAGRAM 7

FLOW DIAGRAM PROGESTERONE ASSAY

SEPARATION

Add 1.0 ml PBS to TC tubes
At $4^{o}C$ and within a total time of 5 min
add 1.0 ml dextran charcoal to all
except TC tubes

Mix. Leave 10 min. $4^{o}C$

Centrifuge 10 min. $4^{o}C$

Decant and drain supernatants to counter vials

Add 10 ml scintillator, shake and count.

for all extracted standards, blanks, and samples as ng/ml uncorrected for recovery. The manual method was as follows: The average count for the BC tubes was determined and designated 100%. All other tubes, except the TC tubes, were expressed as a percentage of this ($\frac{BT}{BC}$ %). The percentage bindings of the standards were then plotted on a semi log scale and the amounts of progesterone in all other tubes were read from this curve. The recovery of progesterone in the assay was calculated from the apparent amounts of progesterone in the extracted standard tubes (S8 - S10), less the value of the blank plasma if this proved above the sensitivity of the assay, as a percentage of the actual amount of hormone in these tubes. Sample results from the standard curve were corrected for recovery and expressed as ng/ml.

(f) Reliability of the assay

Specificity

The treatment of plasma by diethyl ether is known to extract a variety of steroids including progesterone, oestradiol, oestrone, oestriol, testosterone (Short, 1958). Due to the fact that amongst an extensive range of steroids tested only 11α hydroxyprogesterone showed significant cross reaction with the antiprogesterone serum (Table 2), it was considered that the lack of specificity of the extraction technique would be compensated for by the specificity of the antibody.

Sensitivity

Standard curve. The sensitivity of the standard curve was determined

by comparison in 20 assays of the $\frac{BT}{BC}$ % results for each point on the curve. The mean and standard deviation were calculated for each point and Student's 'T' was applied to consecutive points.

Results

A significant difference was found ($p < 0.05$) between all points on the standard curve (Table 4). Due to the fact that in all cases the BC tubes, representing 0 ng, were assigned a value of 100%, it was not possible to determine the significance of the difference between the 0 and 0.05 ng levels of the standard curve. These results indicate that the sensitivity of the standard curve lies, at worst, between 0.05 and 0.1 ng.

Using plasma samples

To give an estimate of the smallest amount of progesterone that could be measured in plasma the formula - Sensitivity (S) = $\frac{2 \times SD}{R \times F} \times 100$ (Abraham, 1969) was applied.

SD - the standard deviation of the mean of blank plasma values in the assay was determined by estimating the apparent progesterone concentration in 500 µl aliquots of ovariectomised cow plasma. In five assays where duplicate determinations were performed the level was 0.07 ± 0.02 ng.

R - the recovery of progesterone in the assay. Although Abraham (1969) estimated recovery by calculating the efficiency by which tritiated hormone was extracted from plasma in this thesis by incorporating

TABLE 4 PROGESTERONE ASSAY. STANDARD CURVE SENSITIVITY AND ACCURACY

Progesterone ng	$\frac{BT}{BC}$ % $\bar{x}$ $n=40$	S.D.	Coefficient Variation	't'	Significance p
0.05	89.8	6.6	7.3		
				3.765	< 0.05
0.1	83.9	7.3	8.7		
				5.417	"
0.2	75.0	7.3	9.7		
				12.301	"
0.5	56.9	5.7	10.0		
				14.893	"
1.0	38.3	5.4	14.1		
				12.619	"
2.0	25.3	3.6	14.2		
				14.603	"
5.0	15.4	2.3	14.9		

blank plasma containing known amounts of progesterone in the assay and processing these as samples, the overall loss of hormone in the method was determined. The mean recovery at the 0.5 ng level in the five assays above was 84%.

F - the fraction of recovered steroid used in the assay. When the extraction of tritiated steroid is used to estimate recovery, a fraction of the extract is used for this calculation and the remainder assayed. In the technique in this thesis the total extraction volume was assayed and therefore F = 1.

Applying these figures Sensitivity (S) = 0.05 ng. When 500 µl aliquots of plasma were used, the minimum amount of hormone that could therefore be distinguished from 0 was 0.1 ng/ml.

Accuracy

To estimate accuracy the following experiment was performed:

0.2, 0.5, and 1.0 ng progesterone were dried down in duplicate in extraction tubes. 500 µl aliquots of ovariectomised cow plasma were then added to each tube, and to 3 empty tubes to give an estimate of the apparent progesterone level in the blank plasma. After mixing, the tubes were left for 30 minutes, at room temperature, to allow protein binding of steroids to take place. The hormone content was then estimated.

After subtraction of the plasma blank, the apparent progesterone level, if above the sensitivity of the assay, in 10 assays was obtained. Comparison of the estimated v. the actual progesterone levels is given

in Fig 5. It can be seen that a linear relationship exists between the progesterone added and that quantified. The deviation from the theoretical correlation coefficient of 1.0 gives an estimate of the error of the method. Table 5 gives details of blank plasma apparent progesterone levels and the recovery of steroid at the 0.2, 0.5 and 1.0 ng levels.

Precision

It is apparent from the results of the replicate standard curves, given in Table 4, that the coefficient of variation between assays varies from 7.3% at the 0.05 ng level to 14.2% where a concentration of 2.0 ng was determined.

Due to the fact that extracted standards were incorporated in triplicate in each assay analysis of the differences in estimates of these standards gave a measure of the precision of the assay. Table 6 gives details of the precision.

2.2.3. Application of assay - Plasma progesterone levels in cycling cows

Experimental design

Animals. The study was carried out using 6 non-lactating cows of different breeds.

Management. The animals were kept in a cowshed in individual stalls but were turned out for 1 hour to a court for behavioural observations

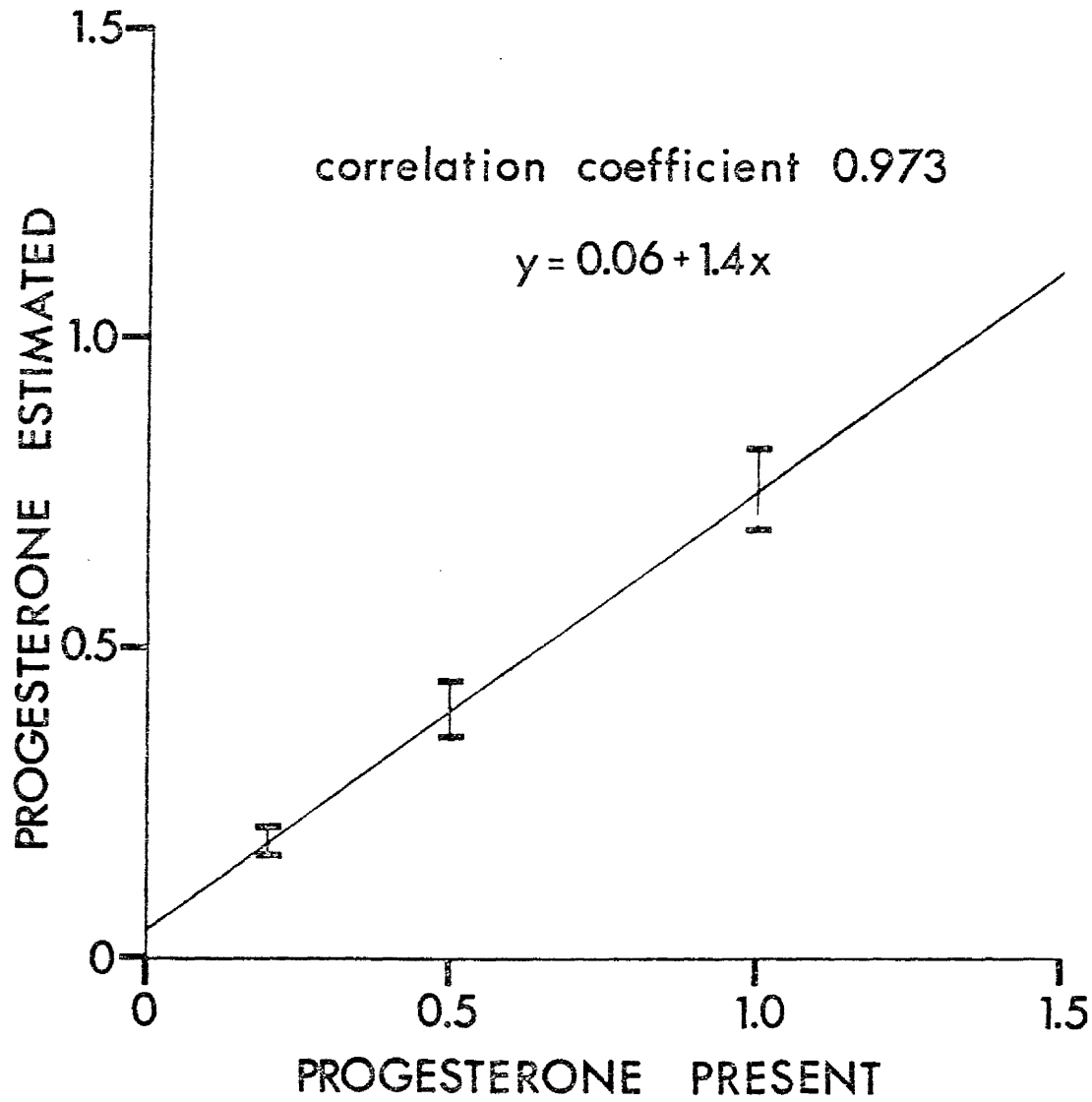


FIG 5 DETERMINATION OF THE ACCURACY OF THE PROGESTERONE ASSAY

TABLE 5 : PROGESTERONE ASSAY. EXTRACTED STANDARDS.

BLANK PLASMA 500 µl	Apparent Progesterone ng	Recovery % $\bar{x}$ ± 1 S.D.
	0.096 ± 0.035	
0.2 extracted standard (S8)	500 µl BLD + 0.2 ng	91.2 ± 16
0.5 extracted standard (S9)	500 µl BLD + 0.5 ng	89.4 ± 14
1.0 extracted standard (S10)	500 µl BLD + 1.0 ng	87 ± 11.8
Overall (S8 - S10)		88.9 ± 13.6

TABLE 6 PROGESTERONE ASSAY. PRECISION

	Extracted standard computed from	
	0.5 ng	1.0 ng
$\Sigma(x_1 - x_2)^2$	0.1037	0.00368
No. duplicate pairs of determinations (n)	20	20
Precision $\sqrt{\frac{\Sigma(x_1 - x_2)^2}{2n}}$	0.0509	0.0607
Overall Precision	0.0594	

each day. Oestrus was judged on the basis of the animal standing to be ridden by other cows. Hay and water were available ad lib.

Blood sampling was carried out from the jugular vein into heparinised evacuated glass tubes (Vacutainer, Becton & Dickinson Ltd.) at the same time each day. Plasma was separated by centrifugation at 4°C and stored at -15°C till assayed.

Hormone levels. The concentration of progesterone in the plasma samples was determined by radioimmunoassay.

Results

Details of the animals used and the duration of their oestrous-oestrous intervals are given in Table 7. The inter-oestrous interval was taken as the period from one oestrus to the next including the days of heat. In no case did oestrus last longer than 1 day. The plasma progesterone levels from the individual cows during the oestrous cycle are shown in Fig 6. It is apparent that in all animals the level of this hormone increased after oestrus, remained elevated for a period and then fell before the next heat. For comparison, the findings of other workers using either competitive protein binding or radioimmunoassay techniques in cycling cows are given in Table 8.

2.2.4. Discussion

Murphy (1967) and Neill, Johansson, Datta and Knobil (1967) demonstrated that progesterone could be estimated in plasma samples

TABLE 7 DETAILS OF THE ANIMALS AND THEIR OESTROUS-OESTROUS INTERVALS

Animal	Breed	Reprod. history	Inter-oestrus interval - days
A	Shorthorn	Heifer	21
B	Ayrshire	Cow	19
C	Shorthorn	Cow	23
D	Ayrshire	Cow	20
E	Friesian	Cow	23
F	Maine Anjou	Heifer	22

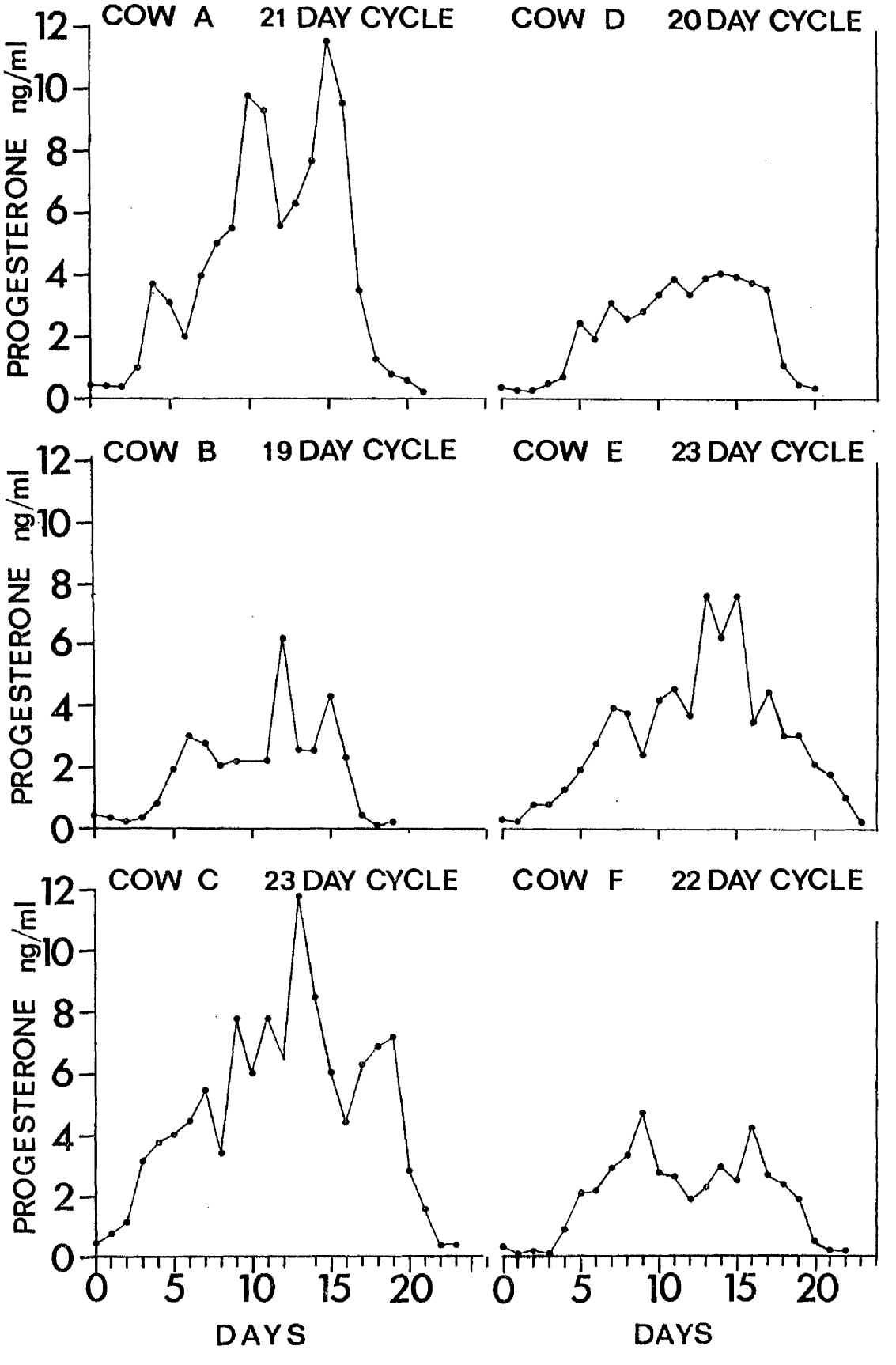


FIG 6 PLASMA PROGESTERONE LEVELS IN COWS DURING THE OESTROUS CYCLE

REFERENCES

1. Rao and Estergreen (1969)
2. Donaldson, Bassett and Thoburn (1970)
3. Shemesh, Lindner and Ayalon (1971)
4. Henricks, Dickey and Niswender (1970)
5. Henricks, Dickey, Hill and Johnston (1972)
6. Swanson, Hafs and Morrow (1972)
7. Gaverick, Erb, Niswender and Callahan (1971)
8. Edgerton and Hafs (1973)
9. Christensen, Hopwood and Wiltbank (1974)
10. Glencross, Munro Senior and Pope (1973)
11. Dobson, Hopkinson and Ward (1973)

TABLE 8 PLASMA PROGESTERONE LEVELS DURING THE OESTROUS CYCLE IN THE COW

Method Assay	Plasma progesterone ng/ml: Around oestrus	Plasma progesterone ng/ml: During dioestrus	Plasma progesterone within the cycle: Increase after oestrus	Plasma progesterone within the cycle: Maximum during dioestrus	Plasma progesterone within the cycle: Decline	Ref.
CPB	2.6 - 2.85			Day 18		1
CPB	0.44	2.9 ± 1.43 (day 6) to 6.8 ± 3.4 (day 14)	Days 2-3	Day 14 (12-15)	Last 4 days	2
TLC + CPB	0.6 ± 0.15	5.4 ± 0.16	Day 3	10 to 6 days before next heat	4 to 3, or 3-2 days before next heat	3
CPB	1.6 ± 0.15		As above			3
CPB	<1.0	5-12	Days 2-4	Day 16	4 to 3 days before next heat	4
CPB	-	7-9*	Day 3	Day 12	From day 12	5
CPB	0.2 ± 1.0	2.8*-3.7 ± 0.6	Day 4	3 days before next heat	3 to 2 days before heat	6
CPB	5.1 ± 0.7 (Days 1-5)	12.6 ± 0.8	Day 2	Between day 11 and 5 days before next heat	5 to 4, or 4 to 3, or 2 to 1 days before next heat	7
CPB	0.3 ± 0.1	7.3 ± 0.6	Day 4	Day 15	From day 15	8
CPB	1.31 ± 0.84	3-7*	Day 4	Day 15	-	9
RIA	0.5 or less	6.0 ± 1.5	Within first 5 days	8 to 4 days before next heat	4 days before next heat	10
RIA	0.3 ± 0.07	7.5 ± 2.0				11

* Estimated from graphical results

using naturally occurring cortisolbinding globulin. Using either these techniques or the modifications suggested by Johansson (1968) the peripheral plasma levels of progesterone in the cow have been determined. Due to the relative lack of specificity of the binding globulin used (Murphy, 1967), purification of the plasma extracts was required to give adequate specificity and sensitivity in the method. Attempts to simplify the extraction/purification system did not lead to a marked variation in high levels of the hormone but to elevated blank values and resulted in an overestimation of low concentrations. Although using column chromatography employing Sephadex LH-20 sensitivities of 0.2 ng were obtained (Swanson, Hafs and Morrow, 1972) in many instances only concentrations in excess of this figure could be accurately distinguished from 0 ng (Henricks et al., 1971; Christensen et al., 1974). Besides the reliability criteria used to describe an immunoassay the value of any technique must also be considered in terms of its practicability (Borth, 1962; Abraham, 1969). This is defined as encompassing estimates of the speed, cost, and skill required to perform the method. Obviously in each instance modifications to make an assay more practical must be reconciled with any simultaneous changes in its reliability. Although reliable assays using CPB methods have been described for use in the cow, such as that of Edgerton and Hafs (1973) their practicability to handling large numbers of samples is poor relative to the later radioimmunoassay methods.

Although a radioimmunoassay technique for determining plasma insulin levels was described by Yalow and Berson (1960), it was not until 1969 that a technique of this type was applied to the measurement

of steroids (Abraham, 1969). The basis of any radioimmunoassay is the availability of a specific antiserum of adequate titre (Ekins, Newman and O'Riorden, 1968; Yalow and Benson, 1968; Thorneycroft, Tillson, Abraham, Scaramuzzi and Caldwell, 1970). The relative affinities of other steroids to the antiprogesterone serum raised in this study compared well to those reported by other workers assaying progesterone by radioimmunoassay. In contrast to the antibody used by Tillson, Thorneycroft, Abraham, Scaramuzzi and Caldwell (1970), which was conjugated through position C20 using an oxime derivative, minimal cross reactivity was observed against the C19 steroids tested with the antiserum in this thesis. The relative affinities of other steroids reported in this thesis are similar to those found by Niswender and Midgley (1970) who also employed conjugation of a hemisuccinyl through C11 to the steroid, and bovine serum albumin as the carrier protein, leaving both ends of the steroid molecule free to act as antigen determinants. In addition the titre of 1:6,000 used in the present study enabled extensive use of the serum from one bleeding of the animal.

Using this antiserum an assay with good accuracy, precision and sensitivity was developed based on the techniques described by Abraham (1968), Tillson et al. (1970), and Abraham, Swerdloff, Tulchinsky and Odell (1970). This technique used a phosphate buffer containing gelatin to minimise non-specific binding to the antibody and to ensure its stability at low concentration. Dextran coated charcoal was employed to separate antibody-bound from free steroid. Due to the specificity of the antibody, extraction with further

purification of the diethyl ether extract of plasma proved unnecessary. In practical terms this represented a significant advantage over the use of celite microcolumns and Sephadex LH-20 chromatography employed by other workers (Abraham et al., 1971; Youssefnejaddian et al., 1971) for radioimmunoassay of progesterone. Assay reliability was as good as that described in competitive protein binding techniques (Swanson, Hafs and Morrow, 1971; Shemesh, Ayalon and Lindt, 1971; Edgerton and Hafs, 1973). When the assay was applied to plasma samples from cycling cows, it was apparent that the results were in agreement with the observations by Donaldson et al. (1970), Swanson et al. (1972) and Edgerton and Hafs (1973) who used CPB methods, and with Glencross et al. (1973) and Dobson et al. (1973) who used RIA techniques. The variability in the time progesterone started to increase after oestrus recorded by other workers (2 - 4 days) was illustrated within the small group of animals in this study. It was apparent from the individual cow results that marked variation could occur between cows during dioestrus and within cows on differing days of dioestrus. This observation is in agreement with the finding of Garverick, Erb, Niswender and Callahan (1971) who reported a highly significant difference between animals in the overall amount of progesterone secreted during the cycle. In general, however, the levels found within animals in this thesis from days 6 - 14 of the cycle, which covered the range 2.0 - 7.7 ng/ml, were similar to those reported in other studies using CPB and RIA techniques. In agreement with the results of other workers, maximum levels of the hormone were found during the latter part of dioestrus - ranging from 12 - 15 days after oestrus. With the

exception of one animal - Cow E - the end of dioestrus when plasma progesterone fell was followed within six days by the following oestrus. It was therefore apparent from the results of these six animals that variability exists in the period between luteal regression and the next oestrus and that this contributed, along with the period of progesterone secretion to the overall oestrous-oestrous interval. Within the cycle, both plateaux and a series of peaks and declines have been recorded in the progesterone levels (Donaldson et al., 1970; Henricks et al., 1971; Shemesh et al., 1971; Garverick et al., 1972). The variability in the occurrence of peaks of secretion recorded by these workers was reflected in the differences in the patterns found in the six cows in this study ranging from Cow A where three well defined peaks were recorded to Cow C where a gradual increase over the complete period of dioestrus was observed.

It therefore appeared that the assay developed in this thesis gave an estimate of the progesterone level in plasma during the oestrous cycle in the cow which agreed with observations of the presence of corpora lutea (Erb et al., 1961), ovarian venous progesterone concentrations (Dobrowolski, Stupnicka and Domanski, 1968) and the results of other workers using CPB and RIA techniques on peripheral plasma samples.

2.3. Determination of total oestrogens in the peripheral plasma of the cow

Prior to the development of competitive protein binding and radioimmunoassay techniques, several studies had been carried out into the peripheral plasma levels of oestrogens in the cow. Following the demonstration by Szego and Roberts (1946) that oestrogens could be detected in the blood of pregnant cows, other workers confirmed the presence of these hormones during late gestation (Bitman, Wren and Sykes, 1958; Higaki, Suga and Fujisaki, 1959; Saba, 1964; Pope, Jones and Waynforth, 1965). The results of these studies were however variable in that oestrogens were not consistently demonstrated in all cows at comparable stages of gestation and where they were detected, the amounts reported ranged from 8.0 ng oestradiol/l to less than 1.0 µg/l. The lack of specificity in these early studies and the influence of the type of biological or chemical assay employed for detection of the hormone has been reviewed by Lorraine and Bell (1966). The conflicting observations found in pregnant animals were also found when attempts were made to apply these chemical and biological assays to studies in cycling animals. Although Duncan, Casas, Emmerson and Melampy (1953) failed to detect oestrogens in the blood of cows during the oestrous cycle, Higaki *et al.* (1959) showed a fluctuation in the oestrogen levels during the cycle with maximum concentrations at oestrus and at days 9 - 10 of the cycle. However, Ayalon and Lewis (1961) found the plasma oestrogen levels to be lower at oestrus than at all other parts of the cycle. In contrast Pope *et al.* (1965) and

Robertson (1969) confirmed the pattern of elevated levels around oestrus though in both studies accurate quantitation of the amounts was inhibited due to the extremely low concentrations involved. Further work on circulating oestrogens in cycling animals necessitated techniques of increased sensitivity which became available with the development of protein binding and immunoassays. From the range of assays of these types that have been developed since 1967, protein binding using plasma protein fractions (Shemesh, Ayalon and Lindner, 1972) and radioimmunoassays using specific antibodies (Henricks et al., 1971; Edqvist et al., 1972; Wettermann, Hafs, Edgerton and Swanson, 1972; Glencross et al., 1973; Smith et al., 1973; Arijie et al., 1974; Agathe and Kolm, 1975), have been applied to studies in cattle.

In this thesis a simple reliable assay for total plasma oestrogens, based on a widely used anti-oestradiol serum, will be described. Validation of the method will in part be based on a comparison of the results obtained when it was applied to studies in cycling cows with studies by other authors in similar animals.

2.3.1. Materials

Ethanol, phosphate buffered saline, PBS gelatin, diethyl ether, charcoal, dextran T-70, and scintillation fluid were as described for the progesterone assay.

1. Oestradiol (Sigma, London)

   1.0 ng/ml and 0.1 ng/ml solutions were prepared in ethanol, and stored at -15°C.

2. (2, 4, 6, 7 (n)-^{3}H) oestradiol (Radiochemical Centre, Amersham, England)

250 µCi, with a specific activity of 40 - 60 Ci/mmol, were dissolved in ethanol and stored at -15°C.

Unless otherwise stated the required amounts of labelled and unlabelled hormone were obtained by evaporation of the ethanol, as previously described, and reconstitution in PBS or other PBS based reagents.

Disposable test tubes as previously described were used as assay tubes.

3. Extraction tubes Quickfit (Jobling, Stone) MF24/15.

2.3.2. Methods

(a) Characterisation of anti-oestradiol serum

In the case of the proposed assay for plasma oestrogens, antiserum against oestradiol was obtained from Dr. B. V. Caldwell (Code AS/029/18). A series of experiments similar to those described for the progesterone assay was performed to establish the optimal conditions for the use of this antiserum.

Determination of the concentration of dextran coated charcoal

To a series of assay tubes containing approximately 4,500 c.p.m. tritiated oestradiol, in 500 µl PBS gelatin, suspensions of charcoal ranging from 0.5 - 0.0325 g/100 ml, with dextran T-70 present at 10% of the charcoal concentration, were added in 500 µl aliquots at 4°C. After mixing the tubes were left at 4°C for 10 minutes after which the

supernatants were removed by centrifugation (4°, 10 minutes, 2,500 g) and the c.p.m. determined by adding them to 10 ml scintillator in plastic vials.

Results

The results presented in Table 9 showed that 0.5 of charcoal/ 100 ml PBS were capable of absorbing the bulk of the tritiated steroid used. No significant advantage was gained by increasing the concentration of charcoal.

Antibody titre

The antibody titre was determined in a similar manner to that described for the antiprogesterone serum except that the dilutions ranged from 1:35,000 to 1:1,120,000 and 500 µl aliquots were used/tube. Tritiated oestradiol containing 0.00166 µCi and representing 4 pg of the hormone, were added to each tube in a volume of 100 µl.

Results

It can be seen from the results in Fig 7 that the antibody dilution binding 50% of the tritiated oestradiol added was 1:230,000 using 500 µl aliquots of diluted antiserum.

Displacement of labelled oestradiol from the antibody

At the same time as the antibody titre was assessed, the displacement of the labelled steroid in the presence of a fixed mass of oestradiol was determined. 50 pg of oestradiol, representing the

TABLE 9 : ADSORPTION OF TRITIATED OESTRADIOL BY DEXTRAN COATED CHARCOAL

Charcoal concentration G/100 ml	Average Initial c.p.m.	Final c.p.m.	Average % c.p.m. adsorbed
1.0	5035	39; 41; 36	99.2
0.5	"	56; 67; 56	98.8
0.125	"	274;271;280	94.5
0.0325	"	1530;1351;1406	71.6

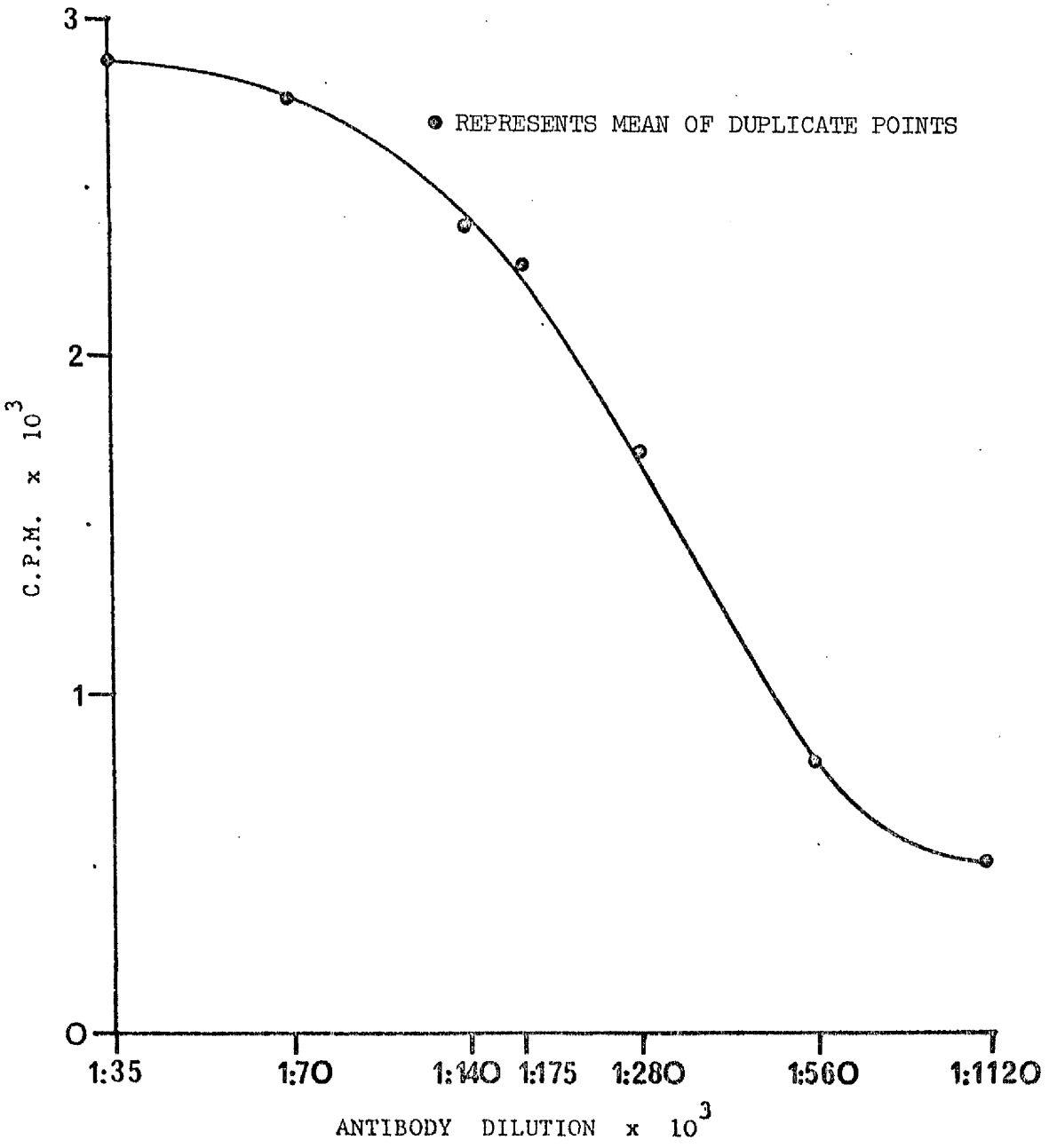


FIG 7 ANTI-OESTRADIOL DILUTION CURVE

mid point of the range of concentrations required to be measured in the proposed assay, were added to duplicate tubes at each antibody dilution. Further treatment of the tubes was as described for the antibody titre.

Results

It can be seen from the results in Fig 8 that maximum displacement of the labelled steroid was obtained at an antibody dilution of 1:175,000. Although 50% binding of the amount of tritiated oestradiol used occurred at an antibody dilution of 1:230,000 to give maximum sensitivity between 0 pg and 50 pg in the eventual assay, 1:175,000 was used as the antibody dilution in subsequent experiments.

Effect of time on the binding of tritiated steroid to charcoal

To establish the stability of the charcoal absorption step in binding free steroid, the following experiment was performed.

Twenty assay tubes containing 500 µl anti-oestradiol serum, at a dilution of 1:175,000 in PBS gelatin and 100 µl oestradiol-H^3 in PBS (approximately 4,500 c.p.m.) were set up. Three additional tubes with the antibody replaced by PBS gelatin gave an estimate of the total amount of tritiated steroid used (TC tubes). After incubating all tubes at $4^{\circ}C$ for 2 hours, 500 µl PBS gelatin were added to the TC tubes. At the same time 500 µl aliquots of dextran coated charcoal, containing 0.5 g charcoal with 0.05 g dextran T-70/100 ml PBS, were added to the remaining tubes. The addition of PBS gelatin or charcoal and all

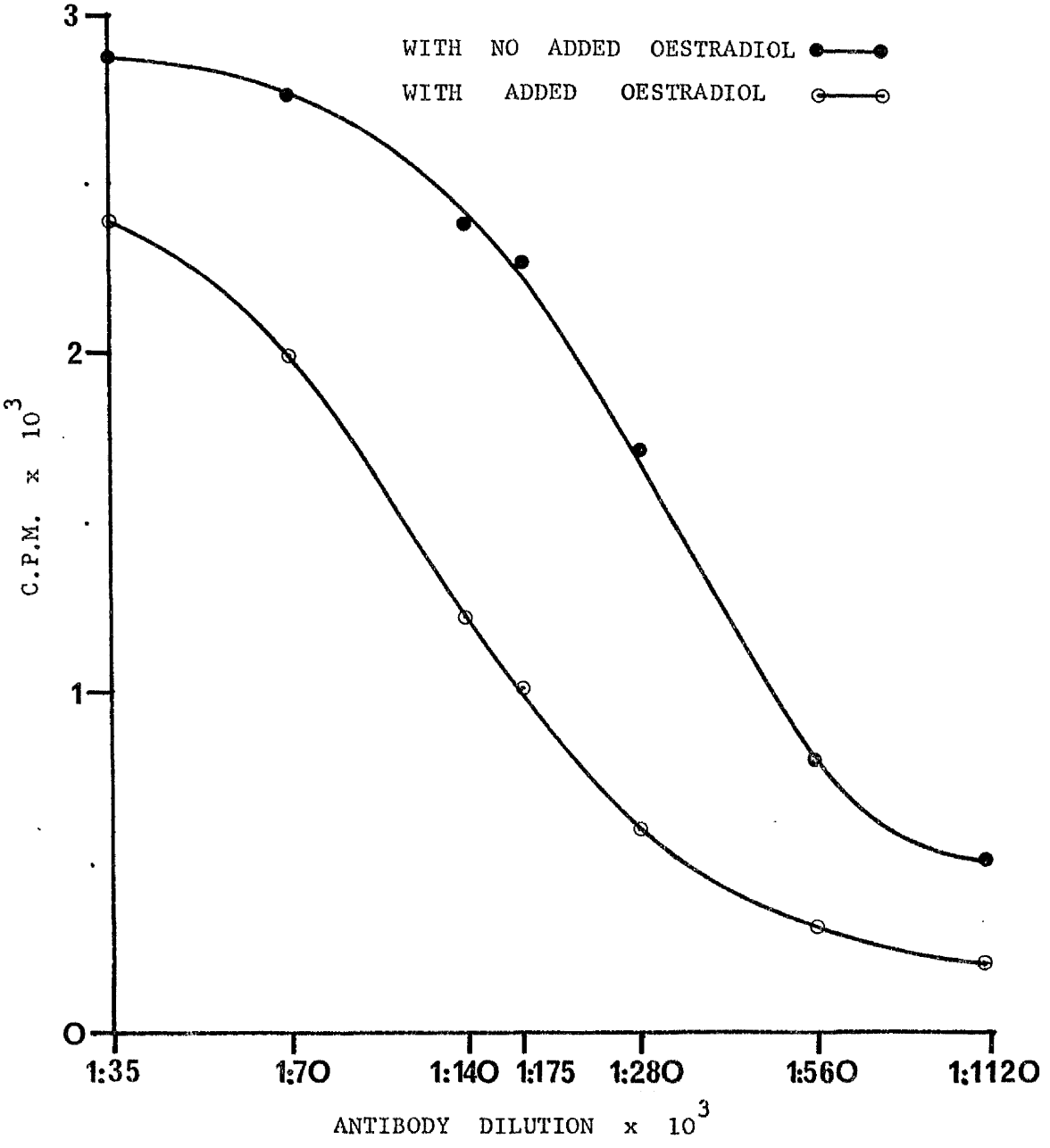


FIG 8 DISPLACEMENT OF TRITIATED OESTRADIOL FROM VARIOUS ANTIBODY DILUTIONS BY THE PRESENCE OF UNLABELLED OESTRADIOL

subsequent steps were performed at 4°C. Immediately after mixing, the supernatants were removed from the three TC tubes and four of the charcoal tubes by centrifugation and were transferred to counter vials - 0 time. Thereafter at 5, 10, 15 and 20 minutes after the addition of the charcoal, a further four charcoal tubes had their supernatant removed. 10 ml scintillator were added to all counter vials and, after shaking, the c.p.m. determined.

Results

The supernatants representing the antibody bound counts are shown for each time in Fig 9. These results indicated that although a large percentage of the free tritiated steroid was adsorbed to the charcoal immediately after its addition, over the following 5 minutes a further significant amount of hormone was taken up. However between 5 and 10 minutes, the amount of hormone adsorbed remained steady. Accordingly in all other experiments and in the eventual assay 10-15 minutes were allowed to elapse between charcoal addition and separation of the supernatant.

(b) Determination of the standard curve

Using the stock solutions of oestradiol in ethanol 2, 5, 10, 20, 50, 100 and 200 pg of the hormone were dispensed to assay tubes in duplicate and the alcohol removed by evaporation. As in the establishment of the progesterone standard curve three tubes were included to give an indication of the amount of hormone bound to the antibody when no competing cold steroid was present (BC). Two total count (TC) tubes were also incorporated. 500 µl 1:175,000 dilution of

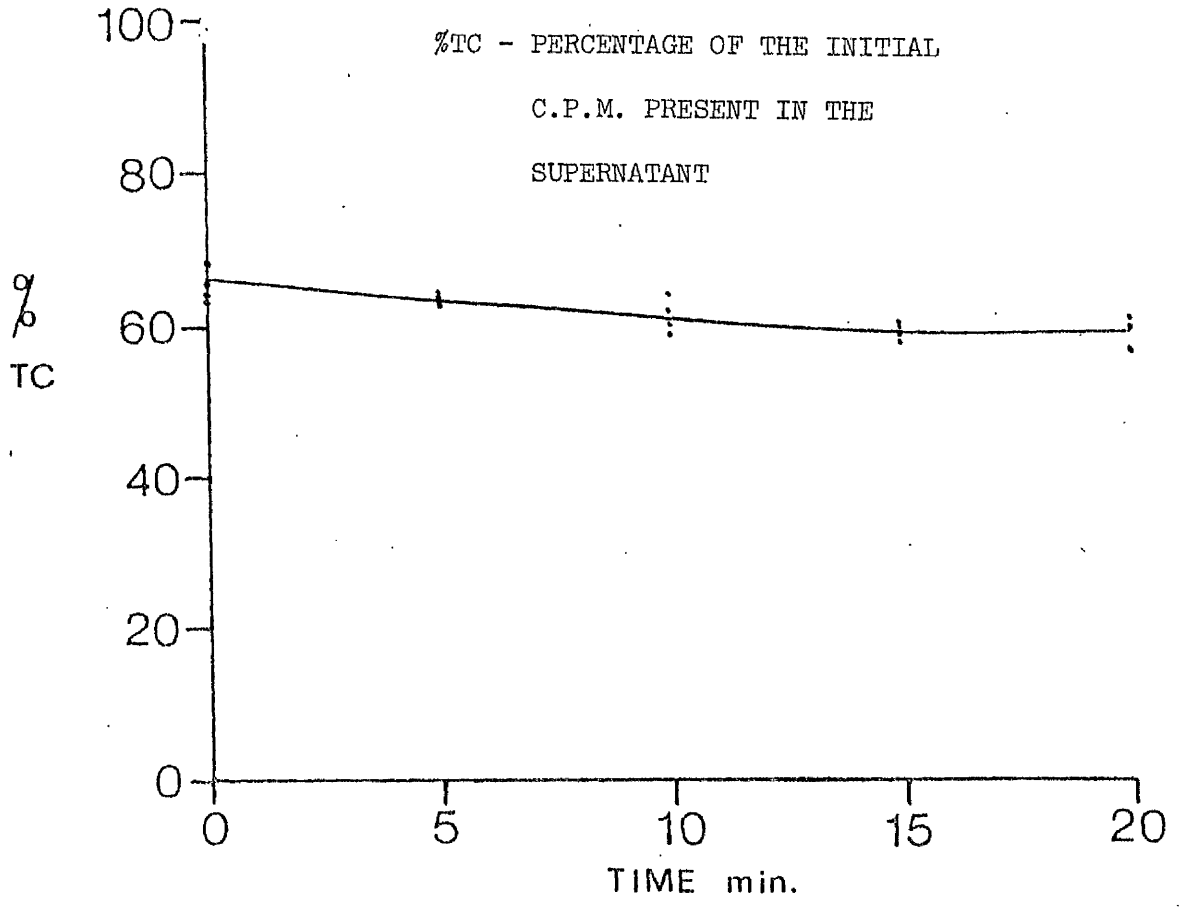


FIG 9 EFFECT OF TIME ON THE BINDING OF LABELLED OESTRADIOL BY DEXTRAN COATED CHARCOAL

the antibody were added to all except the TC tubes which received 500 µl PBS gelatin. 100 µl tritiated steroid in PBS were then added to all tubes. Incubation and separation of the bound from the free hormone were as described for progesterone except that 500 µl of charcoal dextran solution were added.

Results

It is apparent from the results of a typical standard curve presented in Fig 10 that a 40 fold increase in oestradiol was associated with a 77% reduction in the $\frac{BT}{BC}$ %. More especially the steep decline in the amount of antibody bound steroid that occurred over the 5 - 50 pg range was important in the establishment of a sensitive immunoassay.

The effect of modifying the incubation conditions on the standard curve

An attempt was made to improve the sensitivity of the assay by favouring the binding of cold steroid to the antibody. Two standard curves ranging, from 2 - 200 pg oestradiol/tube, were assayed identically except that in one of them after addition of the antibody solution the reaction was allowed to proceed for 30 minutes at room temperature before tritiated oestradiol was added. Both assays were then incubated for 2 hours at $4^{\circ}C$ before being separated.

At the same time to investigate the possibility of performing the incubation step overnight, a further standard curve was left at $4^{\circ}C$ for 18 hours before separation.

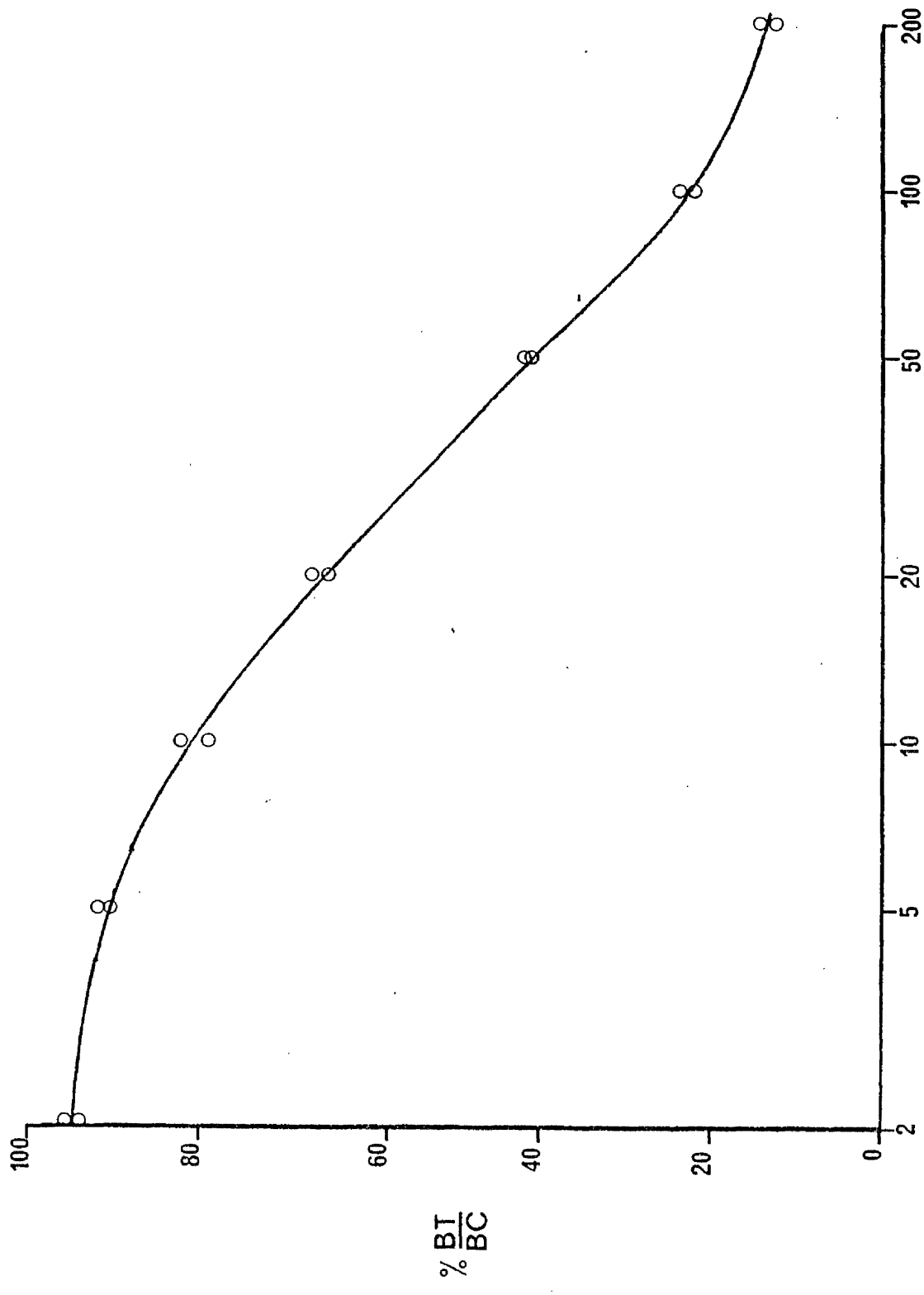


FIG 10 OESTRADIOL STANDARD CURVE

Results

Fig 11 gives the bound counts, expressed as $\frac{BT}{BC}$ % for each of the three standard curves incubated under the different conditions. It was apparent that although the addition of a half hour pre-incubation step slightly increased the gradient of the line between 5 and 10 pg, no difference was observed from the 10 - 50 pg levels. Similar findings were observed between the 2 hour and overnight incubation curves. As the gradient of all lines was similar between 2 and 5 pg, it was felt that no advantage, in terms of increased sensitivity, could be obtained by employing a half hour pre-incubation. All subsequent assays were incubated therefore for 2 or 18 hours at $4^{o}C$.

(c) Antibody specifity

Details of the cross reactivity of a range of steroids with the antibody, given in Table 10, were supplied by Dr. B. V. Caldwell. For all steroids except the oestrogens the displacement of tritiated oestradiol resulting from the incorporation of 1 µg amounts of the hormones was compared with the displacement produced by standard amounts of oestradiol. Results were then expressed as oestradiol equivalents (pg). For the estimation of the degree of cross reaction between oestrone and oestriol and the antiserum, the displacement of tritiated oestradiol by 100 pg of the hormones was determined. Although Caldwell estimated that oestrone and oestriol cross reacted 100% and 10% respectively with the antiserum repetition of the experiment with the antibody dilution determined previously (1:175,000) gave a significantly smaller cross reaction with oestrone (Table 10). All other steroids tested showed 0.3% or less cross reaction.

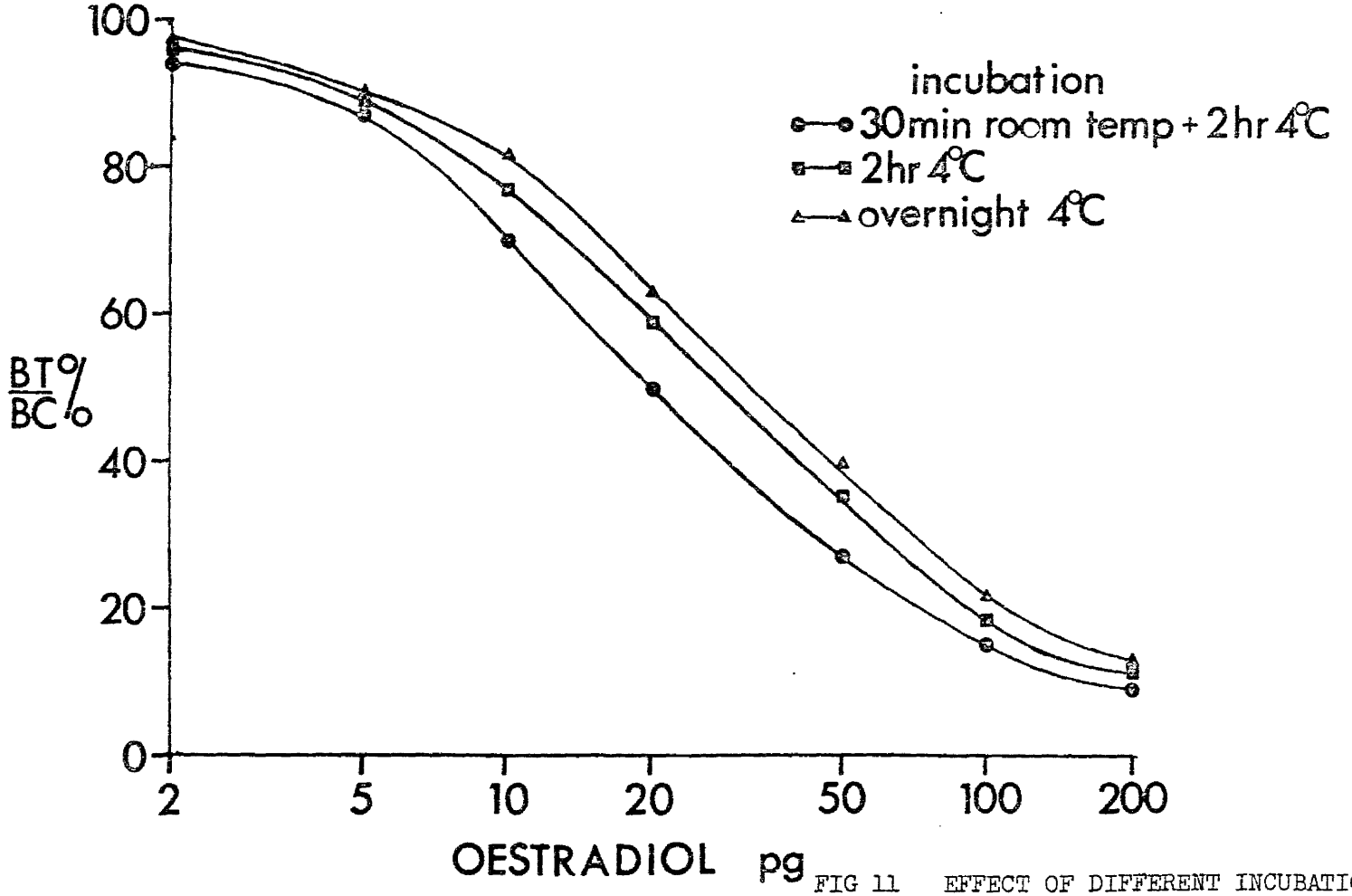


FIG 11 EFFECT OF DIFFERENT INCUBATION CONDITIONS ON THE OESTRADIOL STANDARD CURVE

TABLE 10 CROSS REACTION VARIOUS STEROID (1 µg) WITH CALDWELL OESTROGEN ANTIBODY 029-18

Steroid	Oestradiol Equivalents	Cross Reaction	
	pg	%	%*
Oestradiol	-	100	100
Oestrone	-	100	51
Oestriol	-	10	<10
Cholesterol	0	< 0.01	
Pregnenolone	15	"	
20α-hydroxy-4-pregnenone	2	"	
20α-hydroxy-4-pregnenone	4	"	
Progesterone	5	"	
17α-hydroxyprogesterone	3	"	
Adrenosterone	5	"	
Cortisone	25	"	
Cortisol	13	"	
Deoxycorticosterone	30	"	
Dehydroepiandrosterone	15	"	
Androstenedione	13	"	
Dihydrotestosterone	8	"	
Testosterone	7	"	

*As determined by the method employed in this thesis

(d) Extraction of oestrogens from plasma

Diethyl ether was used for extraction of oestrogens from plasma (Diczfalusy and Lindkvist, 1956).

100 µl tritiated oestradiol in ethanol containing approximately 14,000 c.p.m. were dried in extraction tubes and 4.0 ovariectomised plasma were added to each. After mixing the tubes were left at room temperature for 30 minutes. At the end of this time 5.0 ml diethyl ether were added to each. By rotating the tubes end over end for 10 minutes, followed by centrifugation and freezing the plasma layer, the ether extract was obtained. This was dried in plastic counter vials and the counts present determined by adding 1.0 ml PBS gelatin + 10 ml scintillator to each vial, then counting for 10 minutes or 10,000 counts. The total counts used were obtained by counting 100 µl of the tritiated oestradiol solution with 900 µl PBS gelatin and 10 scintillator. Re-extraction of some of the tubes with a further 5 ml ether was performed as for the progesterone extraction method.

Results

The recovery of tritiated steroid following extraction by 5 ml ether, and after extraction by two 5 ml aliquots of ether are given in Table 11. Only a small increase in the recovery of steroid was obtained by the double extraction method. Routinely 5.0 ml ether was therefore used in a single extraction step.

(e) Assay technique

Unless otherwise stated, the reagents were as described before.

TABLE 11 : EXTRACTION OF OESTRADIOL FROM PLASMA WITH DIETHYL ETHER

Average Initial c.p.m.	Recovered c.p.m. 1 extraction	Extraction efficiency %	Recovered c.p.m. 2 extractions	Extraction efficiency %
14509	11305	78	13261	91
	11617	80	12990	90
	11408	79	13264	91
	10864	75	13229	91

Average extraction efficiency - single 78%

- double 91%

1. Phosphate buffered saline pH 7.0.

2. Phosphate buffered saline 0.1% gelatin.

3. Oestradiol

   20, 50, 100, 200, 500, 1000 and 2000 pg/ml solutions were prepared in ethanol. Routinely 100 µl of these solutions were dried in tubes to give the required amount of hormone. The solutions were stored at $-15^{\circ}C$.

4. (2, 4, 6, 7 (n)-^{3}H) oestradiol

   300 µl of the stock (250 µCi in 100 ml ethanol) evaporated to dryness, were reconstituted in 7 ml PBS. This was prepared for each assay.

5. Anti-oestradiol serum

   A 1:175,000 dilution in PBS 0.1% gelatin was prepared for each assay.

6. Dextran coated charcoal

Charcoal	0.5 g
Dextran T-70	0.05 g
PBS to	100 ml

The assay method was basically the same as described for progesterone. Diagram 8 gives a flow sheet of the technique. The following modifications from the progesterone assay were employed.

DIAGRAM 8 - OESTROGEN RADIOIMMUNOASSAY

Extraction - Ether blank, plasma blank, extracted standard volume

sample volume 4.0 ml

Extracted standards 20, 50, 100 pg

5.0 ml diethyl ether

↓

Extract, remove ether extract and dry in assay tubes.

Incubation - Consisting of the following assay tubes - Total count, bound count tubes

Standards 5, 10, 20, 50, 100, 200, 400 pg E-2

Dried extracts from ether blanks, plasma blanks etc.

↓

Add 500 µl PBS gelatin to T.C. tubes

Add 500 µl antibody to remainder

↓

Incubate 30 minutes at room temperature

↓

Add 100 µl oestradiol H^3 to all tubes

↓

Incubate 2 hours $4^{\circ}C$

Separation - Add PBS to T.C. tubes and dextran coated charcoal to remainder.

Centrifuge and count supernatant.

5 ml diethyl ether were used for the extraction of samples, extracted standards and blanks. The extracted standards contained 20, 50 and 100 pg oestradiol/tube and the seven unextracted standards covered the range 2 - 200 pg/tube. Normally the sample volume, and that of the ether blanks, plasma blanks and extracted standards, was 4.0 ml, but this was reduced if a preliminary assay showed that it contained a greater amount of oestradiol than the upper point of the workable standard curve. 500 µl of the working antibody dilution were added to each assay tube and the incubation carried for 2 hours or overnight at 4^{o}C. Counting of the antibody bound steroid was carried out for either 10,000 counts or 10 minutes. Calculation of results was as previously described.

(f) Reliability of the assay

Specificity

Diethyl ether has been found to extract a range of steroids, including oestradiol, oestrone, and oestriol, from plasma (Short, 1958). Due to the cross reactivity of the antibody with oestrone and oestriol in addition to oestradiol (Table 10), the results given in the assay system represent the total unconjugated oestrogens in the plasma.

Sensitivity

Standard curve

Fig 12 gives the mean ± 1 S.D. of the percentage bound counts for each point in the standard curve in 20 assays. Applying Student's

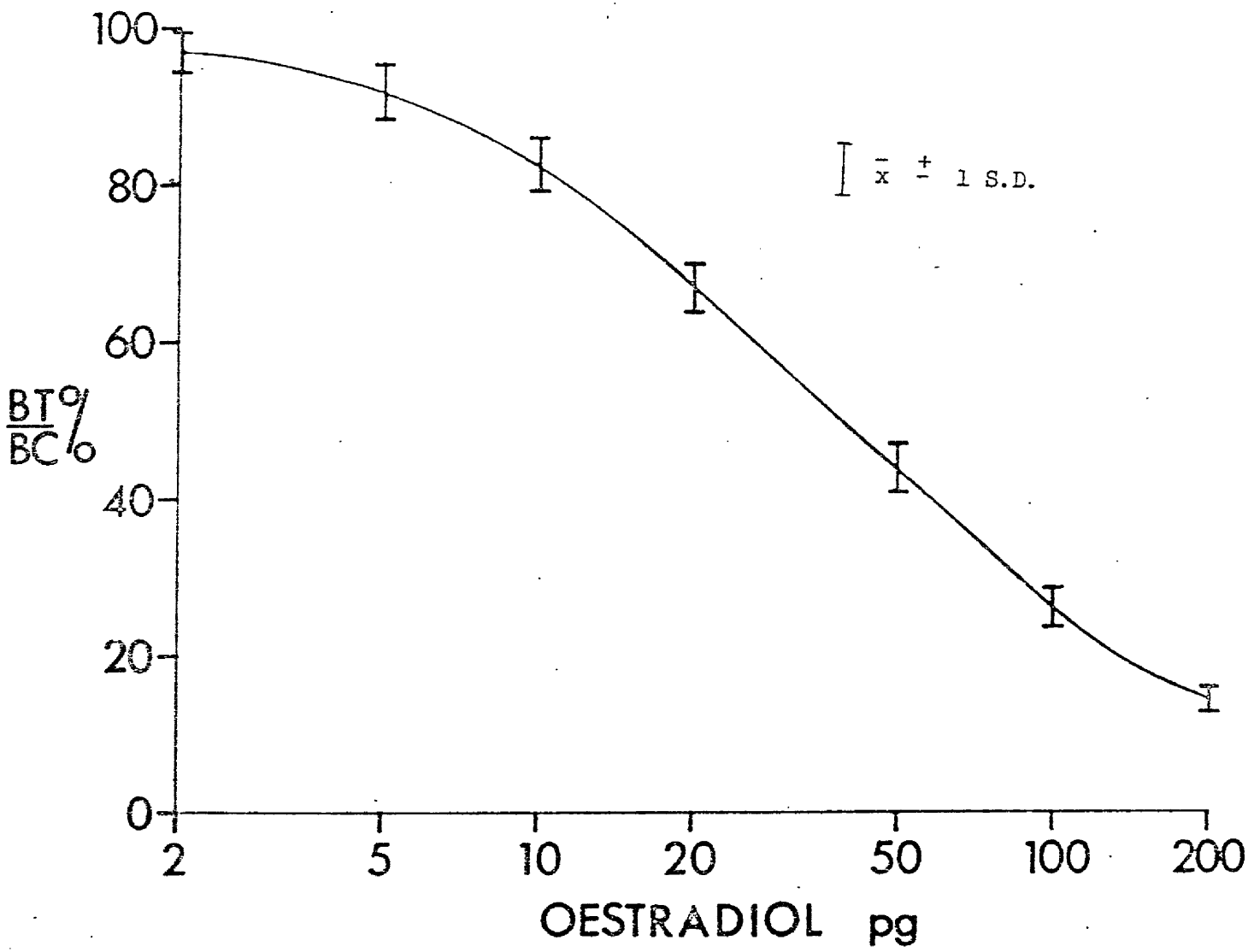


FIG 12 VARIATION WITHIN REPLICATE OESTRADIOL STANDARD CURVES

't' test to consecutive points, a significant difference ($P < 0.05$) was found between the 5 and 10 pg levels of the curve. The sensitivity of the standard curve at worst is therefore between 5 and 10 pg/ml.

Plasma samples

Sensitivity was determined as for the progesterone assay by applying the formula given by Abraham (1969). Table 12 summarises the results used in establishing the sensitivity in pg/ml when the assay was applied to 4.0 ml cow plasma samples.

Accuracy

Accuracy was determined as described for the progesterone assay except that 4.0 ml plasma samples containing 20, 50 and 100 pg oestradiol were used and were extracted with 5 ml ether.

Results

The accuracy determinations are presented in Fig 13.

Precision

Precision was determined by examination of the variation in replicate determinations of S8 and S9 extracted standards in 10 assays.

Results

The estimates of precision are given in Table 13.

TABLE 12 TOTAL OESTROGEN ASSAY. SENSITIVITY WHEN APPLIED TO PLASMA SAMPLES

	$\bar{x}$ S.D.
Blank plasma apparent oestradiol (pg)	= 17.4 ± 2.0
Recovery of oestradiol (S8 - S10)%	= 83 ± 14
Sensitivity (S) pg $\frac{2 \times SD}{R \times F}$	= 4.82
Sensitivity pg/ml	= 1.2

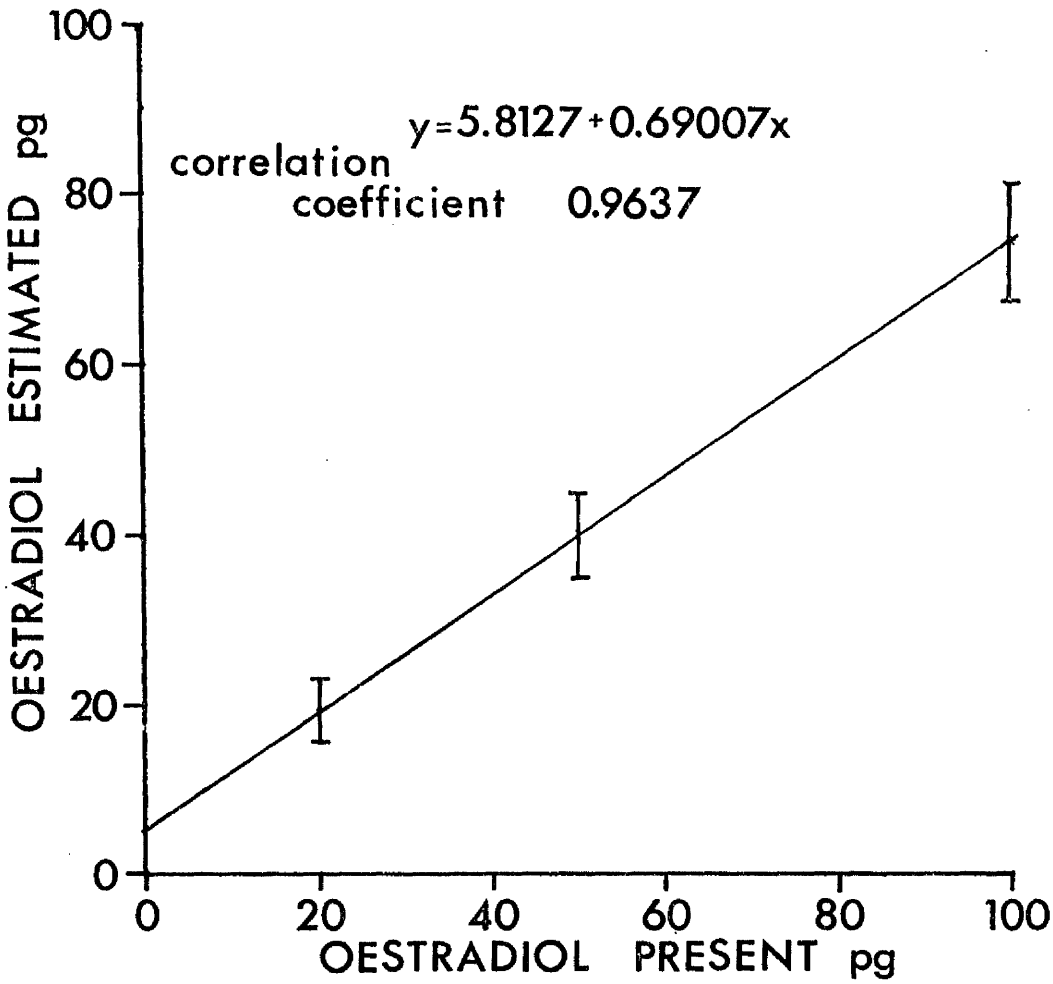


FIG 13 DETERMINATION OF THE ACCURACY OF THE TOTAL OESTROGENS ASSAY

TABLE 13 TOTAL OESTROGEN ASSAY. PRECISION

	Extracted standard computed from	
	50 pg	100 pg
$\Sigma(x_1 - x_2)^2$	297	334
No. duplicate pairs of determinations (n)	10	10
Precision $\sqrt{\frac{\Sigma(x_1 - x_2)^2}{2n}}$	3.8536	4.0866
Overall Precision	3.9717	

2.3.3. Application of the assay - peripheral plasma oestrogens during the oestrous cycle in the cow

The animals used and their management were as described previously. The peripheral plasma oestrogen levels were determined on the same blood samples as those assayed for progesterone.

Results

The manner of expressing the results was as given before. Fig 14 gives details of the peripheral plasma oestrogen levels for the individual animals. It is apparent from these results that an elevation in the circulating oestrogen concentrations was found around oestrus in all animals. In addition in all cows, except Cow F, at least one further peak was recorded during the cycle. For comparison with these results, the findings of other workers who used a range of immunoassay methods in cycling cows are given in Table 14.

2.3.4. Discussion

Ferin, Zimmering, Liebermann and Van Wiele (1968), by immunising a ewe at monthly intervals over a period of 15 months with oestradiol-17-bovine serum albumin, succeeded in producing a very high titre anti-oestradiol antiserum. Using this serum, Abraham (1969) developed a radioimmunoassay for determination of oestrogens in biological fluids based on a solid phase system to bring about separation of bound and free hormone. By incorporating a celite chromatography step, it was possible to determine the specific concentration of oestradiol. A simplified method omitting fractionation

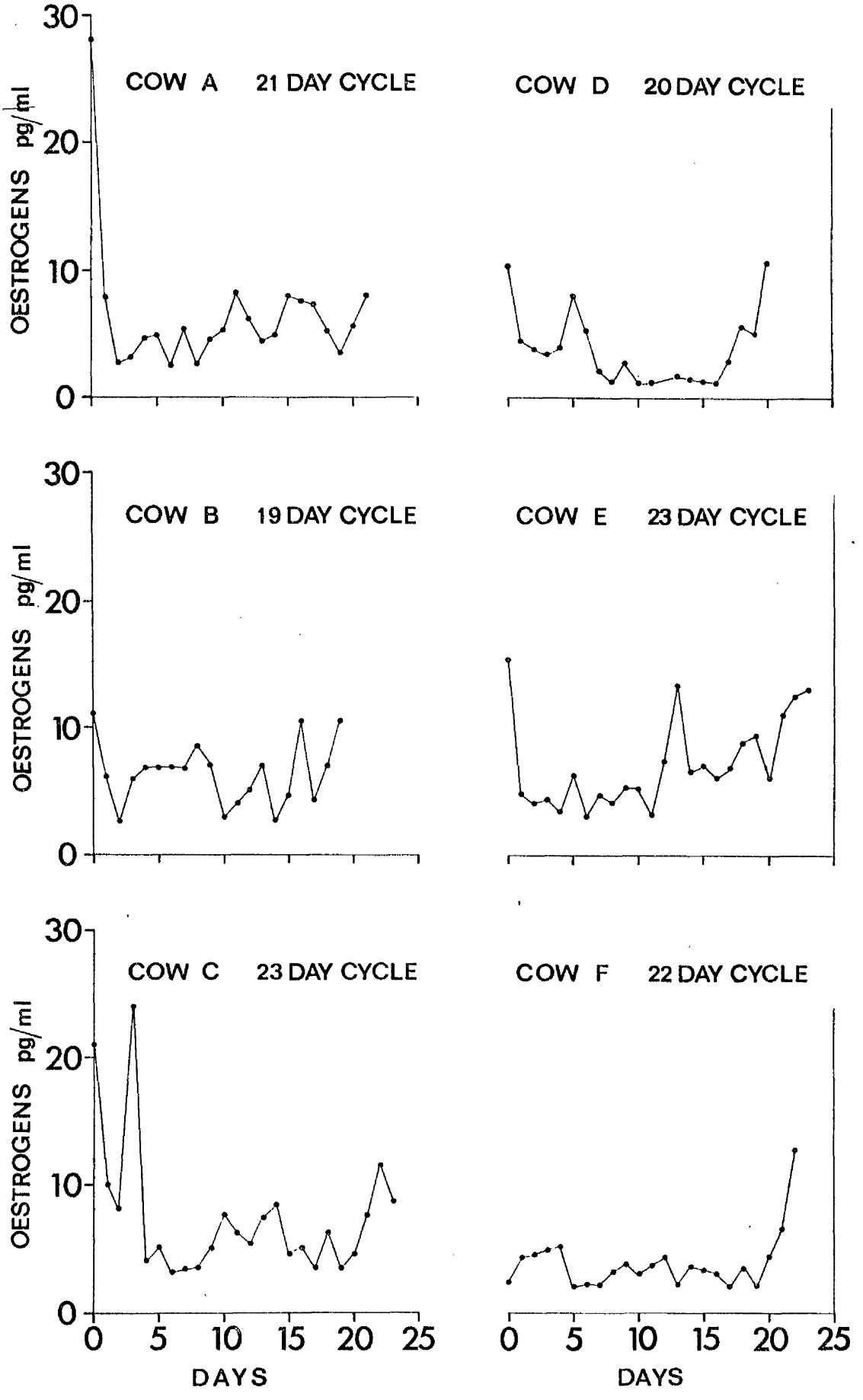


FIG 14 LEVELS OF PLASMA TOTAL OESTROGENS IN COWS DURING THE OESTROUS CYCLE

References

1. Henricks, Dickey and Hill (1971a)

2. Henricks, Dickey and Hill (1971b)

3. Arijie, Wiltbank and Hopwood (1974)

4. Christensen, Hopwood and Wiltbank (1974)

5. Shemesh, Ayalon and Linder (1972)

6. Glencross, Munro, Senior and Pope (1973)

7. Wettemann, Hafs, Edgerton and Swanson (1972)

8. Glencross, Munro, Senior and Pope (1973)

TABLE 14 PERIPHERAL PLASMA OESTROGENS DURING THE OESTROUS CYCLE IN THE COW

		Plasma Oestrogens				
Assay Method	Oestrogens Estimated	Levels around oestrus pg/ml	Time of max. levels around oestrus	Secondary peaks Time	Magnitude pg/ml	Ref.
RIA	Total	15-25	One day before and day of oestrus	-	-	1
RIA	Total	As above	As above	Day 9	10-16	2
Solid phase RIA	Total	500	Two days before oestrus	Day 5	50-500	3
Solid phase RIA	Total	150-175	One to two days before oestrus	Levels of around 100-150 pg/ml for remainder cycle		4
TLC + CPB	Oestradiol	170 ± 19	One day before oestrus	Day 11 Day 4	81 ± 36 40	5
Chromatography + RIA	Oestradiol	5-7*	Two days before to the day of oestrus	Day 4-6	5-7	6
Chromatography + RIA	Oestradiol	8-10				7
Chromatography + RIA	Oestrone	No peak recorded - levels approx. 1.0 pg/ml				

of the oestrogen extract, was found only to slightly overestimate oestradiol in cycling human females and to have a sensitivity of between 25 and 50 pg. Henricks, Dickey, Hill and Niswender (1971) were able to estimate the total oestrogen concentration in the peripheral blood of cycling cows using this antibody of Ferin et al. (1968).

Circulating oestrogens are known to be present in the blood, both bound to plasma proteins and to a very much lesser extent in the free form (Sandberg, Slaunwhite and Antonaides, 1957). In addition biologically inactive conjugates are present (Preedy, 1968). In common with a wide range of mammalian species investigated, both oestrone and 17β-oestradiol have been found in the cow (Dorfman and Ungar, 1965). 17α-oestradiol has also been demonstrated in this species (Klyne and Wright, 1959) although oestriol has not been detected (Dorfman and Ungar, 1965). In common with the solid phase immunoassay described by Abraham (1969) the method reported in this thesis used diethyl ether to extract the oestrogens from plasma. This system, originally proposed by Veldhuis (1953) has been shown to give a good recovery of protein bound and free oestrogens from plasma (Abraham, Odell, Edwards and Purdy, 1970; Mikhail, Wu, Ferin and Van de Wiele, 1970) along with the added advantage that conjugated oestrogens are not extracted (Diczfalusy and Lindkvist, 1956). In this thesis no advantage was gained over a single extraction by using repeated extraction as suggested by Abraham et al. (1970).

As with the anti-oestradiol serum raised by Ferin et al. (1968)

the antibody used in this thesis had been prepared by injecting oestradiol, conjugated through C17 to a hemisuccinate, a configuration shown to result in a high titre of more specific antibody than coupling through other sites e.g. C3.(Thorneycroft, Tillson, Abraham, Scarramuzi and Caldwell, 1970). Due to the specificity of the antibody, it was possible to obtain an accurate estimate of the amounts of oestrogens present in plasma without further purification of the extract. This represents a significant advantage over the competitive protein binding methods such as that used by Shemesh, Ayalon and Lindner (1972) in the cow, which necessitated chromatographic separation of the crude extract. As the purpose of the assay was to enable an extensive investigation to be carried out into aspects of reproductive function in the cow, it was not considered practical to introduce a step such as Celite (Robertson, Smeaton and Durnford, 1972) or Sephadex LH-20 (Mikhail et al., 1970) chromatography to compensate for the lack of specificity of the antibody between oestradiol and oestrone. This decision was also influenced by the observation that oestradiol is the predominant oestrogen in the cow (Dorfman and Ungar, 1965; Erb et al., 1971). Using a sensitive immunoassay in cows, Glencross et al. (1973) has demonstrated the relatively low levels of oestrone in plasma during the oestrous cycle. However, allowing for the fact that this hormone is present, albeit in small amounts, and the observation that the antibody shows a greater affinity for oestradiol, all results have been expressed as total unconjugated oestrogens to accommodate any influence of oestrone in giving an overestimate of the oestradiol concentrations.

The preliminary studies of Henricks et al. (1971) in cows suggested that any assay for use in this species must have a high sensitivity. By minimising the solvent blank contribution and incorporating a preliminary incubation of the antibody with the non-radioactive hormone, the sensitivity of the method in this thesis was below 10.0 pg. By adopting a system where cold hormone was added to blank plasma for recovery estimates, the total extraction volume could be assayed which also contributed to the detection limit of the system of less than 2.5 pg/ml. This level is considerably less than the 20 pg/ml found by Shemesh et al. (1972) using a protein binding system and as low as those reported in other radioimmunoassay systems used in cows (Henricks et al., 1971; Glencross et al., 1973; Dobson et al., 1974). In spite of the incorporation of several decanting steps, for reasons of practicability, which could possibly be expected to influence the precision of the technique, the overall coefficient of variation compares favourably with that of other studies.

The observation that when the assay was applied to studies on cycling cows an elevation in oestrogens was detected associated with oestrus is similar to that reported by other workers using RIA techniques with the exception of Arijie, Wiltbank and Hopwood (1974) (Table 14). The range of levels at oestrus (2.2 - 28 pg/ml) can possibly be explained in part by the observation of Henricks et al. (1971) and Glencross et al. (1973) that individual animals show variability in the timing of maximum levels of these hormones relative to the day of heat. An illustration of the peak level one day before the onset of heat is seen in Cow C. When the average levels of plasma

oestrogens for the days before and after oestrus were calculated (Fig 15), it is apparent that the levels are similar to the 15 - 25 pg/ml concentration around oestrus reported by Henricks et al. (1971) though considerably less than those found by Christensen, Hopwood and Wiltbank (1974). The high levels recorded by Christensen et al. (1974) cannot be explained on the basis of the data they supply on their assay, which used an established method (Abraham, 1969) and an antiserum showing significant cross reaction only with oestrone. It is also apparent that a discrepancy exists when the results of specific oestrogen determinations are compared with the results in this thesis. Combining the observations of Glencross et al. (1973), on the concentrations of oestradiol and oestrone present around oestrus, gives a total oestrogen level of around 6 - 8 pg/ml which is significantly less than the values recorded for the animals in this study, and those of Henricks et al. (1971). As both this study and that of Glencross et al. (1973) used antisera provided by Caldwell, and as accuracy and sensitivity figures are comparable, this apparent anomaly cannot be explained. However this observation does serve to illustrate the necessity for establishing 'normal ranges' with the assay technique available to allow comparison with, and interpretation of, results from animals during periods of reproductive function other than the normal oestrous cycle.

When the results from all animals before and after oestrus are considered, it is apparent that the level of peripheral plasma oestrogens drops markedly between the day of and one day after heat (Fig 15). As ovulation in the cow occurs 10 to 15 hours after the end

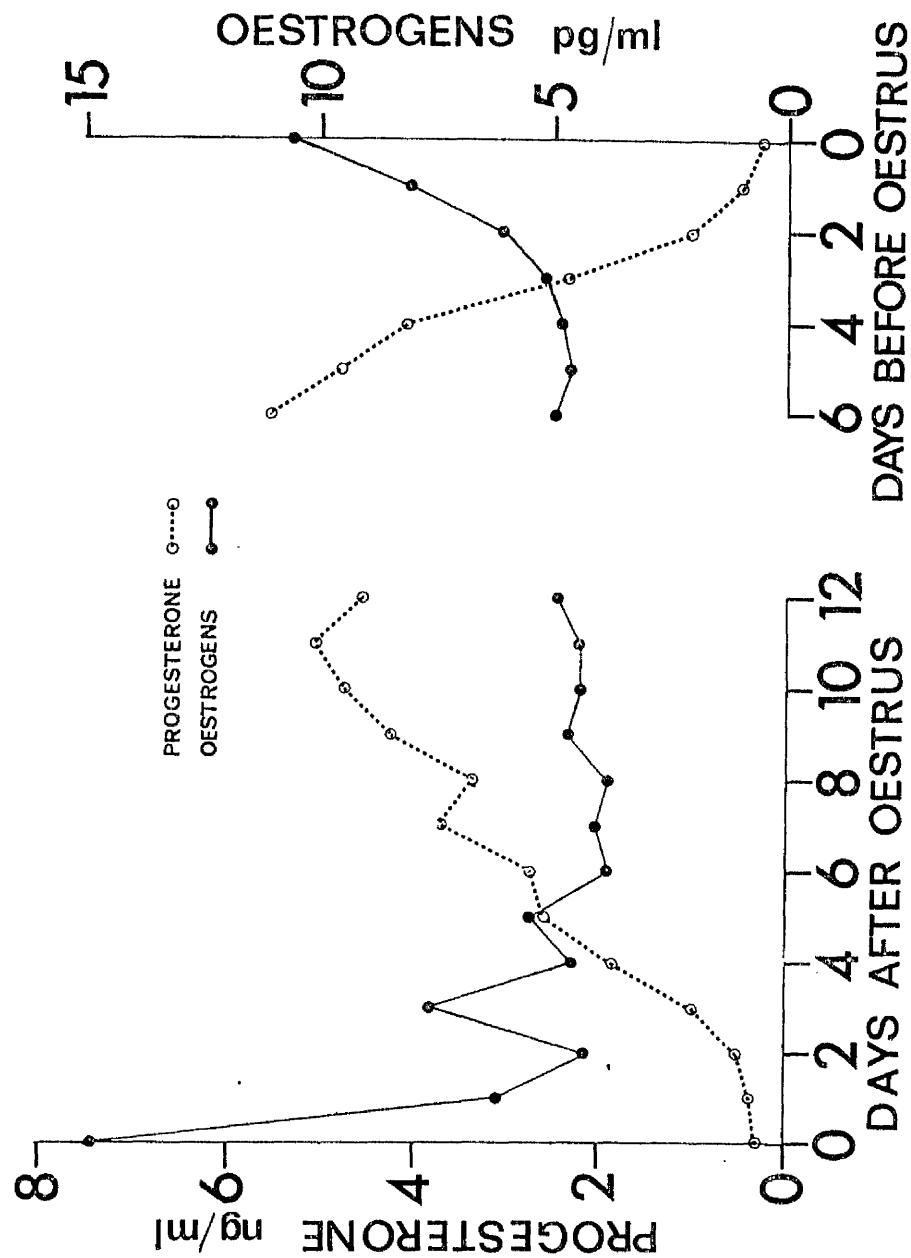


FIG 15 AVERAGE PROGESTERONE AND TOTAL OESTROGEN LEVELS DURING THE OESTROUS CYCLE IN THE COW

of oestrus (Asdell, 1964) the peak of oestrogens precedes this event on average by 36 hours. The pattern of increasing levels of oestrogens from 2 - 3 days before heat, seen in Fig 15, is in agreement with the observations of Henricks et al. (1971), Wetteman et al. (1972) and Glencross et al. (1973). When considered along with the average progesterone concentrations before oestrus it can be seen that functional luteal regression precedes the increase in circulating oestrogens by approximately 3 days (Fig 15).

The elevated levels of plasma oestrogens found at times other than around oestrus have been reported. Table 14 gives details of any secondary peaks of these hormones found in other studies and illustrates the wide variability in timing encountered. The elevated level recorded 3 days after oestrus when all results are taken together (Fig 15) is similar to the post-oestrous elevation reported by Glencross et al. (1973) though slightly earlier in the time of occurrence after heat. It is apparent from the peripheral plasma progesterone results given in Fig 15 that this second peak of oestrogens precedes the development of full luteal function. Within individual animals the magnitude of these inter-oestrous peaks relative to the maximum levels around oestrus are similar in this study to those reported by Henricks et al. (1971) and Glencross et al. (1973). The occurrence of follicular development during dioestrus (Wagner and Hansel, 1969) probably accounts for the origin of these inter-oestrous oestrogen peaks.

2.4. Determination of androgens in the peripheral plasma of the cow

The application of androgen estimations to peripheral plasma samples became possible with the development of competitive protein binding and immunoassay techniques which were more sensitive and specific than previous chemical and biological methods. Competitive protein binding techniques resulted from the recognition that although a large amount of testosterone in the blood appeared to be bound to albumin, human plasma contained a globulin which could bind this and other 17β-hydroxyl steroids with high affinity (Mercier-Bodard, Alfsen and Baulieu, 1970). Testosterone was bound with very much greater affinity than the other 17β-OH steroids. The presence of some other 17β-OH steroid hormones in very much greater concentrations in the blood necessitated extensive purification of plasma extracts before the protein binding of testosterone could be quantified (Vermeulen and Verdonck, 1970).

As an alternative to competitive protein binding systems, the production of steroid protein conjugates, as described by Erlanger, Borek, Beiser and Weberman (1957) allowed the production of anti-testosterone serum and subsequent development of radioimmunoassay techniques (Niswender and Midgley, 1970).

In this thesis an antiserum against testosterone has been raised in sheep and used to develop an immunoassay for estimation of androgens in the peripheral blood of the cow. In contrast to progesterone and oestrogens, only preliminary information exists in

the literature on androgen levels in cows (Saba, Cunningham and Millar, 1975) although extensive studies were previously performed using bioassay techniques (Garm, 1949).

2.4.1. Materials

The materials were as described for the progesterone assay with the exception of :

Testosterone (Sigma, London)

10.0 ng/ml, 1.0 ng/ml, 0.1 ng/ml in ethanol. Stored at -15°C.

(1, 2, 6, 7 (n)^{3}H) Testosterone (Radiochemical Centre, Amersham, England)

250 µCi, of specific activity 80,000 - 119,000 mCi/mmol, were dissolved in 100 ml ethanol and stored at -15°C.

Recovery of unlabelled and labelled steroid were as described for progesterone.

For plasma extraction, the extraction tubes described for the progesterone assay were employed.

Sodium hydroxide AR (B.D.H., Poole, England).

2.4.2. Methods

(a) Preparation of anti-testosterone serum

Immunisation

Conjugation of testosterone to bovine serum albumin was

achieved through the keto group on C-3. An (O-carboxy-methyl) oxime derivative was prepared as an intermediate as described by Erlanger et al. (1957). Using the steroid conjugate concentrations, the same volume of Freund's adjuvant and employing a similar immunisation schedule to that previously described for progesterone, the anti-testosterone serum was prepared in a sheep. The final serum used in subsequent experiments followed the fifth series of injections. The stock antiserum was diluted 1:1 with PBS gelatin and stored at -15°C.

Absorption of tritiated steroid

The optimal concentration of dextran coated charcoal to absorb the tritiated steroid was determined as described for the progesterone assay. In the case of the testosterone-^{3}H, however, the tritiated steroid was dissolved in 500 µl PBS gelatin and 500 µl of the charcoal suspensions added.

Results

The results, presented in Table 15, showed that a 0.125 g/100 ml charcoal suspension was capable of absorbing in excess of 90% of the labelled steroid. This concentration was used in subsequent experiments.

The effect of time on the binding of labelled steroid by charcoal

The effect of leaving the charcoal suspension in contact with the tritiated steroid for varying periods of time was determined as described for the oestrogen assay. 500 µl 0.125 g/100 ml charcoal suspensions were used throughout.

TABLE 15 : ADSORPTION OF TRITIATED TESTOSTERONE BY DEXTRAN COATED CHARCOAL

Charcoal concentration G/100 ml	Average Initial c.p.m.	Final c.p.m.	Average % c.p.m. adsorbed
0.5	3250	47;36;42	98.7
0.25	"	82;86;88	97.4
0.125	"	130;133;132	95.9
0.03125	"	198;183;193	94.1

Results

It is apparent from the results in Fig 16 that a greater amount of steroid was absorbed by the charcoal when the suspension was left for 5 minutes, as opposed to being centrifuged immediately. Although a further change in the amount bound occurred from 5 - 10 minutes between 10 and 15 minutes the rate of absorption declined. In subsequent experiments centrifugation was carried out approximately 15 minutes after adding the charcoal.

Antibody titre

A range of antibody dilutions from 1:20,000 to 1:100,000 were used to determine the dilution binding 50% of an added amount of testosterone-^{3}H. 500 µl of each antibody dilution were used per tube. Tritiated steroid was added in 100 µl PBS.

Results

It can be seen from the counts bound to the antibody, illustrated in Fig 17, that a 1:50,000 dilution of the antiserum bound 50% of the tritiated testosterone added.

Optimisation of antibody dilution

By adding 500 pg testosterone to each of the antibody dilutions used to establish the antiserum titre, the dilution where maximum displacement of the labelled steroid from the antibody occurred was determined.

%TC - PERCENTAGE OF THE INITIAL C.P.M. PRESENT IN THE SUPERNATANT

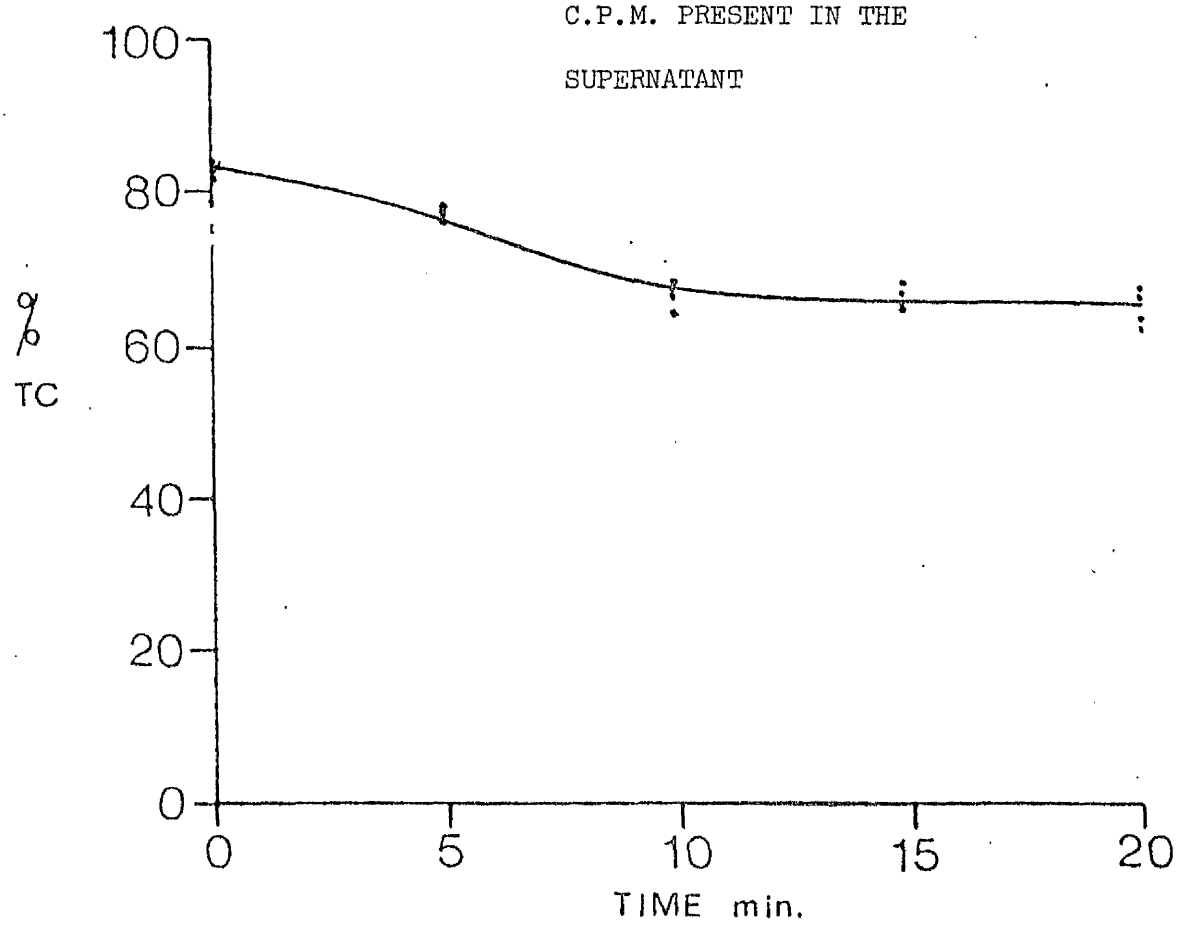


FIG 16 EFFECT OF TIME ON THE BINDING OF LABELLED TESTOSTERONE BY DEXTRAN COATED CHARCOAL

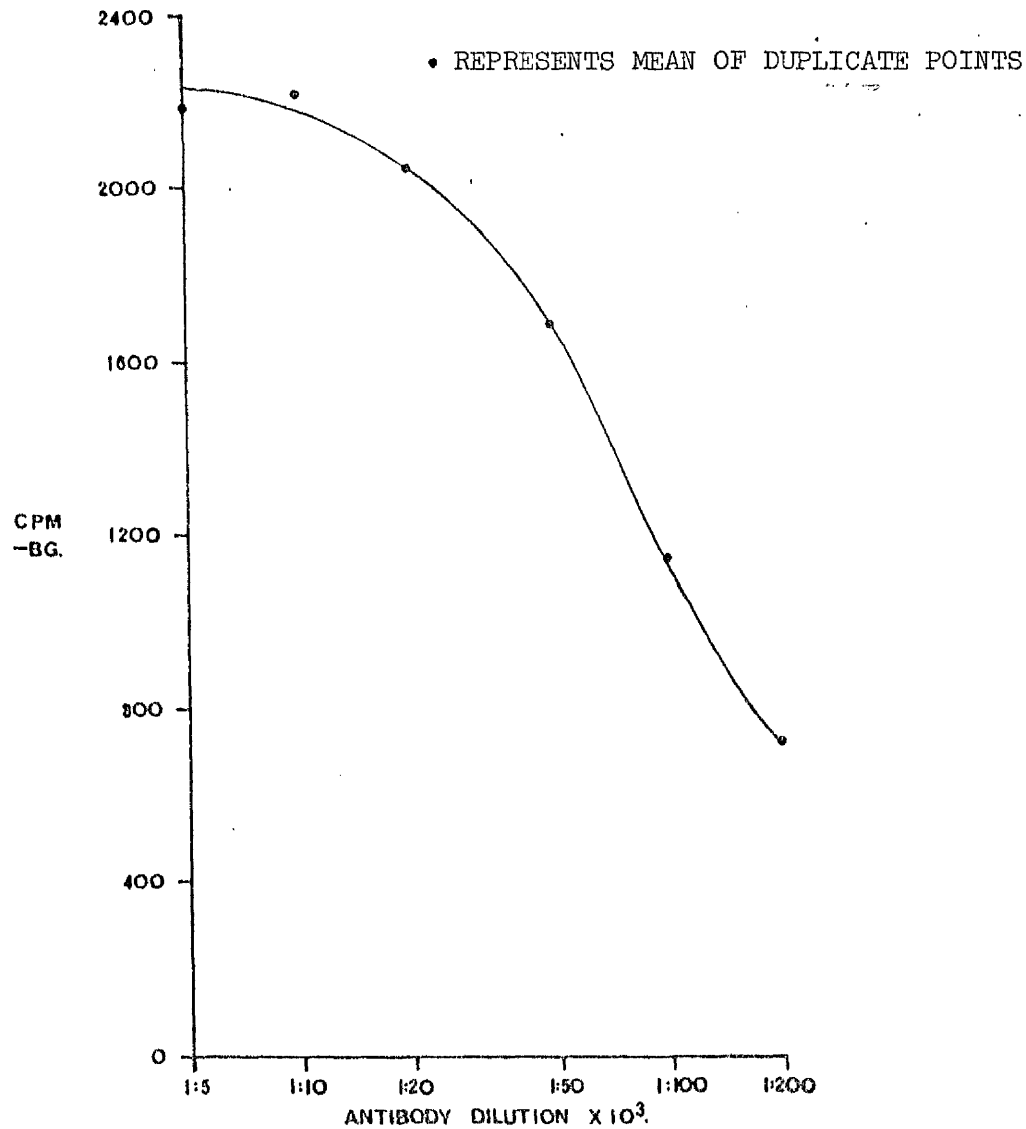


FIG 17 ANTI-TESTOSTERONE SERUM DILUTION CURVE

Results

It can be seen from the results in Fig 18 that maximum displacement of the labelled hormone occurred at an antibody dilution of 1:50,000. This dilution of the antibody contained in a volume of 500 µl was therefore used in the assay.

(b) Standard curve

The displacement of tritiated testosterone from the antibody with unlabelled steroid in amounts ranging from 10 to 1,000 pg was determined as described for the progesterone assay.

Results

It is apparent from the results, presented in Fig 19, that approximately 80% displacement of the labelled steroid occurred over the range 20 - 1,000 pg.

(c) Antibody specificity

The cross reactions of a range of steroids with the antibody were determined as described previously.

Results

The cross reactivities of a range of steroids are given in Table 16. Amongst the steroids tested, the antibody cross reacted strongly with 5α-dihydrotestosterone.

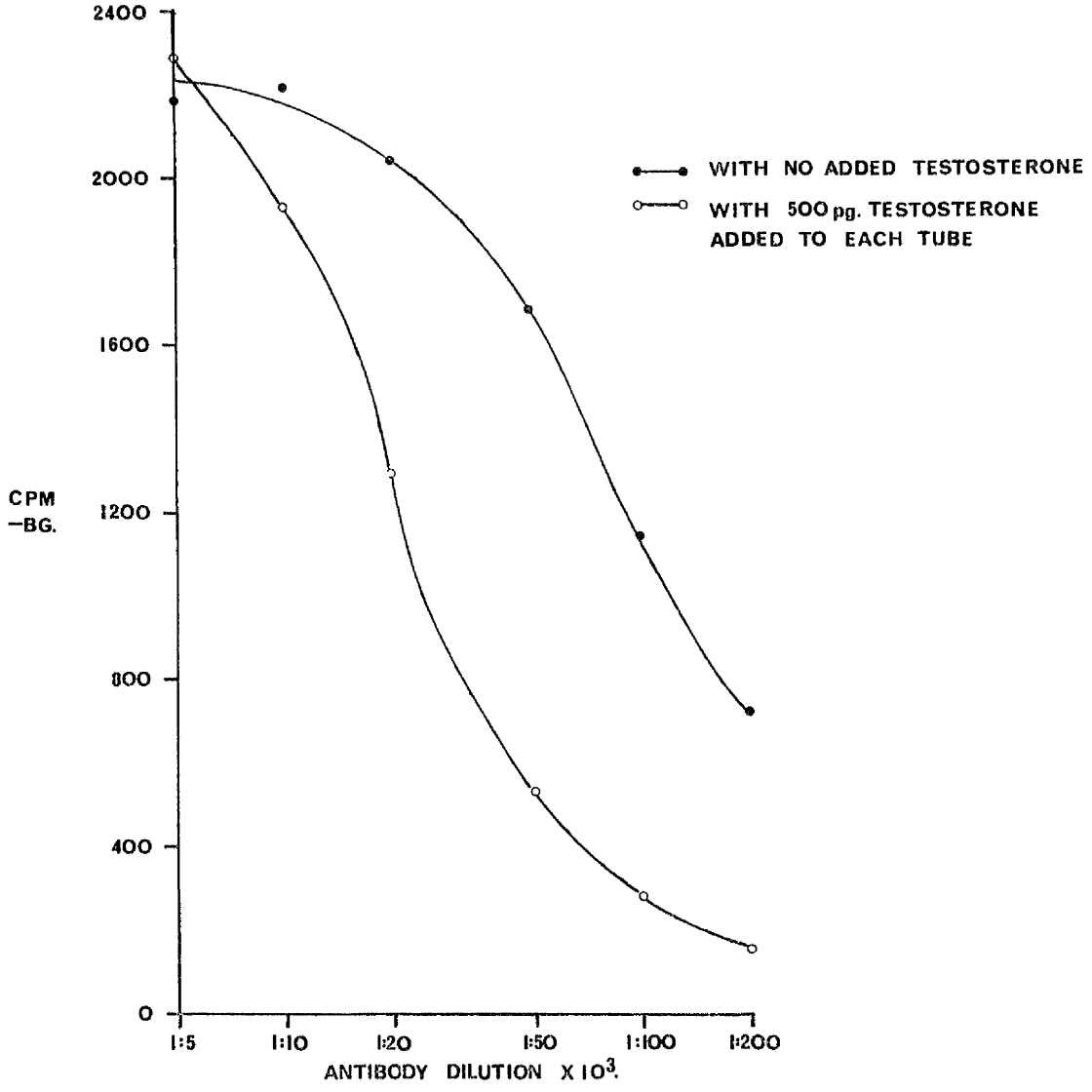


FIG 18 DISPLACEMENT OF TRITIATED TESTOSTERONE FROM VARIOUS ANTIBODY DILUTIONS BY THE PRESENCE OF UNLABELLED TESTOSTERONE

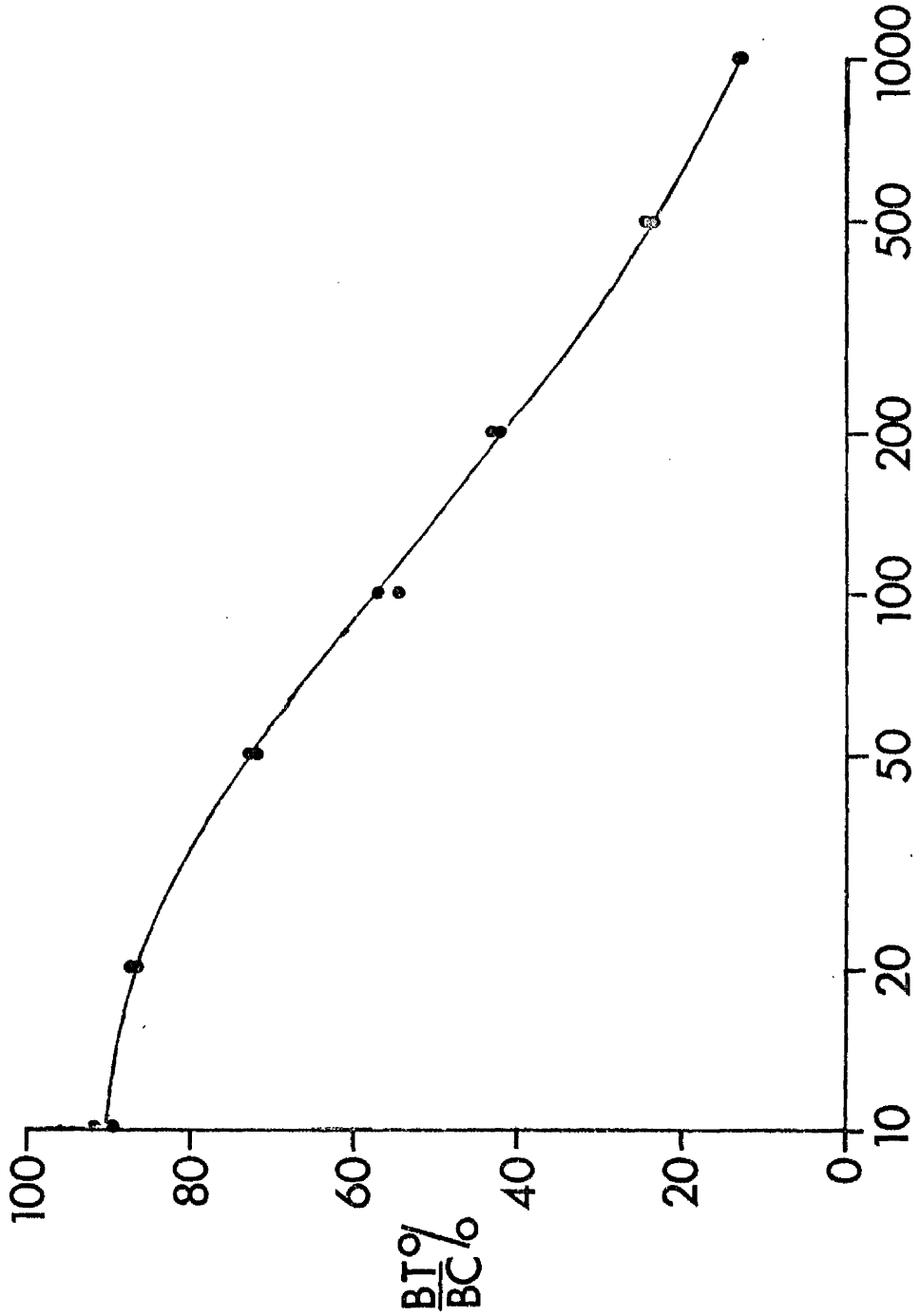


FIG 19 TESTOSTERONE STANDARD CURVE

TABLE 16 : SPECIFICITY OF ANTI-TESTOSTERONE SERUM (Y6/5)

	% cross reactivity
TESTOSTERONE	100
DIHYDROTESTOSTERONE	100
PROGESTERONE	1.6
OESTRADIOL	6.0
OESTRIOL	1.5
OESTRONE	1.4
HYDROCORTISONE	1.6

(d) Extraction of testosterone from plasma

The extraction of testosterone from plasma was determined using the tritiated steroid as described for progesterone, with the following exceptions:

1.0 ml aliquots of plasma were used. Prior to the addition of diethyl ether 100 µl 4% NaOH were added to each tube. 5 ml diethyl ether was used for the extraction.

Results

The recovery of tritiated testosterone is presented for single and duplicate extractions with 5 ml ether in Table 17. As no significant increase in extraction efficiency resulted from the duplicate extraction method a single 5 ml ether extraction was employed in subsequent experiments.

(e) Assay technique

Based on the previous experiments which demonstrated that the antibody had high specificity for androgens and in suitable dilution could be used to measure a wide range of quantities of testosterone, an assay system, similar to those described for progesterone and oestrogens was developed.

Materials

These were as described previously unless otherwise stated.

1. Phosphate buffered saline pH 7.0.

TABLE 17 : EXTRACTION OF TESTOSTERONE FROM PLASMA WITH DIETHYL ETHER AND SODIUM HYDROXIDE

Average Initial c.p.m.	Recovered c.p.m. 1 extraction	Extraction efficiency %	Recovered c.p.m. 2 extractions	Extraction efficiency %
10514	8348	79	8245	78
	7845	75	8008	76
	8192	78	8110	77
	8104	77	8324	82

Average extraction efficiency - single 77%

- double 78%

2. PBS 0.1% gelatin.

3. Testosterone

   10.0, 5.0, 2.0, 1.0, 0.5, 0.2 and 0.1 ng/ml solutions in ethanol were stored at -15°C.

4. (1, 2, 6, 7 (n)-^{3}H) Testosterone

   300 µl testosterone-^{3}H in ethanol (250 µCi in 100 ml) were evaporated to dryness and the residue reconstituted in 15 ml PBS.

5. Anti-testosterone serum

   A 1:50,000 dilution of the antibody was prepared fresh for each assay in PBS 0.1% gelatin.

6. Sodium hydroxide

   40 g/l in glass distilled water.

7. Diethyl ether.

8. Dextran coated charcoal

Charcoal	0.125 g
Dextran T-70	0.0125 g
Water to	100 ml.

9. Blank plasma

   Plasma from an ovariectomised cow was used.

   A flow diagram of the assay protocol is given in Diagram 9.

DIAGRAM 9

Testosterone Radioimmunoassay - Flow Sheet.

(a) Set up the following in extraction tubes

2 -	Ether blanks (BL)	1.0 ml PBS gelatin
3 -	Plasma blanks (BLD)	1.0 ml ovariectomised cow plasma
2 -	Extracted standards S8	50 pg testosterone 1.0 ml blank plasma
2 -	" " S9	100 pg " " " "
2 -	" " S10	200 pg " " " "

Samples (A, B, C, etc.) 1.0 ml plasma

↓

Add 100 µl 4% NaOH to each and leave

30 minutes at room temperature

↓

Add 5.0 ml diethyl ether.

Mix 15 minutes.

Separate phases by centrifugation and freezing.

Pour ether layer to assay tubes and evaporate solvent.

DIAGRAM 9

Testosterone Radioimmunoassay - Flow Sheet (continued)

(b) Assay tubes from (a) + the following assay tubes -

Duplicate unextracted standards (S1 - S7) 10, 20, 50, 100, 200, 500, 1,000 pg testosterone

3 - Bound Count (BC)

2 - Total Count (TC)

↓

Add 500 µl antibody to each tube except TC (500 µl PBS gelatin)

↓

Add 100 µl testosterone H^3 to all tubes. Mix and incubate for 2 hours at $4^{\circ}C$.

↓

Separate antibody bound from free steroid by adding 500 µl dextran charcoal suspension to all except TC tubes (500 µl PBS).

↓

Decant and drain supernatants to counter vials. Add 10 ml scintillator and count for 10 minutes or 10,000 counts.

(f) Reliability of the assay

Specificity

Although the sodium hydroxide-ether system extracts a range of steroids from plasma, the specificity of the antibody used in the assay leads to minimal interference from these other steroids. Although significant cross reactivity was observed with 5β DHT, only small quantities of this androgen have been detected in a range of species studied (Dorfman and Ungar, 1958). Due to cross reaction of the assay system with other androgens the plasma results are expressed as androgens - pg/ml.

Sensitivity

Standard curve

Comparison of the tritiated hormone bound to the antibody in 5 assays showed that a significant difference resulted from the presence of 20 compared to 10 pg testosterone, and between 50 and 20 pg added steroid ($p < 0.01$ in each case). The sensitivity of the standard curve lies therefore at worst between 10 and 20 pg (Table 18).

In plasma samples

The recovery of testosterone (200 pg) in 10 assays was 89.5 ± 8.9% ($\bar{x}$ ± 1 S.D.). The total extraction volume was used in the incubation step therefore $F = 1$. As the blank plasma values consistently fell around the 10 - 20 pg level in accordance with the convention suggested by Abraham (1969), the standard curve sensitivity,

TABLE 18 : ANDROGEN ASSAY. STANDARD CURVE SENSITIVITY.

Testosterone pg.	$\frac{BT}{BC}$ % $\bar{x}$		S.D.	P
5	94.7	±	2.75	
				N.S.
10	92.5	±	3.0	
				< 0.01
20	87.6	±	4.3	
				< 0.01
50	78.3	±	4.22	
				< 0.01
100	62.7	±	5.43	

taken as 15 pg/ml, was used in place of 2 x S.D.

Applying the formula $S = \frac{2 \times S.D}{R \times F} \times 100$ gives an assay sensitivity of 16 pg. As 1.0 ml plasma samples were used in the assay the sensitivity was therefore 16 pg/ml.

Accuracy

The correlation coefficient, determined as described for the progesterone assay, was 0.985 when the amount of hormone estimated was compared with the amount added to ovariectomised cow plasma (Fig 20).

Precision

Using the replicate values of the extracted standards in the assay the precision of the method was determined as described for progesterone.

Results

The results of the precision estimation are presented in Table 19.

2.4.3. Application of the assay - Plasma androgen levels around oestrus in five cows

Animals

Cows A, B, C and D were as described previously. Animal G was a three year old Red Poll. Management, behavioural observations and blood sampling in all animals were as before.

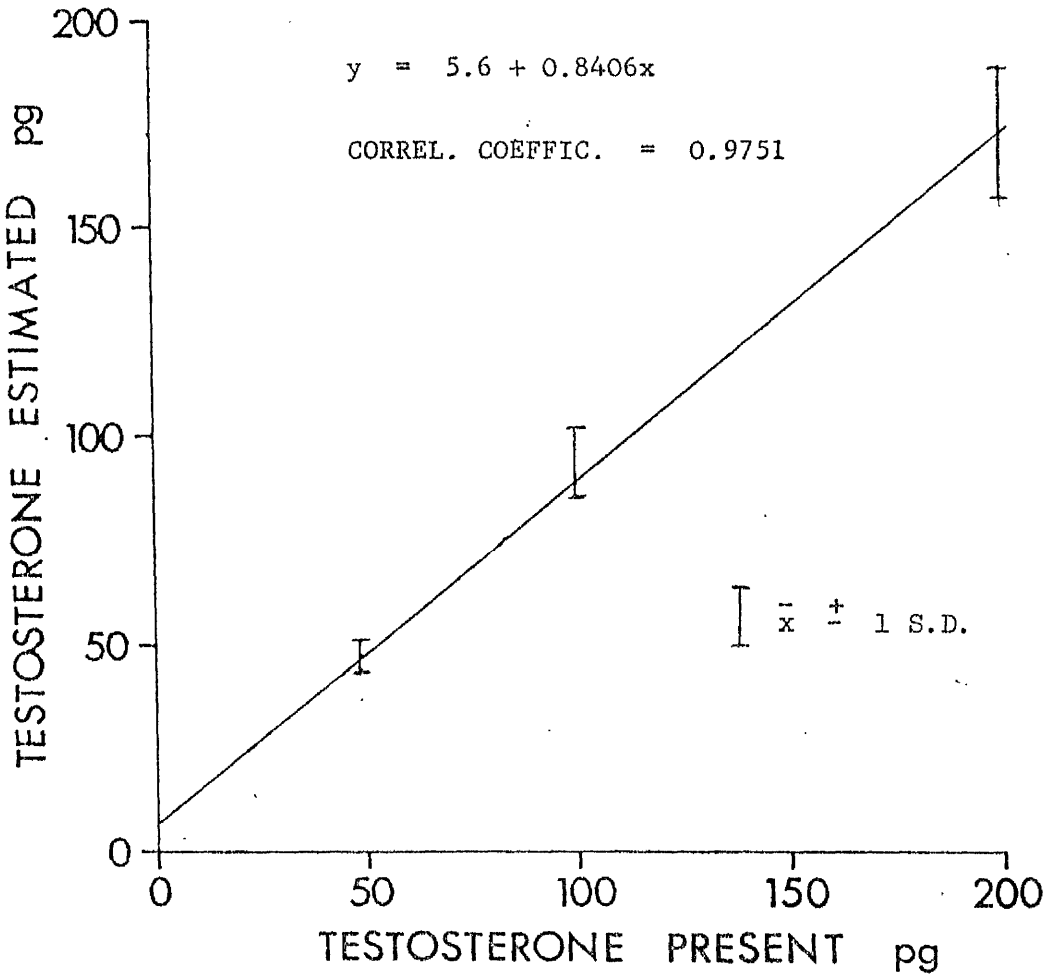


FIG 20 DETERMINATION OF THE ACCURACY OF THE ANDROGEN ASSAY

TABLE 19 ANDROGEN ASSAY. PRECISION

	Extracted standard computed from	
	100 pg	200 pg
$\Sigma(x_1 - x_2)^2$	132	6350
No. duplicate pairs of determinations (n)	8	12
Precision $\sqrt{\frac{\Sigma(x_1 - x_2)^2}{2n}}$	2.87	16.26
Overall Precision	12.73	

Results

The circulating androgen levels from four days before to one day after oestrus are shown for the individual animals in Fig 21. It was apparent from these results that evidence of a peak of androgens around oestrus was found in three cows (A, D, G). No marked elevation similar to that reported for oestrogens was recorded.

2.4.4. Discussion

The assay technique reported in this thesis was simple, and rapid to perform. Although it sacrificed specificity, by omission of a step to separate individual androgens in the plasma extract, the practical advantages obtained by this omission were not felt to prejudice the validity of the method as a means of assessing total androgens in the peripheral plasma of the cow. Unlike the results of determinations of progesterone and oestrogen in plasma samples, which were to be related to and used as a monitor for ovarian structural development, plasma androgen levels were to be determined to provide only preliminary information on the secretion of these steroids in the cow. In spite of the simplicity of the technique described it was comparable in terms of reliability criteria to a range of competitive protein binding assays that have been developed (Nugent and Mayes, 1970).

2.5. Determination of cortisol in the peripheral plasma of the cow

More than 40 steroids have been isolated from human and animal adrenal cortices (Wettstein, 1959). In the cow cortisol and

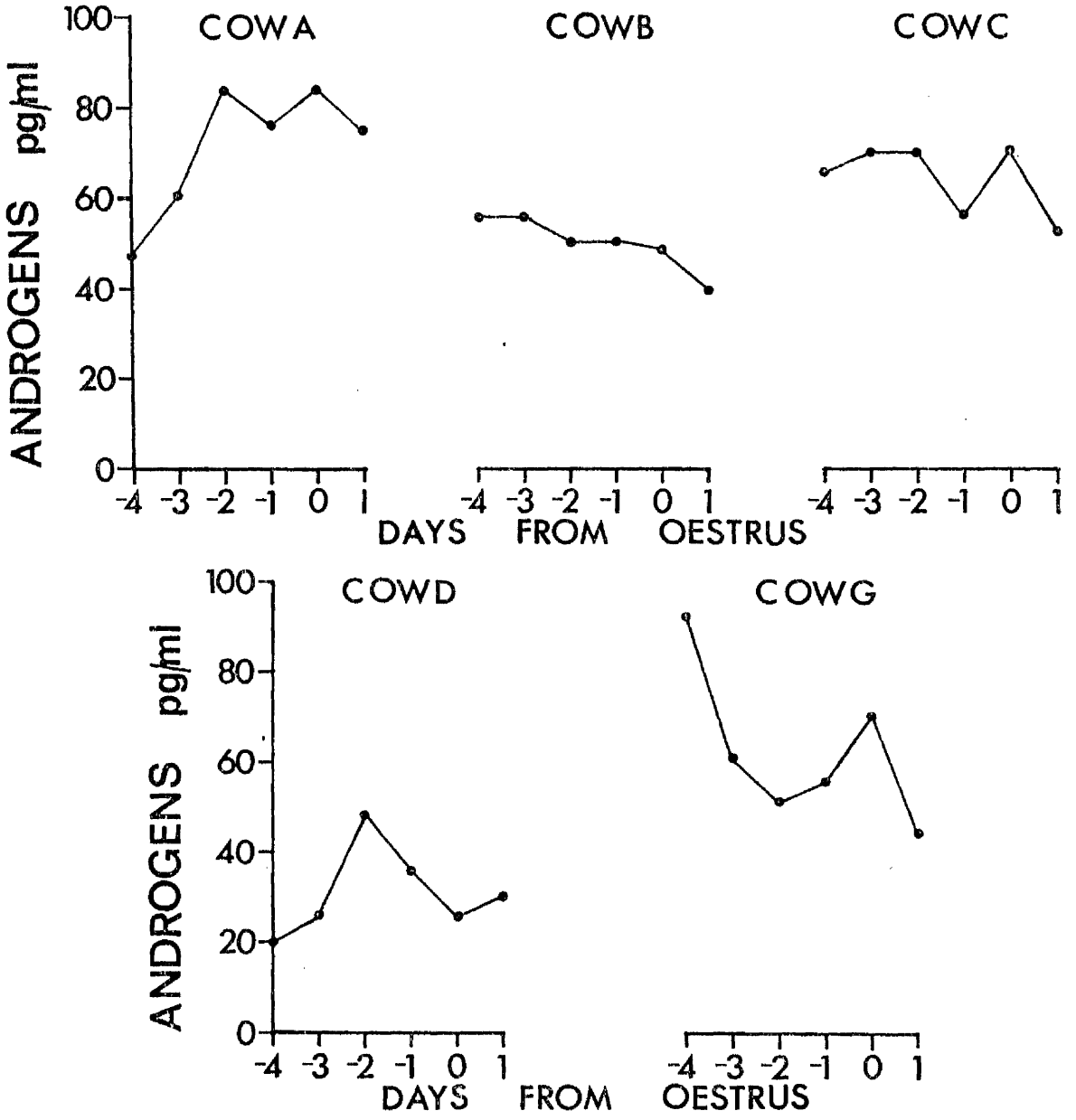


FIG 21 PERIPHERAL PLASMA ANDROGEN LEVELS AROUND OESTRUS IN THE COW

corticosterone have been identified as the major secretory products of the adrenal being present at a ratio of 1:1 in adrenal venous and 2.5:1 in peripheral blood (Bush, 1953; Venkataseshu and Estergreen, 1970). In the plasma corticosteroids are bound to corticosteroid binding globulin (CBG) (Sandberg, Rosenthal, Schneider and Slaunwhite, 1966) and non-specifically to albumin (Westphal, 1961). Very much smaller quantities of these hormones are present in the blood in the free form and can be excreted by the kidney (Peterson and Wyngaarden, 1956; Cope and Black, 1958). Metabolites of cortisol and corticosterone both free and conjugated to glucuronic or sulphuric acid are also present in the circulation prior to their excretion by the bile and urine (Gray, Greenaway and Holness, 1961).

Based on the presence of specific chemical groupings within corticosteroid molecules a variety of assay methods for estimating the levels of these hormones in blood and urine have been developed. (Review by Lorraine and Bell, 1966). Of these the Porter-Silber reaction which involves steroids possessing a dihydroxyacetone side chain (Nelson and Samuels, 1952) has been used in studies on cattle (Robertson and Mixner, 1956; Shaw, Dutta and Nichols, 1960). This method however suffers many drawbacks amongst which are its lack of specificity, its failure to detect corticosterone, and an overall low sensitivity (Marrian, 1955). Many of these disadvantages were overcome with development of a simple reliable technique for estimation of 11-hydroxycorticosteroids in plasma by Mattingly (1962) using a methylene chloride extraction and fluorescence in a sulphuric acid-ethanol mixture to quantitate the hormones.

Although to a certain extent the method of Mattingly (1962) has been superseded by the development of competitive protein binding techniques, using CBG, comparative studies between both methods in the human have revealed a close correlation between the results (Gore and Lester, 1975).

Competitive protein binding techniques have been applied to the estimation of cortisol and corticosterone in cows (Heitzman, Adams and Hunter, 1970; Gwazdauskas, Thatcher and Wilcox, 1972).

In this thesis due to the fact that only a small number of plasma samples were to be examined for the amounts of 11-hydroxy-corticosteroids present the method of Mattingly (1962) was used as this technique was more easily established than CPB methods.

2.5.1. Materials

1. Dichloromethane F.D.P.C. Grade (B.D.H., Poole, England)

2. Fluorescence reagent

   Ethanol (Burrough's) 150 ml

   Sulphuric acid AR (B.D.H., Poole, England) 350 ml.

   At 4°C the sulphuric acid was added dropwise with constant shaking to the ethanol. A fresh solution was prepared monthly.

3. Hydrocortisone (B.D.H.)

   Stock 50 µg/ml in Burrough's ethanol

   Stored at 4°C

   High standard. Prepared by diluting the stock solution 1:100 with distilled water. 500 ng/ml.

Low standard. Prepared by diluting the high standard 1:5 with distilled water. 100 ng/ml.

The high and low standard solutions were prepared for each assay.

4. Control serum

Seronorm (B.D.H.) 11-hydroxycorticosteroid value within the range 130 - 160 ng/ml.

5. Extraction tubes (Quickfit, Jobling, Stone, England) 14/13.

6. Extraction-couvette tubes. Quickfit 14/13 with 10 x 50 mm cylindrical couvettes fused to their bases.

7. Spectrofluorimeter (Aminco Bowman, Silver Spring, Md., U.S.A.) with a xenon lamp. Excitation wavelength 472 mu emission wavelength 525 mu. Sample holder modified to allow combined extraction-couvette tubes to be used.

8. (1, 2, ^{3}H) Cortisol (Radiochemical Centre, Amersham) 250 µCi were dissolved in 100 ml ethanol and stored at -15°C.

9. Scintillator ELS 93 (Koch-Light Laboratories Ltd., Colnbrook, England).

2.5.2. Methods

(a) Extraction of corticosteroids from cow plasma

The extraction of cortisol from cow plasma by dichloromethane was determined by adding the tritiated steroid to plasma and estimating the recovery.

100 µl of 1, 2, ^{3}H-cortisol in ethanol were dried in Quickfit 14/13 tubes. 1.0 ml of ovariectomised cow plasma and 1.0 ml water were added to each tube and after mixing, the tubes left at room temperature for 30 minutes. 10 ml dichloromethane were then added to each tube and the contents mixed by rotating the tubes for 15 minutes. At the end of this period the upper plasma layer was removed by suction and 5.0 ml of the dichloromethane extract transferred to counter vials and evaporated to dryness. 1.0 ml water and 10 ml scintillator were added to each vial and after shaking the c.p.m. determined. Two counter vials in which 100 µl 1, 2, ^{3}H-cortisol had been dried gave, after adding 1.0 ml water and 10 ml scintillator, the amount of tritiated steroid present prior to extraction.

Results

The results of a series of recovery experiments are presented in Table 20. Due to the efficiency of extraction of the tritiated steroid with 10 ml dichloromethane no recovery correction was applied to subsequent experiments.

(b) Determination of the standard curve

Using the stock hydrocortisone solutions in ethanol, a range of concentrations of the hormone from 12.5 - 500 ng/ml were prepared in water. 1 ml of each dilution of hydrocortisone and 1.0 ml water were pipetted to extraction tubes. Two tubes containing 2 ml water served as blanks. Extraction of the tubes was achieved by rotating them end over end for 15 minutes. Following removal of the upper phase (plasma and water), by suction, 5 ml of the dichloromethane

TABLE 20 : EXTRACTION OF CORTISOL FROM PLASMA WITH DICHLOROMETHANE

(1,2 ^{3}H)CORTISOL ADDED c.p.m.	RECOVERED (10 ml extract) c.p.m.	%
51916	44794	86.2
"	44004	84.8
"	44596	85.9
"	45224	87.1
"	44920	86.5
"	45150	87.0
	($\bar{x} \pm$ 1 S.D.)	86.3 $\pm$ 0.8

layer were transferred to extraction couvette tubes. 2.5 ml of the fluorescence reagent were added to each tube and after shaking vigorously for 20 seconds, the % transmission and the photomultiplier meter factor read. The product of the % transmission and the photomultiplier meter factor give the Relative Fluorescent Intensity (RFI). The average RFI values for the water blank tubes were subtracted from the RFI values of all other tubes.

Results

A typical standard curve is shown in Fig 22. A linear relationship between RFI and cortisol concentration was present over the range 12.5 - 500 ng.

(c) Assay technique

The technique used for the determination of cortisol levels in cow plasma samples was, with minor modifications, that of Mattingly (1962). A flow diagram of the assay system is given in Diagram 10.

After determining the RFI of all tubes and correcting these results for the RFI blank value, the cortisol concentration of control and sample tubes was read from the standard curve graph. In the event of the control values agreeing with the actual concentrations (as supplied by the manufacturer), the sample results were accepted. The results were not corrected for recovery.

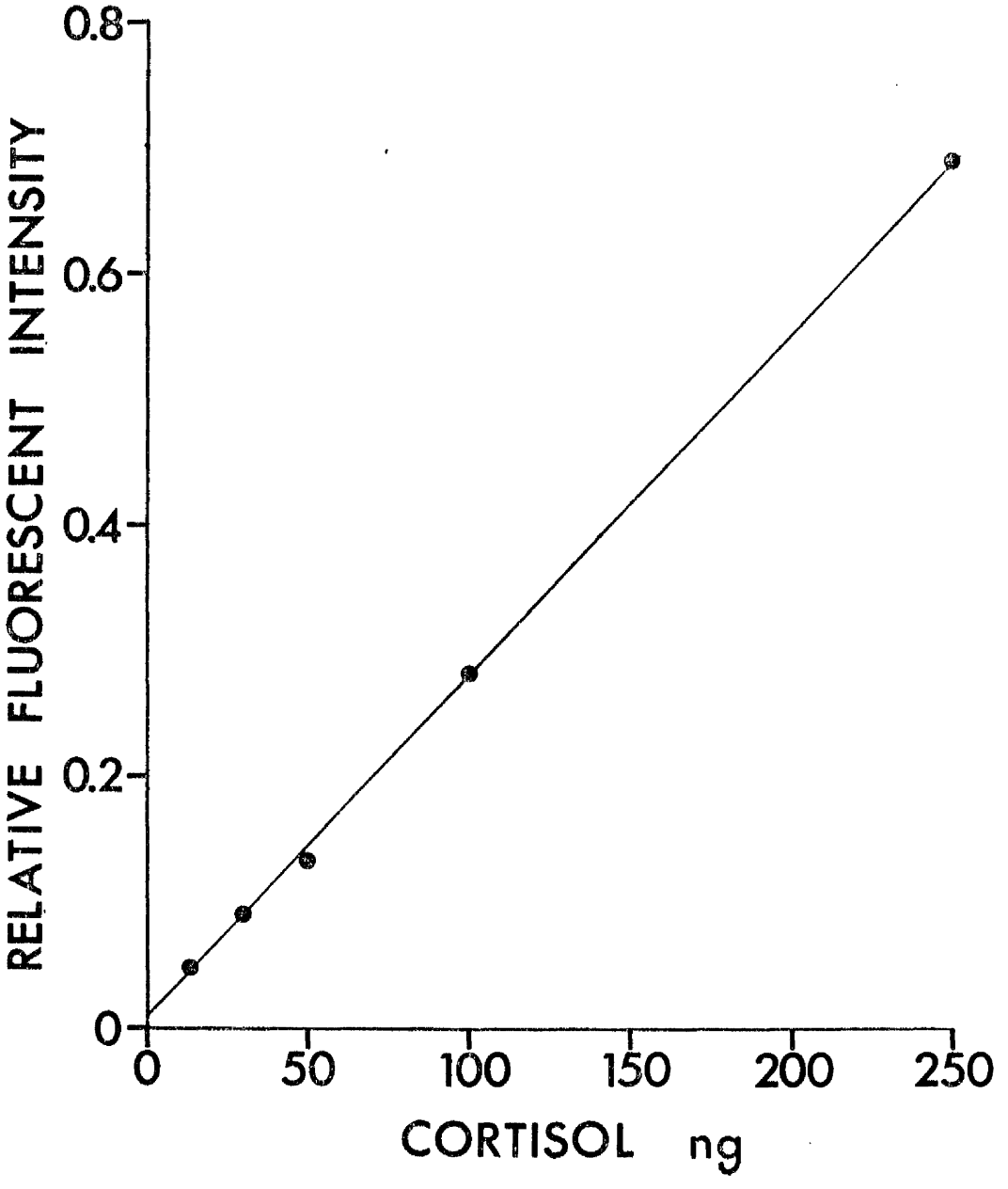


FIG 22 CORTISOL STANDARD CURVE

DIAGRAM 10

Cortisol Estimation - Flow Diagram

Set up the following in extraction tubes:

Tube	Blank	High Standard	Low Standard	Control	Samples
Content	1.0 ml distilled water	1.0 ml 500 ng/ml hydrocortisone	1.0 ml 100 ng/ml hydrocortisone	1.0 ml Seronorm	1.0 ml plasma
No.	2	2	2	2 x 1	(maximum 14)

↓

Add 1.0 ml distilled water to each tube

↓

Add 10 ml dichloromethane

↓

Mix 15 min. Centrifuge 5 min, 1,000 g

↓

Remove and discard upper phase by suction.

Transfer 5.0 ml lower phase to

extraction-couvette tubes

↓

Add 2.5 ml fluorescence reagent.

Shake each tube for 20 seconds.

↓

Determine fluorescence in each tube

exactly 13 minutes after adding

fluorescence reagent.

(d) Reliability of the assay

Specificity

The degree of interference by other steroids when the fluorescence of sulphuric acid-ethanol is used for measurement of 11-hydroxycorticosteroids has been widely investigated (Silber et al., 1958; Mattingly, 1962). Amongst a range of steroids tested, oestrone and oestradiol showed 6 and 23.2% cross-reaction, respectively, compared to cortisol (100%). As in all cases where the technique was applied in this thesis, the level of oestrogens present in plasma was determined, any significant influence of these hormones on the corticosteroid estimations will be apparent.

Sensitivity

The significance of the differences between each point on four standard curves and the sensitivity estimate for the method are presented in Table 21.

Accuracy and precision

The incorporation of duplicate control sera of known cortisol values (Wellcontrol 1 and Seronorm containing 195 and 137 ng/ml respectively) in six assays was used to confirm the accuracy of the method. The mean ± S.D. for each serum were 198 ± 12 and 138 ± 5 ng/ml respectively giving a correlation coefficient of 0.962. Ten

TABLE 21 : CORTISOL ASSAY. SENSITIVITY.

Cortisol ng	No. tubes	RFI* $\bar{x}$	S.D.		P
12.5	4	0.07	0.03		
				———	N.S.
25.0	4	0.07	0.02		
				———	< 0.05
50.0	4	0.17	0.04		
				———	< 0.05
100.0	4	0.27	0.04		

*Corrected for water blank RFI

independent estimates of the apparent level of cortisol in plasma containing 157 ng/ml gave a coefficient of variation for the method of 5% ($\bar{x}$ ± 1 S.D. = 154 ± 7 ng/ml).

2.5.3. Application of the assay - Peripheral plasma cortisol in the cow

The 11-hydroxycorticosteroid level in 8 samples of jugular vein blood from each of 3 adult cows taken at intervals throughout the oestrous cycle was found to be 24.0 ± 5.1; 22.8 ± 7.7, and 22.9 ± 6.5 ng/ml ($\bar{x}$ ± 1 S.D.). These results were similar to the findings of other workers using chemical assay techniques. Paterson (1957) gave values of 17-hydroxycorticosteroids of between 10 and 30 ng/ml, although the technique used was impracticable for studied involving frequent samples as 200 ml aliquots of blood were required. In contrast to studies in the human, competitive protein binding techniques gave lower estimates of cortisol in cows than the values found by the Mattingly (1962) method in this study.

CHAPTER THREE

PARTURITION

3.1. Introduction

Many theories have been advanced to explain why parturition occurs 270 - 296 days after conception in the cow. Both maternal factors, such as age and breed (De Fries, Touchberry and Hays, 1959), and foetal factors, such as the number present, their sex, and genetic constitution (De Fries, 1959; Holm, 1967) have been found to modify the duration of gestation. The role of the foetal hypothalamic-hypophyseal-adrenal system in initiating parturition in the sheep has been established (Bassett and Thorburn, 1969; Liggens, 1972; Nathanielsz, Comline, Silver and Paisey, 1972). Comline, Hall, Lavelle, Nathanielsz and Silver (1974) were unable, in foetal calves, to demonstrate the marked adrenocortical hyperfunction found in late gestation in the lamb. However, in calves prolongation of the period of gestation was found in association with either adrenal abrnomalities (Wilson and Young, 1958; Holm, 1967) or with defects of the hypothalamus-hypophyseal region of the brain (Kennedy, Kendrick and Stormont, 1957). These observations in cattle indirectly suggested a similar involvement of the foetal hypothalamus-pituitary-adrenal system in terminating pregnancy in this species as in the sheep.

Irrespective of the factors initiating parturition, and therefore terminating the period of gestation, expulsion of the foetus demands that various changes occur in what for the previous nine months has been a relatively quiescent uterus with a closed cervix. Uterine contractility is obviously an integral part of foetal expulsion. Allan and Reynolds (1935) demonstrated that uterine

contractions could be stimulated by oxytocin, and that this oxytocic effect could be prevented by the administration of progesterone. Marshall and Moir (1952) suggested that uterine activity therefore reflected the balance between progesterone and the opposing effects of oestrogens and oxytocin. Alteration of this balance by either increasing the amount of oestrogens, or decreasing the amounts of progesterone acting on the myometrium resulted in uterine contractions. Csapo (1956) described hyperpolarisation of the myometrial cells by progesterone, thus explaining uterine quiescence under the influence of this hormone. He related this observation to the process of parturition by proposing that this 'blocking effect' of progesterone must be removed before foetal expulsion could occur. It is apparent, however, from studies carried out into sperm transport at mating, where myometrial activity is directed cranially and is of short duration, that a decrease in the level of progesterone and an associated increase in the amounts of oestrogens acting on the uterus is not necessarily followed by the type of uterine contractions observed at term.

Using bioassay techniques, Gassner (1952) concluded that faecal androgens in the cow increased during pregnancy and declined after calving. The secretion of androgens in late gestation need not necessarily imply that they have a function in relation to the process of birth. However, biological interactions of androgens with progesterone and oestrogens, which are in turn functionally involved in foetal expulsion, have been described (Robson, 1936; Hohn and Robson, 1950; Velardo, Hisaw and Bever, 1956).

This study in cows was designed to investigate the interrelationship of progesterone, oestrogens, androgens and glucocorticoids in the plasma around parturition and to relate these findings to the mechanical process of foetal expulsion.

3.2. Experimental design

Animals

This study was carried out in late May using seven dairy type heifers (Nos. 1, 3, 4, 5, 7, 8, 9) in calf to various unknown sires. As service dates were not available, the cows were selected on the basis of physical changes indicative of late gestation such as abdominal distension, mammary development and slackening of the pelvic ligaments. To provide additional information on hormone levels around parturition, a further 2 similar animals were obtained (Nos. 12 and 13). Studies using these latter 2 cows were carried out in September.

Management

All animals were housed in an open court over the periparturient period. The first group of 7 cows was maintained together as a group, whereas cows 12 and 13 were kept in the company of cycling non-pregnant cows. The animals were removed from the court twice daily for concentrate feeding in an adjoining cowshed. Hay and water were made available ad lib. No attempt was made to isolate calving cows from the remainder of the animals in the group, if calving was observed to be proceeding normally. Only in the event of a cow showing apparent

difficulty in parturition was it removed from the court to an adjoining cowshed where any assistance required could be given. The cows were frequently observed both throughout the day and at least twice during the night, at approximately 23.00 and 5.30, for any signs of imminent calving or other behavioural alterations.

Blood samples

Blood samples were taken at the same time each day from the jugular vein into heparinised evacuated glass tubes (Vacutainer, Beckton and Dickenson). The plasma was separated at $4^{\circ}C$ and stored at $-15^{\circ}C$ till assayed.

Hormone levels

The concentrations of progesterone, oestrogens and androgens in plasma samples were determined by radioimmunoassay. Plasma cortisol levels were estimated by a fluorometric technique. The details of the individual assays used were given in chapter 2.

3.3. Results

Outcome of gestations

The breed of the cow and the outcome of their parturitions are given in Table 22. All animals calved without assistance with the exception of Cow 1. In this animal a dystocia, associated with a posterior presentation of the foetus in the breech position, necessitated correction of the postural defect under epidural anaesthesia and removal of the foetus by traction.

TABLE 22

DETAILS OF OUTCOME OF PARTURITION IN THE COWS

Cow No.	Breed	Calf	Comment
1	Ayrshire	1 dead calf	Dystocia. Manual removal 1 recently dead calf. Fostered on calf that was accepted by dam. Retention placenta and post-partum endometritis. Antibiotic therapy.
3	Ayrshire	1 live calf	Calving normal. Expelled placenta normally.
4	Ayrshire	1 live calf	Calving normal. Expelled placenta normally.
5	Ayrshire	1 live calf	Calving normal. Expelled placenta normally.
7	Shorthorn/ Ayrshire Cross	1 live calf	Calving normal. Expelled placenta normally.
8	Friesian	1 live calf	Calving normal. Retained placenta. No adverse clinical signs noted.
9	Friesian	1 live calf	Calving normal. Expelled placenta normally.
12	Ayrshire	1 live calf	Calving normal. Expelled placenta normally.
13	Ayrshire	1 live calf	Calving normal. Expelled placenta normally.

Plasma progesterone levels

Fig 23 shows the plasma progesterone levels from Cows 1, 3, 4, 5, 7, 8 and 9, within the period from 8 days before to 6 days after calving. It was apparent from these results that pre-partum plasma progesterone levels varied widely among cows but that in all cases a precipitous decline occurred within the last 2 days of pregnancy. This marked decrease in progesterone levels was noted 2 days before birth in 5 of the cows (Nos. 1, 3, 5, 7 and 8) and 1 day pre-partum in the remaining 2 animals (Nos. 4 and 9).

Fig 24 gives details of the progesterone levels in Cows 12 and 13 over the period from 12 days before, to 6 days after, calving. It was apparent that plasma progesterone concentrations remained in excess of 2.0 ng/ml till 1 day before calving (No. 12) or 2 days pre-partum (No. 13) when a decline occurred.

Plasma oestrogen levels

Fig 25 illustrates the levels of plasma oestrogens of Cows 1, 3, 4, 5, 7, 8 and 9. It was apparent that the circulating concentrations of these hormones sharply decreased around calving. Among cows variability in the pre-partum levels and in the timing of the decline, relative to foetal expulsion, was observed. In 5 of the 7 animals plasma oestrogen levels were still markedly elevated on the day of calving. Fig 26 gives the periparturient oestrogen levels of Cows 12 and 13. In the case of Cow 12 maximum oestrogen levels were found between 4 days before birth and term. Although a slight

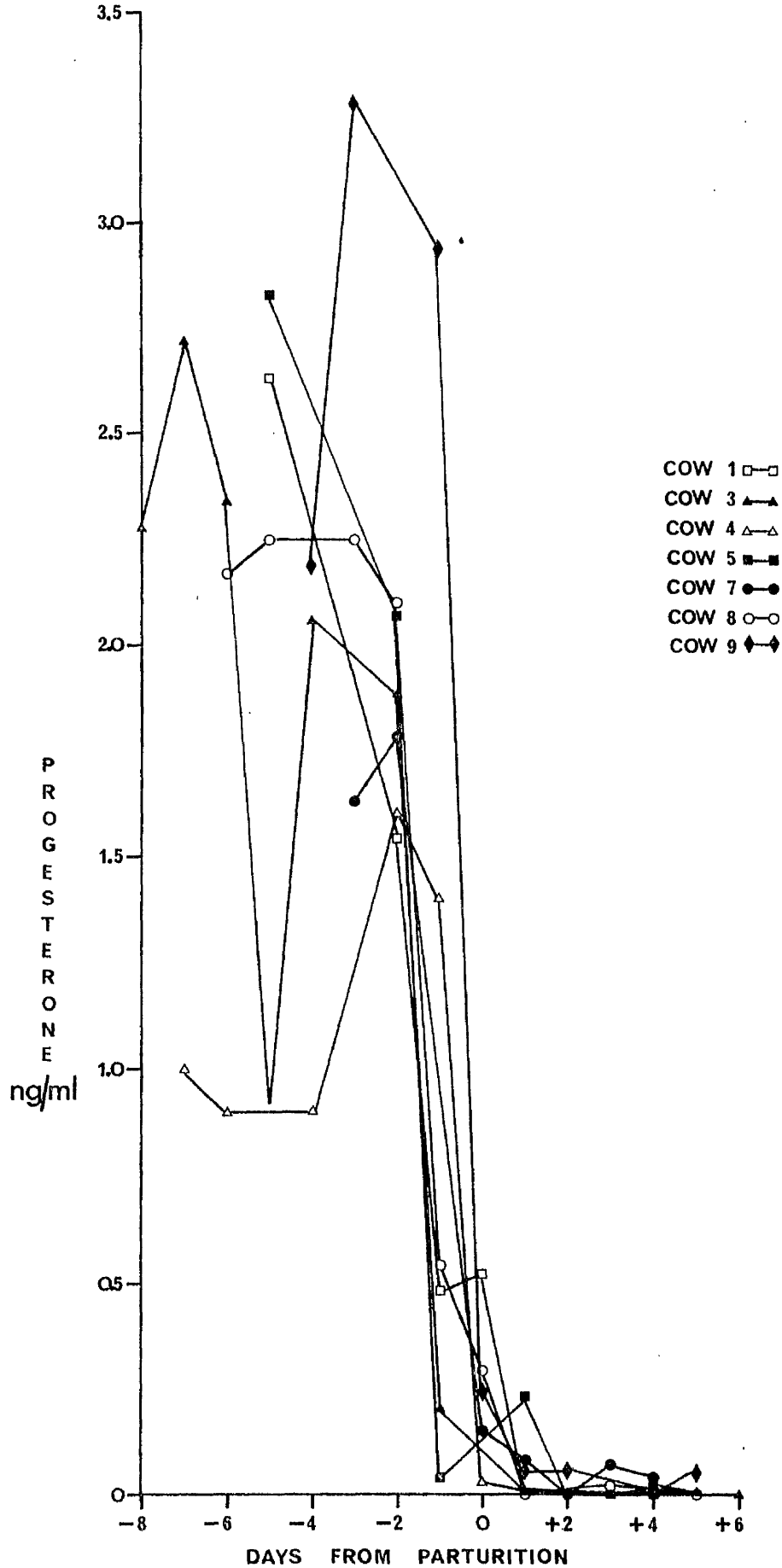


FIG 23 PERIPHERAL PLASMA PROGESTERONE LEVELS AROUND PARTURITION IN SEVEN COWS

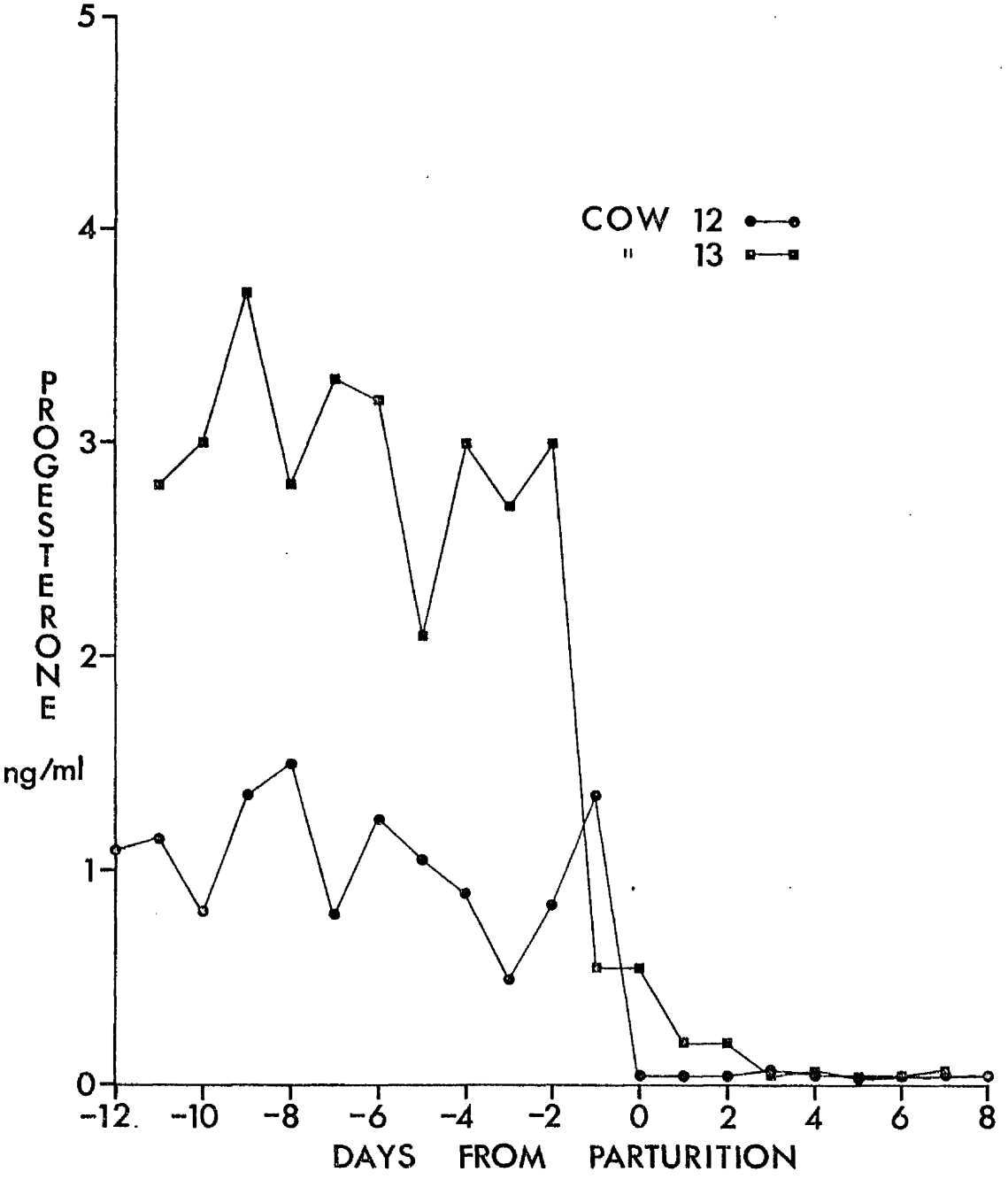


FIG 24 PERIPHERAL PLASMA PROGESTERONE LEVELS AROUND PARTURITION IN COWS 12 AND 13

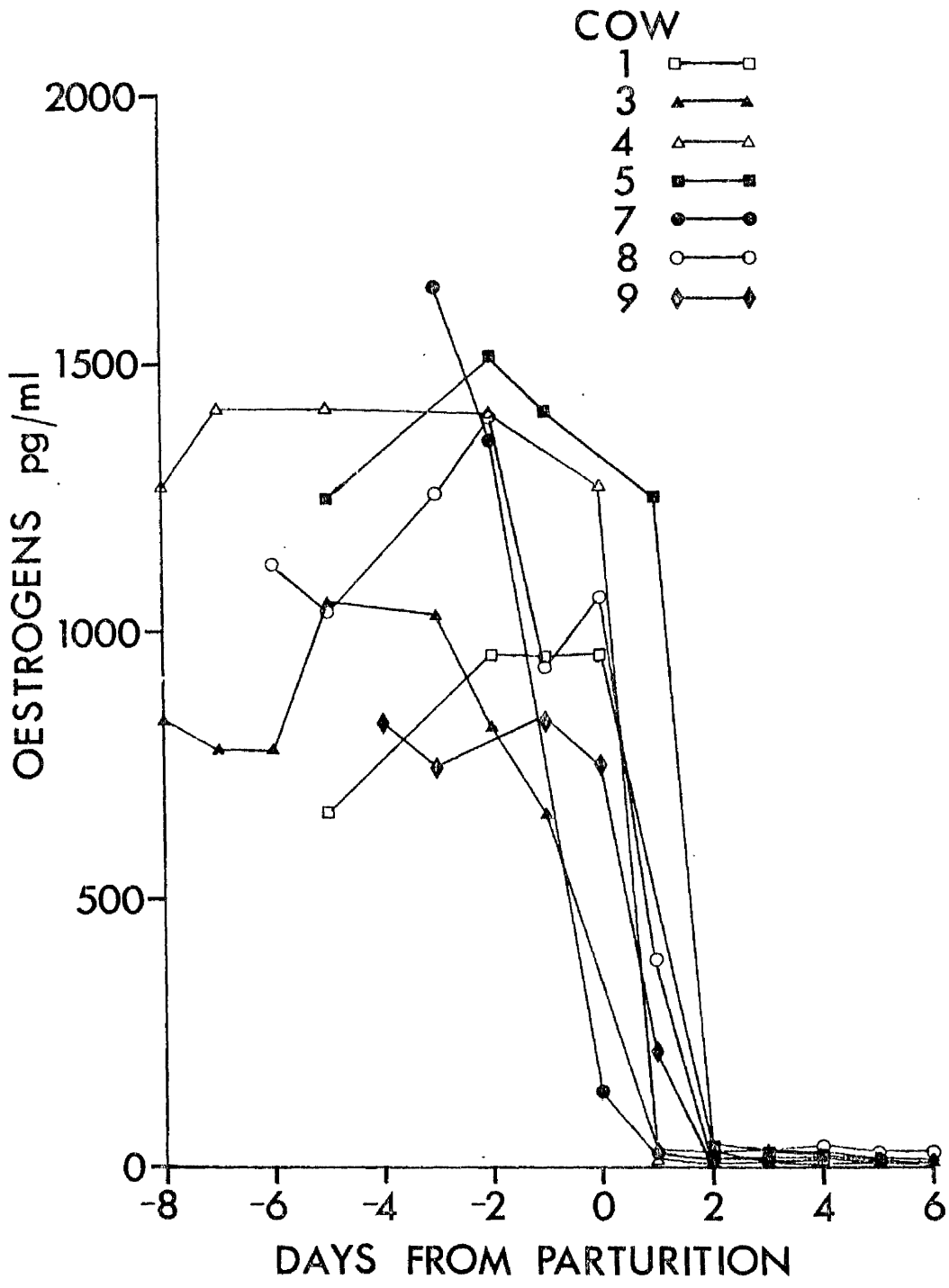


FIG 25 LEVELS OF PERIPHERAL PLASMA TOTAL OESTROGENS AROUND PARTURITION IN SEVEN COWS

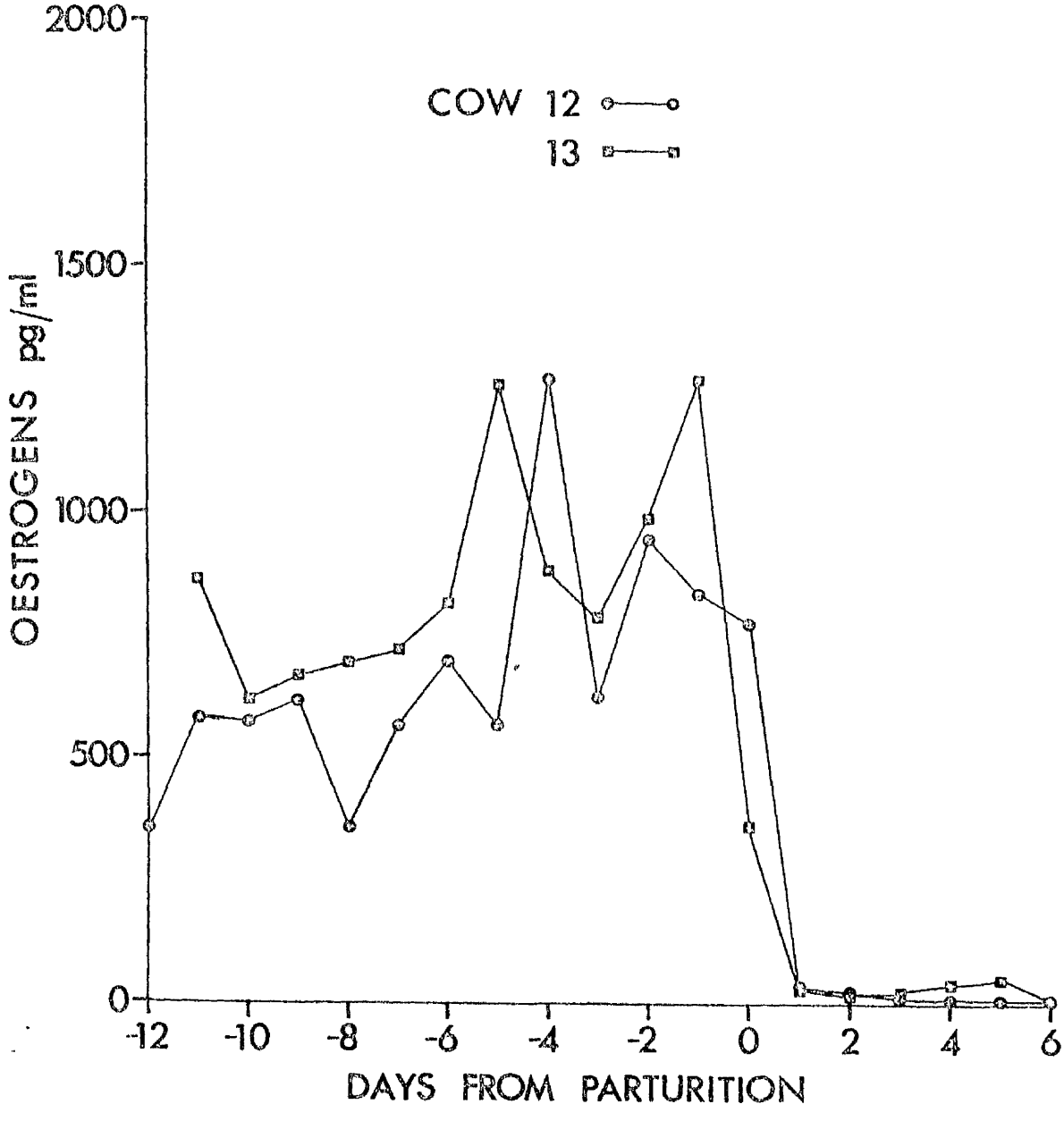


FIG 26 LEVELS OF PERIPHERAL PLASMA OESTROGENS AROUND PARTURITION IN COWS 12 AND 13.

decrease in the level was observed from 2 days before calving, the rate of decline was especially marked after expulsion of the foetus. Similarly in Cow 13, although plasma oestrogen levels fell from 1 day pre-partum, basal concentrations were not reached till the day after calving.

Plasma androgen levels

Fig 27 shows the plasma androgen levels of Cows 1, 3, 4, 5, 7, 8 and 9. The results demonstrated a decline in the circulating concentrations of these hormones over the periparturient period. Fig 28 illustrates the plasma androgen levels of Cows 12 and 13 over the period from 12 days before to 6 days after calving. In Cow 12 the plasma androgen levels fluctuated between 100 and 200 pg/ml during the pre-partum period. Commencing on the day of calving, an overall decrease in the levels was noted. On the other hand Cow 13, after having shown a pattern of overall decline within the period up to parturition, exhibited a temporary increase in circulating androgens on the day of calving, followed by a decline post-partum.

Plasma cortisol levels

Fig 29 gives details of the plasma cortisol levels of Cows 1, 3, 4, 5, 7, 8 and 9. It was apparent that no consistent pattern of change in the levels of these hormones, relative to the time of foetal expulsion, was exhibited.

Fig 30 illustrates the plasma cortisol levels of Cows 12 and 13. Although in the case of Cow 12 maximum concentrations of these

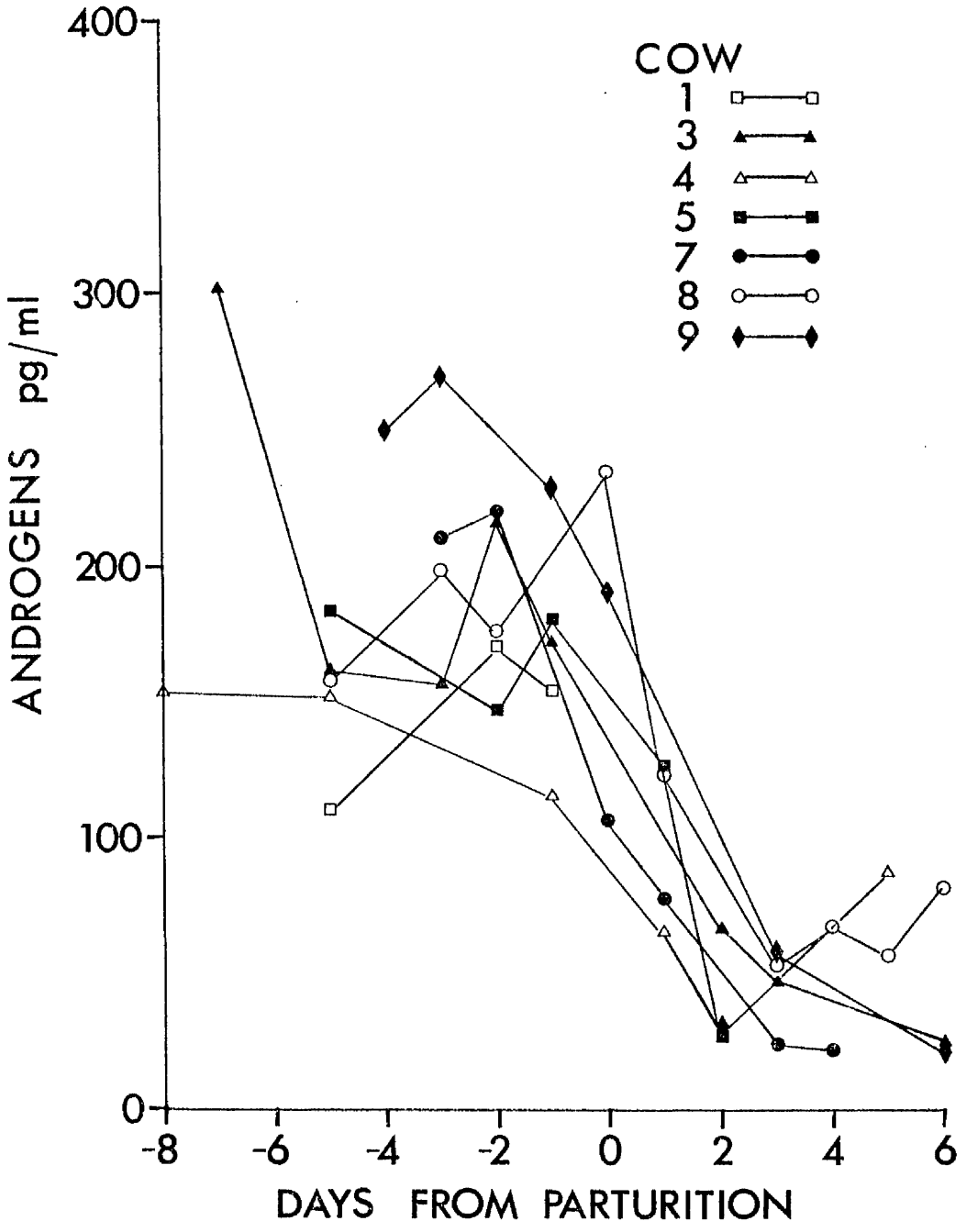


FIG 27 PERIPHERAL PLASMA ANDROGEN LEVELS AROUND PARTURITION IN SEVEN COWS

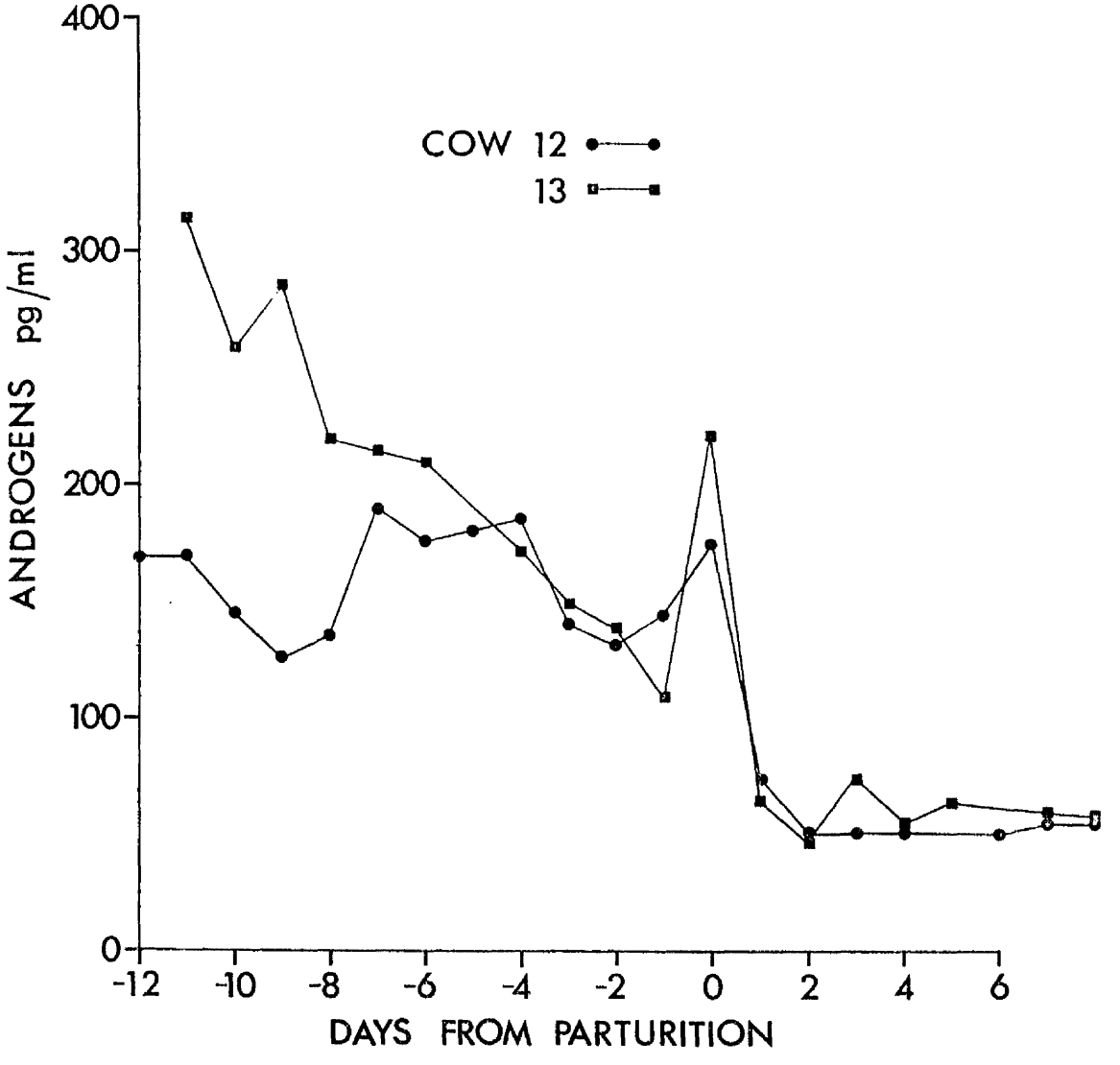


FIG 28 PERIPHERAL PLASMA ANDROGEN LEVELS AROUND PARTURITION IN COWS 12 AND 13

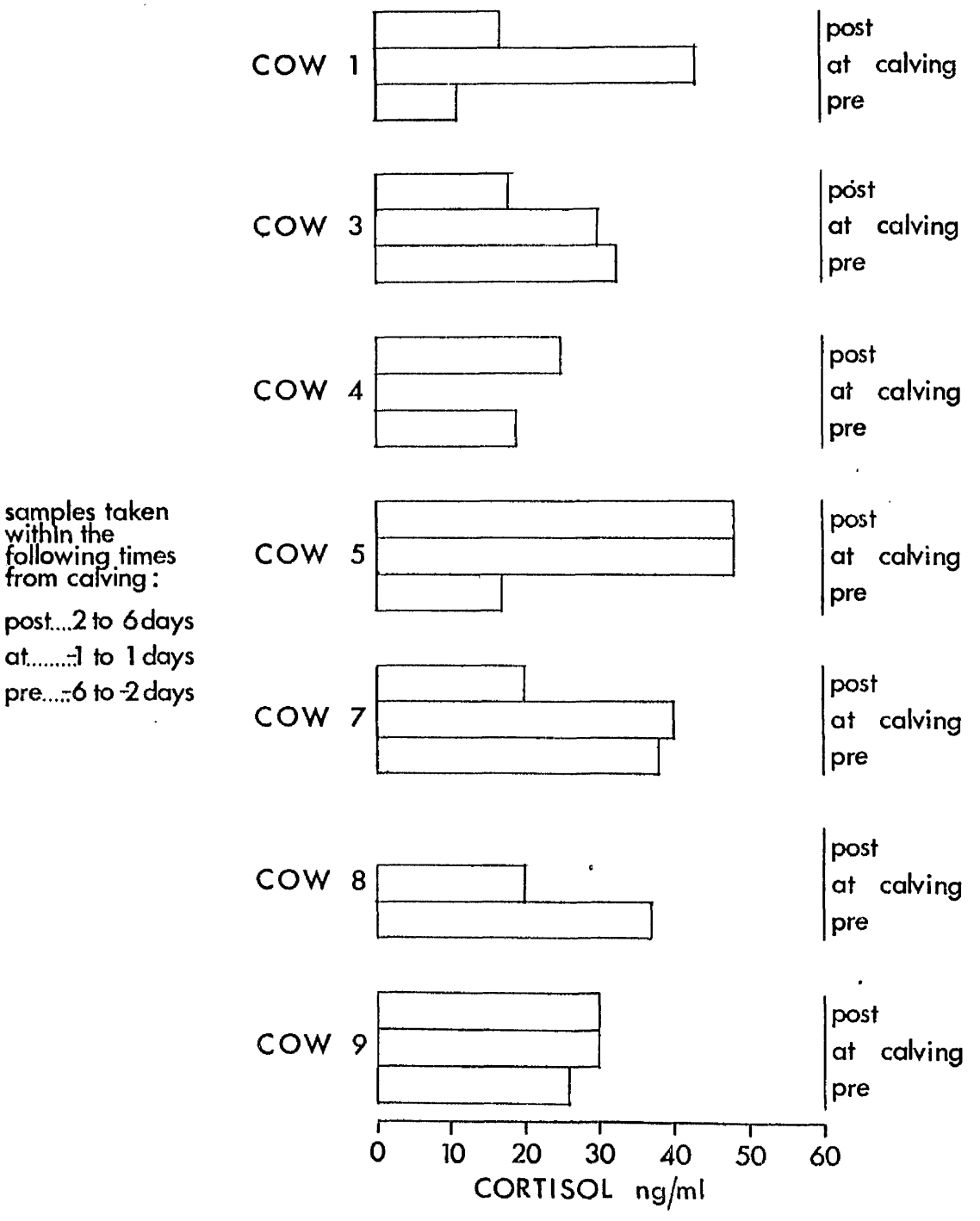


FIG 29 PERIPHERAL PLASMA 11-HYDROXYCORTICOSTEROID (CORTISOL) LEVELS AROUND PARTURITION IN SEVEN COWS

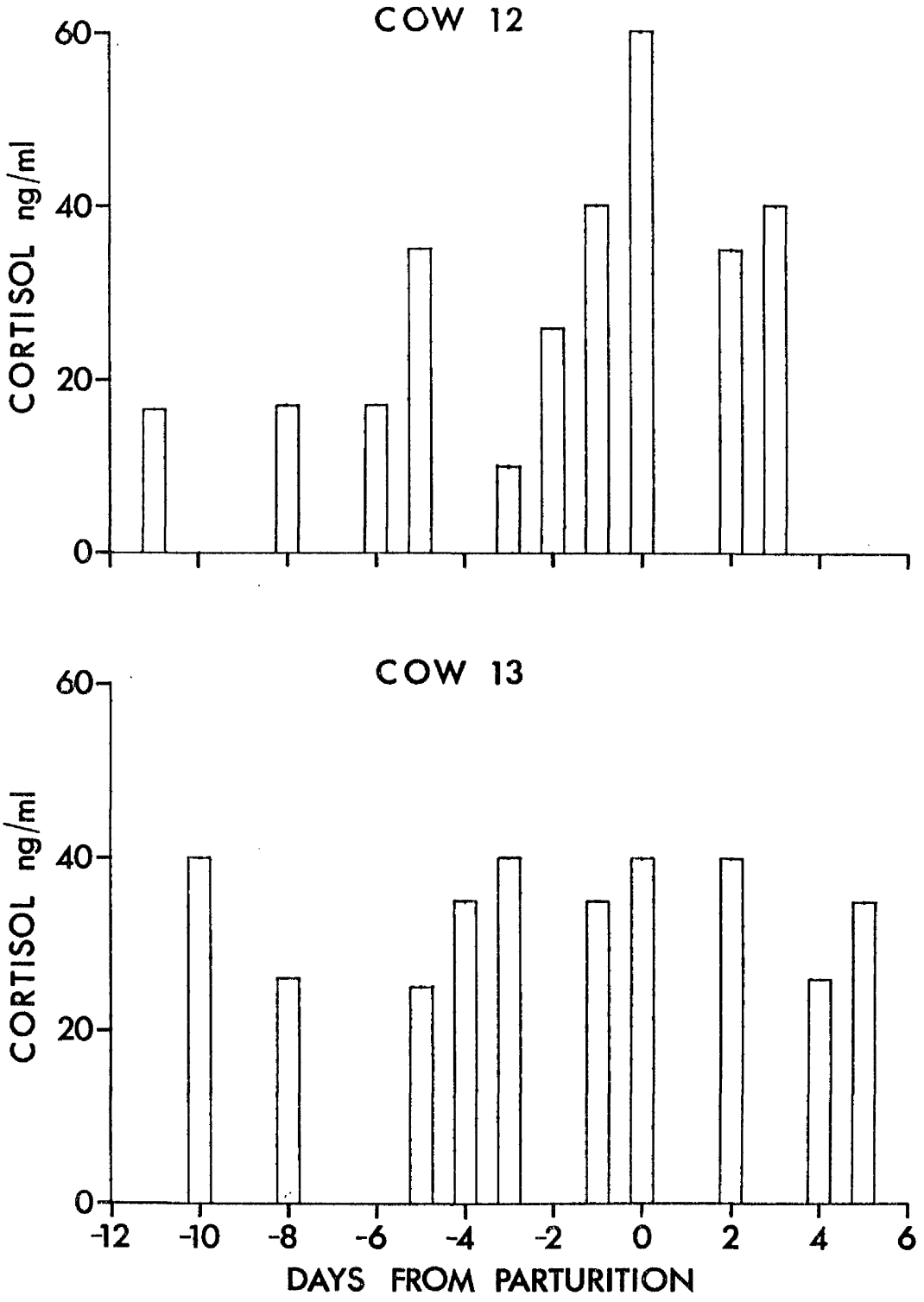


FIG 30 PERIPHERAL PLASMA 11-HYDROXYCORTICOSTEROID (CORTISOL) LEVELS AROUND PARTURITION IN COWS 12 AND 13

hormones were recorded on the day of foetal expulsion, in Cow 13 the cortisol levels were as great both pre- and post-partum as those on the day of calving.

3.4. Discussion

Numerous workers including Short (1960), McCracken (1963), Pope, Gupta and Munro (1969) and Stabenfeldt, Osburn and Ewing (1970) have reported that progesterone is secreted in significant amounts during late pregnancy in the cow and that the concentration in plasma declines some time within the last 34 days of gestation. McDonald, Nichols and McNutt (1959) showed that in the cow removal of the corpus luteum from day 160 of gestation did not interfere with the course of pregnancy. In this species the corpus luteum has been shown to persist in late gestation and to contain appreciable quantities of progesterone (Melampy, Hearn and Rakes, 1959; Labhsetwar, Tyler, Collins and Casida, 1964). In contrast both adrenal and placental tissue were found to contain relatively insignificant quantities of progesterone at this time (Labhsetwar et al., 1964). It can therefore be deduced that the peripheral plasma progesterone levels recorded within the last 2 weeks of pregnancy in the cows in this thesis represented almost exclusively the secretions of corpora lutea.

Due to the dominant effect of progesterone in maintaining quiescence of the myometrium, uterine contractions to bring about parturition were liable to be prevented till such time as the circulating progesterone level was reduced or its effect overcome.

It has previously been proposed that due to an inherent ageing of the corpus luteum in late gestation the amount of progesterone secreted declines and this is associated with a removal of the progestational block to myometrial contractility (Labhsetwar et al., 1964). In the animals studied in this thesis, it was apparent that when the average daily levels of progesterone were considered, no significant pattern of decline could be seen between 8 days and 2 days before parturition (Fig 31). In all cases, irrespective of the progesterone levels within the preceding 6 days, foetal expulsion did not take place till a dramatic reduction to levels of 0.5 ng/ml or less occurred. These results were taken to indicate that the act of foetal expulsion necessitated a mechanism to reduce rapidly the amount of circulating progesterone. A similar late decline in plasma progesterone levels in the pregnant cow has been recorded by Henricks et al. (1972). The results in this thesis supplement their observations and demonstrated that a variable period of 1 - 2 days elapsed between a decline in progesterone levels and foetal expulsion. Luteal function is terminated at the end of dioestrus in the cycling cow by the intervention of a uterine luteolytic agent (Rowson et al., 1972). Examination of the rate of decrease in plasma progesterone levels in animals at the end of dioestrus with the marked reduction in progesterone levels within 1 to 2 days of birth suggested that the same lytic mechanism may operate (Fig 32). In animals where gestation was abnormally prolonged due to defects of the hypothalamo-hypophyseal-adrenal axis parturition occurred at a variable time after the normal period of pregnancy. It may be that in these animals the luteolytic mechanism did not operate due to its requiring a normal foetus to

FIG 31 AVERAGE PERIPHERAL PLASMA LEVELS OF PROGESTERONE, TOTAL OESTROGENS, ANDROGENS, AND CORTISOL OVER THE PERIPARTURIANT PERIOD IN NINE COWS

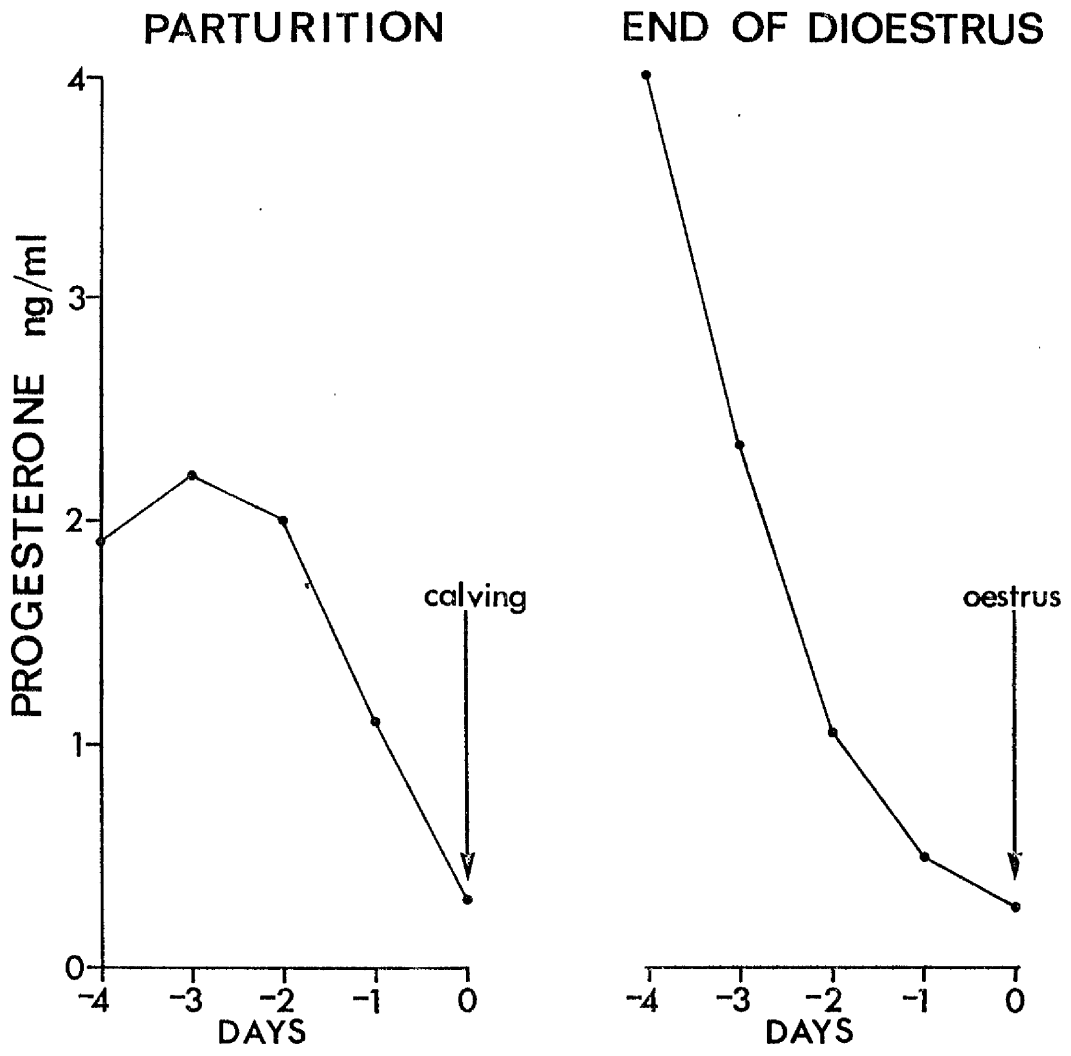


FIG 32 DECLINE IN PLASMA PROGESTERONE LEVELS AT PARTURITION, AND AT THE END OF DIOESTRUS IN CYCLING COWS

trigger its release, and that ultimate foetal expulsion represented the variable, more prolonged period for luteal function to be sufficiently reduced due solely to an inherent ageing process.

It was apparent from the results of the animals in this thesis that although the average peripheral plasma oestrogen levels in the periparturient period followed a similar pattern to that observed for progesterone, i.e. being high pre-partum and declining markedly around the time of foetal expulsion, the timing of the precipitous decline differed (Fig 31). On the day of calving, the plasma oestrogen levels, in contrast to those of progesterone, were still elevated in the majority of cows. This was reflected in an alteration in the progesterone:oestrogen ratio, in favour of oestrogens, commencing two days pre-partum with the most marked change on the day of foetal expulsion (Fig 33). Just as variability was noted in the timing of the progesterone decline before calving, so also was variation shown by individual cows in the time their oestrogen levels fell. In some animals a decline prior to the day of foetal expulsion was observed although even in these cows the levels were still high on the day of parturition (Cows 3, 7 and 13). Within individual animals there appeared to be no relationship between the day progesterone decreased and the time the precipitous decline in oestrogens occurred e.g. in Cows 8 and 9 plasma progesterone declined from 2 days and 1 day before parturition respectively, whereas in both cases plasma oestrogens fell from the day of calving. These results suggested that progesterone and oestrogens are secreted independently during late gestation and that differing mechanisms are responsible for the alterations in their

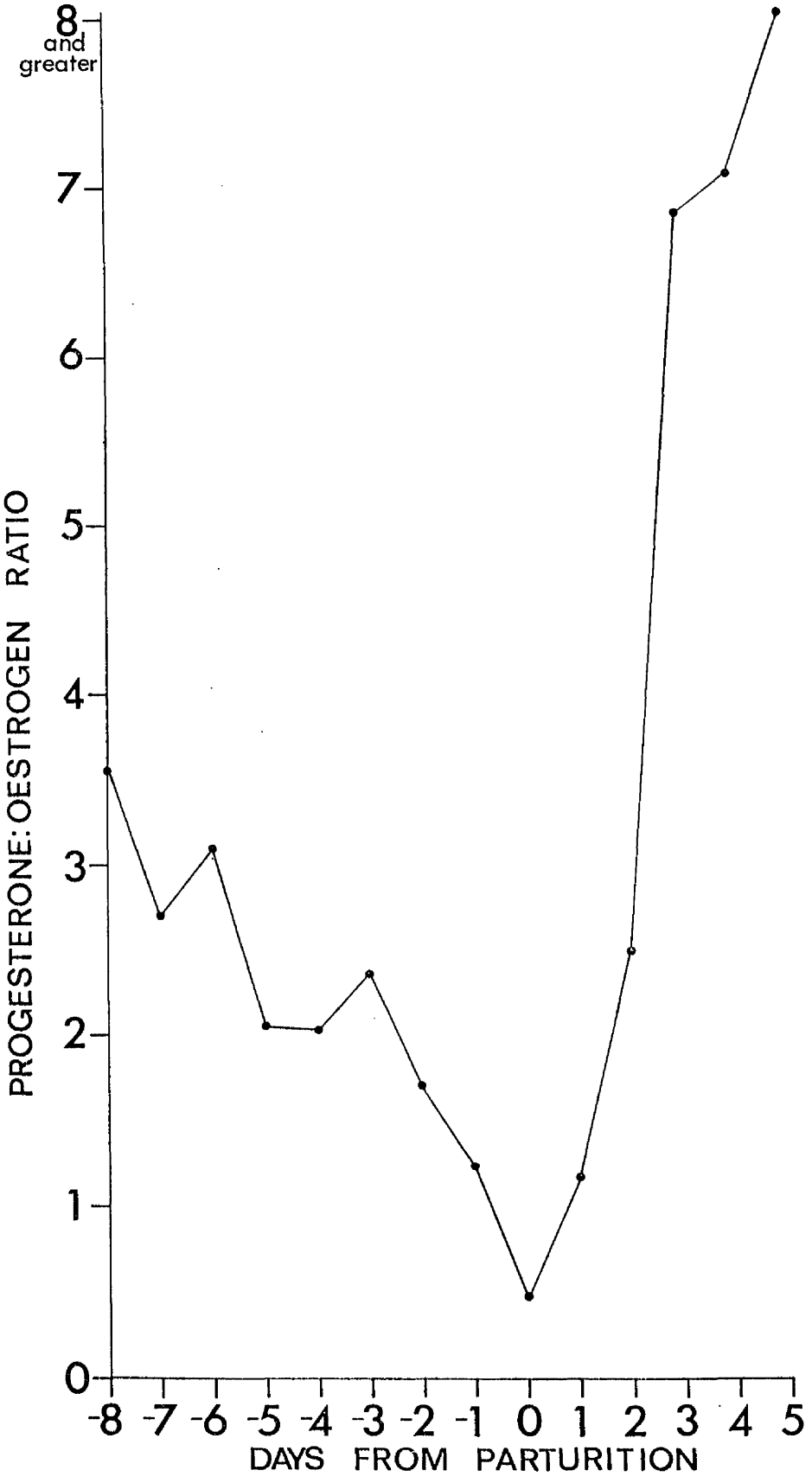


FIG 33 AVERAGE PROGESTERONE:OESTROGEN RATIOS OVER THE PERIPARTURIENT PERIOD IN NINE COWS

circulating concentrations around term. Both the adrenal and the placenta of the cow have been shown to contain oestrogens though much greater concentrations have been recorded in the latter tissue (Meyer, 1955; Gorski and Erb, 1959). Veenhuizen, Erb and Gorski (1960), by studies of tissue hormone levels, concluded that the foetal cotyledons are the major source of oestrogens during pregnancy in the cow. It has previously been suggested that the decline in progesterone levels at term represents the intervention of a luteolytic agent. During the oestrous cycle in the cow prostaglandin F2α has been tentatively identified as the luteolytic agent (Louis, Hafs and Seguin, 1973). In species where significant quantities of progesterone are secreted in late gestation by the placenta - such as the sheep (Stabenfeldt, Drost and Franti, 1972) - a rapid increase in plasma oestrogen levels occurred a few days before parturition (Challis, 1971). In the human where progesterone is produced by the placenta, infusion of this tissue in vivo with prostaglandins lead to increased oestrogen secretion (Alsat and Cedard, 1973). From the results in this thesis, it was apparent that in the cow within the 1 - 2 day period pre-partum when progesterone levels markedly declined, no associated dramatic increase in the already elevated oestrogen levels was found (Fig 31). It, therefore, appears that although prostaglandins may be involved in triggering the alteration in progesterone:oestrogen ratio in both sheep and cattle, the mechanism of producing this change, in favour of oestrogens, differs and that it is the decline in plasma progesterone that is of prime importance in determining the onset of the process of foetal expulsion in the cow. This suggestion is, however, not compatible with the observation by Henricks et al. (1972) that in normal

cows with gestation lengths longer than average progesterone declined at the same time in both these long and average length pregnancies. In contrast oestrogens continued to rise in both groups until the day of parturition. These authors, therefore, concluded that the trigger to myometrial contractility was the level of oestrogens rather than the absence of significant amounts of progesterone. Obviously, due to this discrepancy between the observations of Henricks et al. (1972) and the results in this thesis, additional studies into the role of oestrogens in relation to foetal expulsion are required. The suggested importance in this thesis of the decline in progesterone to the onset of labour is not intended to imply that the elevated oestrogen levels present around term are not functionally involved in the process of birth. Administration of glucocorticoids to cows 260 days pregnant, or more, leads to expulsion of a viable foetus. However, prior to day 260 injection of these steroids frequently results in foetal death (Edqvist et al., 1972). It may be that the effectiveness of glucocorticoids in inducing expulsion of a viable foetus depends, at least partly, on the elevated levels of oestrogens present after day 260 of gestation.

An overall alteration in the progesterone:oestrogen (P:E) ratio, in favour of oestrogens occurs at the time of oestrus in the cycling cow. Comparison of the P:E ratios at the time of parturition with those found around oestrus revealed a marked difference at the two stages (Table 23). It may be that the very much lower ratio at the time of calving was associated with the fact that myometrial contractions differ at oestrus and at term. The higher ratio may be

TABLE 23 COMPARISON OF THE PROGESTERONE:OESTROGEN RATIOS AROUND PARTURITION WITH THOSE AROUND OESTRUS

DAYS FROM OESTRUS OR PARTURITION	P:E*	
	PARTURITION	OESTRUS
-5	2.05	1032
-4	2.03	845
-3	2.36	442
-2	1.7	172
-1	1.24	62
0	0.47	28
1	1.18	56
2	2.48	116
3	6.83	131
4	7.03	330

*calculated as $\frac{\text{pg/ml progesterone}}{\text{pg/ml oestrogens}}$ from the average results of the cows studied in section 2 and section 3.

partly responsible for the fact that at parturition uterine contractions are more prolonged and of greater magnitude.

The available evidence implicating the foetal placenta as the source of oestrogens in late gestation in the cow was of particular relevance to two of the animals in this study that retained their placenta - Cows 1 and 8. Amongst the many factors that have been considered in relation to the aetiology of this condition, hormonal abnormalities have been suggested as a possible cause (McDonald, McNutt and Nichols, 1954). It was apparent from the results of the individual animals in this thesis that no difference existed in either the levels or time of decline of progesterone and oestrogens in these two animals compared to the remainder that expelled their placentae normally. These animals, therefore, do not substantiate the hypothesis of McDonald et al. (1954) that retention of the placenta is associated with a relative insufficiency of progesterone in late gestation. Neither are they in agreement with the observation by Agathe and Kolm (1975) that oestrogens fell at a more gradual rate in animals that retained their placentae. The results in this thesis indicated that expulsion of the foetus in the cow terminates placental function irrespective of whether or not this tissue remained in utero. The variable findings of the studies referred to above and those in this thesis reflect the fact that the condition of retention of the placenta may in some cases, but not in others, be associated with endocrine abnormalities.

Plasma androgen levels in the cow in relation to parturition have not been recorded previously. The results in this thesis

indicated that the levels of these hormones followed a similar sequence to that observed for progesterone and oestrogens - being high pre-partum and sharply decreasing around the time of expulsion of the foetus. In contrast to progesterone, in the majority of animals the levels of androgens were still elevated at the time of calving. It was also apparent that although the average level of plasma oestrogens declined slightly from one day before parturition, the average level of androgens at this time did not fall. However, both androgens and oestrogens exhibited a marked decrease between calving and one day post-partum (Fig 31). These results suggested that androgens, as with oestrogens, are derived from an alternative tissue source than progesterone. Riley and Hammond (1942), using bioassay techniques, detected androgenic activity in the faeces of pregnant cows. Further work by Gassner (1952) showed that faecal androgens increased in late gestation but declined markedly after calving. Gassner (1952) considered that faecal androgens were elevated when a corpus luteum was present in the ovaries - a concept elaborated by Miller and Turner (1955), who postulated that these compounds were derived from extra-glandular conversion of progesterone. The results in this thesis demonstrating a continued elevation of androgens in the absence of high levels of plasma progesterone suggested that these earlier deductions were incorrect. The similarity in pattern of plasma oestrogens and androgens in this thesis suggested the possibility that both these hormones are secreted by placental tissue. Androgens have been detected in small amounts in the adrenal of the cow (Garn, 1949; Short, 1960). It may be that the differences observed between the pattern of androgens and oestrogens before calving in some cows

such as No. 13, reflect the fact that the adrenal contributes to the total plasma androgen levels. It was apparent from the average plasma cortisol levels (Fig 31) that a transient increase in adrenocortical activity occurred on the day of calving. This temporary increase in adrenocortical function may have been associated with a transient increase in androgen secretion which gave rise to the observation that plasma androgens remained elevated when oestrogens started to decline. Additional evidence that tissue other than the placenta contributed to the level of plasma androgens was based on the observation that following expulsion of the foetus and the placenta, the level of androgens remained elevated relative to the amount of oestrogens present in the blood. The effect of androgens on the activity of the myometrium is not known. It may be that these hormones are not actively involved in the process of foetal expulsion but are simply present as by-products of the activity of steroid producing tissues. However, one of the surprising features of parturition in the cow is that no oestrous behaviour is seen around the time of birth. In cycling cows a decrease in progesterone levels in the blood, along with an elevation in the concentration of oestrogens, is found at the time of oestrus (See Chapter Two). It may be that the elevated levels of androgens in the pre-partum cow where progesterone levels were low and plasma oestrogens were elevated were involved in blocking the oestrous inducing effect of the female sex hormones.

Recent studies have suggested that removal of the progestational block at the myometrium in the terminal phase of gestation in the cow may reflect, at least in part, the activation of placental enzyme systems as a result of increased foetal adrenal steroidogenesis. In the sheep, where progesterone is synthesised by the placenta, an increase in 17α-hydroxylase activity has been noted in placental homogenates obtained from animals following pretreatment of the foetus with glucocorticoids (Anderson, Flint and Turnbull, 1974). It has been proposed that in this species the presence of 20α-hydroxysteroid dehydrogenase coupled with the induced 17α-hydroxylase activity results in the formation of 17α 20α-dihydroxypregn-4-en-3-one. The net effect of this increased catabolism of pregnenolone and progesterone is postulated as being the mechanism underlying the drop in utero-ovarian progesterone at the end of gestation in the sheep. Although Fairclough, Hunter and Welch (1975) failed to demonstrate an appreciable placental contribution to the overall peripheral plasma levels of progesterone in the cow, in agreement with the earlier studies of Labhsetwar et al. (1967), the potential ability of this structure to carry out in vitro synthesis of progesterone has been demonstrated (Ainsworth and Ryan, 1967). It may be that small quantities of placental progesterone are produced in this species and that they exert a local effect on the myometrium. The existence of such a source of this hormone may be of relevance to the maintenance of pregnancy in ovariectomised cows in which continuation of gestation occurs under substantially reduced peripheral plasma levels of this steroid (Edqvist, Ekman, Gustafsson and Johansson, 1973). It is possible that foetal glucocorticoids in the terminal stages of

gestation in the cow bring about an activation of 17α-hydroxylase activity and that this reduces the availability of placental progesterone. The observation that systemically administered progesterone failed to delay parturition in normal cows (McDonald and Heys, 1958) is possibly associated with the removal of substantial local progesterone biosynthesis due to the prior action of foetal glucocorticoids on the placenta. Obviously further study is warranted on the inter-relationship of luteal and placental progesterone synthesis and metabolism during the last few weeks of pregnancy in the cow.

Working with ovine placentae Steele, Flint and Turnbull (1975) found that labelled 17α-hydroxyprogesterone could be converted to oestrone indicating the presence of C17-20 lyase activity in this tissue. Due to the fact that this activity was present in placentae from glucocorticoid induced and natural parturitions they deduced that induction of this enzyme system resulted from increased foetal adrenal activity. The situation in late pregnancy in the sheep therefore suggests that enzymic induction by glucocorticoids brings about increased catabolism of progesterone and in addition facilitates production of oestrogens from this substrate.

In the sheep foetal oestrogens rise sharply during the last few days of gestation (Currie, Wong, Cox and Thorburn, 1973). In contrast in the calf plasma levels of both free and sulphated oestrogens show little consistent change before calving (Hunter, Welch, Fairclough, Barr and Seamark, 1974). Although the bovine placenta as previously noted secretes oestrogens for a considerable period of time prior to parturition it is possible that induction of similar enzymes to those

recorded in the sheep may have a minor contributory role in production of the late pre-partum increase in the levels of these steroids in the utero-ovarian vein (Peterson, Hunter, Welch and Fairclough, 1975).

The administration of stilboestrol to pregnant ewes elevates the levels of PGF in the maternal placenta, myometrium and utero-ovarian vein within 24 hours (Liggins, 1973). It may be that in the cow increased placental biosynthesis of oestrogens, resulting from the action of glucocortocoids on this tissue, leads to a release of prostaglandin F (PGF) which can then act as a luteolysin. Diminution of a progesterone block to PG following luteolysis would allow the marked increase in PGF in the utero-ovarian vein recorded by Fairclough et al. (1975) within the last 48-24 hours of pregnancy in the cow.

CHAPTER FOUR

POST-PARTUM REPRODUCTION

4.1. Introduction

After calving, besides making provision for care of the newborn, the cow must reactivate various systems to allow conception to recur and, if a suitable environment in the reproductive tract is available, a subsequent pregnancy to become established. Over this transitional period from one pregnancy to the next, profound anatomical, physiological and endocrinological changes take place.

Numerous studies have been carried out on the period of time from calving to conception (e.g. Olds, Morrison and Seath, 1949; Edwards, 1950; Trimberger, 1956; Wiltbank and Cook, 1958). The duration of this period is governed, to a large extent, by the variable interval between calving and first overt heat. Morrow, Robert and McEntee (1969) have reviewed the literature noting the effects of some maternal, environmental and managemental factors on this interval. Within the post-partum period before overt oestrus occurs, many cows ovulate and form corpora lutea. This phenomenon is referred to as 'silent heat.' The effect of several factors in the incidence of these so-called 'silent heats' has been studied (Kidder, Barrett and Casida, 1952). Of these many factors the management of lactation has been most widely investigated (Saiduddin, Riesen, Tyler and Casida, 1968; Moller, 1970; Smith and Vincent, 1972).

Studies of ovarian morphology between calving and first ovulation have demonstrated that although the ovaries at calving appear quiescent, follicular development occurs prior to the first post-partum ovulation (Casida, Meyer, McShan and Wisnicky, 1943; Saiduddin *et al.*,

1968; Moller, 1970). Attempts to breed cows early in the post-partum period have been largely unsuccessful (Trimberger, 1954; Whitmore, Tyler and Casida, 1974). These studies suggested that other factors, apart from overt heat and ovulation, are involved in the re-establishment of pregnancy.

Against a background of morphological events within this period, limited physiological and endocrinological studies have been carried out with a view to explaining some of the mechanisms involved. The release of ovulatory amounts of pituitary gonadotrophins has been studied in the cycling cow (Snook, Saatman and Hansel, 1971; Garverick et al., 1971). It is not apparent why follicular development in the early post-partum period does not proceed to ovulation. Although limited studies have been carried out on the level of circulating oestrogens associated with normal overt heat (Henricks et al., 1971; Glencross et al., 1973; Christensen et al., 1974) it has not been established why in post-partum cows ovulation commonly occurs without the behavioural changes of oestrus being manifest. The structural development of corpora lutea, their progesterone content and the circulating levels of progesterone have been investigated in normal cycling cows and to a lesser extent in animals in the post-partum period prior to the time of first overt heat (Foote, Zimbelman, Loy and Casida, 1959; Erb and Stormshak, 1961; Naves, Zimbelman and Casida, 1962; Gomes, Estergreen, Frost and Erb, 1963; Stabenfeldt, Ewing and McDonald, 1969; Morrow, Roberts and McEntee, 1969; Pope et al., 1969; Moller, 1970; Garverick et al., 1971). However, these observations have not been correlated with the accepted difficulties in re-establishing pregnancy in the post-partum period.

Attempts have been made to decrease the interval between calving and conception by endocrine treatments. Progesterone and progestagens alone, and in combination with oestrogens or gonadotrophins, have been shown to decrease the period to first heat but have had variable effects on subsequent fertility (Foote and Hunter, 1964). Similarly although the use of gonadotrophins or gonadotrophic hormone releasing factors could advance the time of first ovulation, the time of first heat was unaffected (Foote and Hunter, 1964; Britt, Kittok and Harison, 1974). It would appear, therefore, that before a logical system for regulation of the calving-conception interval can be elucidated, information on the normal endocrine events within this period is essential.

This study was designed to allow intensive investigation of follicular and luteal function over the period (1) from calving to conception (Chapter 4) and (2) from conception to confirmation of pregnancy (Chapter 5). The findings were integrated with results of ovarian structural changes and behavioural observations. In the light of the well-accepted effect of suckling on post-partum events, this study was carried out on cows nursing calves. It was hoped that the results of this work would help to explain many of the findings of previous studies and the variable success rates of the empirical hormone regimes applied to post-partum cows before this time.

4.2. Experimental design

Animals

Ten dairy type heifers were used for this experiment. Seven

of the cows (Nos. 1, 3, 4, 5, 7, 8 and 9) were those studied over the period of calving - the results of this having been reported in Chapter three. A further three animals (Nos. 2, 10 and 11) were acquired at the same time as the other animals and maintained over calving. Cows Nos. 2, 10 and 11 were an Ayrshire, a Friesian and a Friesian respectively. Parturition in two of the animals was normal in cows 2 and 10; Cow 11 gave birth to a stillborn calf but did not retain her placenta. Cow 11 however was not suckled by her own but by a fostered calf which she totally accepted. Parturition in all cows occurred over a period of three weeks from mid-June to mid-July. The experiment commenced 1 day after calving in each case and was continued until such time as all animals with one exception (Cow 9) had shown at least one overt heat and had been inseminated. Due to the variation in the time of parturition coupled with the fact that the complete group was to be kept together till the end of the experiment, the individual periods of study ranged from 102 to 125 days. Each animal over the period of study was suckled by its own, or a fostered calf. No attempt was made to restrict the suckling activities of the calves only to their dams. The animals were maintained as a group in an open court in the company of a 3 year old vasectomised Ayrshire bull of established good libido.

Nutrition

Hay and water were available ad lib to all animals. In addition summer concentrate pellets (Parker's Bronze Label nuts) were fed to the cows, in individual stalls, at the rate of 1.8 kg/day +

1.8 kg per estimated gallon milk produced/day. Supplementary weaner pellets (Parker's) were also made available to the calves from approximately 6 weeks of age onwards.

Cows and calves were weighed weekly. The first weight of the cow was taken as 100% and the differences at subsequent weighings were expressed as % of the initial weight. The mean % weight change was then calculated by dividing the total weight gain or loss by the number of weighings. In the case of the calves the weight gains were expressed as kg/day.

Behaviour

Behavioural observations were classified as follows:

(+) bull showing some interest in the cow, but making no attempt to mount.

(++) bull attempting to mount cow but cow not standing.
(+) and (++) behaviour were referred to as perioestrous behaviour.

(+++) overt or standing oestrous behaviour comprising more than one occasion when the bull mounted the cow and the cow stood to be ridden.

The observations were made either

(a) by direct observation of the group at least 3 times daily for a period of not less than 20 minutes on each occasion. For the most part, these observations were carried out around 06.30, 12.00 and 21.00 although the frequency was increased in the event

of any signs of pro-oestrous or oestrous behaviour by a cow in the groups.

(b) by time lapse photography. A 16 mm time lapse camera (Vinton Scientific) loaded with Panchromatic X film (Kodak Ltd.) was mounted so that the entire area of the court was included in the frame. Pictures were taken from just before daylight to just after dark. Examination of the films was carried out by projection at 18 frames/second. If required, more detailed assessment of specific sections was carried out by projection at 2 frames/second by means of a time lapse projector.

Any animal showing oestrous behaviour was removed from the court, leaving the bull in the company of the remaining cows. However, to estimate the duration of oestrus, the response of the cow to the bull was examined by turning her back into the court at intervals till oestrous behaviour was no longer observed. The cow was then returned to the group. If a cow was in oestrus over the hours of darkness, the court was illuminated to enable her behaviour to be monitored. At all other times natural lighting was used.

Examination of the reproductive tract

At the time of rectal palpation inspection of the vestibular mucous membrane was carried out for any abnormalities and the presence of any gross vulvar discharge. To avoid any possibility of ovarian palpation affecting the time of ovulation, the ovaries of cows showing oestrous behaviour were not examined per rectum. Rectal palpation of the reproductive tract was carried out at least twice weekly.

Examination of the ovaries proved impossible in all animals for a variable period of time at the start of the experiment.

The size and position of any ovarian structures were recorded by making drawings at the time of rectal palpation and subsequently carrying out measurements from these drawings. In addition the surface nature and consistency of any ovarian structures present was noted. Based on these observations, and provided that the structures were greater than 0.5 cm in diameter, they were classified as corpora lutea, follicles or cysts. Total follicular corpora lutea, and cyst surface diameters were calculated.

The presence of any tone in the uterine wall was recorded on an arbitrary scale ranging from zero to +++ (maximum). Due to the well recognised fact that uterine turgidity can be stimulated by rectal palpation, this parameter was assessed prior to any further examination of the reproductive tract being carried out. The diameter of the uterine horns at a point approximately mid-way along the horizontal part of the horns was also determined by palpation. Uterine horn diameter was considered to be 'normal' on the first occasion after calving when a consistently reduced size was found.

Blood samples

Blood samples were obtained from the jugular vein into heparinised evacuated glass tubes (Vacutainer - Becton and Dickinson) at the same time each day. Standardisation of the procedure and personnel used minimised any disturbance caused to the animals. Immediately after collection, the blood was stored at $4^{\circ}C$, the plasma

separated within 2 hours and then stored at -15°C till the hormone assays were carried out.

Hormone assays

The concentrations of progesterone, total oestrogens and androgens were determined in the plasma samples by the radioimmunoassay techniques described in Chapter two.

4.3. Results

The results presented in the following figures with comments for the individual cows were those obtained over the complete period of study. In all animals parturition was designated day 0. Although all results are given at this point they will be discussed firstly up to the point at which conception, which subsequently became an established pregnancy, occured - Chapter four - and secondly from conception to the end of the period of study - Chapter five. The division of the overall study into each of these two periods is given on Figs 34 to 63 for the individual animals with the former period being designated 'non pregnant' and the latter 'pregnant.'

Cow 1

The results for this animal are given in Figs 34 - 36.

Oestrous and perioestrous behaviour

Although within the period 26 - 40 days, the bull showed interest in the cow, and tried to mount her on three occasions, overt heat was not exhibited till day 93 after parturition. The cow was not inseminated at this heat.

Plasma progesterone levels

From calving to day 4, and from day 16 to day 92, plasma progesterone levels were basal. Following a transient elevation to 0.7 ng/ml on day 93 the levels fell again. From day 100 the levels rose again to greatly in excess of those previously found - a maximum level of 4.5 ng/ml being recorded on day 108.

Corpora lutea

A well developed corpus luteum of 1.6 cm diameter was found at the time of first examination on day 16. Following its disappearance after day 27, no further luteal tissue was detected until day 102. The structure first found at this time was still present at the end of the experiment.

Cystic ovarian structures

None was recorded over the experimental period.

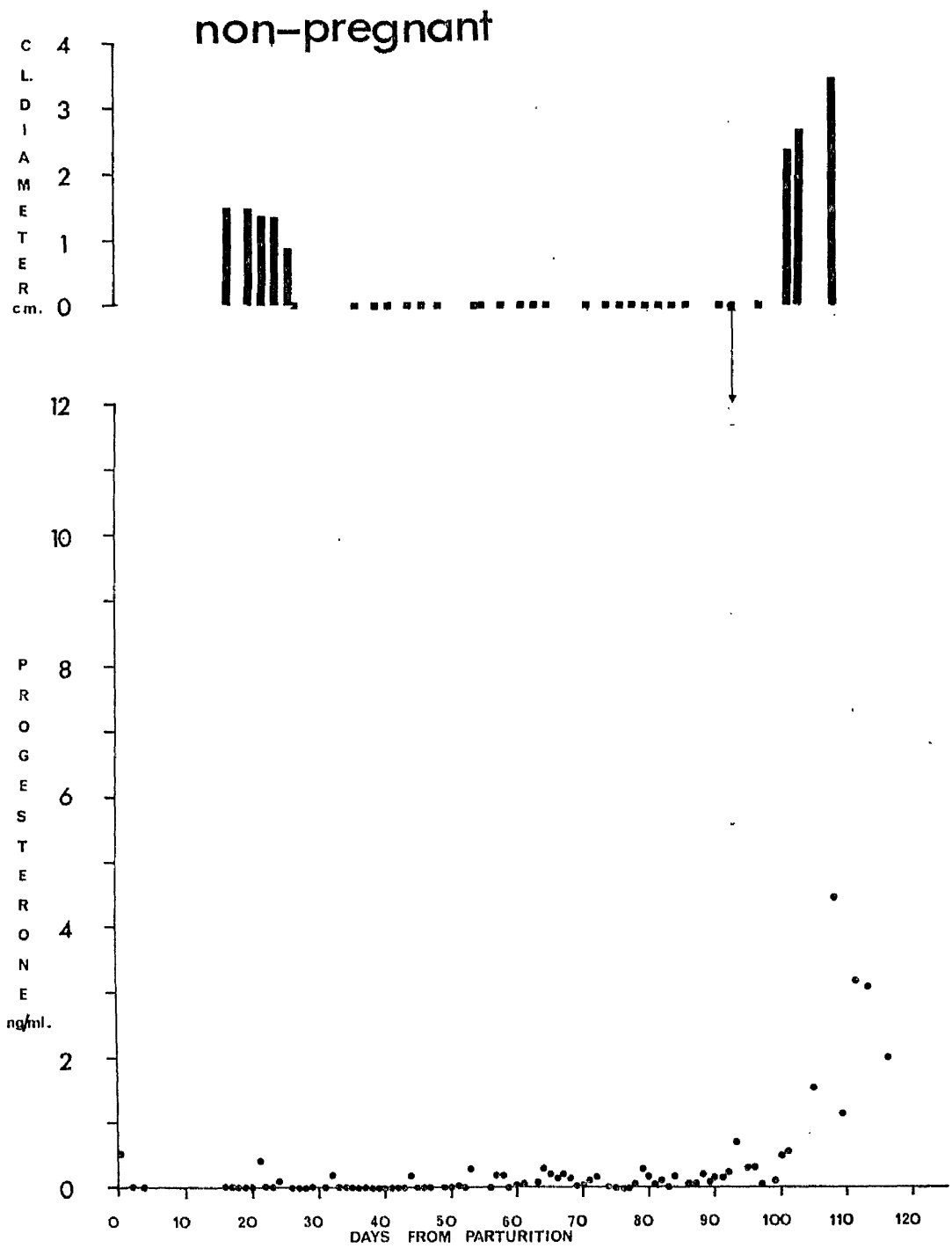


FIG 34 COW 1

PERIPHERAL PLASMA PROGESTERONE LEVELS, AND THE PRESENCE OF CORPORA LUTEA AND CYSTS OVER THE COMPLETE PERIOD OF STUDY

Plasma oestrogen levels

Wide fluctuations in the levels of these hormones were recorded within the period of study. Although the majority of results were less than 6.0 pg/ml, peaks in excess of this concentration were recorded on days 31, 36, 61, 78, 82, 87 and 98.

Follicles

Follicles with a total surface diameter of more than 2.0 cm were recorded at the time of first examination of the ovaries on day 16. Maximum follicular sizes of around 3.0 cm were detected on days 20, 71, 78 and 96. Up to day 80 on the twenty three occasions the ovaries were examined only on one of these were no follicles found. After this, however, absence of follicular development was recorded on 8 out of 11 examinations.

Uterine tone

(+++) uterine tone was found on days 66 and 99. (++) uterine turgidity was recorded within the periods 41 to 46, and 76 6o 94 days. In addition over 4 days before the first occurrence of (+++) tone on day 66, an obvious increase in turgidity was noted.

Plasma androgen levels

Although within one week of calving the level of these hormones was above 160 pg/ml, by the second week they had decreased to around 50 pg/ml. From this time to the end of the experiment a marked decline in the concentrations occurred on weeks 10 and 11 and a substantial increase on week 15.

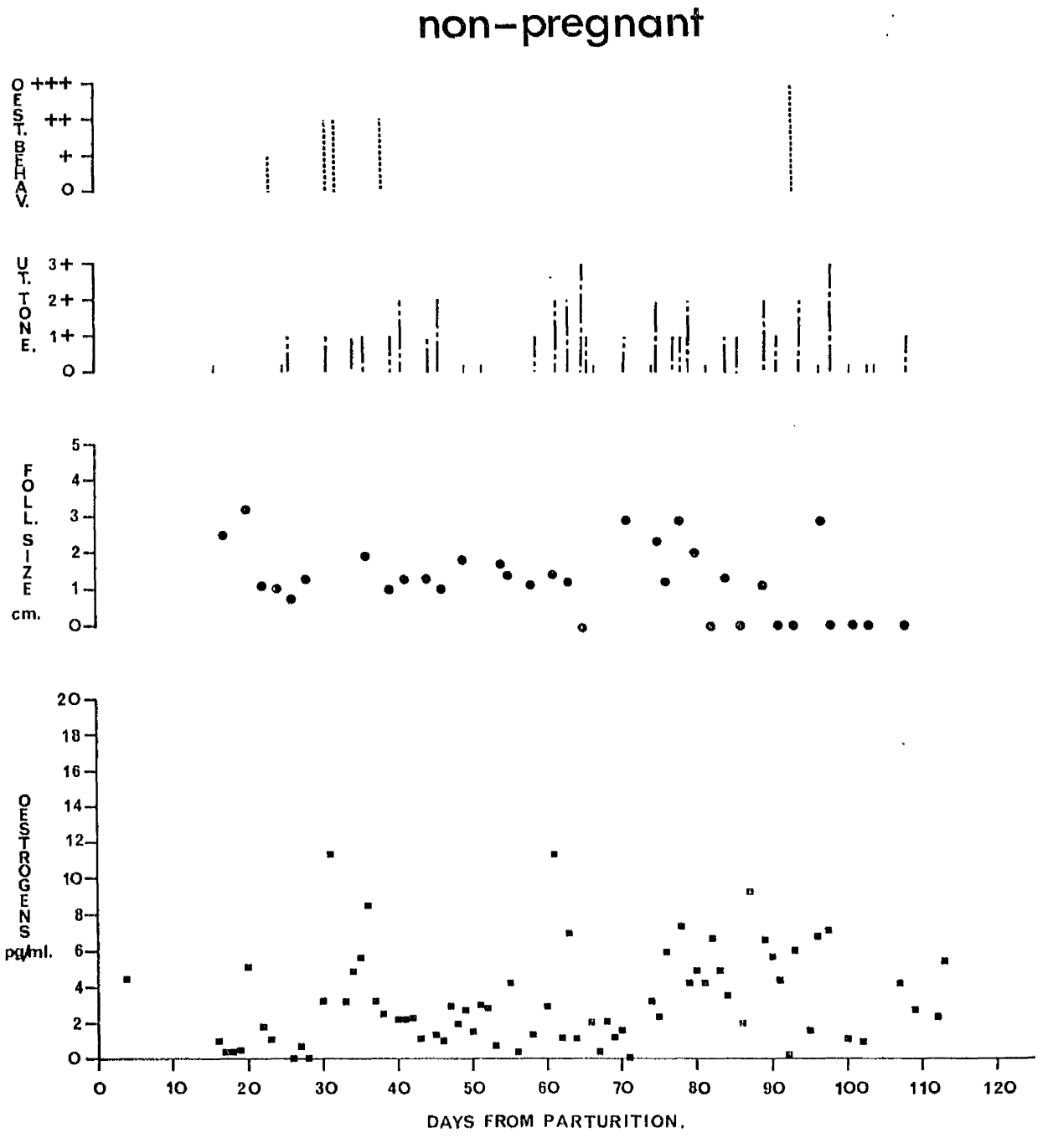


FIG 35 COW 1

LEVELS OF PERIPHERAL PLASMA OESTROGENS, PRESENCE OF FOLLICLES, UTERINE TONE, AND THE OCCURRENCE OF OESTROUS AND PERIOESTROUS BEHAVIOUR OVER THE COMPLETE PERIOD OF STUDY

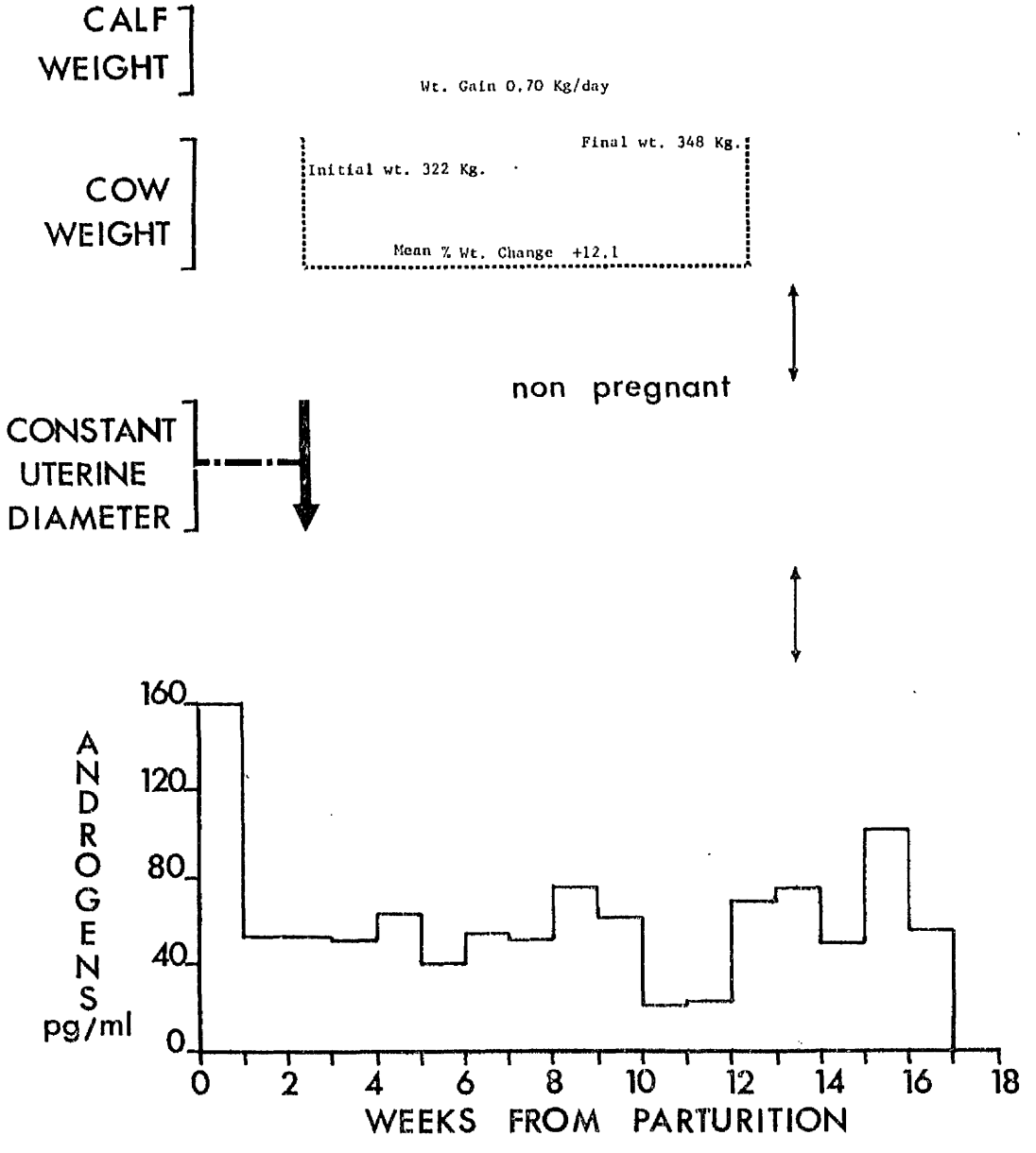


FIG 36 COW 1

PERIPHERAL PLASMA ANDROGEN LEVELS OVER THE COMPLETE PERIOD OF STUDY, THE TIME WHEN A CONSTANT UTERINE HORN DIAMETER WAS FIRST RECORDED AFTER CALVING, AND WEIGHT CHANGES IN THE COW AND CALF

Uterine horn diameter

By 17 days after parturition uterine horn diameter was 4.0 cm. Over the following 19 days this did not significantly change.

Body weight changes

Within the period from 17 to 89 days after calving the weight of the cow showed a net increase.

Additional comments

A bloody vulvar discharge was observed in this animal 7 days after calving. No increase in temperature or inappetence was noted. Following parenteral administration of oxytetracycline (Terramycin, Pfizer) on 2 consecutive days, the discharge ceased.

Cow 2

The results for this animal are given in Figs 37 - 39.

Oestrous and perioestrous behaviour

With the exception of day 18 when the bull showed slight interest in the cow, no behavioural change was recorded between calving and day 78. At this time overt heat occurred, and the cow was inseminated. Pregnancy to this insemination was subsequently confirmed by rectal palpation.

Plasma progesterone levels

Until day 70 progesterone levels remained basal. An elevation in the concentration of this hormone was then recorded between days 70 and 76. Within this period peak values of around 2.0 ng/ml were found. Following this basal levels were again recorded from days 76 - 79. Between this latter time and the end of the experiment the concentration of this hormone was observed to increase and then remain at values in excess of 2.0 ng/ml.

Corpora lutea

A corpus luteum was not detected till 71 days after calving. This structure was then replaced by a further corpus luteum. Although this latter structure could be felt within the ovaries from day 80 to 88, no corpus luteum could be distinguished on day 90. A corpus luteum was then recorded from days 93 to 100 and after a further apparent absence on day 102, again from day 107 to the end of the experiment.

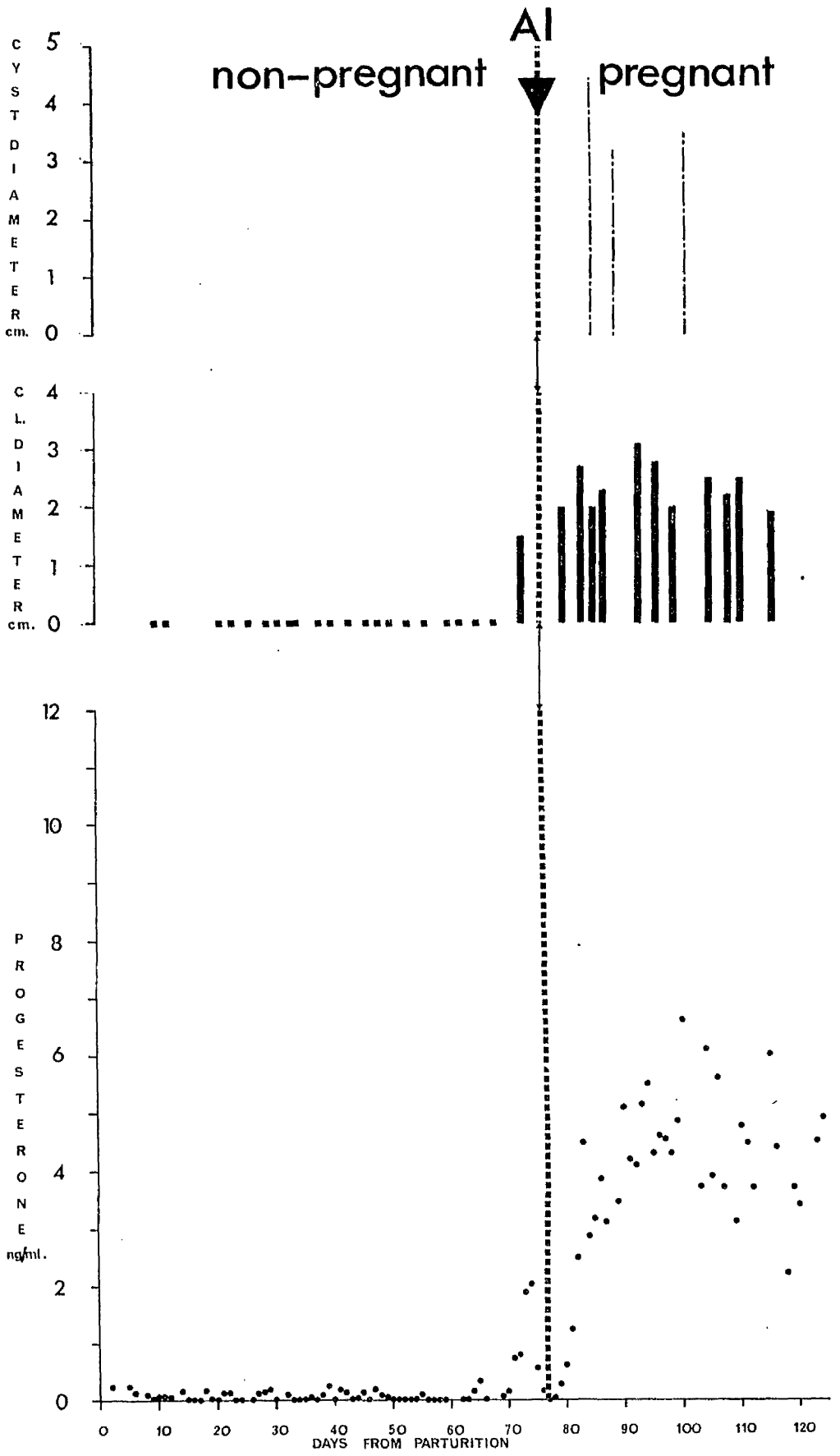


FIG 37 COW 2

PERIPHERAL PLASMA PROGESTERONE LEVELS, AND THE PRESENCE OF CORPORA LUTEA AND CYSTS OVER THE COMPLETE PERIOD OF STUDY

Cystic ovarian structures

Large structures classified as cysts could be palpated on days 85, 90 and 102.

Plasma oestrogen levels

Maximum peak levels of these hormones were recorded within the first half of the experiment. Within the period 0 to 56 days, 6 peaks of greater than 8.0 pg/ml were found, whereas from day 57 to the end, only one peak of comparable magnitude was detected.

Follicles

No evidence of follicular activity was obtained till day 22 when follicles with a total surface diameter of 2.0 cm were palpated. After this time, however, on all but three occasions follicles with diameters of 1.0 cm or more were recorded. Maximum follicular diameters in excess of 3.0 cm were found within the periods 61 - 76 and 91 - 101 days.

Uterine tone

Although (+++) uterine tone was only recorded on days 78 and 79, (++) tone was noted on numerous occasions between days 18 and 41. In addition on days 57, 60, 75 and 96 (++) uterine turgidity was also recorded.

Plasma androgen levels

Concentrations of these hormones remained around 60 - 70 pg/ml

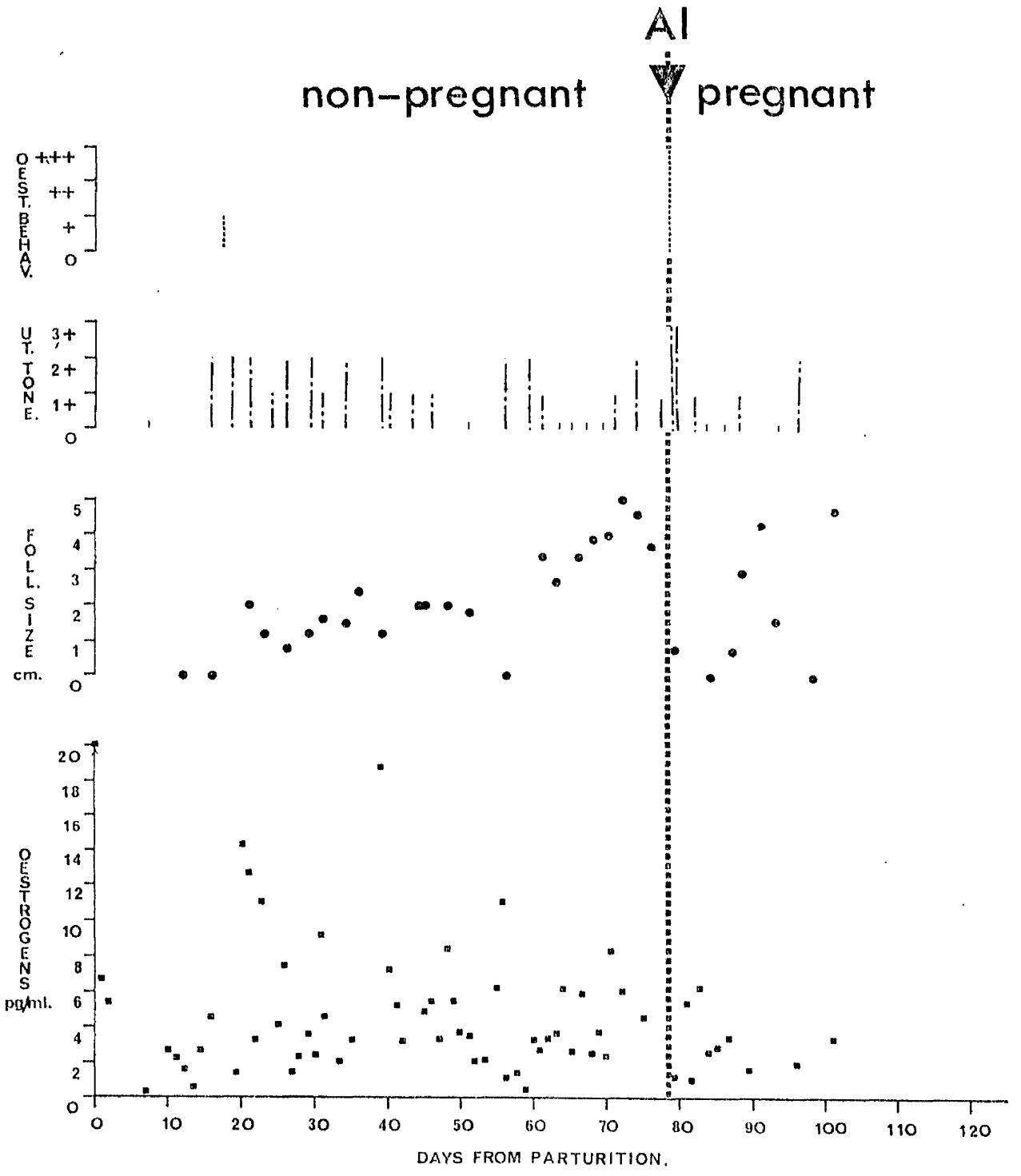


FIG 38 COW 2

LEVELS OF PERIPHERAL PLASMA OESTROGENS, PRESENCE OF FOLLICLES, UTERINE TONE, AND THE OCCURRENCE OF OESTROUS AND PERIOESTROUS BEHAVIOUR OVER THE COMPLETE PERIOD OF STUDY

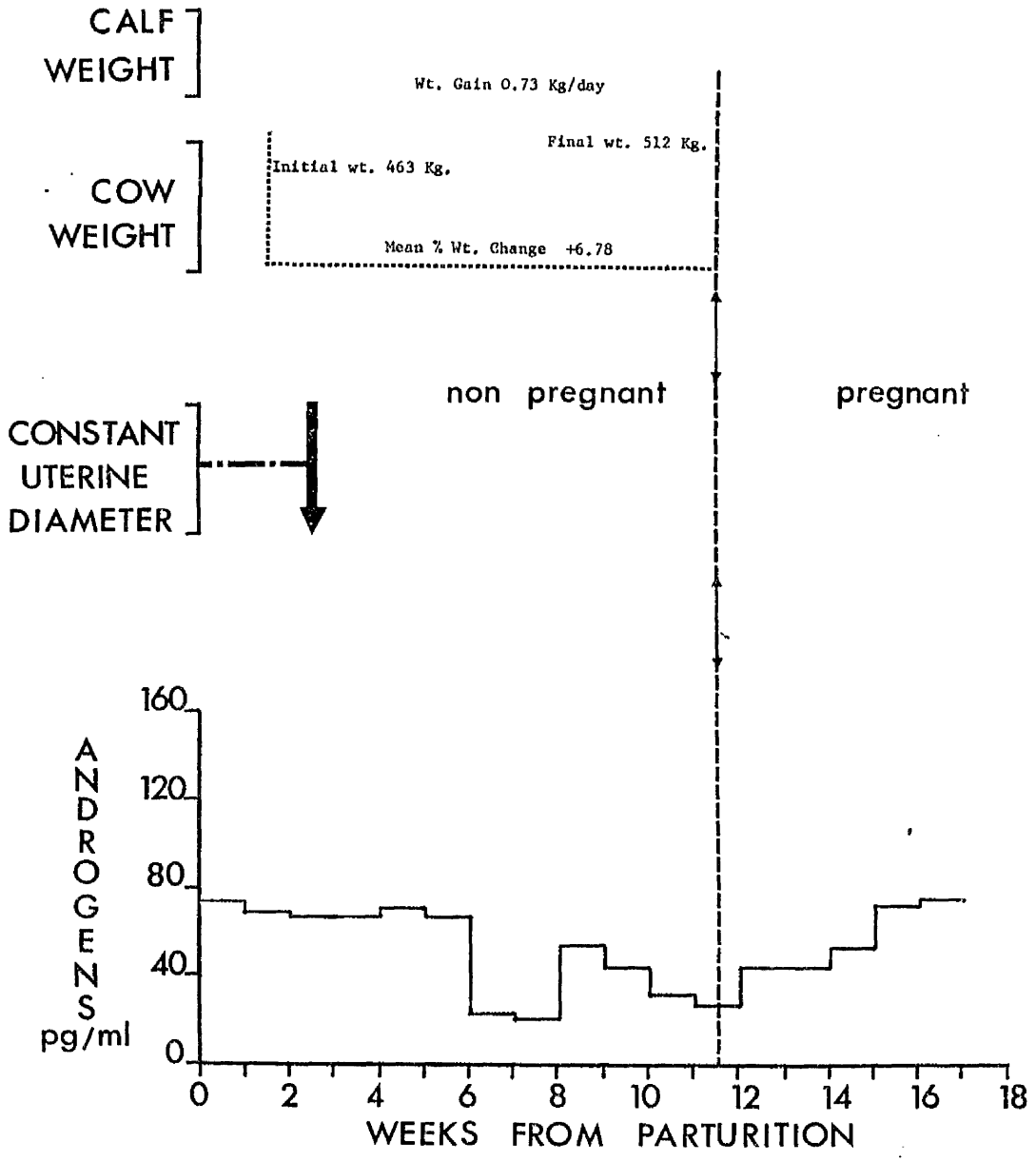


FIG 39 COW 2

PERIPHERAL PLASMA ANDROGEN LEVELS OVER THE COMPLETE PERIOD OF STUDY, THE TIME WHEN A CONSTANT UTERINE HORN DIAMETER WAS FIRST RECORDED AFTER CALVING, AND WEIGHT CHANGES IN THE COW AND CALF

till 6 weeks after calving. After this a marked decline was observed on weeks 6 and 7. Following a transient elevation on weeks 8 and 9 the levels again fell before finally rising from week 12 to the end of the experiment.

Uterine horn diameter

A constant uterine horn diameter of 4.0 cm was reached 19 days after parturition.

Body weight changes

A net increase in body weight occurred between 23 and 81 days post-partum.

Cow 3

The results from this animal are shown in Figs 40 - 42.

Oestrous and perioestrous behaviour

Slight interest was shown by the bull in the cow on days 35 to 37. Following one day when the bull attempted to mount the cow but she would not stand (day 43), overt heat was shown on day 44. Over the remainder of the experimental period the cow returned to heat on day 69. Although the cow was inseminated at this time it did not become pregnant but returned to heat on day 96. Following a second insemination however, on this day pregnancy was established.

Plasma progesterone levels

Except for an elevation in levels to 0.7 ng/ml on day 30, the concentration of this hormone remained basal until day 33. Following this elevated levels were found between days 33 and 43, 48 and 67, 72 and 94, and from day 98 to the end of the experiment. Maximum concentrations in excess of 2.0 ng/ml were found over several days during each of these periods of increased levels.

Corpora lutea

Corpora lutea were found in the ovaries on day 34, between days 52 and 67, and from day 74 to 93. Following regression of this last structure by day 98 a fourth corpus luteum was detected on day 102. No marked size differences were apparent amongst these structures.

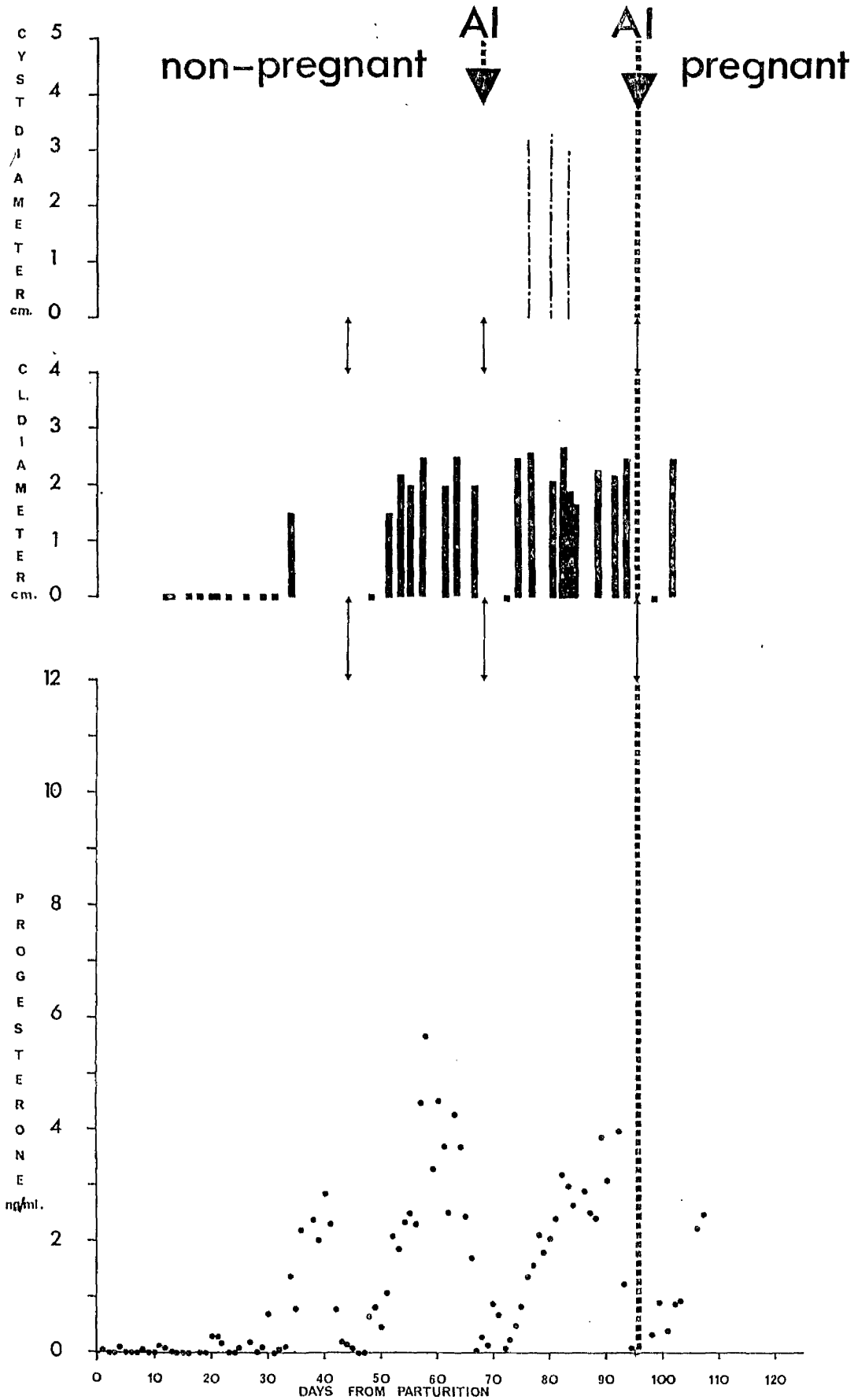


FIG 40 COW 3

PERIPHERAL PLASMA PROGESTERONE LEVELS, AND THE PRESENCE OF CORPORA LUTEA AND CYSTS OVER THE COMPLETE PERIOD OF STUDY

Cystic ovarian structures

Large cystic structures were detected in the ovaries over the period from 76 to 83 days.

Plasma oestrogen levels

Marked fluctuations were recorded in the level of these hormones over the experimental period. Peaks reaching values in excess of 8.0 pg/ml were found on days 29, 46, 76, 79, 94 and 102.

Follicles

Follicles were present from the time of first examination of the ovaries on day 7. Wide fluctuations in the total surface diameter of these structures were found throughout the experimental period, peak diameters occurring on days 21, 51, 62, 72 and 80. Maximum total follicular sizes of between 4.0 and 5.0 cm were recorded during the second half of the experiment.

Uterine tone

Over the complete study what was considered to be (+++) uterine tone was recorded on eight occasions, five of which occurred within the period 23 to 41 days, two on days 67 and 70, and the last on day 93. Between days 23 and 93 (++) uterine turgidity was noted on several occasions.

Plasma androgen levels

Against a mean level of around 40 pg/ml significant increases

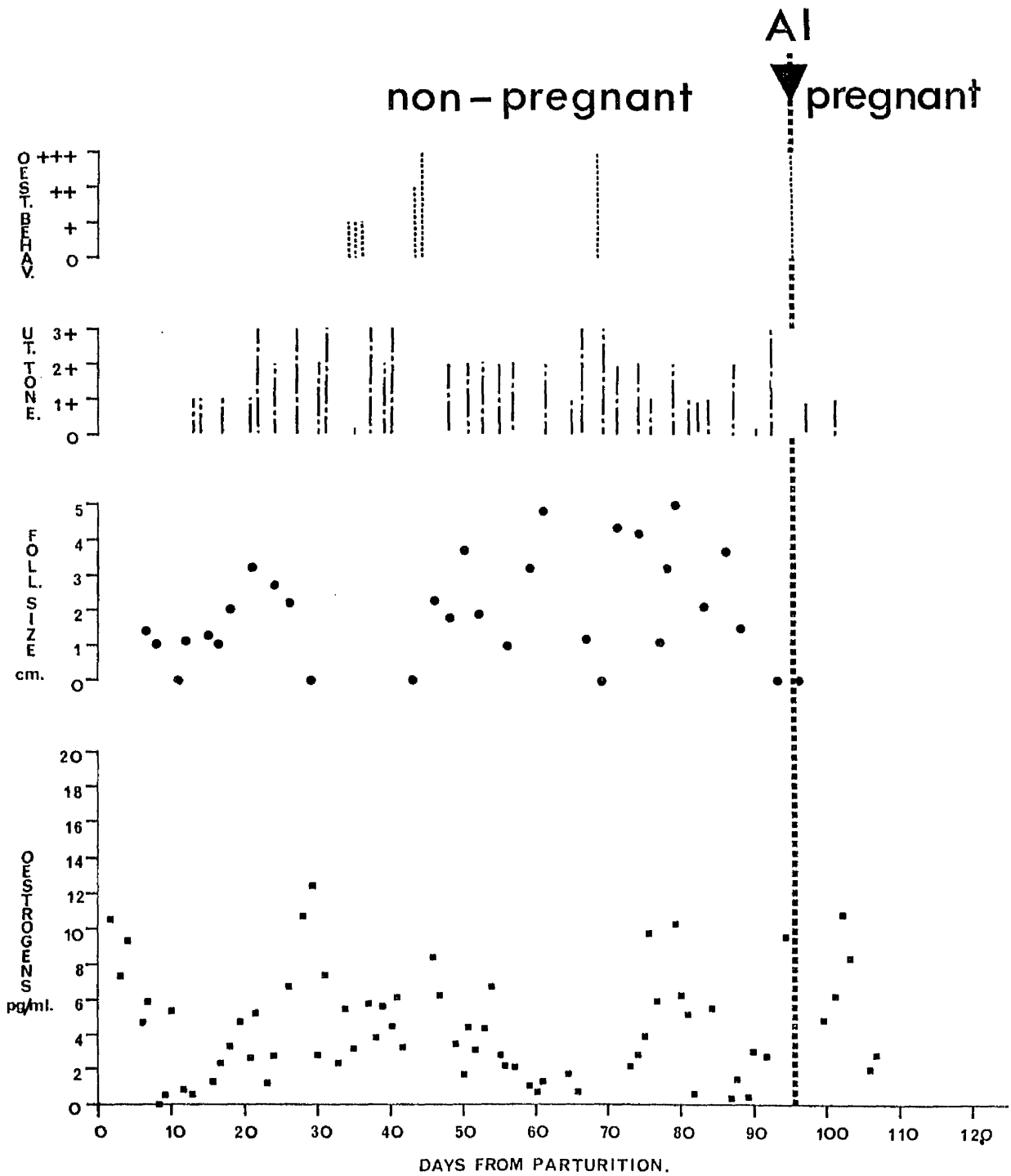


FIG 41 COW 3

LEVELS OF PERIPHERAL PLASMA OESTROGENS, PRESENCE OF FOLLICLES, UTERINE TONE, AND THE OCCURRENCE OF OESTROUS AND PERIOESTROUS BEHAVIOUR OVER THE COMPLETE PERIOD OF STUDY

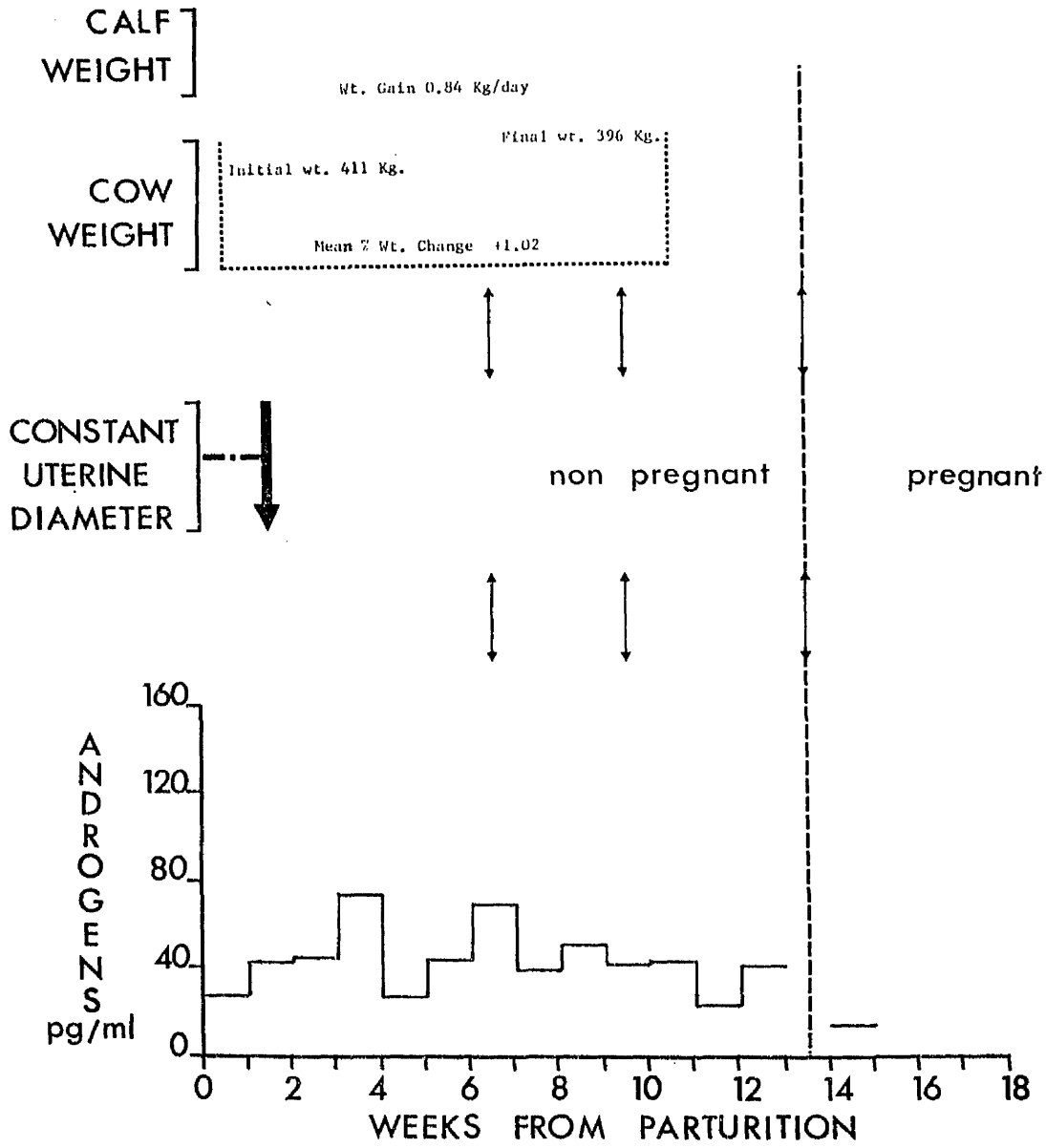


FIG 42 COW 3

PERIPHERAL PLASMA ANDROGEN LEVELS OVER THE COMPLETE PERIOD OF STUDY, THE TIME WHEN A CONSTANT UTERINE HORN DIAMETER WAS FIRST RECORDED AFTER CALVING, AND WEIGHT CHANGES IN THE COW AND CALF

were recorded in the levels of these hormones on weeks 3 and 7, and marked decreases on weeks 0, 4, 11 and 14.

Uterine horn diameter

Between calving and day 12 uterine horn diameter decreased to a final size of 4.0 cm. Over the following 22 days no further significant reduction in size was noted.

Body weight changes

Over the period 1 to 73 days a slight net decrease in body weight of the cow was recorded. However, due to the fact that within part of this time the body weight did increase, the mean weight change relative to the initial weight showed a slight increase.

Cow 4

The results for this animal are given in Figs 43 - 45.

Oestrous and perioestrous behaviour

Although slight interest was shown by the bull in the cow on days 22, 27 and 67 and attempts were made to mount her on day 39, overt heat was not exhibited till 78 days after calving, at which time the cow was inseminated and became pregnant.

Plasma progesterone levels

Between calving and day 51, basal levels of this hormone were recorded with the exception of two transient elevations, reaching levels of less than 0.5 ng/ml, on days 39 and 43. Between days 51 to 55, a small increase reaching a maximum concentration of 0.8 ng/ml was found. Following a return to basal concentrations on day 55, two further periods of increased blood levels, with an intervening basal period were found. In the first of these periods of increase, which lasted from 60 to 75 days, maximum levels in excess of 2.0 ng/ml were found. Similar concentrations greater than 2.0 ng/ml were detected during the second period which commenced on day 81 and continued to the end of the experiment. However, absolute maximum levels in the first reached 6.1 ng/ml, whereas in the second the maximum concentration recorded was 4.3 ng/ml on day 93.

Corpora lutea

A small but well defined corpus luteum was recorded between

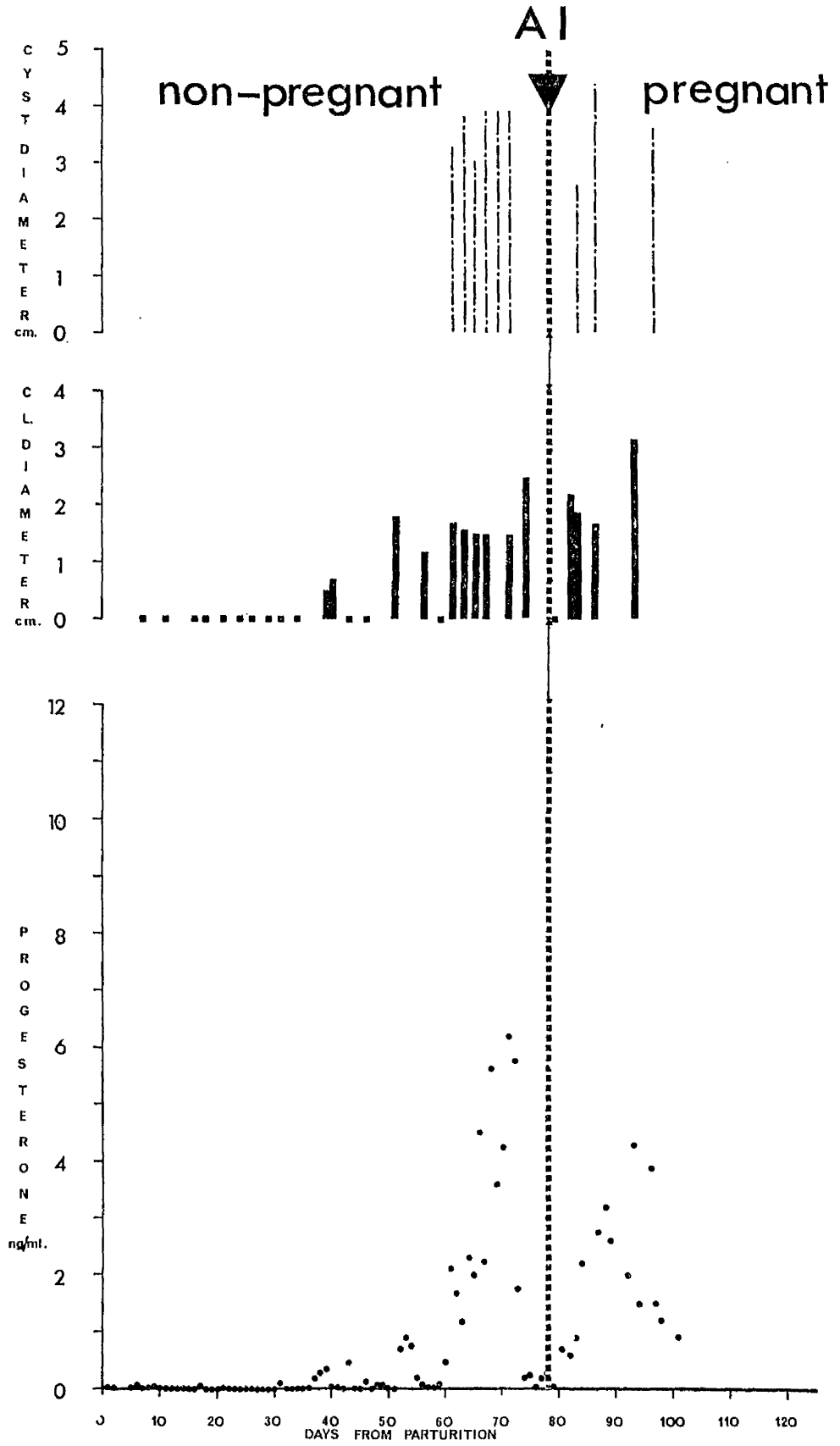


FIG 43 COW 4

PERIPHERAL PLASMA PROGESTERONE LEVELS, AND THE PRESENCE OF CORPORA LUTEA AND CYSTS OVER THE COMPLETE PERIOD OF STUDY

days 38 and 40. Following its regression, another corpus luteum was detected from days 51 to 57. Further corpora lutea were detected from 62 to 75 days, and then from days 82 to 93.

Cystic ovarian structures

Structures classified as being cystic were present over the period from 62 to 71 days. Except for day 68 these could be differentiated from the corpus luteum. No cysts were recorded between days 75 and 82. Further cystic structures were found on days 84 to 87, regressed and cysts were again present on day 97.

Plasma oestrogen levels

Marked fluctuating levels of these hormones were recorded over the period of the experiment. Peaks well in excess of the majority of levels, which lay below 5.0 pg/ml, were recorded on days 45, 63, 90 and 98. Maximum peak concentrations ranged from 7.5 to 16.0 pg/ml.

Follicles

No follicles were recorded till 22 days after calving. Over the remainder of the experimental period widely different total follicular diameters ranging from 0 to a maximum of 5.0 cm, on day 86, were recorded.

Uterine tone

(+++) uterine tone was only recorded on day 79. (++) turgidity was noted five times before this, on days 24, 47, 57, 62 and 73, but on only one occasion after day 79.

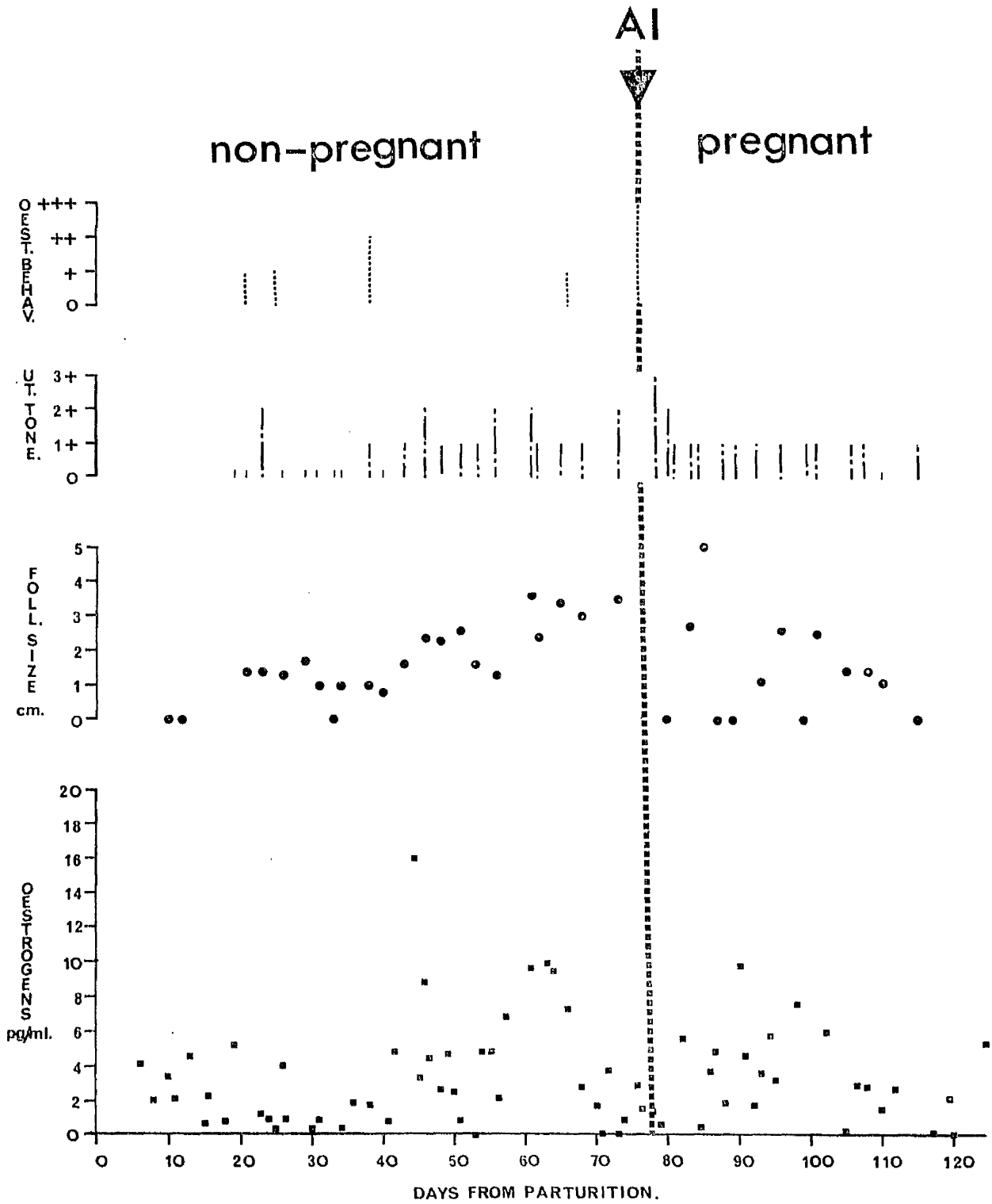


FIG 44 COW 4

LEVELS OF PERIPHERAL PLASMA OESTROGENS, PRESENCE OF FOLLICLES, UTERINE TONE, AND THE OCCURRENCE OF OESTROUS AND PERIOESTROUS BEHAVIOUR OVER THE COMPLETE PERIOD OF STUDY

Plasma androgen levels

Marked fluctuations in the concentrations of these hormones were observed over the period of study. Basal levels from 23 to 53 pg/ml were recorded between peaks of from 101 to 160 pg/ml.

Uterine horn diameter

Uterine horns reached a consistent size of 4.5 cm by 26 days after calving.

Body weight changes

A slight increase in weight of the cow was recorded between 6 and 78 days after calving.

Additional comments

On day 8 after calving, slight necrosis of the vulvar mucous membrane was observed associated with a slight mucopurulent vulvar discharge. In addition the mammary gland appeared turgid and body temperature was increased to 106.5°F. On day 9 a change in the appearance of the milk suggested the presence of mastitis. However, following parenteral oxytetracycline treatment on days 8 and 9 the conditions in both the vulva and udder rapidly regressed.

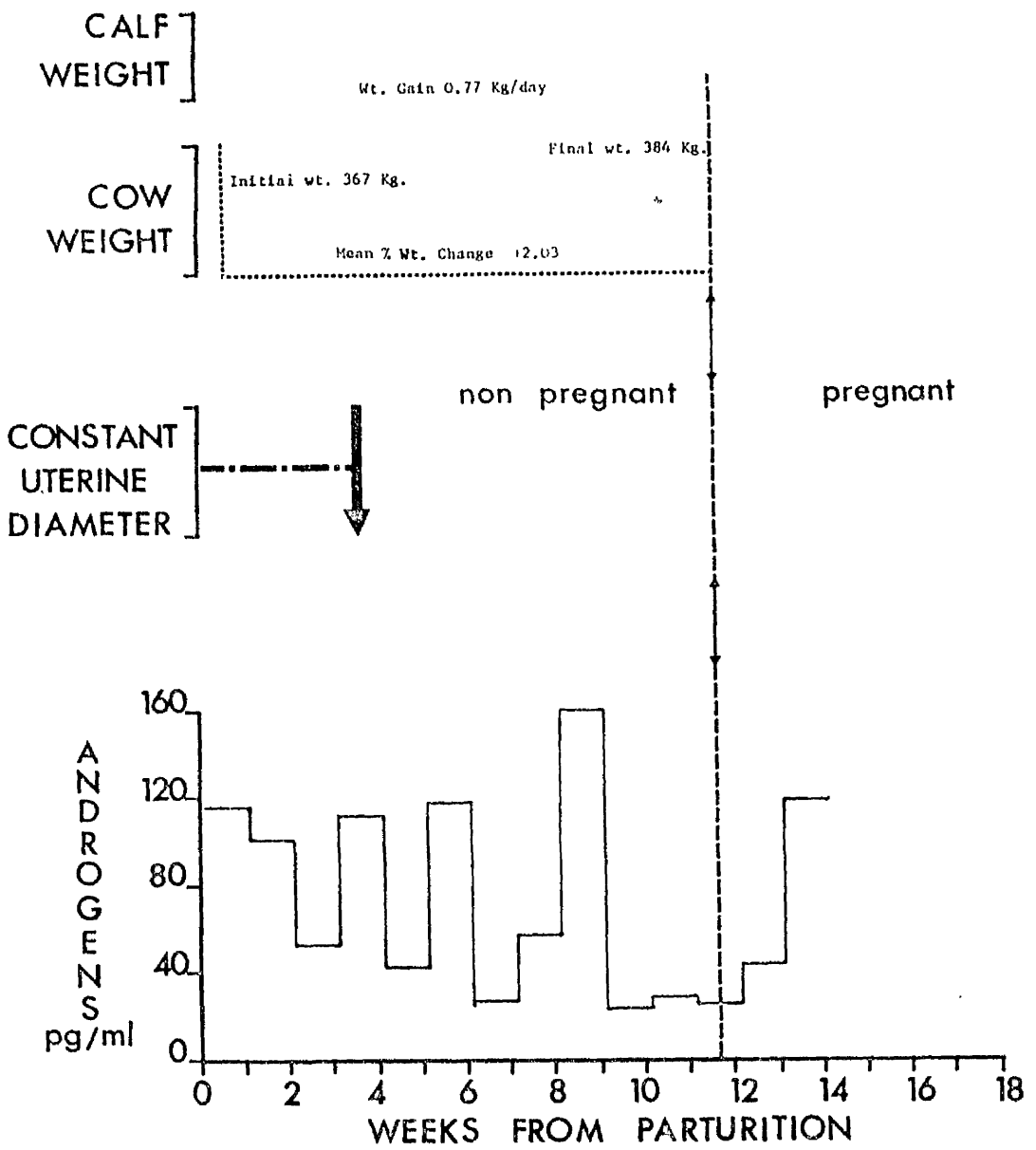


FIG 45 COW 4

PERIPHERAL PLASMA ANDROGEN LEVELS OVER THE COMPLETE PERIOD OF STUDY, THE TIME WHEN A CONSTANT UTERINE HORN DIAMETER WAS FIRST RECORDED AFTER CALVING, AND WEIGHT CHANGES IN THE COW AND CALF

Cow 5

The results for this animal are given in Figs 46 - 48.

Oestrous and perioestrous behaviour

It can be seen that the first overt heat after calving occurred on day 70. Prior to this on day 48, the bull exhibited a slight degree of interest in the cow. Pregnancy was established as a result of artificial insemination on day 70.

Plasma progesterone levels

With the exception of a transient increase to 0.8 ng/ml on day 37, the concentration of this hormone remained basal between calving and day 52. From day 53 to 58, and 64 to 69, plasma progesterone levels were elevated above the concentrations previously recorded. At the end of both of these periods the concentration of plasma progesterone returned to basal values. A third increase was noted commencing on day 72. Unlike the previous two periods of increased concentrations, the progesterone level in this case did not fall after a few days but remained elevated to the end of the experiment. In addition the levels found within this last period, from day 75, were with one exception all in excess of those reported previously in this animal.

Corpora lutea

A corpus luteum was found on day 3 at the time of first examination of the ovaries. This had regressed by day 6 and between

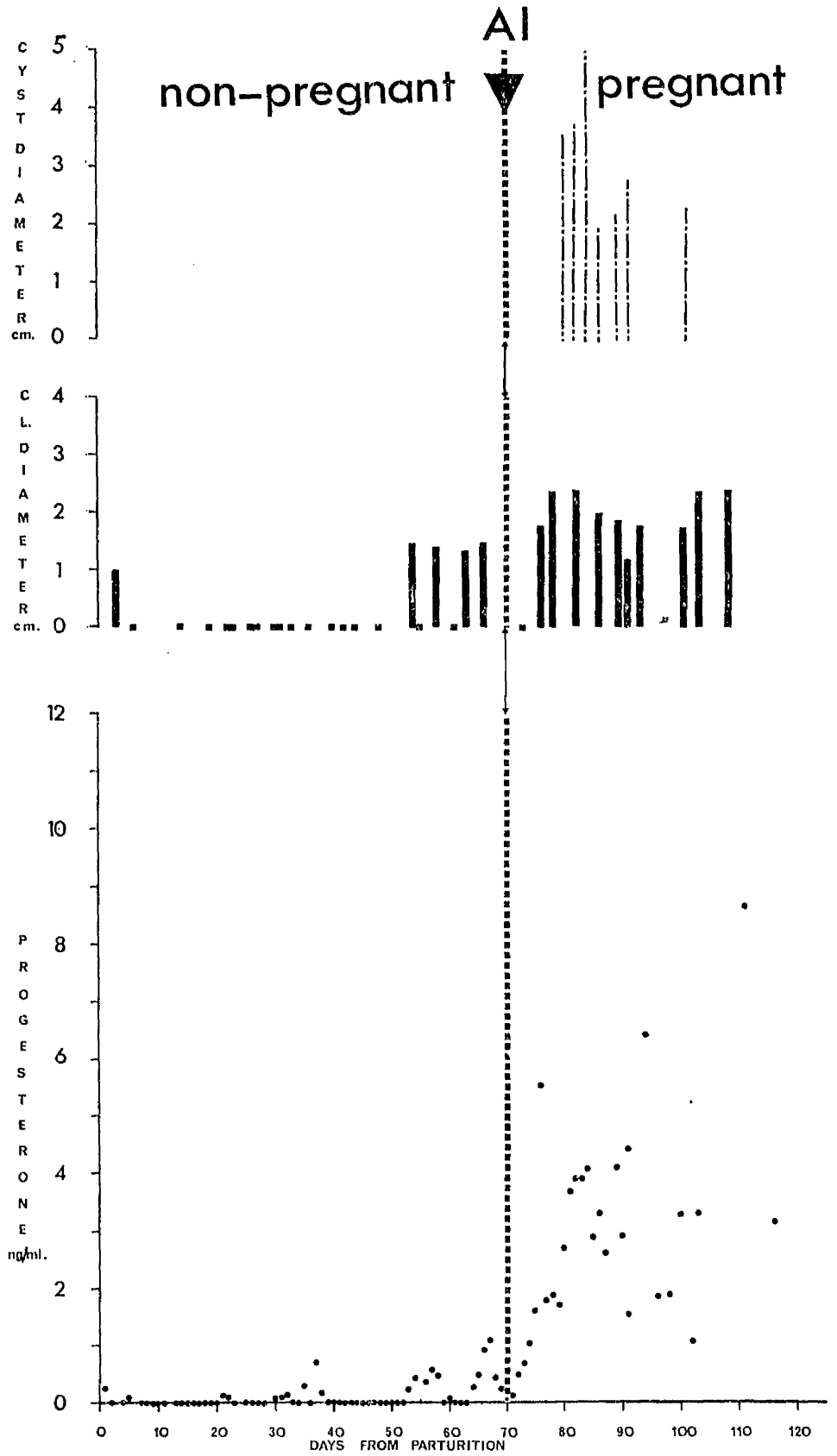


FIG 46 COW 5

PERIPHERAL PLASMA PROGESTERONE LEVELS, AND THE PRESENCE OF CORPORA LUTEA AND CYSTS OVER THE COMPLETE PERIOD OF STUDY

this time and day 48 no corpus luteum was present. A corpus luteum was found on days 54, 59, and from days 63 to 68. These structures had regressed by days 56, 62 and 74 respectively. Following on from the disappearance of the last of these, a new corpus luteum was detected on day 76 and was recorded as persisting to the end of the experimental period.

Cystic ovarian structures

A cystic structure which could be differentiated from the corpus luteum on all occasions except one was present at every rectal examination between days 79 and 101.

Plasma oestrogen levels

Over the experimental period a large number of peaks of these hormones were recorded. On ten occasions peak levels of in excess of 6.0 pg/ml were found. Ninety percent of these peaks occurred within the period 30 to 90 days after calving.

Follicles

No follicles were found at the time of first examination of the ovaries on day 3. Following this from day 12 to the end of the experiment, follicles with a total surface diameter of 1.0 cm or more were found on seventy percent of occasions on which rectal examinations were performed. Follicular diameters of 3.0 cm or more were recorded on day 66 and from days 80 to 84.

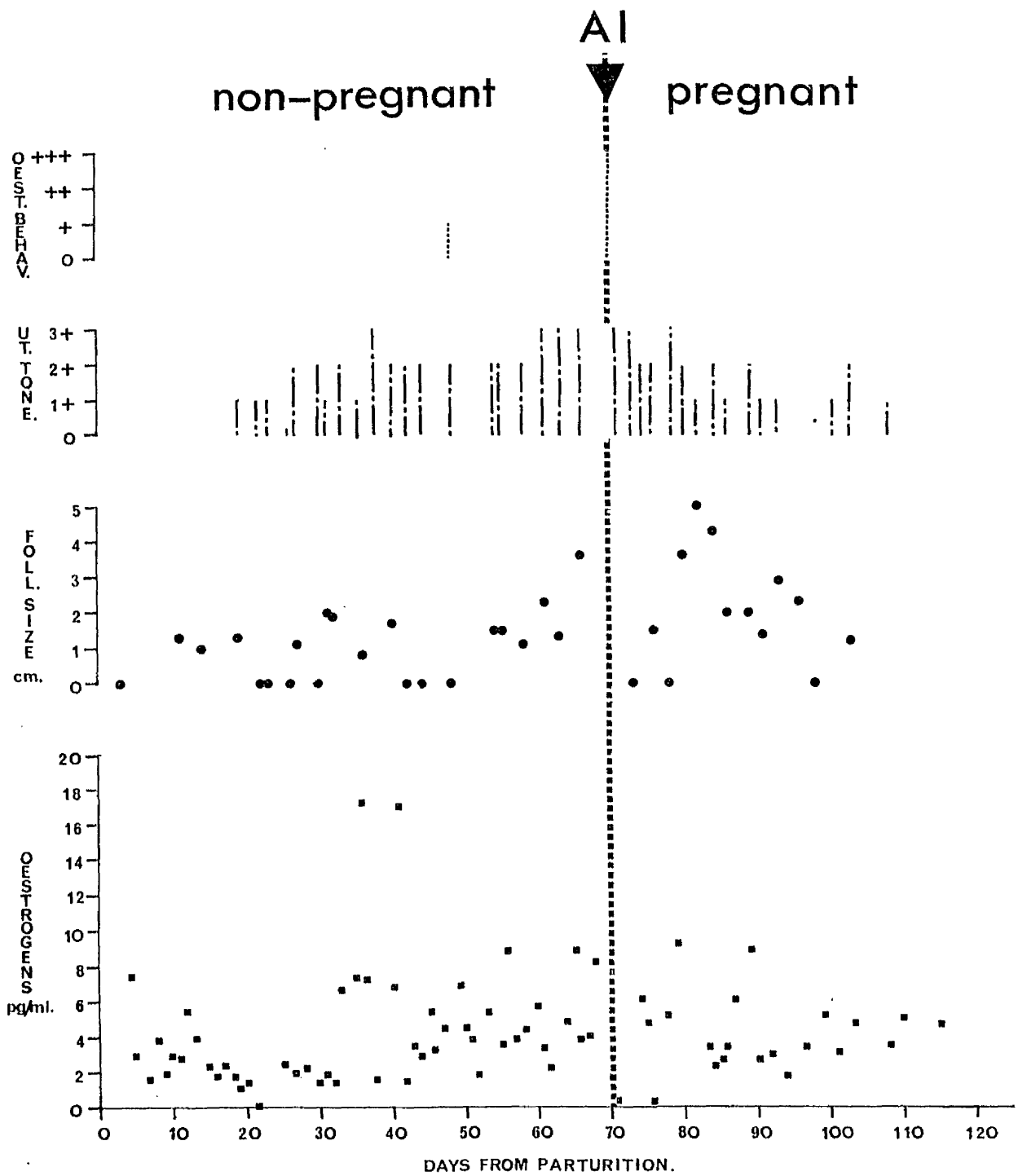


FIG 47 COW 5

LEVELS OF PERIPHERAL PLASMA OESTROGENS, PRESENCE OF FOLLICLES, UTERINE TONE, AND THE OCCURRENCE OF OESTROUS AND PERIOESTROUS BEHAVIOUR OVER THE COMPLETE PERIOD OF STUDY

Uterine tone

Over the experimental period significantly increased uterine tone was recorded on the majority of occasions on which rectal palpation was performed. (+++) turgidity was detected on day 38, from days 62 to 71 and again on day 78.

Plasma androgen levels

Against a background level of between 20 and 60 pg/ml, marked increases in the concentration of these hormones were noted on weeks 2 and 3, and again on weeks 9 and 10.

Uterine horn diameter

Between calving and day 22, uterine horn diameter decreased. Over the following month no further significant reduction from the size of 4.0 cm was recorded.

Body weight changes

A slight increase in the body weight of the cow was noted between days 17 and 75.

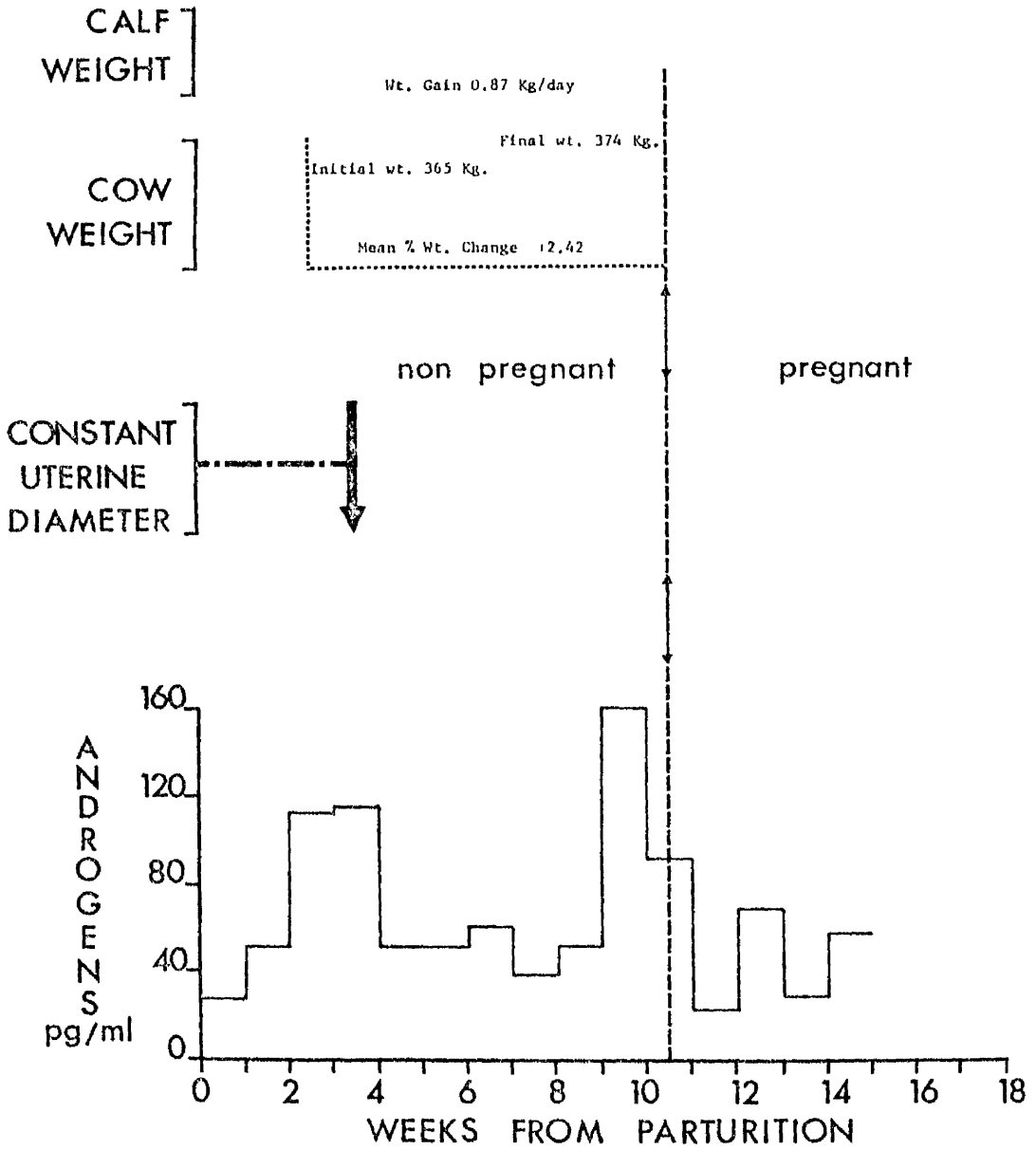


FIG 48 COW 5

PERIPHERAL PLASMA ANDROGEN LEVELS OVER THE COMPLETE PERIOD OF STUDY, THE TIME WHEN A CONSTANT UTERINE HORN DIAMETER WAS FIRST RECORDED AFTER CALVING, AND WEIGHT CHANGES IN THE COW AND CALF

Cow 7

The results for this cow are given in Figs 49 - 51.

Oestrous and perioestrous behaviour

It can be seen from the results that the first oestrus after calving occurred on day 32. Overt heat was again shown by this cow on day 64, on which day the animal was inseminated and subsequently confirmed as being pregnant. Prior to the first heat, interest was shown by the bull on days 10 and 26, and attempts were made to mount her, but she refused to stand, on days 30 and 31.

Plasma progesterone levels

Although over the period from calving to day 58 the great majority of levels of this hormone were basal, temporary increases were recorded. Not until after day 58, however, was the level increased to greater than 1.0 ng/ml. This elevation, from day 59, lasted until day 63 when basal values were again found. Following this, a marked increase occurred commencing on day 66 and reaching concentrations in excess of 2.0 ng/ml by day 71. After day 73 to the end of the experiment, the level was greater than 3.0 ng/ml on most occasions.

Corpora lutea

A corpus luteum was not detected in the ovaries until day 61. Following this a corpus luteum was recorded over the period from days 69 to 90 and again on day 102.

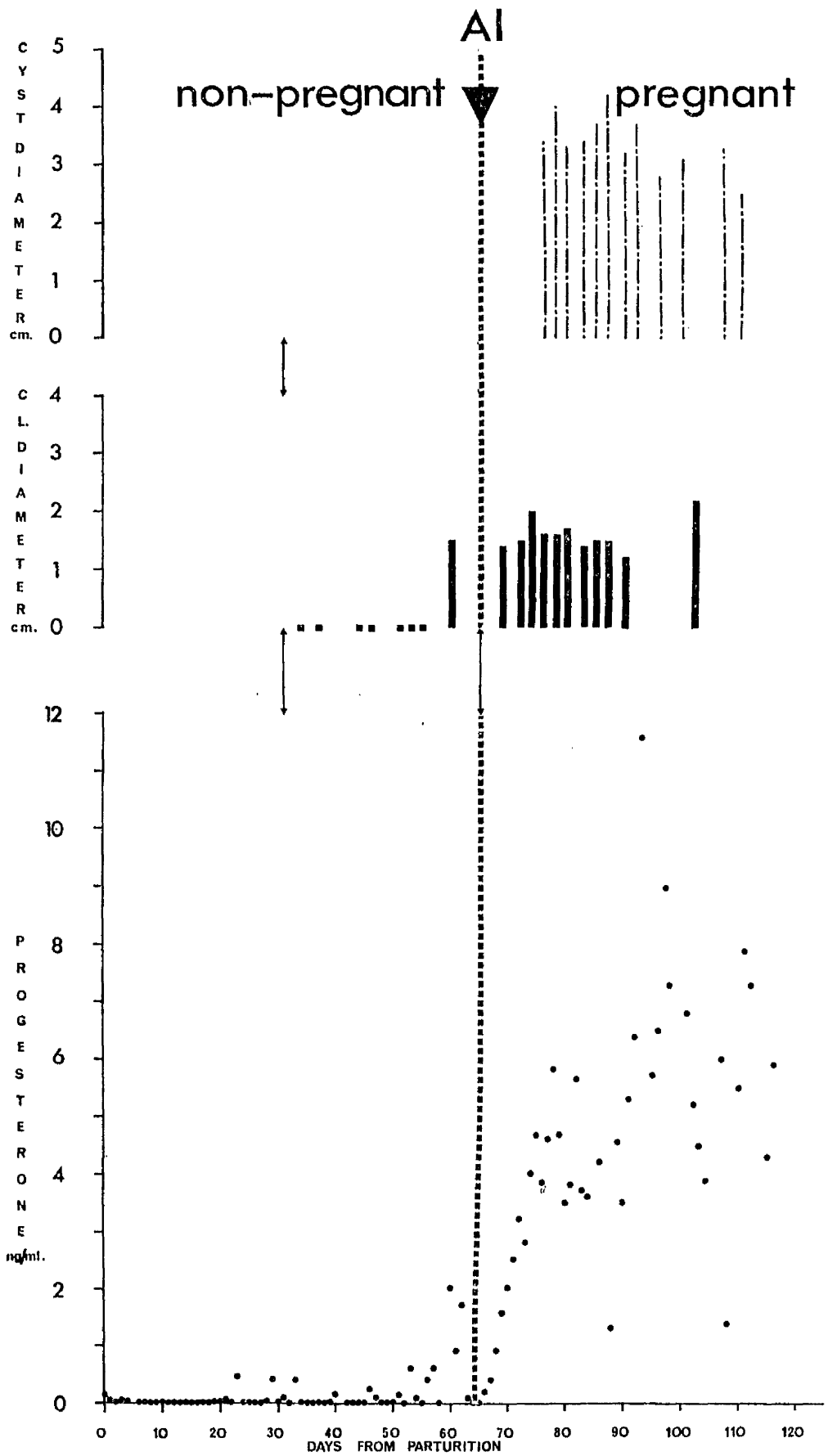


FIG 49 COW 7

PERIPHERAL PLASMA PROGESTERONE LEVELS, AND THE PRESENCE OF CORPORA LUTEA AND CYSTS OVER THE COMPLETE PERIOD OF STUDY

Cystic ovarian structures

A large cystic structure was found between days 76 and 102 and again from days 108 to 116.

Plasma oestrogen levels

Widely fluctuating levels of these hormones were recorded over the experimental period. With the exception of the very high levels recorded for 2 days after calving, elevated levels were found in the periods 27 to 38, 42 to 59, 60 to 76, 80 to 91, and 105 to 109 days. Peak concentrations within these latter times ranged from 9 to 20 pg/ml.

Follicles

Two days after calving no follicles were present in the ovaries. On day 12, however, follicles with a total surface diameter of about 1.0 cm were recorded. From day 19 to the end of the experiment, in twentyfive out of a total of thirty examinations, follicles in excess of 1.0 cm diameter were detected. Maximum follicular size of around 3.0 cm or more was found between days 72 and 100.

Uterine tone

(+++) uterine tone was recorded on days 65 and 66. On more occasions prior to this time uterine tone was greater than it was between day 66 and the end of the experiment.

Plasma androgen levels

With the exception of weeks 2 to 3 and 12 to 13, the levels of

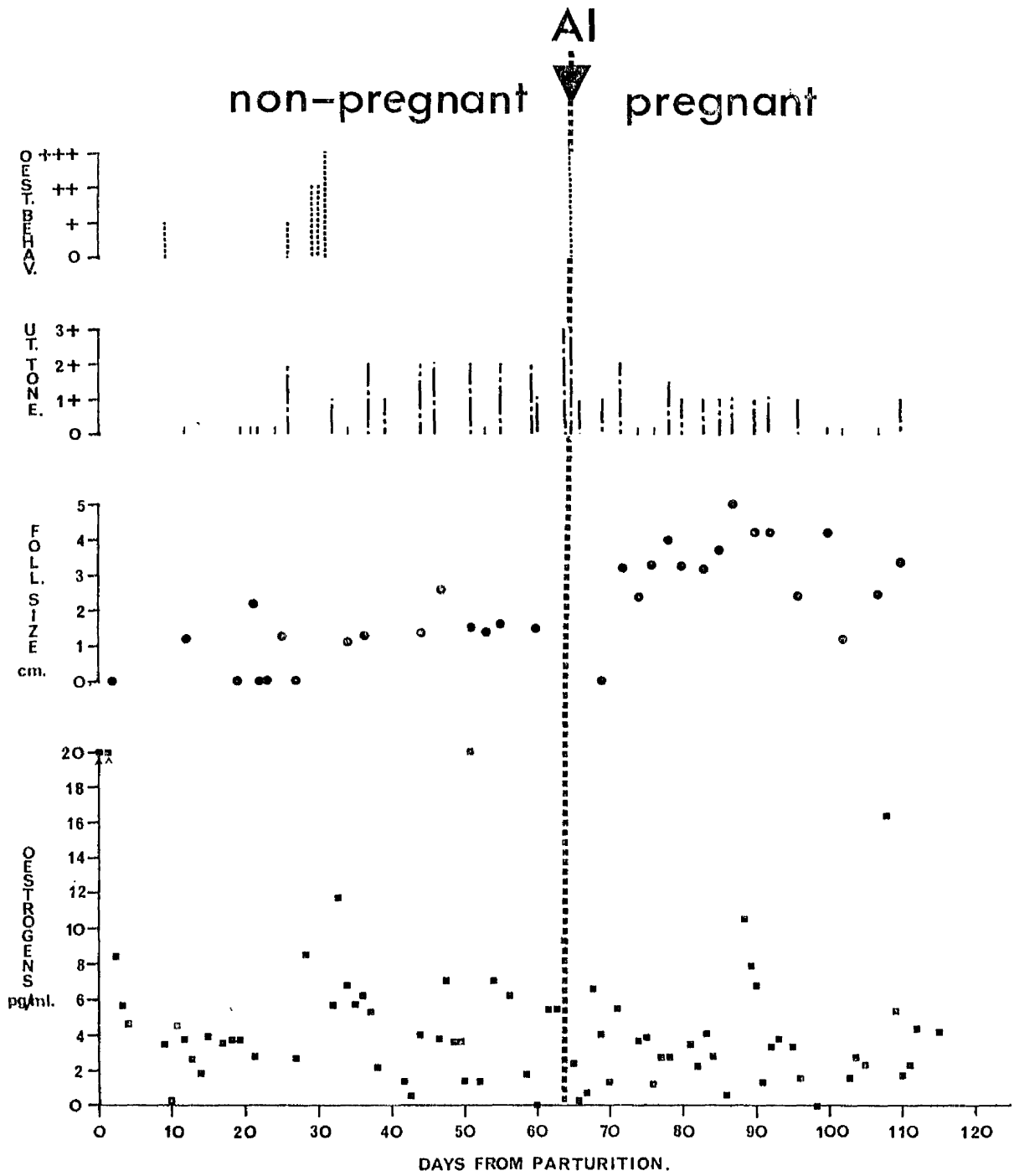


FIG 50 COW 7

LEVELS OF PERIPHERAL PLASMA OESTROGENS, PRESENCE OF FOLLICLES, UTERINE TONE, AND THE OCCURRENCE OF OESTROUS AND PERIOESTROUS BEHAVIOUR OVER THE COMPLETE PERIOD OF STUDY

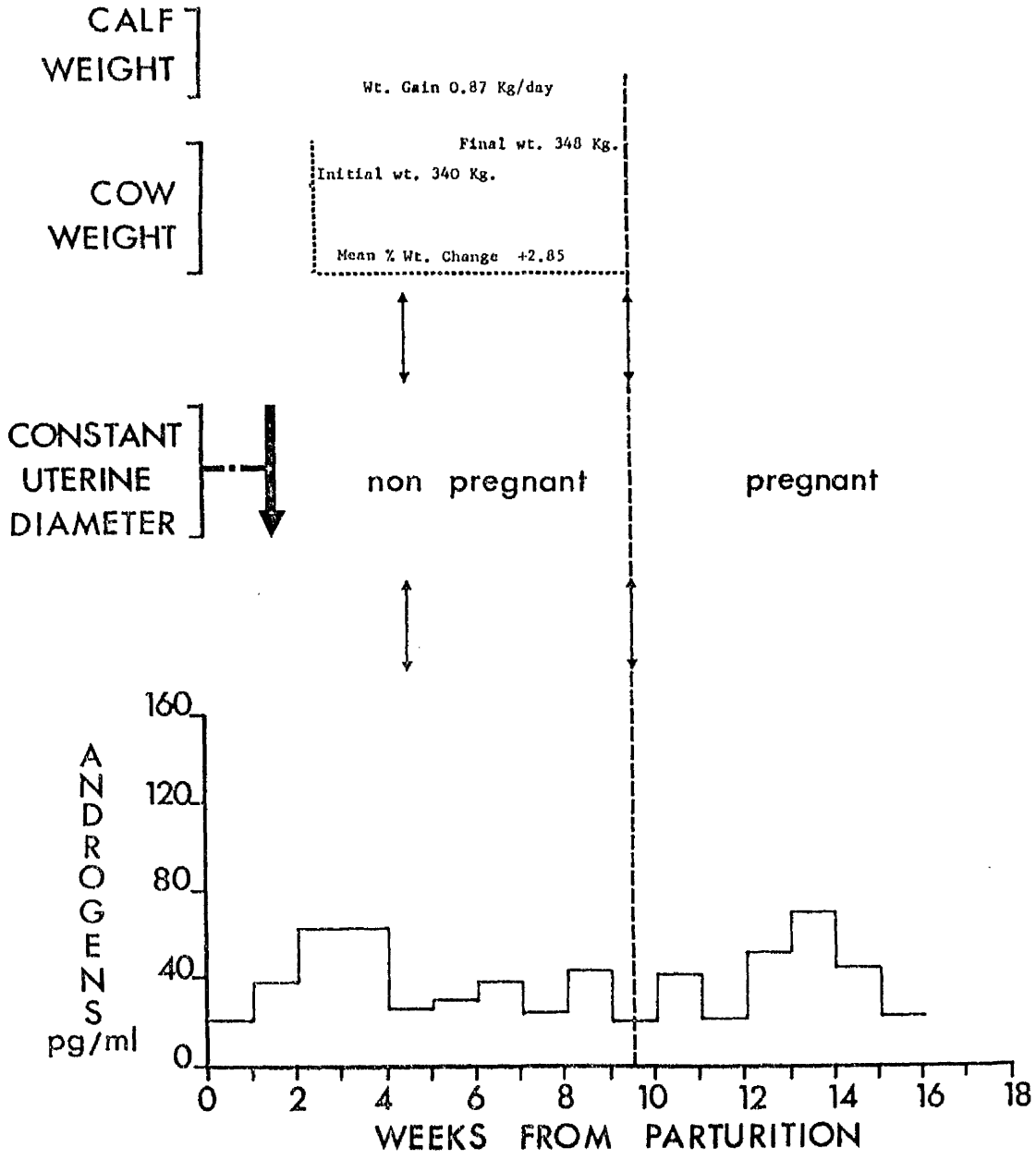


FIG 51 COW 7

PERIPHERAL PLASMA ANDROGEN LEVELS OVER THE COMPLETE PERIOD OF STUDY, THE TIME WHEN A CONSTANT UTERINE HORN DIAMETER WAS FIRST RECORDED AFTER CALVING, AND WEIGHT CHANGES IN THE COW AND CALF

these hormones remained around 20 to 40 pg/ml throughout the experiment. Increases in concentration to 63 pg/ml and to 69 pg/ml occurred on weeks 3 and 13 respectively.

Uterine horn diameter

A consistent uterine horn diameter of 4.0 cm was reached 12 days after calving.

Body weight changes

Weekly weighing over the period from 16 to 66 days showed a slight net increase in the weight of the cow.

Cow 8

The results for this animal are given in Figs 52 - 54.

Oestrous and perioestrous behaviour

Following some interest by the bull in the cow on day 44, overt heat was shown for the first time 46 days after calving. This animal returned to oestrus again on day 89, was inseminated, and a pregnancy established.

Plasma progesterone levels

With the exception of a temporary increase to 0.9 ng/ml on day 27, basal levels of this hormone were recorded between days 0 and 83. Following day 83 increased concentrations were found from day 84 to 88 and again, after 4 days of basal values, from day 92 to the end of the experiment. During the second sustained period of increased values, levels greatly in excess of those during the first were detected.

Corpora lutea

Following a period to day 36 when no structure of this nature was present in the ovaries, a small corpus luteum of about 0.5 cm in diameter was detected on days 38 and 41. By day 43 this had regressed and it was not until day 87 that the next corpus luteum was found. This was followed by another corpus luteum on day 94 and this structure continued to be present to the end of the experiment.

Cystic ovarian structures

No cystic structures were found in this animal at any time during the experiment.

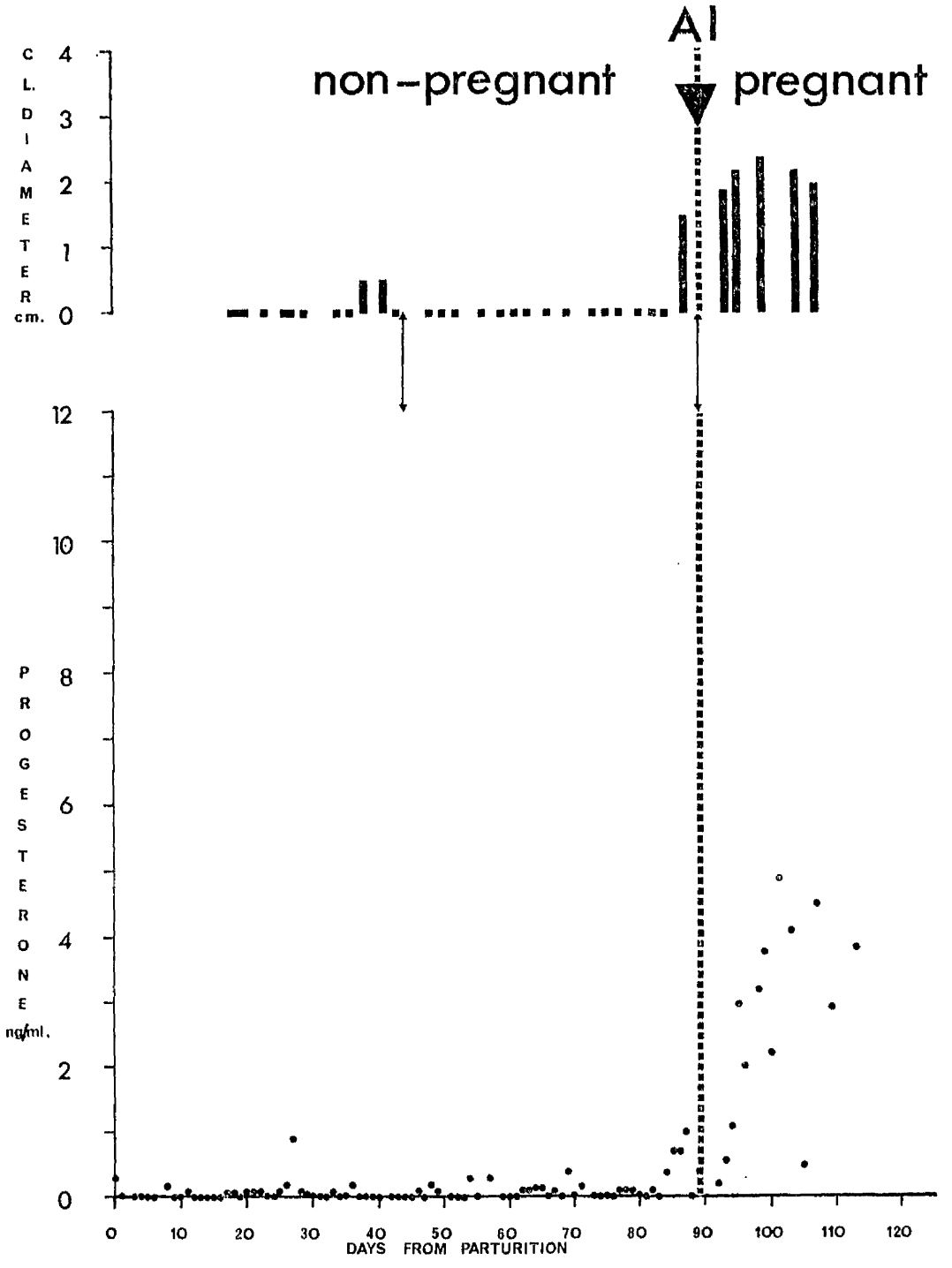


FIG 52 COW 8

PERIPHERAL PLASMA PROGESTERONE LEVELS, AND THE PRESENCE OF CORPORA LUTEA AND CYSTS OVER THE COMPLETE PERIOD OF STUDY

Plasma oestrogen levels

Levels of these hormones in excess of 20 pg/ml were recorded between days 0 and 4. By day 6 the concentration had fallen and during the remainder of the experiment several peaks were detected. Against a background where the great majority of values was 4.0 pg/ml or less, peaks of greater than 6.0 pg/ml were recorded within the times 24 to 26, 31 to 36, 47 to 49, 61 to 63, 71 to 73, 81 to 84, 85 to 87, and 108 to 112 days.

Follicles

Although follicles were present with a total surface diameter of 1.0 cm or more on the majority of occasions up to day 87, at only one time did the diameter exceed 2.0 cm. Between days 89 and 104 no follicles were detected in the ovaries.

Uterine tone

(+++) uterine tone was recorded only on days 89 and 90. However (++) uterine tone was present at intervals throughout the experiment.

Plasma androgen levels

Over the experimental period wide fluctuations in the concentration of these hormones were recorded. Against a background level of approximately 40 - 60 pg/ml, marked increases were found on weeks 1, 5 to 8, 11, and from 13 to the end of the experiment.

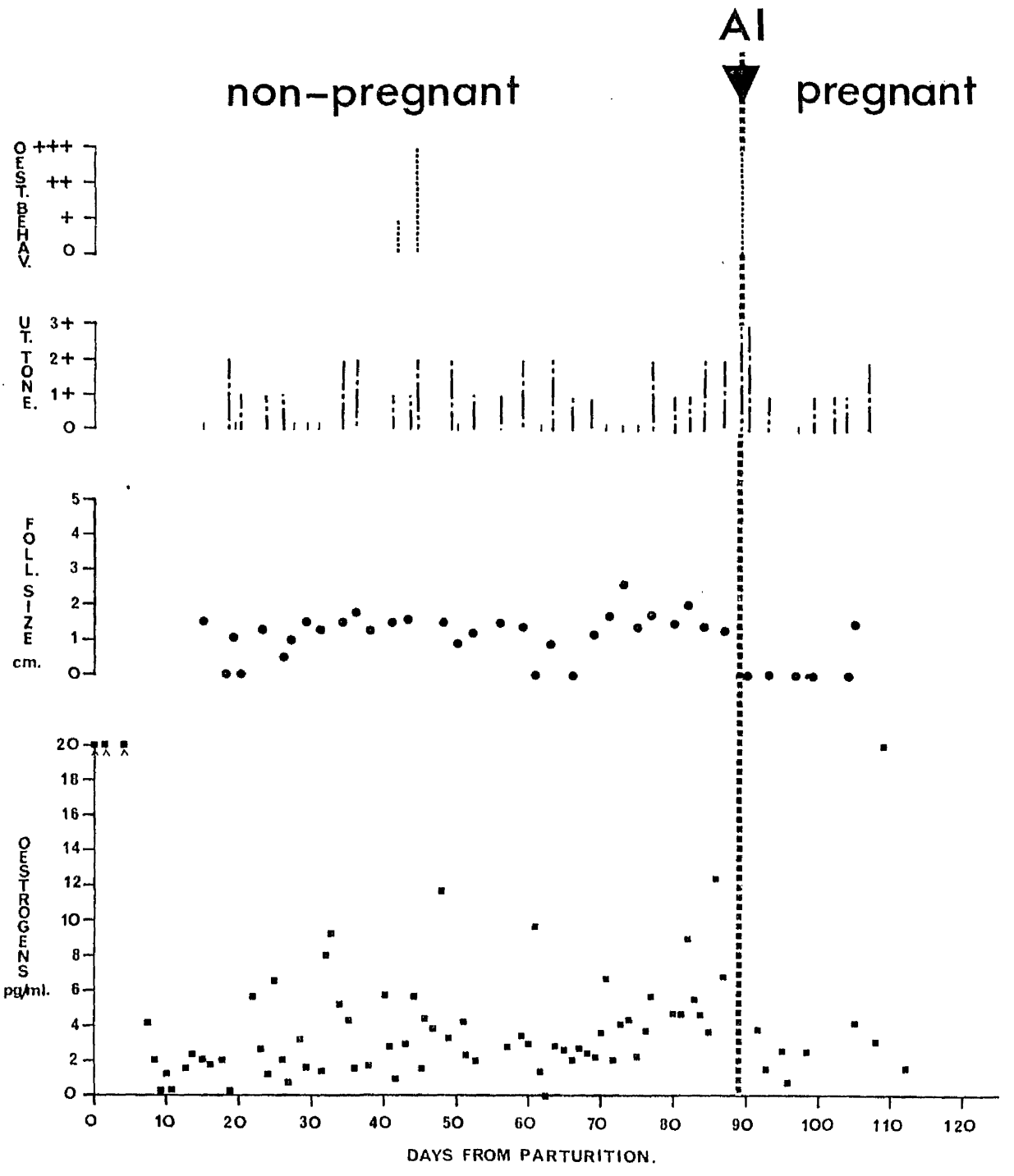


FIG 53 COW 8

LEVELS OF PERIPHERAL PLASMA OESTROGENS, PRESENCE OF FOLLICLES, UTERINE TONE, AND THE OCCURRENCE OF OESTROUS AND PERIOESTROUS BEHAVIOUR OVER THE COMPLETE PERIOD OF STUDY

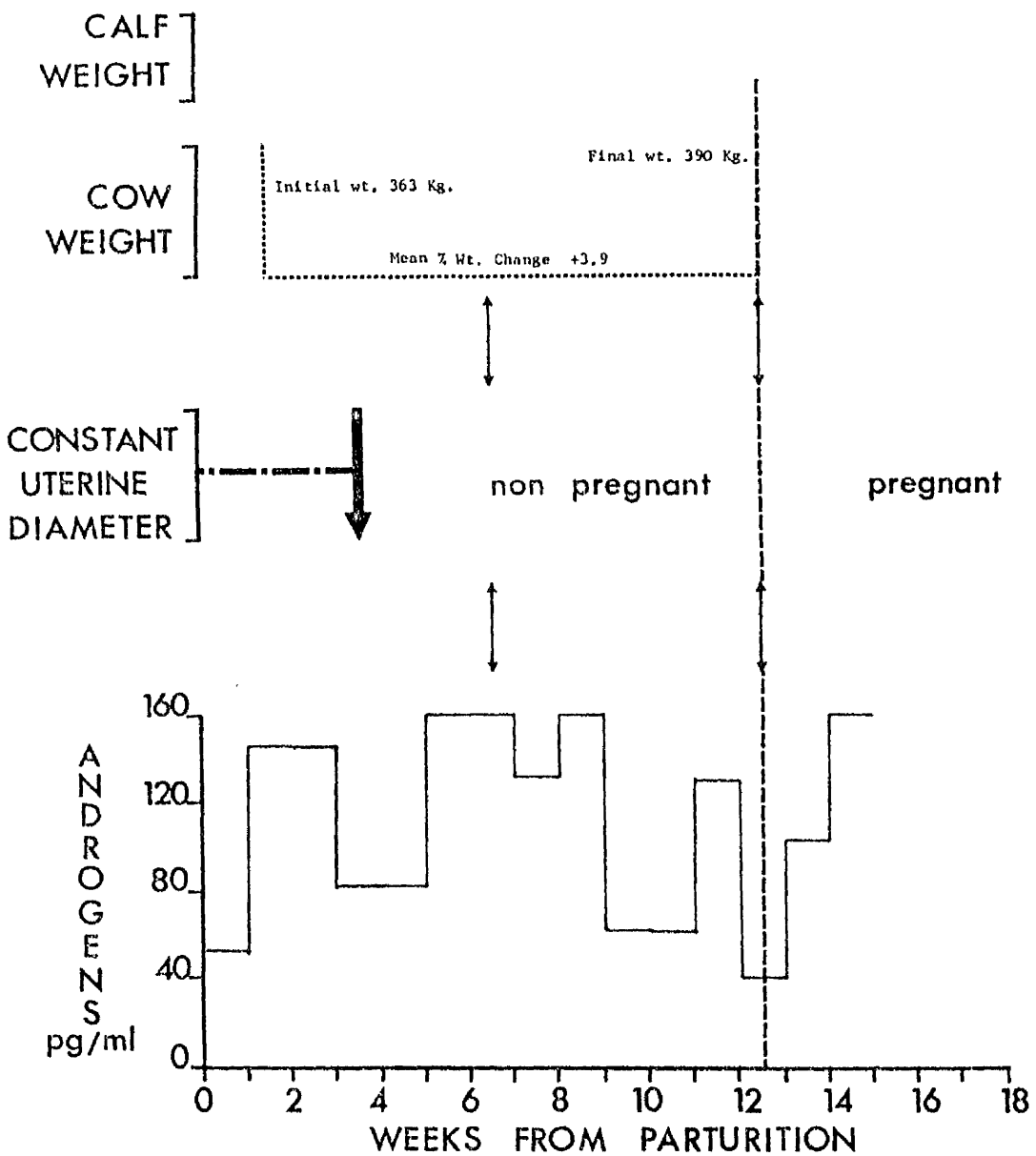


FIG 54 COW 8

PERIPHERAL PLASMA ANDROGEN LEVELS OVER THE COMPLETE PERIOD OF STUDY, THE TIME WHEN A CONSTANT UTERINE HORN DIAMETER WAS FIRST RECORDED AFTER CALVING, AND WEIGHT CHANGES IN THE COW AND CALF

Uterine horn diameter

By 23 days after parturition, uterine horn diameter was 3.0 cm. No further significant decrease in size was recorded.

Body weight changes

Over the period from 12 to 84 days after calving, body weight of the dam increased. Due to the rapid increase in size of the calf over this period, it could not be accommodated in the weighing arrangements available and therefore a figure for the mean weight gain was not obtained.

Additional comments

On day 38 this cow's calf was observed to have a fracture of the radius. This was immobilised by a plaster of paris cast and the calf returned to its dam. No adverse effects were noted over the recovery period.

Cow 9

The results for this animal are given in Figs 55 - 57.

Oestrous and perioestrous behaviour

No behavioural change of this nature was observed during the 114 days after calving on which this animal was studied. In the absence of oestrous behaviour, artificial insemination was not carried out.

Plasma progesterone levels

With the exception of transient elevations to 0.6, 0.7 and 0.8 ng/ml on days 28, 62 and 77 respectively, basal levels of this hormone were recorded during the remainder of the experiment.

Corpora lutea

Although a structure with a surface diameter 0.5 cm was palpated on day 33 and classified as a corpus luteum, this was not detected on re-examination on the following days, but was recorded as being present again on day 37. With these two exceptions, no corpora lutea were detected over the experimental period.

Cystic ovarian structures

No structures of this nature were found.

Plasma oestrogen levels

Following a transient increase to 7.5 pg/ml on day 18, a period

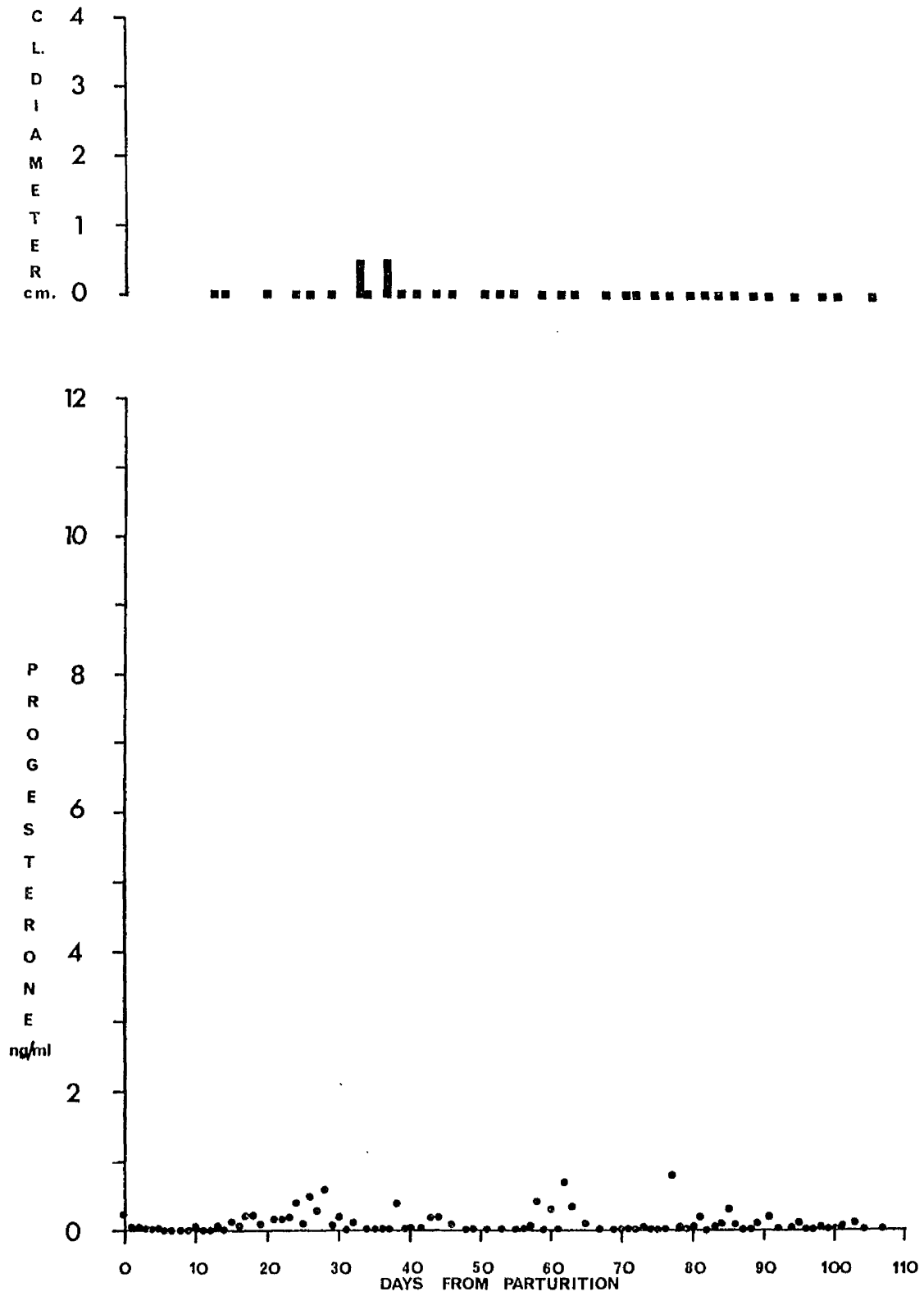


FIG 55 COW 9

PERIPHERAL PLASMA PROGESTERONE LEVELS, AND THE PRESENCE OF CORPORA LUTEA AND CYSTS OVER THE COMPLETE PERIOD OF STUDY

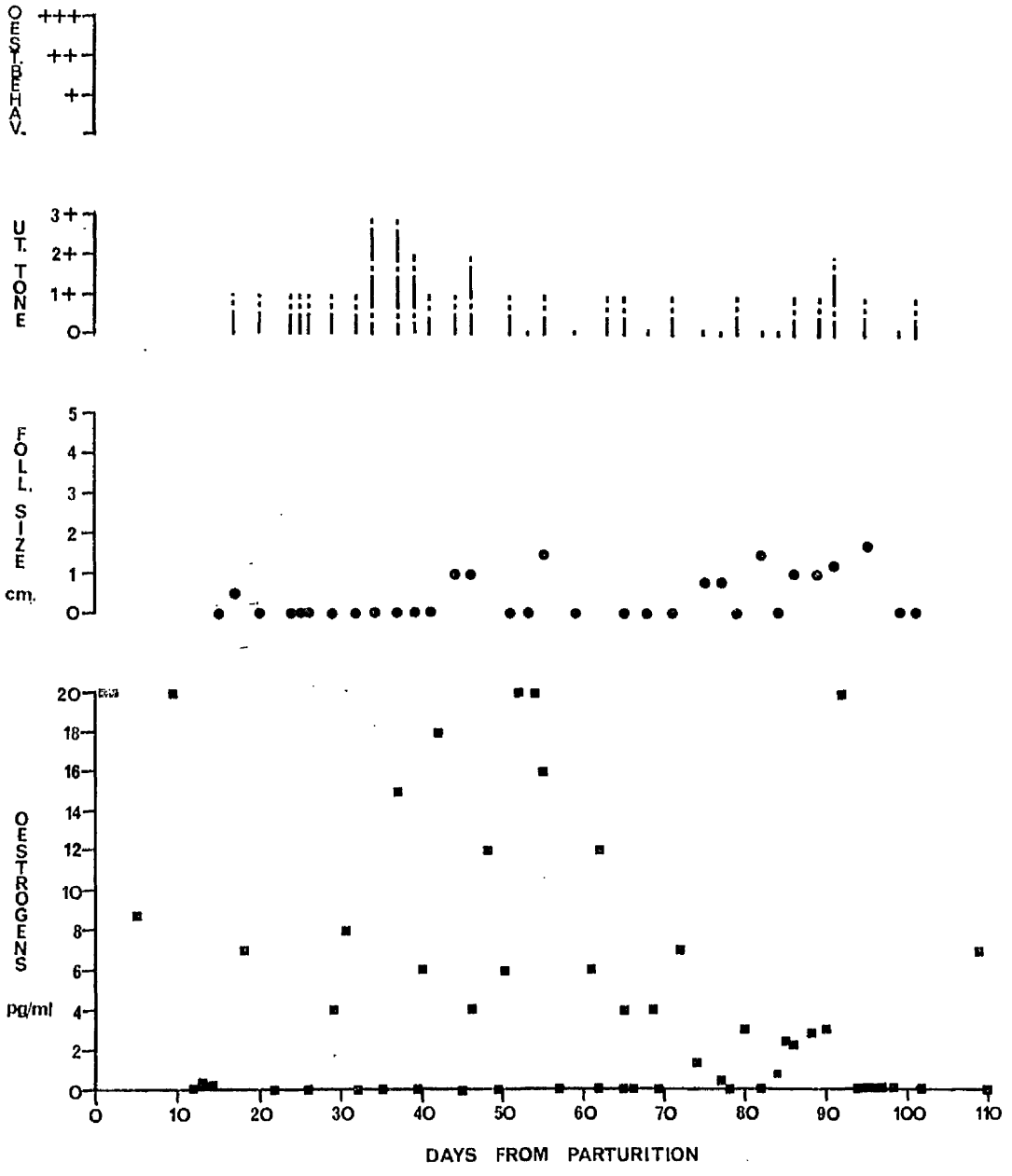


FIG 56 COW 9

LEVELS OF PERIPHERAL PLASMA OESTROGENS, PRESENCE OF FOLLICLES, UTERINE TONE, AND THE OCCURRENCE OF OESTROUS AND PERIOESTROUS BEHAVIOUR OVER THE COMPLETE PERIOD OF STUDY

of elevated oestrogen levels was recorded between days 40 and 57. Within this time maximum concentrations were found on days 52 and 54. A second marked increase in the levels of these hormones was detected with a peak level of 20.0 pg/ml on day 92.

Follicles

On the majority of occasions this animal was examined no follicles were palpated in the ovaries. A follicle of 0.5 cm diameter was recorded 17 days after calving. However, in addition, follicles with a total surface diameter of 1.0 cm or more were recorded on days 44 - 46, 55, 82 and between days 86 and 95. Over the experimental period the maximum follicular diameter observed was 1.7 cm on day 95.

Uterine tone

On most occasions the animal was examined a degree of uterine tone was noted. Although this was generally only slight (+), on three occasions (++) tone was recorded, and on days 34 and 37 marked (+++) tone was present.

Plasma androgen levels

A marked elevation in the level of these hormones was noted on week 2 after calving. At all other times the levels of these hormones were very much less, lying between 20 - 60 pg/ml.

Uterine horn diameter

A constant uterine horn diameter of approximately 4.0 cm was detected for the first time 25 days after calving.

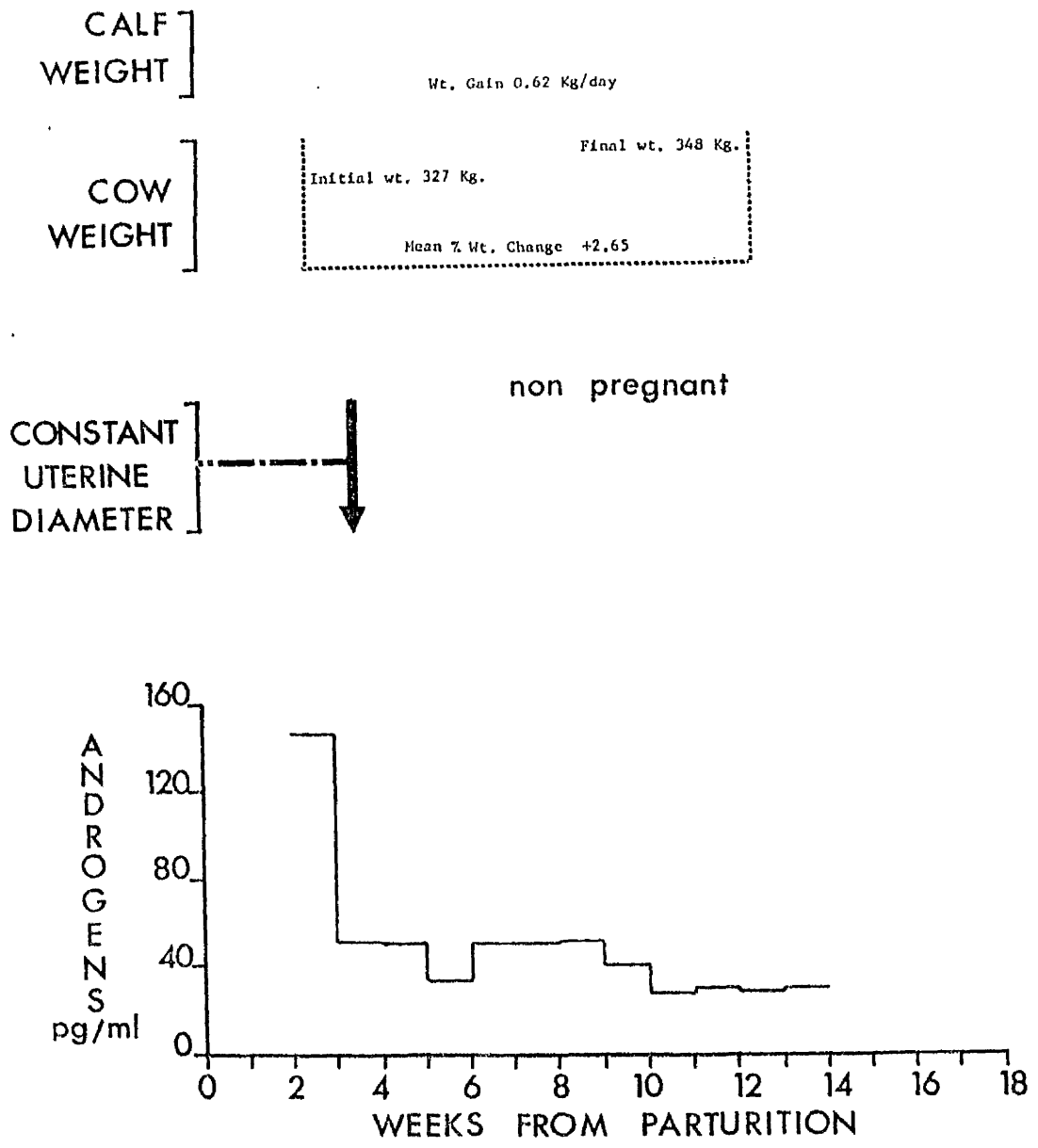


FIG 57 COW 9

PERIPHERAL PLASMA ANDROGEN LEVELS OVER THE COMPLETE PERIOD OF STUDY, THE TIME WHEN A CONSTANT UTERINE HORN DIAMETER WAS FIRST RECORDED AFTER CALVING, AND WEIGHT CHANGES IN THE COW AND CALF

Body weight changes

Over the period from 15 - 87 days after calving the body weight of the cow showed a net increase of 2.65%. Over the same period its calf exhibited an average weight gain of 0.62 kg/day.

Cow 10

The results for this animal are given in Figs 58 - 60.

Oestrous and perioestrous behaviour

Although the bull attempted to mount the cow on day 34, she would not stand. Following this on days 42 to 43 and on day 51, some interest was shown by the bull but overt oestrus was not seen until day 52. The cow was again observed in standing heat on day 72, and was inseminated on this day and subsequently proved to be pregnant.

Plasma progesterone levels

Basal levels of this hormone were found until day 47. Between 47 and 52, 56 and 72, and from 74 days to the end of the experiment, progesterone levels were increased. Maximum levels at the time of the first of these increases were, however, considerably less than those found later.

Corpora lutea

A corpus luteum was detected for the first time after calving on days 47 and 50. Thereafter a structure of this nature was present between days 55 and 70. Again from day 80 a corpus luteum was present in the ovaries. With the exception of days 86, 93 and 102 a corpus luteum was palpable until the end of the experiment.

Cystic ovarian structures

Between days 80 and 95, 98 and 102, and again on day 107, structures which were classified as cysts were present in the ovaries.

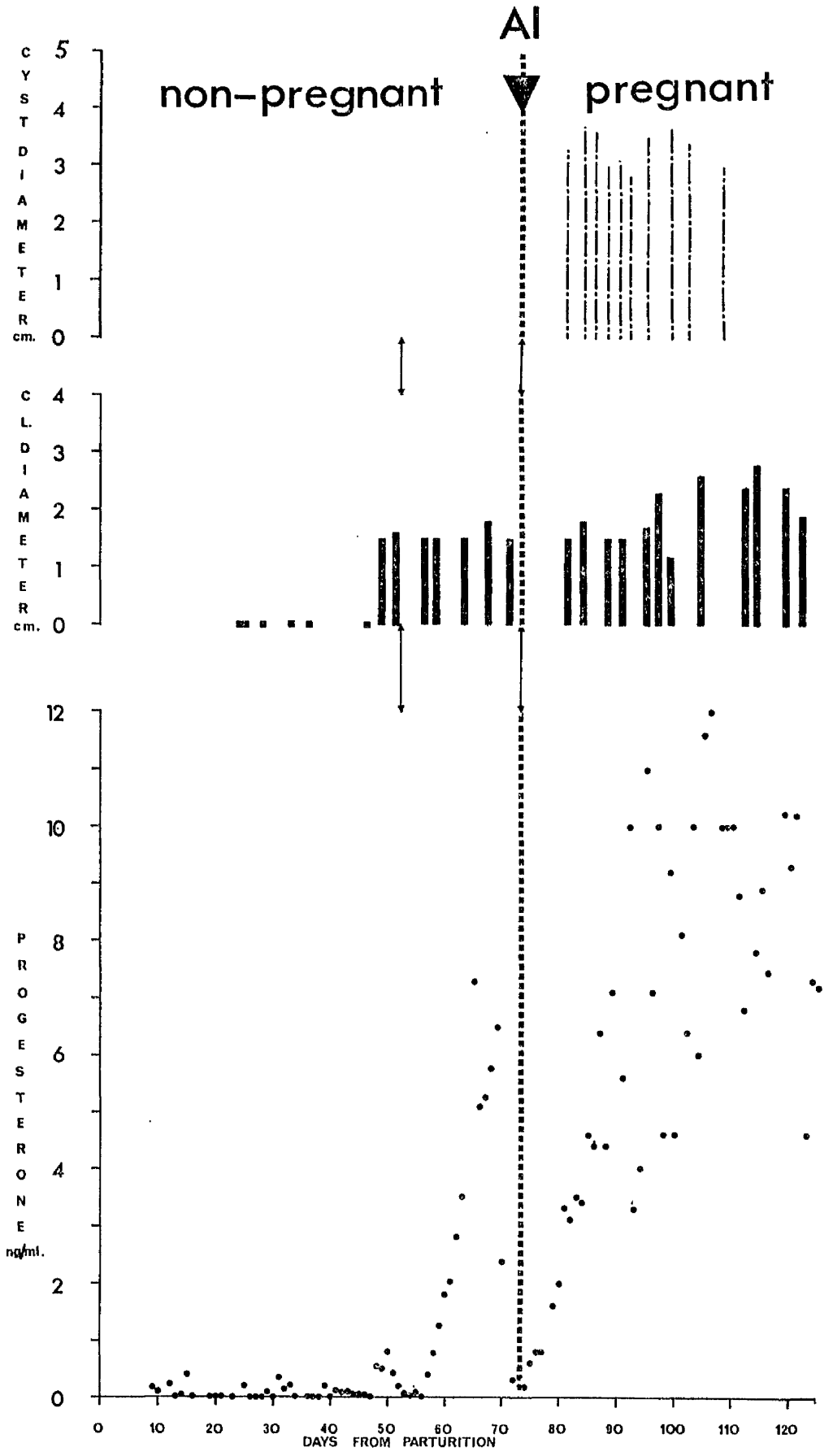


FIG 58 COW 10

PERIPHERAL PLASMA PROGESTERONE LEVELS, AND THE PRESENCE OF CORPORA LUTEA AND CYSTS OVER THE COMPLETE PERIOD OF STUDY

Plasma oestrogen levels

Widespread fluctuations in the concentration of these hormones occurred during the experiment but the majority of results was less than 6.0 pg/ml. Prior to day 45 only slight elevations above this level were recorded on days 10, 15 and 20. In contrast after day 45 peaks ranging in magnitude from 8 to more than 20 pg/ml were detected on days 46, 52, 65, 69, 75, 96 and 102.

Follicles

Follicles were not detected until 26 days after calving. Between this time and day 102, with the exception of days 57 and 98, follicles with a total surface diameter of between 1.0 and 4.0 cm were found. However, between day 104 and the end of the experimental period only on day 114 were follicles detected in the ovaries.

Uterine tone

(+++) uterine tone was recorded on days 50 and 56, and again on day 77. Immediately before and after each of these occasions uterine tone was (++). Outwith this overall period on only one occasion, between days 99 and 103, was a similar degree of uterine turgidity noted.

Plasma androgen levels

Within weeks 1 to 4 after calving, levels of these hormones were around 60 - 70 pg/ml. Following a marked decline to 25 pg/ml on week 5, several periods of increased concentrations of these hormones,

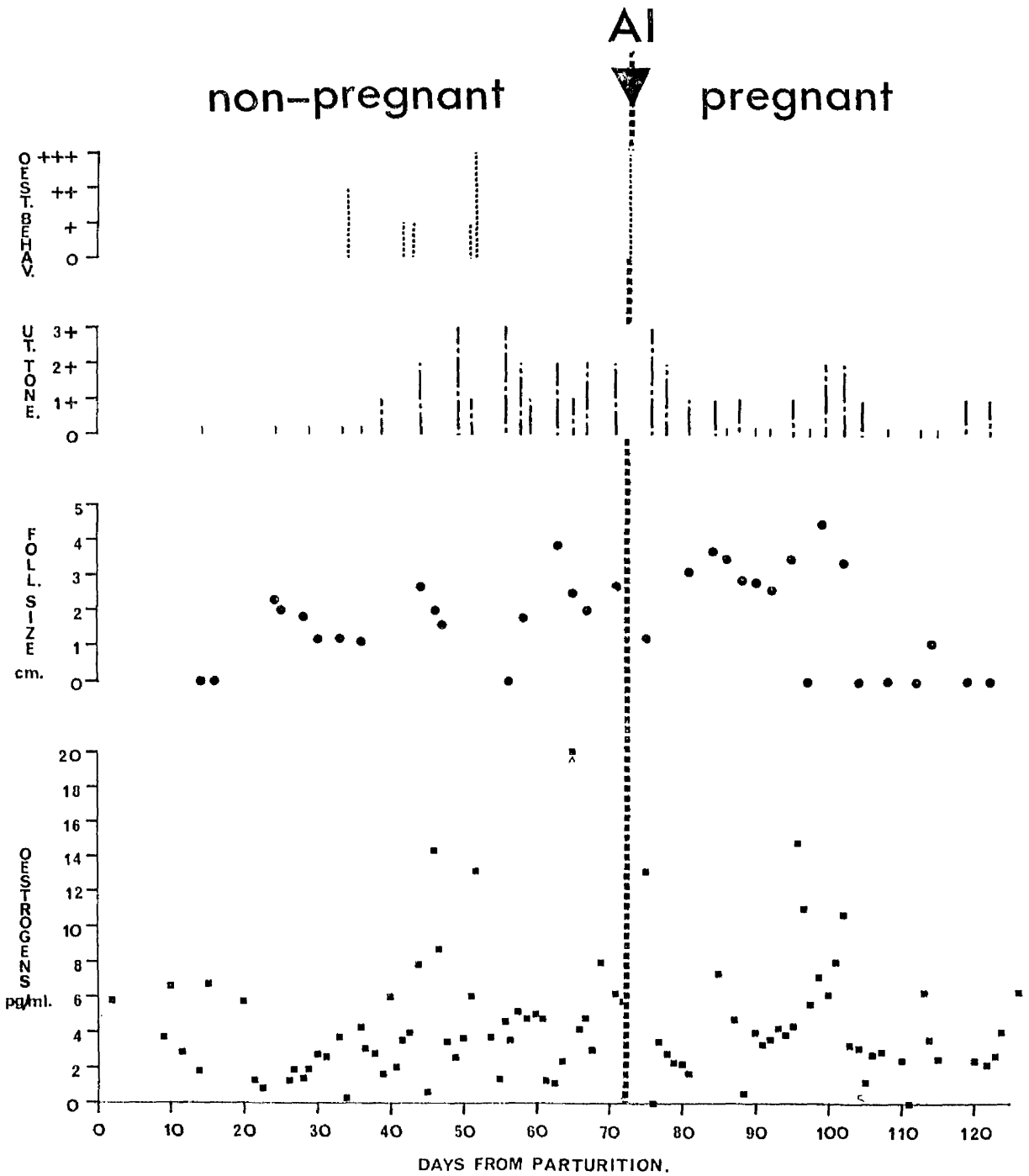


FIG 59 COW 10

LEVELS OF PERIPHERAL PLASMA OESTROGENS, PRESENCE OF FOLLICLES, UTERINE TONE, AND THE OCCURRENCE OF OESTROUS AND PERIOESTROUS BEHAVIOUR OVER THE COMPLETE PERIOD OF STUDY

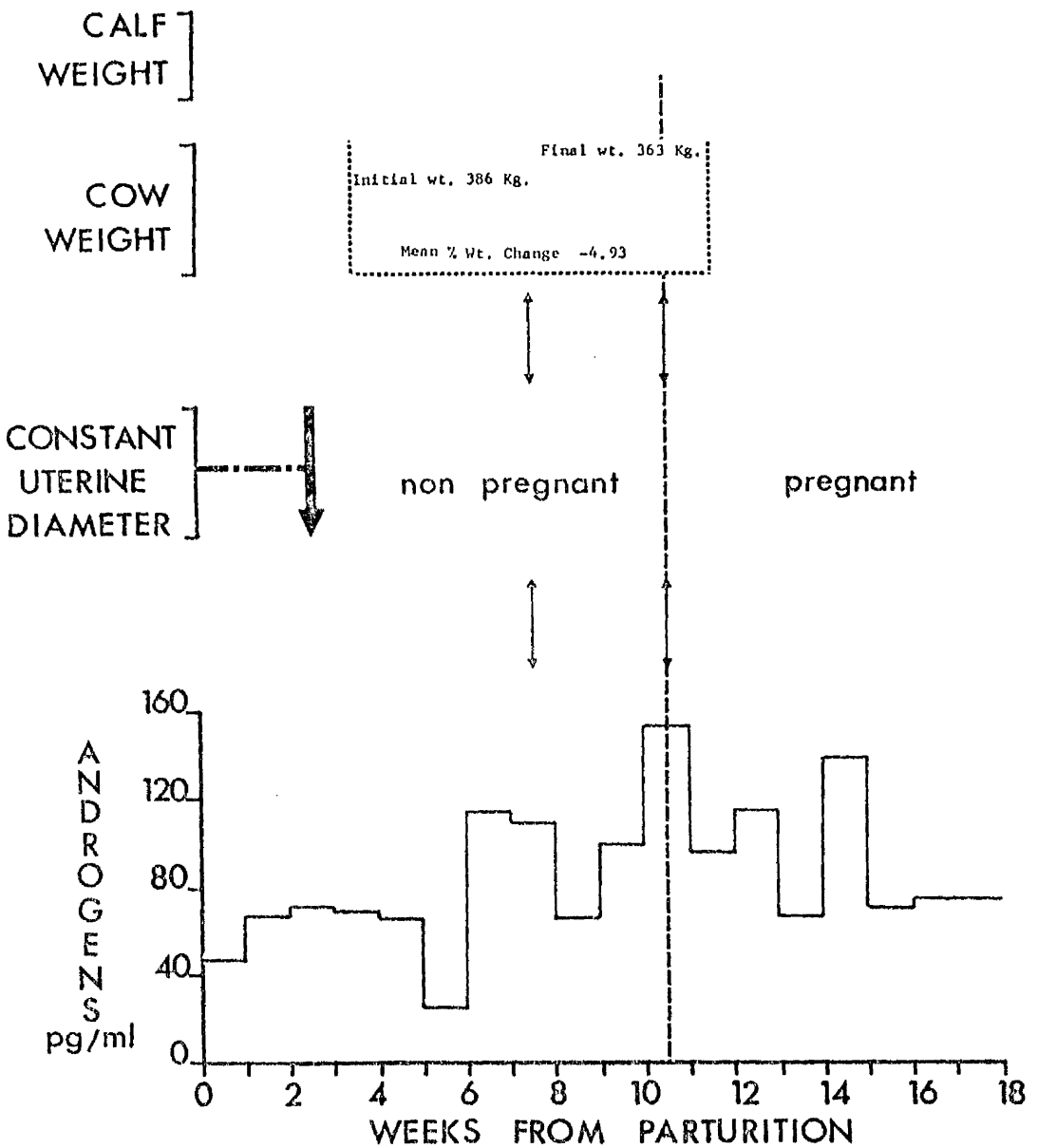


FIG 60 COW 10

PERIPHERAL PLASMA ANDROGEN LEVELS OVER THE COMPLETE PERIOD OF STUDY, THE TIME WHEN A CONSTANT UTERINE HORN DIAMETER WAS FIRST RECORDED AFTER CALVING, AND WEIGHT CHANGES IN THE COW AND CALF

in excess of the values previously found, were recorded. Maximum values of 152 pg/ml and 140 pg/ml were found on weeks 10 and 14 respectively.

Uterine horn diameter

A constant uterine horn diameter of 4.0 cm was recorded for the first time 14 days after parturition.

Body weight changes

Over the period 27 to 77 days post-partum, the body weight of the cow was observed to decrease by 23 kg. Due to the rapid growth rate of the calf, it was not possible to carry out weekly weighing over this time. A figure for the weight gain of the calf is therefore not available.

Cow 11

The results for this animal are given in Figs 61 - 63.

Oestrous and perioestrous behaviour

A slight degree of interest was shown by the bull in the cow on day 54. Overt heat did not, however, occur until 70 days after calving. Oestrus at this time lasted over 2 days. Following this, the cow returned to heat on day 78. Pregnancy resulted from artificial insemination on this day.

Plasma progesterone levels

Until day 72 progesterone levels were basal with the exception of a transient increase to 1.0 ng/ml recorded on day 65. Increased levels of this hormone were then found between 73 and 80 days, and from 82 days to the end of the experiment. Although peak levels during the first of these periods reached values between 1.1 - 2.3 ng/ml, during the second the levels recorded from day 87 were all in excess of 3.5 ng/ml.

Corpora lutea

A corpus luteum was recorded for the first time at 76 and 78 days after calving. Following this, a luteal structure was detected on day 91 and again on days 111 to 121. No marked size difference was apparent among any of these structures.

Cystic ovarian structures

A cystic structure was found in the ovaries between days 89 and 104 and again on day 118.

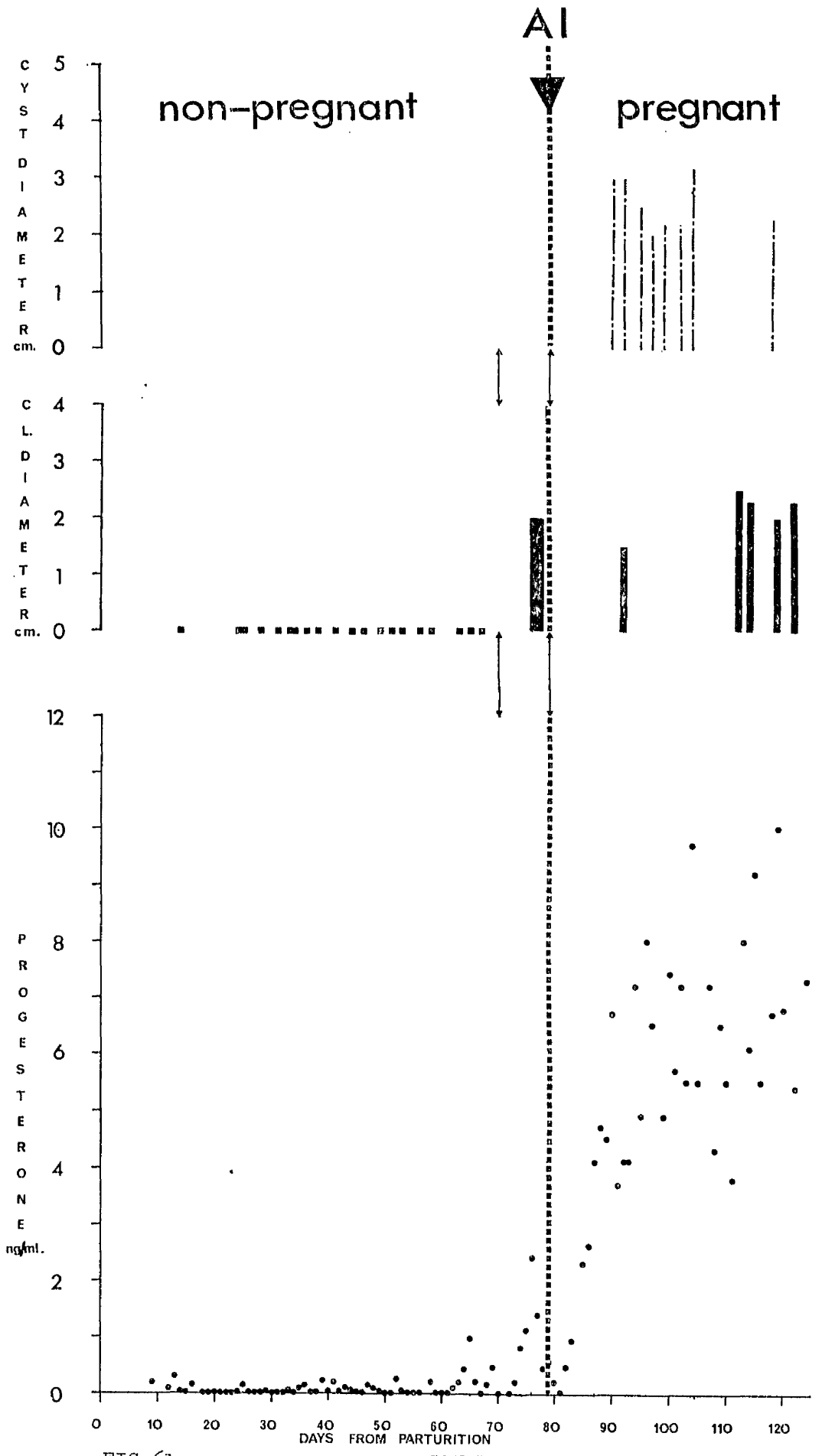


FIG 61 COW 11

PERIPHERAL PLASMA PROGESTERONE LEVELS, AND THE PRESENCE OF CORPORA LUTEA AND CYSTS OVER THE COMPLETE PERIOD OF STUDY

Plasma oestrogen levels

Widely fluctuating levels of these hormones with the great majority of values being 6.0 pg/ml or less were recorded over the experimental period. Peaks in excess of this level were detected on days 31, 40, 46, 54 and 58. A further four peaks with levels ranging from 7.5 to 13.0 pg/ml were recorded within the period 63 - 74 days. In the latter part of the experimental period a further two elevations above 6.0 pg/ml were found on days 99 and 122.

Follicles

Over the experimental period follicles were detected on all but five occasions. Maximum follicular surface diameters were found during the second half of the experiment reaching sizes from 3.0 to 5.0 cm between days 78 and 96.

Uterine tone

(+++) uterine tone was recorded on days 65, 73 and 81. Outwith these times, marked, but only (++), uterine turgidity was mainly restricted to the period from 53 to 93 days.

Plasma androgen levels

Marked fluctuations in the levels of these hormones were recorded over the period of study. Within the period of markedly increased concentrations, levels ranging from 109 pg/ml on week 15 to more than 160 pg/ml on weeks 4, 11 and 12 were observed. Between these periods the level dropped to around 60 - 70 pg/ml.

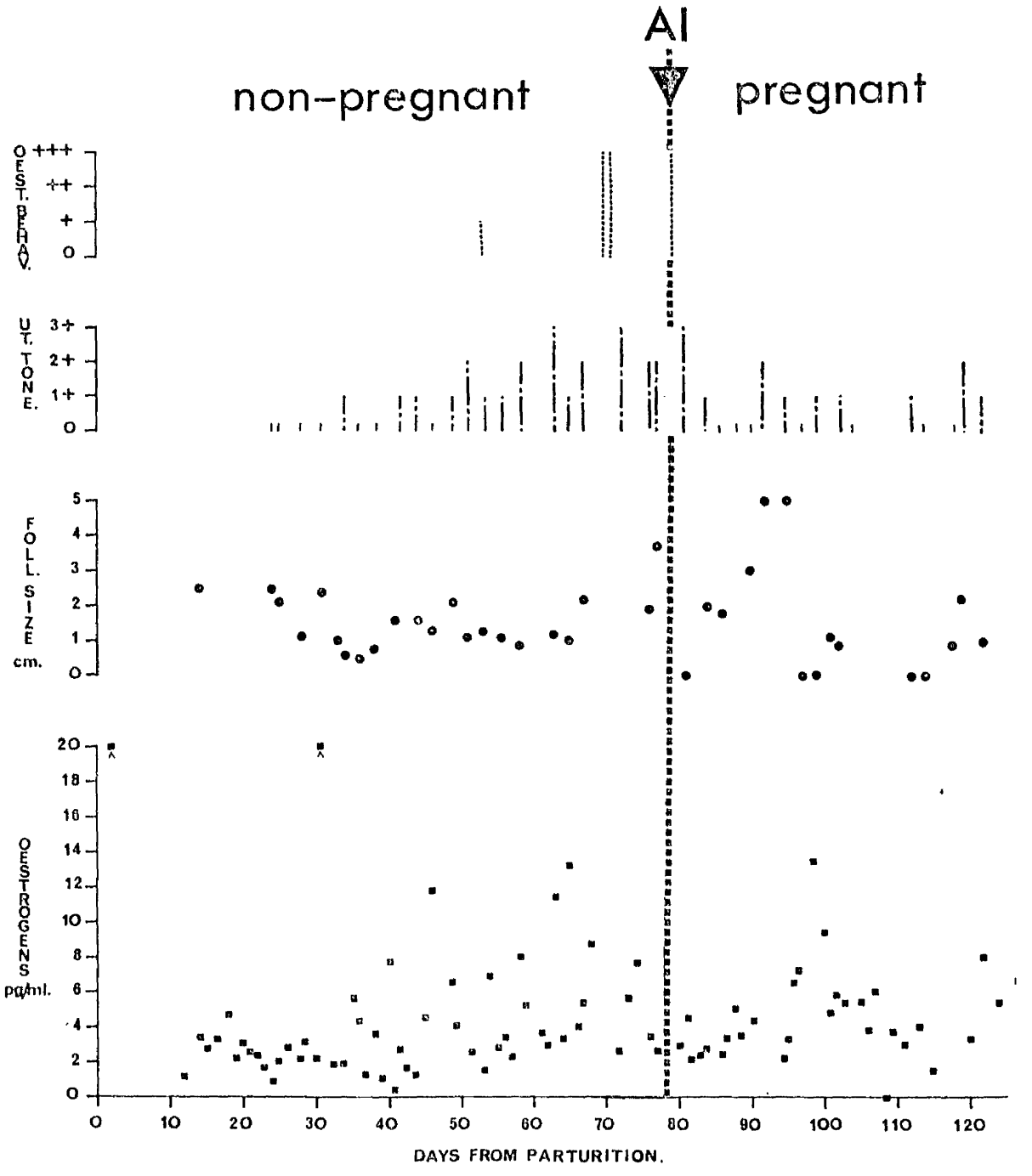


FIG 62 COW 11

LEVELS OF PERIPHERAL PLASMA OESTROGENS, PRESENCE OF FOLLICLES, UTERINE TONE, AND THE OCCURRENCE OF OESTROUS AND PERIOESTROUS BEHAVIOUR OVER THE COMPLETE PERIOD OF STUDY

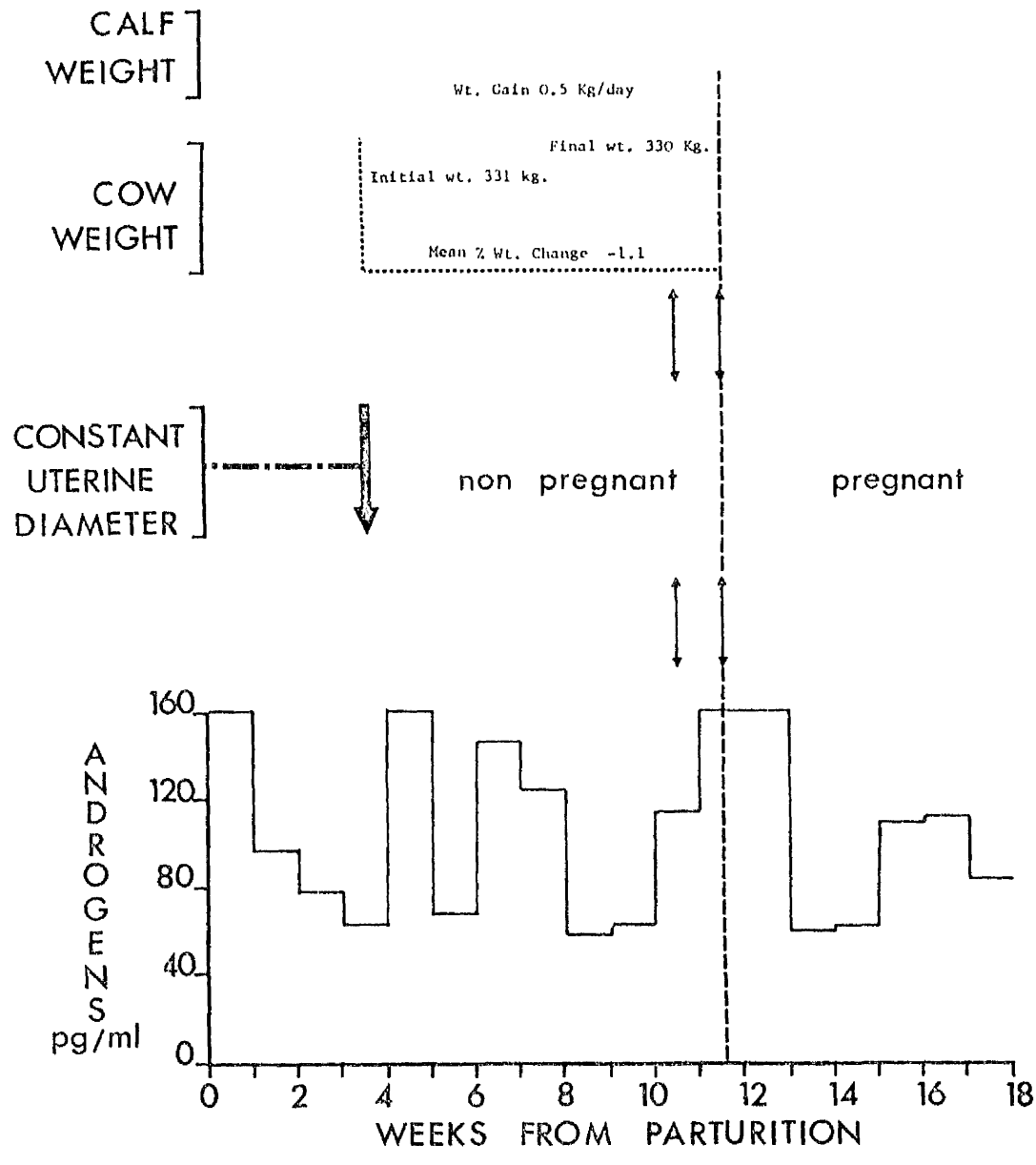


FIG 63 COW 11

PERIPHERAL PLASMA ANDROGEN LEVELS OVER THE COMPLETE PERIOD OF STUDY, THE TIME WHEN A CONSTANT UTERINE HORN DIAMETER WAS FIRST RECORDED AFTER CALVING, AND WEIGHT CHANGES IN THE COW AND CALF

Uterine horn diameter

Between calving and day 24 uterine horn diameter decreased to 4.0 cm. No further decrease was recorded after this time.

Body weight changes

Although the mean percentage weight change over the period of 28 to 78 days showed a net decrease, the final weight of the cow was similar to the initial weight.

4.4. Discussion

The re-establishment of pregnancy in the post-partum period involves numerous changes in body form and function. The various behavioural, morphological and functional events that contribute towards normal reproduction must not only occur but must be integrated in a manner conducive to fertilisation and subsequent development of the conceptus. Due to the obvious necessity for coitus under natural conditions, to allow at least the possibility of fertilisation, a return to the pregnant state after calving is prevented at least until such time as the cow attracts the male and stands to be mated, i.e. until the cow shows overt heat.

Economics dictate that, in the post-partum cow, pregnancy must be re-established within approximately three months. The potential limitations posed on this by the absence of overt heat for a period after calving have led to extensive studies into the duration of post-partum anoestrus. In the experimental work described in this thesis an attempt was made to standardise many of the factors that have been implicated in affecting the parturition to first overt oestrus-interval. In view of the reports on the influence of age (Hammond and Sanders, 1923) and parity (Herman and Edmondson, 1950) the animals selected were of approximately the same age and were all first calf heifers. The seasonal effect described by Chapman and Casida (1934) did not apply as all the animals were studied over the same period of the year. As the duration of post-partum anoestrus appears to be related not only to milk production but also the method

of removal of milk from the udder (Clapp, 1937; Wiltbank and Cook, 1958; Moller, 1970; Carmen, 1955; Olds and Seath, 1953) the animals chosen were all dairy type and were all suckled by one calf. Further management practices, such as the efficiency of methods for the detection of oestrus (Donaldson, 1968) will undoubtedly influence the apparent duration of this period. In spite of the precautions taken in selection of animals, and the fact that a constant system of management was applied to all cows in this experimental group, wide variability in the period of post-partum anoestrus was recorded (Table 24).

As noted above, it is generally accepted that differences in the duration of post-partum anoestrus are associated with differing characteristics in the animals studied, and with different systems of management. However it is apparent from the review by Morrow, Roberts and McEntee (1969a) on the duration of this period that no matter how extensive the selection criteria, nor how constant the management, individual cows still showed marked differences in the time from calving to first overt heat. The reasons behind this individual variability are not understood. A genetic basis for the duration of post-partum anoestrus has been demonstrated by Saiduddin et al. (1968). It may be that individual variability within this period, recorded both in this and in all other experiments, reflects the heterogeneous nature of the animals used. However the heritability of the period from calving to first overt heat is low (Carmen, 1955). Although Chapman and Casida (1937) considered that individual cows always tended to have a similar parturition to first overt heat-interval studies on

TABLE 24 PARTURITION TO FIRST OVERT OESTROUS INTERVAL

Cow No.	First overt heat days post-partum
1	93
2	78
3	44
4	78
5	70
7	32
8	46
9	> 114
10	52
11	70
Average (excluding Cow 9)	62.6

the influence of age and parity on this period (Hammond and Sanders, 1923; Herman and Edmondson, 1950) were at variance with this concept. Also against the hypothesis of Chapman and Casida (1937) was the later observation by Mares, Menge, Tyler and Casida (1961) that the repeatability of the post-partum anoestrus period was low. Even when in-bred animals were studied, although the heritability of the duration of this period of anoestrus increased, marked variability between individuals was still apparent (Olds and Seath, 1953). Therefore although the genotype plays a part in determining the duration of the interval from calving to first overt heat the wide variability in this period between cows cannot adequately be explained on a purely genetic basis.

The occurrence of overt heat has been accepted as terminating the period after calving when reproduction is not possible. However, this is not intended to imply that the manifestation of oestrus at this time will ensure a successful re-establishment of pregnancy should the animal be mated. To establish a pregnancy demands the integration of a series of physiological and morphological changes in the animal. The initial structural development, which constitutes a pre-requisite to all subsequent events, is the occurrence of follicular growth progressing to the state of ovulation with release of an oocyte. To permit fertilisation semen must be transferred to the female and the gametes brought together in an environment conducive to development of the ovum. Subsequent establishment of the pregnancy necessitates that the ovum passes to a uterus where conditions are suitable for the continued growth of the conceptus. These events, involving many parts

of the body, are induced and co-ordinated by means of the endocrine system. In the pre-ovulatory period modifications occur in the female to ensure that the interest of a male is aroused, and that he remains in close proximity to her until such time as she accepts his attentions and allows mating. Synchronisation of this behavioural pattern in the female with the gametogenic aspect of reproduction is achieved through the production of oestrogens by the developing follicles. In addition to this the secretion of these hormones is associated with changes in the tubular genital tract - along which the male gametes will pass, and in which fertilisation will take place. The release of the oocyte is in turn synchronised to these behavioural and structural modifications by employing the common trigger of increased levels of oestrogens to induce an ovulatory discharge of gonadotrophins. Following ovulation a corpus luteum forms. By virtue of this structure producing progesterone and by the action of this hormone on the oestrogen primed uterus a suitable uterine environment for development of the conceptus is ensured. Furthermore until such time as the pregnancy proves to be established, or not, the continued secretion of progesterone suppresses further ovulations and oestrous behaviour. Although if a pregnancy does not result the corpus luteum morphologically and functionally regresses, if the animal is pregnant this mechanism of luteolysis does not operate and the corpus luteum continues to function.

Although the above represents an extremely basic picture of the changes necessary for normal reproductive function it does serve to illustrate the requirement for many aspects of body function to be

activated, or suppressed, and for these events to be integrated together in a controlled manner. In the post-partum cow therefore expression of the first overt heat after calving represents a return to the period when normal reproduction is possible only if this behavioural change occurs as a part of the overall sequence of events. Alternatively it may be that the absence of overt heat for a period of time after calving indicates that none of the events constituting normal reproduction are taking place.

In this experimental group of animals a variable period of time elapsed between parturition and the first examination of the ovaries. This was due to the difficulty experienced in certain individual animals in raising the ovaries to a position where they could be carefully palpated. Allowing for this it is apparent that within 3 - 4 weeks of calving in all animals, except Cow 9, follicles which either individually, or added together, had a surface diameter of at least 1.0 cm were palpated (Table 25). In general from the time of first detection of follicles to the time of first overt heat follicles with a surface diameter of 1.0 cm or more were usually present in the ovaries. Other workers have studied the morphological development of follicles in the post-partum cow (Labsetwar, Tyler and Casida, 1963; Morrow et al., 1969b; Wagner and Hansel, 1969; Moller, 1970; Arijie et al., 1974). The results in this thesis are in agreement with these other workers in demonstrating a rapid re-activation of the mechanisms of follicular growth after calving. We therefore have a situation where, long before the first expression of overt heat, changes which would normally lead to the complete sequence of events involved in

TABLE 25 FIRST PALPATION OF FOLLICLES WITH A TOTAL SURFACE DIAMETER OF AT LEAST 1.0 cm

Weeks after calving	Cows in which follicles > 1.0 cm recorded		
0 - 1	3		
1 - 2	5	7	11
2 - 3	(1)* 2	4	8
3 - 4	10		
> 4	9		

* not examined till this time after calving

reproduction have occurred in the ovaries. Evidence of the presence of these structures within the ovaries should not of course be taken to imply that these follicles exhibit normal endocrine function.

During the immediate post-partum period marked alterations in the peripheral plasma levels of progesterone, oestrogens, and androgens have been recorded. Within 6 days of calving, however, the change in the circulating levels of all of these hormones on a day-to-day basis was small (Fig 64). It was therefore considered that by 6 days post-partum the amounts of these hormones present in the blood no longer reflected the specific endocrinological changes of the pregnant/parturient state. With reference to follicular function it was apparent that in all cows widely fluctuating levels of oestrogens were recorded between day 6 and the first post-partum oestrus (Table 26). The 'basal' levels found in the animals in this study were similar in magnitude to those detected 9 days after calving by Smith et al. (1973), and during the early post-partum period by Henricks et al. (1972) but were very much less than the levels of around 200 pg/ml reported by Arijie et al. (1974). In passing it should be noted that the level of plasma oestrogens found at all periods of productive function by Arijie et al. (1974) are much greater than those found in this experiment and also by other workers (Henricks et al., 1972; Glencross et al., 1973).

From the results of the individual cows given in Figs 34 - 63 it was apparent that within the period from calving to first overt heat in all cases peaks of oestrogens were recorded (Table 27). Although these peaks were of varying magnitude within, as well as

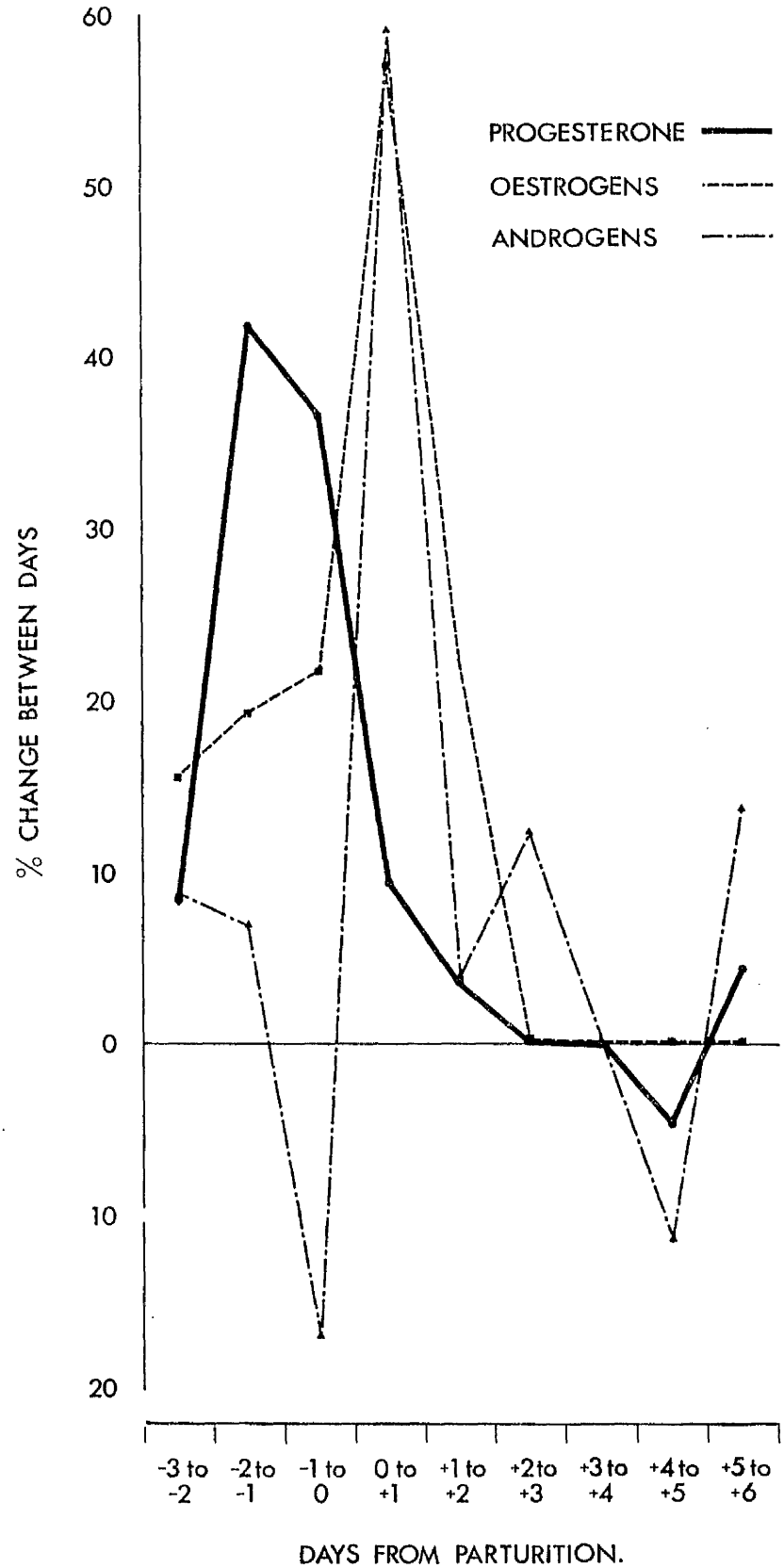


FIG 64 PERCENTAGE CHANGES IN CIRCULATING LEVELS OF PROGESTERONE, OESTROGENS, AND ANDROGENS AROUND CALVING

TABLE 26 PLASMA PROGESTERONE AND OESTROGENS DURING POST-PARTUM ABSOLUTE ANOESTRUS*

Cow No.	Progesterone ng/ml $\bar{x}$ ± 1 S.D.	Oestrogens pg/ml $\bar{x}$ ± 1 S.D.
1	0.1 ± 0.1	3.1 ± 2.7
2	0.1 ± 0.12	3.1 ± 3.2
3	0.1 ± 0.1	2.6 ± 2.0
4	0.05 ± 0.11	5.1 ± 4.4
5	0.1 ± 0.17	4.3 ± 4.2
7	0.04 ± 0.1	3.5 ± 2.4
8	0.1 ± 0.17	2.8 ± 2.4
10	0.11 ± 0.17	2.9 ± 1.9
11	0.1 ± 0.15	4.5 ± 4.1

* Levels calculated from day 7 after calving to 4 days before the first post-partum ovulation.

TABLE 27 : THE OCCURRENCE OF OESTROGEN PEAKS DURING THE PERIOD PRECEDING THE FIRST OVERT HEAT.

COW NO.	PARTURITION TO FIRST OVERT HEAT (DAYS)	OCCURRENCE OF OESTROGEN PEAKS DURING POST-PARTUM ANOESTRUS % PARTURITION - FIRST OVERT HEAT INTERVAL
1	93	33 (5) 39 (25) 66 (17) 84 (9) 94
2	78	26 (11) 40 (8) 50 (9) 62 (8) 72 (15) 91
3	44	23 (12) 50 (7) 66 (2) 70
4	78	56 (19) 81
5	70	51 (5) 59 (15) 80 (10) 94
7	32	88
8	46	72
10	52	19 (5) 29 (5) 38 (20) 77 (4) 85 (3) 90
11	70	44 (15) 66 (19) 93

( ) Interval in days between successive oestrogen peaks.

between, cows, it appeared that the levels found early in the period were at least as great as those detected later on around the time overt heat was shown. On a few occasions rectally palpable follicles were found not associated with a peak of oestrogens, e.g. Cow 1 day 71, Cow 4 day 29, Cow 5 day 19, Cow 7 day 21. However due to the fact that follicles were present on the majority of occasions the cows were examined it was apparent that the peaks of oestrogens within the period of post-partum anoestrus occurred against a background of follicular development. Although these two parameters may bear a temporal relationship to each other, in view of the well recognised function of follicles in production of oestrogens it is probable that these peak levels of oestrogens reflected period of follicular growth. In addition it has been established that in animals where there was no possibility of follicular development - because they were ovariectomised - that the levels of oestrogens were considerably less than the peak values recorded in these cows. (An example of this is given by the blank plasma hormone levels in Chapter Two).

By making sketches of the ovaries at the time of rectal palpation it was possible to gain some idea of the persistence of follicular structures within the period leading to the first overt heat. As a representative example the total follicular diameters of Cow 2, given in Fig 38, have been broken down to illustrate the period of persistence of the individual follicles (Table 28). These results combined with the fluctuating patterns of plasma oestrogens are taken to represent a rapid turnover in both structural and functional follicles

TABLE 28 PERSISTENCE OF INDIVIDUAL FOLLICLES DURING THE PERIOD FROM CALVING TO FIRST OVULATION - COW 2

Days post-partum	Left ovary		Right ovary		
12	-		-		
16	-		-		Period of persistance
21		(9)		(12)	( ) surface diameter follicle - mm
23	-	-		(13)	
26		-	-	(8)	
29		-	(12)		
31		-	(17)	-	
33		-		(15)	
34	(11)		-	(13)	
37	-	-	(12)		
39		(10)	-	-	
42	-	(13)	-	(8)	
45	(17)		(5)		
47	(16)		(6)	-	
50	(18)			-	
52	(9)			-	
55	-	-		-	
60	-	13		20	
61	12	-		14	
64	-	8		20	

Ovulation day 65.

within the ovary by rectal palpation studies undoubtedly gives only an approximate indication of the presence of individual structures. Although this is in part due to the infrequency of examinations the fact that only gross changes in the presence or absence, or position, of follicular structures could be recognised with any certainty leads to a large potential error. The difficulty of following individual ovarian follicles over their period of growth is exemplified by the studies of Donaldson and Hansel (1968). Even with direct observations by endoscopy these authors found that it was not always possible to identify follicles from one examination to the next. To obtain accurate information on the turnover of follicles during anoestrus will probably necessitate the use of a technique for marking these structures with e.g. indian ink, coupled with frequent laproscopic or endoscopic examinations.

The situation was therefore reached in the early post-partum period where the development of functional follicles occurred. It has previously been noted how under normal circumstances follicular development is associated with a behavioural change in the animal, and leads to ovulation and the formation of a corpus luteum. Evidence that this follicular development in the early stages of post-partum anoestrus did not proceed through ovulation to formation of corpora lutea was obtained by rectal palpation of the ovaries coupled with estimations of the peripheral plasma progesterone levels. Over this initial period of follicular development after calving with the exception of Cows 1, 4, 5, 8 and 9, structural corpora lutea were not present in the ovaries and progesterone levels generally remained basal.

However transient elevations in the level of progesterone without associated evidence of the presence of a corpus luteum were recorded in many animals within this period e.g. Cow 5 day 37, Cow 7 day 23 and Cow 8 day 27. Although these elevations may represent luteinisation of follicles, as suggested by Moller (1970), an alternative possibility was that they indicated fluctuations in the extragonadal secretion of this hormone. Although as has previously been stated the level of progesterone in the ovariectomised or immature cow is considerably less than 0.5 ng/ml stimulation of the adrenal can result in increased peripheral plasma levels of progesterone (Cwaazdauskas et al., 1972). The possibility therefore exists that these short-lived elevations in circulating progesterone reflected alterations in adrenocortical function possibly associated with transient stresses imposed on the animal. Further substantiation of the hypothesis that they reflect extragonadal secretion of this hormone comes from the fact that no consistent pattern of increased oestrogens was recorded in the days preceding the elevated progesterone levels. This is taken to suggest that they did not arise as a consequence of follicular growth. The basal level of plasma progesterone recorded for a period of time after calving in every cow is similar in magnitude to the concentrations reported by other workers (Henricks et al., 1971; Smith et al., 1973; Arijie et al., 1974) (Table 26). These observations are at variance with the findings of Hunter et al. (1970), who reported that between 8 and 13 days after calving levels of progesterone similar to those in mid-dioestrous cows were found. However in the case of Hunter et al. (1970), an examination of their data reveals that both in the last

month of gestation and at calving their reported concentrations of progesterone were well in excess of those found in this study and by other workers (See Chapter Three). In coming to the above conclusion on relationship of the 2 weeks post-partum progesterone levels to dioestrous levels of this hormone they compared their post-partum results with those of other workers who used different assay techniques and studied cyclic animals. Due to the inherent variability between different assay methods, which these authors acknowledged, it superficially appeared that when the above comparison was made, cows in absolute anoestrus could have plasma progesterone levels similar to those found in animals where a functional corpus luteum was present. To prevent false deductions of this nature, it is obviously vital to establish the specificity and sensitivity of any assay method and to interpret findings and draw comparisons only in the light of these parameters.

Within the early part of the post-partum period, as can be seen in Fig 34, a well defined corpus luteum was present in the ovaries of Cow 1 from the time of first examination on day 16. This structure persisted until day 27. It was apparent from the plasma progesterone levels in this cow that before, during and immediately after the morphological presence of this structure, basal concentrations of this hormone were found. This observation may suggest that this structure did not develop as a consequence of an ovulation within the post-partum period, but that it represented a morphological but not functional persistence of the corpus luteum of pregnancy. Widely differing estimates exist of the period of time after parturition over which the

corpus luteum of pregnancy can be palpated per rectum. Moller (1970) considered that it could be detected up to 3 weeks after calving when examinations were carried out at weekly intervals. In a more detailed study Morrow et al. (1969b) found that at the time of first rectal palpation around 4 to 7 days post-partum, this structure could often be palpated as a small firm elevated mass on the surface of the ovary, but that by day 14 it had become indistinguishable from the overall ovarian outline. The size of the structure recorded in Cow 1, however, was abnormally large for the period over which it was palpated - assuming that it was as previously stated , the corpus luteum of pregnancy. It is interesting to note that in this cow, following a dystocia and retention of the placenta, a purulent post-partum metritis developed. As has been hypothesised previously, regression of the corpus luteum at the time of parturition is thought to involve the intervention of a luteolysin. The source of this uterine luteolysin has been demonstrated to be the endometrium (Ginther, 1968). It may be that in this animal due to a pathological condition in the endometrium the release of the luteolytic factor was sufficient to suppress only luteal function without markedly altering cellular structure. Persistence of the corpus luteum of the cycle over an abnormally prolonged period of time occurs in animals with uterine abnormalities (e.g. pyometra). The alternative explanation for the presence of this structure at this time was that an ovulation had occurred before day 16. This was considered unlikely in view of the report by Morrow et al. (1969b) that the mean parturition to first ovulation interval was substantially prolonged beyond day 15 in animals

that experienced difficulties at calving and had clinical abnormalities of the puerperium. Finally however it may be that the classification of this structure in Cow 1 as a corpus luteum may have been incorrect. It is well recognised that the accuracy of this technique of rectal palpation of the ovaries with respect to determining what structures are present has a fairly large potential error (Dawson, 1975). By slaughtering animals and examining their ovaries after having recorded rectal palpation findings Dawson (1975) found with regard to corpora lutea an overall accuracy of around 80%.

Based on information on the concentration of progesterone in animals where luteal tissue was absent (cows in oestrus) it was decided in this thesis to consider plasma progesterone levels of 0.5 ng/ml or more on at least three consecutive days as giving functional evidence of luteal tissue. It was considered that the palpation of a 1.0 cm diameter corpus luteum in Cow 5 two days after parturition but not from day 4 onwards (Fig 46) represented a normal disappearance of the corpus luteum of pregnancy as described by Morrow et al. (1969).

Besides cows 1 and 5 the palpation of a structure classified as a corpus luteum was made in a further 3 cows within the period of anoestrus without collaborative evidence of its functional presence (Cow 4 from 38 - 41 days, Cow 8 from 37 - 42 days and Cow 9 on day 33 and again on day 37). In contrast to the situation in Cow 1, a considerable period of time elapsed between calving and detection of these corpora lutea in Cows 4, 8 and 9 (Figs 43, 52, 55). Plotka,

Erb, Callahan and Gomes (1967) have reported on one cow that had a non-functional corpus luteum over a period of time. It may be that in the case of Cows 4 and 8 these structures were further examples of a non-functional corpus luteum. However in Cows 4, 8 and 9 and possibly also the case described by Plotka et al. (1967) it may be again that the classification of these ovarian structures as corpora lutea was incorrect.

Allowing for these specific instances noted above it was apparent from the results of the individual cows that in animals 2, 3, 4, 5 and 10 morphological and functional corpora lutea were palpated within the period of anoestrus (Figs 37, 40, 43, 46 and 58). This finding was taken as evidence that in at least a proportion of the cows studied follicular development and oestrogen secretion did proceed through ovulation to formation of a corpus luteum without oestrous behaviour having been shown.

In this study the presence of a corpus luteum was taken as indirect evidence that an ovulation has occurred. Other workers have attempted by more frequent rectal palpation to detect the process of ovulation directly. However as has been pointed out by Wiltbank et al. (1967) and Marion et al. (1950) this method suffers from the disadvantage that rupture of the follicles may have been caused not by ovulation but by the technique of rectal palpation. To overcome this possibility some studies have reported alternative methods for obtaining direct evidence of ovulation - such as endoscopy (Donaldson and Hasel, 1968). Rectal palpation of corpora lutea is a commonly

used method of obtaining evidence of ovulation in post-partum cows (Wiltbank and Cook, 1958; Morrow et al., 1969b; Moller, 1970). In this thesis in addition to the morphological presence of corpora lutea evidence of their presence was obtained from plasma progesterone levels.

Obviously the overall results and interpretation of the findings in this thesis and of other studies into post-partum reproduction were dependent to a large extent on the efficiency of the techniques used for oestrous detection. Tanabe and Almquist (1960) have reviewed the literature on the relationship between the duration of the period of observation and the results obtained in the detection of oestrus in cycling animals. In some instances study of the post-partum period in cows has involved only twice daily examination of the animals for oestrous behaviour (Wagner and Hansel, 1969). Wishart (1972) monitored cycling cows for oestrous behaviour every 2 hours. Although the majority of animals in Wishart's study had an oestrous duration of between 12 and 16 hours (54%), 16% were in heat for less than 12 hours, and 4.4% for less than 8 hours. This confirms the observation of Donaldson (1968) that a significant number of animals have short oestrous periods. Twice daily examination of animals for oestrous behaviour is therefore inadequate. Besides the frequency of examination of animals for oestrous behaviour the duration of the period of observation and the criteria used for detection of oestrus are important.

In this study detection of oestrus was by the presence of a bull. By continuous monitoring of the behaviour of the group by time

lapse photography over the period of daylight and in addition at least two periodsof direct observation during the period of darkness, an attempt was made to overcome the deficiencies associated with watching the animals for a limited period of time on several occasions throughout the day. The validity of using the time-lapse photography system to determine whether oestrous behaviour was shown by a cow was established early in the course of the experiment. A cycling cow was introduced to the group and continually observed for oestrous behaviour. Plate 1 illustrates a sequence from the time lapse film - the 15 frames having been taken over a period of 3 min 45 sec. It is apparent from this plate that the bull being the only animal with horns was easily identified. In addition, due to the individual markings of the cows, each of these could also be identified. Within the plate frame 1e shows the bull and cow standing parallel to each other in the nose to tail position. This position, characteristic of perioestrous/oestrous behaviour, was repeated in frame 2c. In frame 2e the cow was observed to mount the bull. Mounting of this oestrous cow by the bull and standing by the cow, was noted in frame 3b. Throughout the 3 min 45 sec period covered by the film this was the only occasion the bull, by direct observation, was seen to mount the cow.

The study by Donaldson (1968) confirmed the finding of Anderson (1944) that teasing three times a day by a vasectomised bull was very effective in detecting heat - 93.1% of possible cycles were noted as against only 81% when mounting by other cows was relied upon. Based on the finding by Wishart (1972) that in 68 oestrous periods only one was of less than 6 hours duration (1.5%) coupled with the reports on the

PLATE 1 TIME LAPSE SEQUENCE

COW IN OESTRUS

suitability of a vasectomised bull for detection, it was considered that the system employed here gave the maximum chance, short of continuous direct observation, of detecting oestrous behaviour. A further advantage attached to the use of a vasectomised bull has been reported by Marion, Smith, Wiley and Barrett (1950) who found that heifers would stand for mounting for longer by a bull than they would with other cows. The precautions which were taken minimised the chance of missing oestrous behaviour. The results indicated that corpora lutea were formed in the ovaries of cows 2, 3, 4, 5, 8 and 10 in this study without a preceding period of oestrous behaviour, i.e. a silent heat had occurred.

It was desirable to estimate the day of ovulation to give a reference point for endocrinological and morphological comparisons between ovulations with and without associated oestrous behaviour. In the case of overt heat the endocrinological changes can be related to the behavioural change, whereas in the case of silent heats it became necessary to know the day on which oestrus would have been shown if it had occurred. Different groups of workers have used differing criteria for determining whether ovulation has taken place. Wagner and Hansel (1969), for example, slaughtered groups of cows at 7, 14 and 30 days after calving. The presence of corpora lutea within the ovaries was taken as evidence that ovulation had occurred some time between calving and slaughter. A more accurate estimate of the day of ovulation was obtained by examination of the ovaries for the presence of corpora lutea at weekly (Wiltbank and Cook, 1958; Moller, 1970) and twice weekly (Morrow et al., 1969b) intervals. It is recognised that

increased oestrogen levels are associated with the behavioural change of overt heat and, as previously stated, after the behavioural change approximately 4 days elapse before a corpus luteum becomes rectally palpable. In this thesis therefore in relation to silent heats it was decided to take the day when a peak of plasma oestrogens occurred immediately prior to the rectal and functional presence of a corpus luteum, as the day on which if the heat was 'normal' the behavioural change would have been seen. A further condition on deciding on this day was that at least 3 to 4 days elapsed before a corpus luteum was structurally and functionally detected, i.e. ovulation has taken place 3 days before. The assumption was therefore made that silent heat was in some ways endocrinologically similar to overt heat. It can be seen therefore that in the case of Cows 2, 3, 4, 5 and 10 where the first ovulation occurred without associated oestrous behaviour that this silent heat was noted from 7 to 32 days before the first expression of overt heat (Table 29). However in the case of Cows 1 and 11 the first post-partum ovulation was associated with overt heat. In addition it was apparent that although Cows 7 and 8 showed overt heat, apparently terminating the period of post-partum anoestrus, when the hormone levels and rectal palpation findings were examined after these heats there was no evidence of ovulation having occurred (Figs 49 and 52). Oestrous behaviour without associated ovulation is commonly referred to as 'false heat.' Further, it could be seen that in these cows (7 and 8) structural and functional evidence of ovulation was not found until days 60 and 85 respectively, and since no associated oestrous behaviour was seen, the heats preceding these ovulations and

TABLE 29 : TIME AFTER CALVING, OF THE FIRST OVULATIONS, WITH SUBSEQUENT LUTEINISATION, OF COWS 2, 3, 4, 5 AND 10.

COW NO.	PARTURITION TO HORMONAL PRESENCE LUTEAL TISSUE* - INTERVAL (DAYS)	OESTROGEN PEAK PRECEDING LUTEAL TISSUE* DETECTION ≡ ESTIMATED DAY OF SILENT HEAT (DAY POST PARTUM)	OESTROGEN PEAK TO LUTEAL TISSUE* PRESENCE - INTERVAL (DAYS)
2	71	65	6
3	34	29	5
4	52	48	4
5	65	60	5
10	48	46	2

*FIRST DAY OF AT LEAST 3 SUCCESSIVE DAYS ON WHICH PLASMA PROGESTERONE LEVELS WERE 0.5 ng/ml OR GREATER.

luteal tissue formation were classified as silent. Table 30 summarises for convenience the first detection of functional luteal tissue in all cows, and the day of associated oestrous behaviour if this was overt, or the estimated day of oestrous if silent heat had occurred.

Throughout the experimental period Cow 9 did not ovulate. Cow 1 had only one ovulation on day 94. Cows 2, 3, 4, 5, 7, 8, 10 and 11 had at least one ovulation subsequent to the first before termination of the experimental period. Table 31 summarises the occurrence of silent and overt heats associated with ovulation in all animals over the period of the experiment. It can be seen that although the majority of first ovulations were associated with silent heat, by the second ovulation only one heat out of the seven recorded was silent (Cow 4). Furthermore it was apparent that just as marked variability was observed between cows in the time of first overt heat (32 - 93 days) so also do the first ovulations (taken as 1 day after the day of heat) occur over a wide period of time (47 - 94 days) (Table 31).

It is not understood why a period of time elapses between calving and the first ovulation post-partum. Neither has it been fully explained what factors are responsible for the wide variability recorded within, and between groups of cows in the time of first ovulation. In addition the reason why some of these first and subsequent ovulations are associated with no oestrous behaviour, whereas others are preceded by a period of standing heat, has not been explained.

TABLE 30 TIME, AFTER CALVING, WHEN FUNCTIONAL LUTEAL TISSUE WAS FIRST DETECTED, AND THE ESTIMATED OR ACTUAL DAY OF OESTRUS

Cow No.	Functional luteal tissue days post-partum	Associated Oestrus Behaviour	Associated Oestrus Days post-partum
1	100	Overt	93
2	71	Silent	65
3	34	Silent	29
4	52	Silent	48
5	65	Silent	60
7	60	Silent	54
8	85	Silent	82
9	-	-	-
10	48	Silent	46
11	74	Overt	70

TABLE 31 THE TIME OF OCCURRENCE OF OESTRUS*, AND THE INTERVALS** BETWEEN HEATS IN THE NON-PREGNANT POST-PARTUM PERIOD

Cow No.	Oestrus No. 1 Days post-partum	Interval Days	Oestrus No. 2 Days post-partum	Interval Days	Oestrus No. 3 Days post-partum	Interval Days	Oestrus No. 4 Days post-partum
1	93	-	-	-	-	-	-
2	65	14	78	-	-	-	-
3	29	16	44	26	69	28	96
4	48	9	56	23	78	-	-
5	60	11	70	-	-	-	-
7	54	11	64	-	-	-	-
8	82	8	89	-	-	-	-
9	-	-	-	-	-	-	-
10	46	7	52	21	72	-	-
11	70	9	78	-	-	-	-
Average Intervals		10.6		23.3			

* Accompanied by subsequent ovulation and luteinisation.

** Calculated as being the interval from one oestrus to the next, including the days of heat.

Within the period from calving to first ovulation two distinct phases could be identified, at least in some of the cows in this study. Although the bulk of the period was occupied by a phase of 'ovarian activity' where follicles were developing in the ovaries and oestrogens were being secreted, this was preceded in some animals by a phase of 'ovarian inactivity' where no follicles were detected within the ovaries e.g. Cows 2, 4, 5 and 7. Although in the animals in this study this period of lack of follicular development constituted only part of the period to first ovulation,in other groups of cows it is apparent that it may be of greater importance. Smith and Vincent (1972) found that in 79 beef cows 16% had no detectable ovarian activity by 30 days after calving. In contrast all animals in this thesis had follicles with a total surface diameter of 1.0 cm, or more, present, with the exception of Cow 9 early in the post-partum period. It is generally accepted that follicular development and function is regulated by the release of pituitary gonadotrophins. Labhsetwar et al. (1963) have demonstrated that the ovaries of 1 day post-partum cows were capable of responding to exogenous gonadotrophins suggesting that the ovarian inactivity at this time was as a consequence of a deficiency either in the synthesis or release of these hormones rather than a primary ovarian insensitivity. It has been suggested that the endocrine environment at the end of the period of gestation is involved in a possible blockage of gonadotrophin release for a period of time post-partum (Labhsetwar et al., 1964). It was, however, difficult to see how this postulated blocking effect of high levels of oestrogens could lead to the extremely variable period of ovarian inactivity recorded in this and other studies. The possible relationship of the hormone

levels at the end of gestation to subsequent ovarian activity could be obtained by terminating pregnancy at times when the level of oestrogens was considerably less than the high levels found at term.

It was apparent that possibly in addition to the overall effect of lack of gonadotrophins on ovarian activity an intra-ovarian mechanism governs events in the early post-partum period. It has been found that when ovarian activity does commence after calving it almost invariably is most marked on the ovary opposite the one that contained the corpus luteum of pregnancy. The possible mechanism of action of this effect is not understood.

It was apparent from the results of the animals in this study that the major part of the period leading to the first ovulation is occupied by a period when follicular growth was occurring and oestrogens were being secreted. Within the period to first post-partum ovulation it has been suggested by Morrow et al. (1969b) that the size of the largest follicle found within the ovary increased with increasing time from calving. Similarly Moller (1970) considered that the overall ovarian size increased. These findings imply that over the period of anoestrus follicular development increased with time and ultimately resulted in their development progressing to a stage when ovulation occurred.

Examination of the peripheral plasma oestrogen levels of the animals in this study demonstrated that peaks of these hormones are found long before the time of first ovulation (Figs 35 - 62 summarised in Table 32). In addition it could be appreciated from the results for

TABLE 32 PEAK LEVELS OF PLASMA OESTROGENS WITHIN THE PERIOD FROM CALVING TO FIRST POST-PARTUM OESTRUS WITH SUBSEQUENT OVULATION AND LUTEINISATION

Cow No.	Plasma oestrogens (pg/ml) and day detected after calving ( )					First oestrus with ovulation and luteinisation -days post-partum
1	11.9 (31)	9.0 (36)	11.9 (61)	7.7 (78)	9.7 (87)	93
2	15.4 (20)	9.9 (31)	20.2 (39)	9.0 (48)	11.9 (56)	65
3	5.7 (10)	5.5 (22)				29
4	16.9 (44)					48
5	18.3 (36)	18.0 (41)				60
7	8.6 (28)	12.1 (33)	7.3 (47)			54
8	9.7 (33)	12.1 (48)	10.1 (61)			82
10	6.7 (10)	6.8 (15)	5.8 (20)	6.0 (40)		46
11	20 (31)	11.9 (46)	13.2 (65)			70

the individual cows, summarised in Table 32, that the magnitude of the oestrogen peaks within the period of anovulation did not increase with time. Neither was there any evidence of a consistent enlargement in total follicular surface diameters with increasing time within the period preceding the first ovulation (Table 33). Neither was an increasing pattern of folliclular diameters seen with increasing percentages of the post-partum period (Table 34). These results suggested that within the period of anovulation, follicular development, in terms of both structure and function, did not conform to the pattern of gradual increase suggested by Moller (1970). It appeared that from the results in this thesis, follicular development up to a point just before ovulation should be considered as all or none, and that within the period, provided the ovaries are not inactive, ovulation should be capable of occurring prior to it actually taking place. This was obviously what was observed in studies where the stimulus to ovulation was provided, within the anovulatory period, either directly by administering gonadotrophins, or indirectly through GnRH (Foote and Hunter, 1964; Britt et al., 1974). The results in this thesis on follicular development and function during the period of anovulation provided a possible explanation for the observation of Foote and Hunter (1964) and Britt et al. (1974). Ovulations induced by these authors on injection of LH or GnRH were possible because of the normal pre-ovulatory development of follicles during post-partum anoestrus. It is obvious that these results do not agree with the concept of Moller (1970) which suggested that follicular development increased over the period of anovulation. If follicular development was a

TABLE 33 TOTAL FOLLICULAR SURFACE DIAMETER WITH INCREASING TIME FROM PARTURITION TO THE FIRST POST-PARTUM OVULATION

Days post-partum	Follicular diameter cm $\bar{x} \pm 1$ S.D.	P	Animals included
0 - 10	0.49 ± 0.7		1 2 3 4 5 7 8 10 11
		> 0.05	
11 - 20	0.95 ± 0.96		1 2 3 4 5 7 8 10 11
		> 0.05	
21 - 30	1.23 ± 0.91		1 2 3 4 5 7 8 10 11
		> 0.05	
31 - 40	1.25 ± 0.56		1 2 - 4 5 7 8 10 11
		> 0.05	
41 - 50	1.45 ± 0.73		1 2 - 4 5 7 8 10 11
		> 0.05	
51 - 60	1.25 ± 0.41		1 2 - - 5 7 8 - 11
		> 0.05	
61 - 70	1.25 ± 1.06		1 2 - - - - 8 - 11
		> 0.05	
71 - 80	2.15 ± 0.66		1 - - - - - 8 - 11
		> 0.01	
81 - 90	0.80 ± 0.74		1 - - - - - 8 - -

TABLE 34 TOTAL FOLLICULAR SURFACE DIAMETER WITH INCREASING PERCENTAGES OF THE INTERVAL FROM CALVING TO FIRST POST-PARTUM OVULATION

% Calving to first ovulation interval	Follicular diameter cm $\bar{x} \pm 1$ S.D.	P
0 - 20	0.96 ± 1.10	
		> 0.05
21 - 40	0.98 ± 0.81	
		> 0.05
41 - 60	1.28 ± 0.62	
		> 0.05
61 - 80	1.3 ± 0.86	
		> 0.05
81 - 100	1.43 ± 0.89	

gradual process it would be unlikely for ovulation to be produced at an early stage of post-partum anoestrus by the administration of either LH or GnRH, as pre-ovulatory follicular development would be incomplete.

It was also apparent from the consideration of the peak levels of oestrogen present in the anovulatory period that they were at least as great as those which were found at normal ovulation (Table 35). This would indicate that during anovulation, peaks of oestrogens were present which should be capable of causing ovulation. It therefore appears that the absence of ovulation within the period of post-partum anoestrus is not due to a lack of oestrogenic stimulation. Moller (1970) suggested that within the period up to first ovulation ovarian cycling not based on corpora lutea but solely as a result of fluctuations in follicular development occurs. In contrast the results in this thesis indicated that no apparent increase in either total follicular diameters, nor in follicular function, as indicated by plasma oestrogen concentration, occurred with increasing time. This could lead to the conclusion that follicles were potentially capable of ovulating at any time.

The observation by Ulberg and Lindley (1960) that oestrogens administered in early post-partum anoestrus could advance the time of first ovulation may appear initially to be at variance with the above suggestion. Evidence has been presented demonstrating that the positive feedback effect of oestrogens in triggering the ovulatory discharge of LH involves the overcoming of a threshold before release

TABLE 35 PEAK LEVELS OF PLASMA OESTROGENS IN THE ANOVULATORY PERIOD, AND THE MAGNITUDE OF THE PEAKS OF THESE HORMONES ASSOCIATED WITH LATER OVULATIONS

Cow No.	Peak levels of oestrogens (pg/ml) recorded in the anovulatory period - range or individual values	Peak levels of oestrogens (pg/ml) associated with subsequent ovulations** - average ± 1 S.D.
1	7.7 - 11.9	
2	9.0 - 20.2	
3	5.5, 5.7	
4	16.9	
5	18.0, 18.3	10.2 ± 3.8
7	12.1, 7.3	
8	9.7 - 12.1	
10	5.8 - 6.8	
11	11.9 - 20.0	

* Cow 9 excluded as it did not ovulate during the experimental period.

** The maximum level of plasma oestrogens from 2 days before to the actual, or estimated day of oestrus.

of the necessary amounts of GnRH occurs (Convey, 1973). If there was an increase in the threshold barrier to oestrogenic stimulation of the release of GnRH then this would account for the fact that when phramacological quantities of oestrogens were present in the blood, such as those produced by Ulberg and Lindley (1960), this elevated threshold could be overcome and ovulation induced. It is postulated that in the untreated animal only the lowering of this threshold will determine when the endogenous oestrogens will trigger an ovulatory discharge of LH. This hypothesis therefore suggests that the period between calving and first ovulation, although initially involving a period of ovarian inactivity was for the most part controlled not by the ovaries, nor by the pituitary but by factors governing the potential excitability of the hypothalamus. It would appear that further progress in both an understanding of the aetiology of the condition of post-partum anovulation and artificial modification of its duration will involve an investigation of the mechanisms between oestrogens and the release of gonadotrophic hormone releasing factors.

The neural activity of the hypothalamus is recognised as being able to be modified by progestagens (Flerko, 1966). It may well be that the observation that progesterone administration during early post-partum anoestrus could lead to an advancement of the time of first ovulation can be explained not in terms of the unproven 'Rebound Phenomenon' (Lakshman and Nelson, 1963) but by the progesterone modifying the threshold to oestrogens at this time. Furthermore by virtue of the involvement of the hypothalamus in many body functions it may be that many of the factors noted previously as having an effect on the

parturition-first ovulation interval act by modification of this proposed threshold.

In the normal cycling animal follicular development and oestrogen secretion are followed not only by ovulation but by the associated behavioural change referred to as oestrus. The fact that in the post-partum cow ovulation occurs dissociated from oestrus (silent heat) is well recognised. Trimberger and Fincher (1956) found, by rectal palpation of corpora lutea coupled with various regimes of oestrous detection, that the overall incidence of silent heats in a survey of 500 ovulations after calving was 18.6%. A similar figure has been reported by Labhsetwar et al. (1963). However it is apparent that wide variations in the incidence of this phenomenon are found ranging from figures such as those above, through the 44.3% recorded by Kidder, Barret and Casida (1952) in a study of closely related Friesian heifers, to the 100% found by Moller (1970) in ovulations before 60 days post-partum in suckled cows. It has been demonstrated that the occurrence of silent heats appears to be related to the number of ovulations after calving, decreasing from the first to the second, and the second to the third (Morrow et al., 1968). Although Morrow et al. (1969b) recorded 78.8% of first ovulations in normal cows being unassociated with any sign of heat, Saiduddin et al. (1968) considered that overt heat was not shown in 46.5% of first ovulations. However in this latter study a further 32.5% of the animals, although exhibiting some signs associated with oestrus did not stand to be ridden. Although these cows were not considered to have shown silent heats, they should, in the view of

this thesis, have been included in this category as 'oestrus' which should by definition be restricted to animals standing to be ridden and 'silent heat' should be applied to any heat that constitutes an incomplete part of oestrous behaviour.

A genetic (Saiduddin et al., 1968) and a seasonal effect (Labhsetwar et al., 1963) on the level of heat behaviour associated with post-partum ovulations have been described. The influence of the level of milk production has been noted demonstrating that high producers can be expected to have a greater frequency of silent heats (Wiltbank and Cook, 1968). The effect of the method of milk removal has also been extensively studied. Moller (1970) in a comparative investigation involving milked and suckled cows found that in milked cows of all ovulations occurring before 60 days after calving 56.8% of heats were silent, whereas after this time 23.1% of ovulations were not associated with oestrus. It was also apparent from the data presented by Moller (1970) that in suckled cows if only the first ovulations were considered the chance of these being associated with silent, rather than overt, heat was influenced by the time they occurred after calving. In Moller's study all suckled cows ovulating for the first time before day 56 (14.3%) did not show overt heat at the time of ovulation. However in the case of suckled cows whose first ovulation occurred after day 56, 66.3% were associated with overt heat. These results would suggest that in suckled animals the majority of first ovulations occurred along with the expression of standing heat. Within the different regimes for milk removal these observations agree with the finding by Labhsetwar et al. (1963) that

there is a highly significant negative correlation between the occurrence of silent heats and the period of time that elapses from calving to ovulation. However it is apparent that this correlation does not appear to occur when milked and suckled animals are taken together. It therefore appears to be the case that genotype, season and milk production affects the degree of oestrous behaviour accompanying ovulations. In addition it seems that the signs of heat are a function of the number of the ovulations after calving and of the time the ovulations occur. It was not the intention in this thesis, due to the small number of animals used, to provide additional incidence figures on the occurrence of various degrees of oestrous expression with post-partum ovulations.

Examination of the results in this thesis was carried out to establish how they relate to the observations by other workers on ovulations and associated heats after calving. From the time of occurrence of heats associated with ovulations and whether they were overt or silent, given for all cows over the complete period of the experiment (Tables 36 and 37) it was apparent that the individual cows had from 0 - 4 ovulations over the period of study and that the ratio of silent/overt heats was 8/13. Furthermore it could be seen that the majority of first ovulations were associated with silent heat (78%) whereas the majority of second ovulations were accompanied by overt heat (88%). Of first ovulations occurring before 56 days (Cows 3, 4, 7 and 10) 100% were silent, whereas of first ovulations occurring after 56 days (Cows 1, 2, 5, 8 and 11) 40% were associated with absence of overt heat. It appears that this small experimental group of animals,

TABLE 36 : THE OCCURRENCE OF SILENT AND OVERT HEATS ASSOCIATED WITH SUBSEQUENT LUTEINISATION OVER THE EXPERIMENTAL PERIOD.

% TOTAL HEATS	HEAT NUMBER 1st	2nd	3rd	4th
SILENT	78	13	0	0
	(9)	(8)	(3)	(1)
OVERT	22	87	100	100

( ) Number of heats recorded.

TABLE 37 THE EXPRESSION OF HEAT ASSOCIATED WITH THE OVULATIONS OF ALL COWS OVER THE EXPERIMENTAL PERIOD

Cow No.	Heat No. and Type of Behaviour 1	2	3	4
1	Overt			
2	Silent	Overt		
3	Silent	Overt	Overt	Overt
4	Silent	Silent	Overt	
5	Silent	Overt		
7	Silent	Overt		
8	Silent	Overt		
9	-	-	-	-
10	Silent	Overt	Overt	
11	Overt	Overt		

although agreeing with the observation that the degree of heat behaviour is a function of the number of ovulations after calving, did not fit into the concept that with increasing time after calving first ovulations show a transition from silent to overt heat. To attempt to elucidate the aetiology of silent as opposed to overt heat the plasma progesterone and oestrogen levels were examined over the times preceding both of these periods.

It is well established that oestrogens play an integral part in the production of oestrous behaviour. By administration of oestrogens to ovariectomised cows, a proportion of these were induced to show oestrous behaviour (Asdell, de Alba and Roberts, 1945; Holy and Hrivnak, 1965). However following the demonstration by Dempsey, Hertz and Young (1936) that more normal sexual receptivity could be induced in ovariectomised rodents if progesterone was given along with oestrogens, a role for this former hormone in the production of heat has been investigated in a wide range of species (e.g. hamster - Frank and Fraps, 1945). The observation by Edgar (1953) that progesterone was present in the follicular fluid of pre-ovulatory follicles in the cow was interpreted as substantiating the possibility that this hormone was secreted around oestrus and that it synergised with oestrogens in the production of heat. Although Henricks, Dickey and Niswender (1970) and Plotka, Erb, Callahan and Gomes (1967) found an increase in plasma progesterone levels at the time of overt heat, several other studies have failed to confirm their findings.

Independently of this concept of progesterone being involved as

an integral part of the trigger to oestrus, a further role for this hormone in the expression of overt heat has been postulated. Several of the techniques that have been used to decrease the interval from parturition to first ovulation have also decreased the period of post-partum anoestrus. Those that have been successful have involved the administration of a progestagen alone (Ulberg and Lindley, 1960; Saiduddin, Quevedo and Foote, 1968), progestagens with either oestrogens or gonadotrophins (Henricks et al., 1972) and the injection of oestrogens alone (Ulberg and Lindley, 1960). Overall the time of induction of first ovulation preceded the time of first overt heat. It was thought therefore that the production of overt heat involved a period of progesterone priming - the implication being that silent heat with resulting progesterone secretion had to take place before overt heat could be exhibited. Added weight was given to this hypothesis of progesterone priming as a pre-requisite to overt heat by the studies of Carrick and Shelton (1969) in ovariectomised cows. These workers demonstrated that treatment with progesterone over a period of 5 days resulted in an apparent increased sensitivity to oestradiol benzoate after the progesterone treatment was stopped. It should be noted however that this effect was observed in animals that had become refractory to the oestrous-inducing effects of large repeated doses of oestradiol benzoate. It was considered by these workers that the ending of this refractory state was not brought about by synergism between residual progesterone and the injected oestrogens but rather by a preconditioning of neural centres to allow the expression of overt heat. The concept of whether or not an effect of this nature is

involved in the return to oestrus after calving is still not settled. Donaldson et al. (1970) recorded a transient increase in plasma progesterone 3 - 5 days before the first post-partum ovulation in dairy cows. However this elevation was noted irrespective of whether or not this ovulation was accompanied by overt or silent heat. A similar pattern of increase in progesterone levels was recorded in post-partum cows prior to first ovulation when this was accompanied by overt heat (Henricks et al., 1972). Arijie et al. (1974) found that progesterone increased steadily from 7 days before the expression of post-partum oestrus, reaching peak levels of around 2.0 ng/ml 3 days before the expression of heat, and then falling sharply to very low levels on the day of oestrus. In Arijie et al's. study, no corpora lutea were recorded in the ovaries prior to the first overt heat and all cows that ovulated showed overt heat to the first post-partum ovulation, which occurred on average 98 days after calving. It was concluded from these studies that the establishment of a pattern of progesterone secretion was a pre-requisite to the expression of overt heat, but that the establishment of the pattern did not guarantee that the heat would be overt.

It appears therefore that at least two factors determine whether an ovulation that is associated with oestrus is an overt or silent heat. Firstly, a dose-response effect of oestrogens such that silent heat is associated with insufficient quantities of these hormones to trigger the behavioural change (Asdell et al., 1945); secondly an alteration to the response induced by oestrogens following the prior action of progesterone.

Summarising the results of the individual cows in Figs 35 - 62 shows that, as previously noted, oestrogen peaks of comparable magnitude to those found at ovulation were present in all animals except Cows 3 and 10 in the anovulatory, anoestrous period (Table 32). In addition when oestrogen levels of ovulations accompanied by overt heats were taken together (Cow 1 - ovulation 1; Cow 2 - ovulation 2, etc.) and compared to the levels associated with silent heats (Cow 2 - ovulation 1; Cow 3 - ovulation 1, etc.), it was apparent that there was no significant difference in either case between the average levels over 2 days preceding the behavioural change, or between the maximum levels within this period. These results indicated that there is no evidence of a dose effect relationship between circulating oestrogens and the level of heat behaviour. In addition, these findings suggested that if only the presence of a suitable quantity of oestrogens in the blood was involved in the behavioural change of oestrus, then heat should have been shown in all animals long before the first post-partum ovulation. There was no evidence in this study of an increase in plasma progesterone levels, compared to the basal levels during the anovulatory phase (Table 38), at the time of either silent or overt heat. In addition within the anovulatory period no elevation of progesterone levels was recorded at the times peaks of oestrogens were detected (Table 27). These results suggested that, irrespective of the type of heat behaviour associated with ovulations, there was no significant pre-ovulatory progesterone secretion in the cow and were therefore in agreement with the observations of Hansel and Snook (1969), Stabenfeldt et al.(1969) and Christensen et al. (1974) on overt heats.

TABLE 38 PLASMA OESTROGENS AND PROGESTERONE ASSOCIATED WITH SILENT AND OVERT HEATS (EXCLUDING FALSE HEATS)

	Oestrogens pg/ml $\bar{x} \pm 1$ S.D.		Progesterone*** ng/ml $\bar{x} \pm 1$ S.D.
	Average level*	Peak level**	
Silent heat	7.3 ± 3.7	9.0 ± 2.9	0.13 ± 0.1
Overt heat	8.2 ± 4.7	10.9 ± 4.2	0.27 ± 0.2

* Calculated from the average levels of the day before and the day of heat for the individual heats.

** Calculated from the maximum concentrations of oestrogens recorded within the period 2 days before, and the day of the individual heats.

*** Calculated from the individual progesterone levels at the times of peak oestrogen concentrations.

The results in this thesis indicated that neither the occurrence of silent heat nor the failure of elevated oestrogens to trigger oestrus in the anovulatory period could be explained on the basis of an absence of pre-ovulatory progesterone secretion.

It can be seen from the results of the individual cows (Figs 34 - 61) that there was no evidence of the type of transient progesterone increase recorded by Pope et al. (1969), Donaldson et al. (1970) and Arijie et al. (1974) immediately prior to the first post-partum heat. In these other studies the elevated progesterone levels were considered not to be due to the presence of corpora lutea resulting from previous ovulations. Although Donaldson et al. (1970) considered that the pattern of progesterone secretion recorded in their study represented pre-ovulatory luteinisation of follicles, that could not be detected by rectal palpation, the source of the increased secretion of progesterone in this and the other studies has not been established. In contrast in this thesis elevated progesterone levels were always associated with the presence of what was considered to be a corpus luteum in the ovaries, although the converse was not always the case. In relation to the first overt heats after calving of Cows 2, 3, 4, 5 and 10, an established pattern of progesterone secretion was present before the expression of heat. This was, however, due to the prior occurrence of at least one silent heat in these individuals. In contrast, Cows 1 and 11 did not have a period of increased progesterone levels before their first overt heats, which coincided with their first ovulations. False heats exhibited by Cows 7 and 8 on days 32 and 46 were also preceded by a period when progesterone levels

were indistinguishable from those recorded throughout the overall anovulatory phase (Figs 50 and 52). However, in both these animals, their first normal overt heats, i.e. when ovulation and subsequent luteinisation occurred, were recorded only after a period of increased progesterone levels due to the fact that both experienced silent heats after their false heats.

The results in this study suggested that although an established pattern of increased progesterone secretion was normally found prior to the first overt heat after calving, this did not appear to be an absolute pre-requisite to the expression of overt heat.

Sawyer (1960) has shown that although the hypothalamus is necessary for the expression of heat and the release of pituitary gonadotrophins, the triggering of both of these changes involves different groups of cells within this region. Under normal circumstances, oestrogens excite both groups of cells together thus synchronising the behavioural change with final follicular development. The failure of the pre-ovulatory oestrogens to trigger the behavioural state of oestrus, while effectively inducing ovulation, demonstrated that, in the post-partum cow, the two different groups of cells in the hypothalamus could respond independently of each other. The results in this thesis have shown that the level of circulating oestrogens, constituting the exciting factor, was not distinguishable irrespective of whether ovulation was, or was not, associated with oestrus. The response to a hormone is a product of the magnitude of the trigger factor and the potential excitability of the target organ (Phoenix, 1964). It is therefore suggested that in the post-partum cow the

response to pre-ovulatory oestrogen secretion varied according to the potential excitability of the groups of target cells in the hypothalamus. It has already been suggested that in relation to ovulation the variable response of a constant trigger could be explained by a temporary elevation in the threshold to oestrogens. A similar, but independent, threshold involving the basal hypothalamic cells involved in oestrous behaviour could explain the occurrence of silent heats. Increasing the potential excitability of the cells controlling gonadotrophin release, without a corresponding modification occurring in the cells associated with the behavioural change, would allow follicular development to proceed to ovulation. As the trigger factor remains the same, ovulation with overt heat necessitates the removal of the refractory state in the basal hypothalamic cells. This increase in potential excitability of the basal cells may, in some individuals, involve a pre-conditioning of these cells by progesterone secreted, not at the time of oestrus, but prior to the behavioural change. The numerous factors such as genotype, season and lactation, recognised as influencing the occurrence of silent heat, may all exert their effects not by modification of the secretion of oestrogens, but by altering the potential excitability of the hypothalamus.

In terms of the overall period from calving to the first occasion when conception, under natural conditions, became possible, the sequence of changes in the hypothalamus was therefore considered to be:

1. Refractoriness of the basal cells and of the cells controlling gonadotrophin release giving rise to the anovulatory, anoestrous period.

2. An increase in the potential excitability of the cells controlling gonadotrophin release only, allowing ovulation but no behavioural change.

3. Removal of the refractory state of the basal cells permitting pre-ovulatory oestrogen secretion to trigger both ovulation and oestrus.

It was apparent from the above hypothesis that the refractoriness in the cells regulating gonadotrophin release was normally removed before that in the basal cells, giving rise to the fact that the majority of first heats were silent. However, it appeared that the transition from total refractoriness of the two groups of cells to potential excitability of both could occur concurrently giving rise to the first post-partum ovulation being associated with overt heat (Cows 1 and 11). In addition, the manifestation of false heats (Cows 7 and 8) suggests that temporary modifications in the cells regulating the behavioural change could occur within the overall period when the excitability of both hypothalamic centres were suppressed. It may be that the temporary alteration allowing this response was as a consequence of some independent factor acting on the cells in the hypothalamus.

It is well recognised that in the normal cycling cow various morphological changes occur around the time of oestrus that are considered also to be induced by the presence of elevated levels of oestrogens in the blood. Amongst these the change in character of the uterine wall, referred to as tone, is well established. The previous concept of why animals do, or do not, respond to increased circulating

oestrogens was based on a hypothesis involving a temporary refractoriness in one of the target organs for oestrogens, i.e. the hypothalamus. It was considered that it would be of interest to determine if this was reflected in a similar temporary refractoriness in a further target organ for oestrogens, i.e. the uterus. Examination of the results for the individual cows, given in Figs 35 - 62, showed that in the case of Cows 1, 3, 5, 9 and 10, uterine tone of (+++), i.e. comparable to that considered to be present at oestrus, was recorded within the anovulatory period. Marked, though slightly less pronounced tone, i.e. (++) was recorded within this period in the remaining 5 animals - Cows 2, 4, 7, 8 and 11. However, it was also apparent from the findings on uterine turgidity that although (+++) tone was usually recorded at around oestrus the turgidity was classified as only (++) at this time in two animals (Cows 1 and 8). These results were taken to suggest that the responsiveness of the uterus to oestrogens did not reflect the postulated hypothalamic refractoriness to these hormones. The relationship between various degrees of uterine tone and progesterone and oestrogen levels over the non-pregnant period in all cows is summarised in Fig 65.

In addition to the accepted role of oestrogens in the production of oestrus a role for androgens has been suggested in subhuman primates (Baird, 1974). It has been suggested that in these animals, although oestrogens are considered to produce changes in the female making her attractive to the male, androgens are responsible for the triggering of sexual receptivity to the advances of the male. In the human female, it has been demonstrated that although ovariectomy is

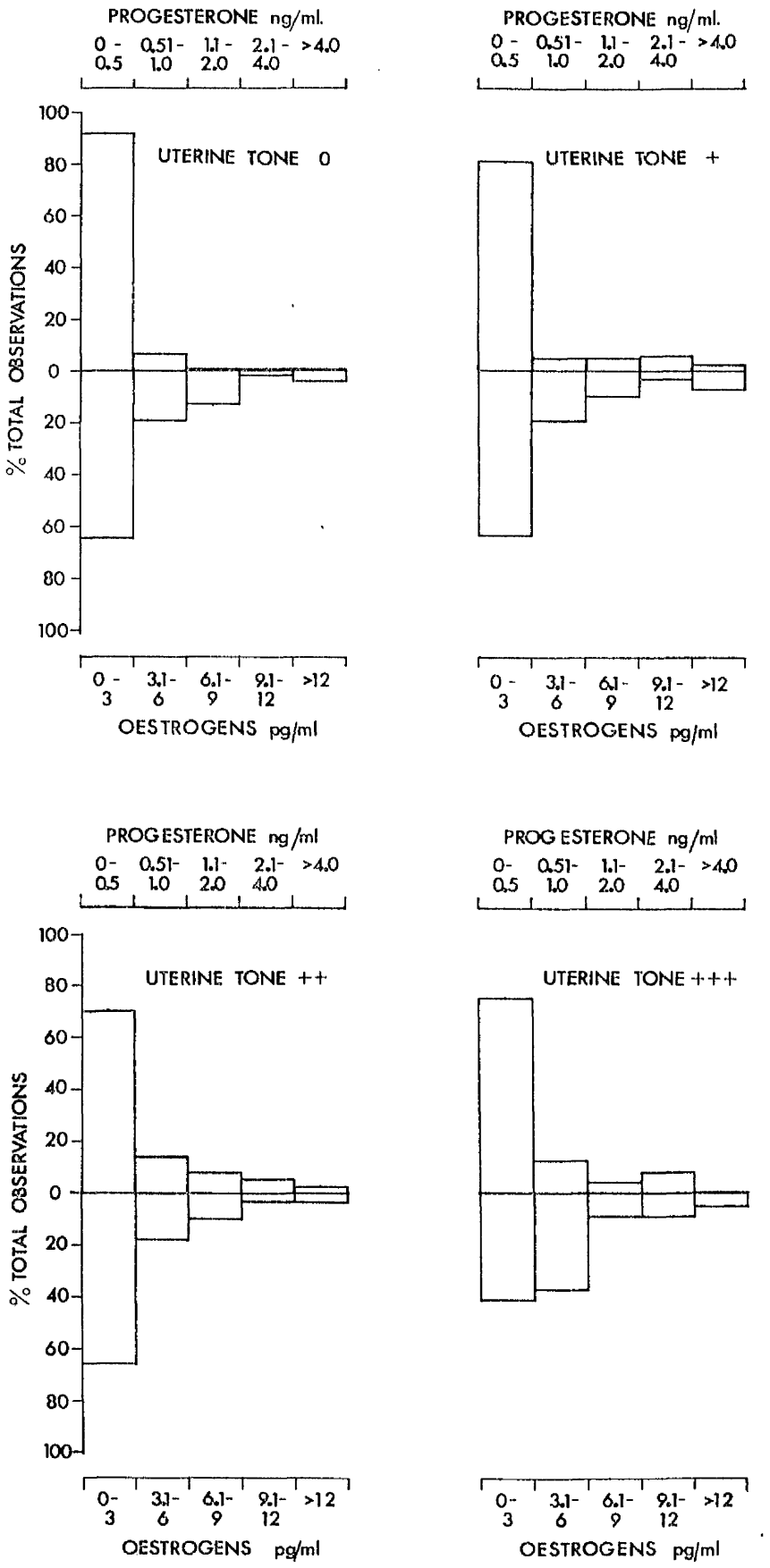


FIG 65 THE RELATIONSHIP BETWEEN UTERINE TONE AND THE PLASMA LEVELS OF PROGESTERONE AND OESTROGENS IN NON-PREGNANT COWS

not associated with a lack of libido adrenalectomy abolishes what is in fact a continuous state of oestrus in this species. Evidence suggests that this change of libido is associated with the removal of the major source of androgens in the human female, i.e. the adrenal (Baird, 1974). A possible role for the involvement of androgens at oestrus in the sheep has been suggested by the observation that the major steroid secreted by pre-ovulatory follicles in this species is the weak androgen androstenedione (Moore et al., 1966). The work, previously referred to, in ovariectomised cows is considered to provide sufficient evidence that oestrogens are an integral part of the mechanisms inducing the behavioural modification of oestrus (Asdell, de Alba and Roberts, 1945). However, in view of the variability noted in oestrous expression in the post-partum state, androgen levels were determined over the period of study to determine if fluctuations in the secretion of these hormones was related to the expression of heat. Results for the individual cows (Figs 36 - 63) demonstrate that elevated levels, from those on either side, were recorded in Cows 1, 2, 3, 7, 8, 10 and 11 at the time of first ovulation, but not in Cows 4 and 5 (Cow 9 not having ovulated at any time). Similarly elevated levels were found at the time of first overt heat with subsequent luteinisation in the case of Cows 3, 5 and 10 plus Cows 1 and 11 where the first ovulation was associated with overt heat. Increased concentrations were not, however, recorded at the time of first overt heat in Cows 2, 4, 7 and 8. Subsequent overt heats showed a similar divergence of results - in Cows 10 and 11 increased levels were found, but in Cow 3 there was no evidence of elevated

secretion of androgens at the times of subsequent overt heats. These results suggest that in general elevated androgens were found within the period around ovulation, irrespective of whether or not it was associated with silent or overt heat. It should be emphasised that these levels were from blood samples taken at weekly intervals over the experimental period. It may be that increased androgen levels around ovulation are of such a duration that an elevation in the hormone levels in the blood may have been missed due to the infrequency of sampling. This may provide an explanation for the inconsistency in results such as increased levels before the first ovulations in Cows 4 and 5. Obviously more frequent sampling over the period of pre-ovulatory follicular development will be required before it can be established whether the tentative suggestion given above, i.e. that androgens are secreted at this time, holds true. What was apparent from these studies is that, accepting the above limitations, levels of androgens at least as great as those found later, were recorded in the anovulatory, anoestrus period in all cows. These results suggest that the secretion of androgens was probably not related to the absence of oestrus during the post-partum period in the cow. It is apparent that similar studies to those involving oestrogens should be carried out in ovariectomised cows to provide an indication of whether or not androgens can elicit any part of the behavioural change constituting oestrus.

All cows within this study had more than one ovulation over the experimental period, with the exception of Cow 1 which had one ovulation, and Cow 9 which failed to ovulate. Of the animals whose

first heat was silent, it was apparent that an average of 12.9 days (range 6 - 30 days) elapsed before the first overt heats. Cow 11 was the only individual where the first ovulation was accompanied by overt heat and where subsequent heat was recorded within the experimental period. The interoestrous interval in this case was 8 days.

Both the mean interval from first to second oestrus and the range summarised in Table 36 were considerably less than that reported for 'normal' cycling cows. Wishart (1972) found that in 211 oestrous cycles in Friesian heifers the average inter-oestrous interval was 26.17 days during a similar period of the year to that when this study was carried out, and it is generally agreed that 60 - 90% of cows show an oestrous interval of between 17 and 25 days (Vandermark, 1952). Reduction in the time from first to second heat after parturition, compared to later inter-oestrous periods, has been noted by Morrow et al. (1968) and Menge et al. (1962). The fact that the duration of the first oestrous interval was significantly shorter in the animals in this study compared to those of Morrow et al. (1968) may be related to the very much later time of first post-partum ovulation in these suckled cows. In contrast to this hypothesis Morrow et al. (1968) found that there was an apparent relationship between the interval from parturition to first oestrus and the duration of the first oestrus-oestrus interval. Provided the cows were reproductively normal, prolongation of the period to first oestrus was more likely to be followed by a first inter-oestrous interval of more normal duration. In addition, within the animals in this study, analysis of the limited data available failed to demonstrate any

significant correlation between these two parameters, i.e. time from parturition to first ovulation, and time from first ovulation to second ovulation (Table 39).

In progressing along the path to a re-establishment of normal reproductive function after calving, the point is reached where at least conception becomes possible when the animal has its first overt heat. However, as previously noted, the re-establishment of pregnancy involves not only conception but the provision of a suitable uterine environment for further development of the conceptus. The absolute requirement for progesterone to maintain the pregnant state in the cow has been well established. Removal of the source of progesterone, i.e. the corpus luteum, at any time up to at least 160 days of gestation, has been shown to be associated with abortion or a return to oestrus (McDonald et al., 1958). That this abortion or return to heat was due, in the cow, to a deficiency of progesterone was confirmed by preventing these sequelae by administration of this hormone (McDonald et al., 1958).

It is considered that it is the action of progesterone from the corpus luteum on a uterus that has previously been under the influence of oestrogens that produces the type of uterine environment conducive to development of the conceptus (Corner and Allen, 1929).

It is recognised that, although cows are cycling in the post-partum period, it is more difficult to establish pregnancy in the earlier part of this period than later on (Groves, Lauderdale, Hauser and Casida, 1968). Thatcher and Wilcox (1973) found that as the

TABLE 39 : THE RELATIONSHIP BETWEEN THE PARTURITION TO FIRST OVULATION INTERVAL AND THE FIRST INTER-OESTROUS INTERVAL IN ALL COWS

Cow	Parturition to first ovulation* days	First to second oestrous interval days
2	66	14
3	30	16
4	49	9
5	61	11
7	55	11
8	83	8
10	47	7
11	71	9
Average	57.8	10.6

Correlation coefficient = -0.4448

*Taken as one day after the first post partum oestrous when luteinisation followed ovulation.

number of oestrous periods before 60 days post-partum increased, the number of matings after this time, necessary to achieve conception, decreased. Fertility, judged on the basis of ability to become pregnant, therefore, appears to be dependent not simply on the re-establishment of ovulations with subsequent luteinisation, but also on the time after calving and the number of previous oestrous cycles. Amongst the numerous factors involved in establishing a conceptus in the uterus is luteal function, or the secretion of progesterone after ovulation. Information on the possible involvement of the level of luteal function on potential fertility in the post-partum period was obtained by observations in both normal cows and animals given exogenous hormones. Working with slaughter-house specimens, it was found that corpora lutea, removed 15 days after oestrus, tended to be much smaller when removed from animals soon after calving than later in the post-partum period (University Wisconsin Research Bulletin 280, 1968). In addition to size, the progesterone content of these early corpora lutea was decreased. These observations suggested that rather than luteal function being an all-or-none phenomenon, it developed with increasing time after calving. It was suggested that the level of luteal function may be related to the ability to re-establish pregnancy in the post-partum cow. A further role for progesterone in relation to fertility after calving was demonstrated by Huertas, Vega, Britt and Ulbert (1972). By feeding dairy cows the potent, orally active progestagen, melengesterol acetate, from 14 days after calving, i.e. prior to the time these animals first ovulated, they showed that fewer services were required to achieve conception in these treated

cows than in similar control animals. These findings were interpreted as suggesting that there may be a requirement for a period of cyclicity, or rather of progesterone secretion, prior to their becoming pregnant.

On this basis the results of luteal structure and function in the individual cows in this thesis have been analysed: firstly with regard to all cycles commencing within increasing periods of time after parturition; secondly from the point of view of all first, second and third cycles, post-partum irrespective of their time of occurrence.

Asdell (1943) considered that the life span of the corpus luteum could be divided into two periods. The period after ovulation when the luteal tissue was forming and acquiring function was referred to as metoestrus. This led into the time when a fully functional corpus luteum was present in the ovaries, which was called dioestrus. Although this terminology will be employed in this thesis, it will be related to the direct observations on secretory function of luteal tissue in the post-partum cow.

The half life of progesterone in the cow is very short, being between 15 - 30 min (McCracken, 1963; Imori, 1967). Coupling this rapid turnover of the hormone in the circulation with the fact that only very small, if any, quantities of progesterone are derived from extraglandular conversion in the non-pregnant female (Baird, 1974) means that the level of this hormone in a peripheral blood sample gives an accurate indication of the secretory function of luteal tissue.

In the non-pregnant cows in this thesis, differences were apparent in the pattern of secretion of progesterone during cycles commencing from 0 - 49 days, 50 - 69 days and 70 - 89 days after calving (Fig 66). Taking the interoestrous intervals as 100%, a relatively longer percentage of the cycle elapsed before peripheral plasma progesterone concentrations reached a level of 2.0 ng/ml in cycles commencing between 0 - 49 days then in later cycles. In addition, although the peak concentrations in each group of cycles were similar, the timing of the decline in function showed differences between earlier, and later cycles. Only in the case of cycles commencing on or after day 50 was a precipitous decline recorded towards the end of the cycle. It therefore appeared that cycles early in the post-partum period were not associated with a relative deficiency of progesterone at the time of maximum function, but that the period before maximum function was relatively prolonged, and that the termination of dioestrus was not so abrupt.

Comparison of non-pregnant cycles on a chronological basis revealed that a marked difference was apparent between the peripheral plasma progesterone levels during first, as opposed to subsequent, cycles (Fig 67). These results demonstrate that at no time did the average plasma progesterone concentration reach 2.0 ng/ml during the first cycles, whereas this level was greatly exceeded during at least a part of succeeding cycles. In spite of these differences in the level of luteal function, however, it was apparent that in all cases the pattern of secretion shows similarities. Metoestrus seemed to be relatively prolonged during the first cycles as was evident from the

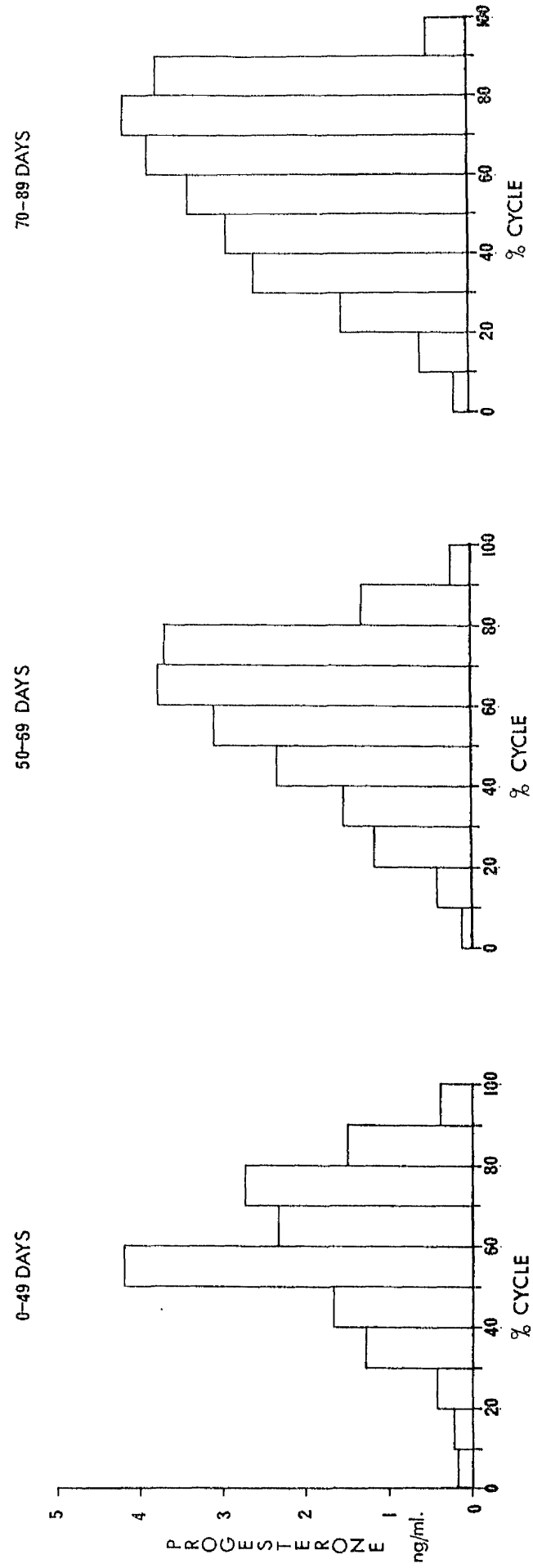


FIG 66 PROGESTERONE SECRETION DURING OESTROUS CYCLES COMMENCING WITH INCREASING TIME FROM CALVING

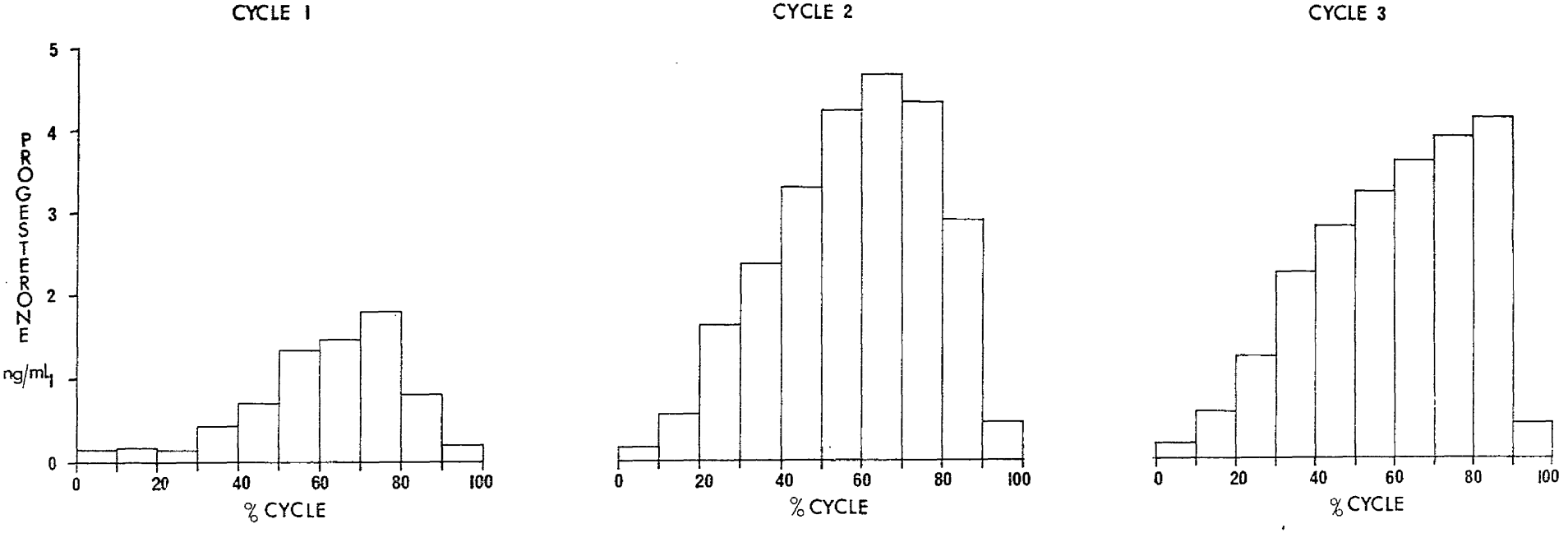


FIG 67 PROGESTERONE SECRETION DURING SUCCESSIVE OESTROUS CYCLES AFTER CALVING

increased percentage of the inter-oestrous interval that elapses before progesterone secretion rose after ovulation. However, in all cases maximum secretory function was found towards the terminal part of dioestrus, indicating that these early corpora lutea have secretory function in many ways similar to the 'normal' but at a lower level. These results suggested that although the secretory function of luteal tissue was in some ways reduced in cycles occurring earlier rather than later after calving, this was overshadowed by the marked effect of the first as opposed to subsequent cycles.

An increasing pattern of progesterone secretion during consecutive oestrous cycles has been recorded in the post-partum cow by Edgerton and Hafs (1973). However, the results from these workers were based on observations during the first and second cycles after overt heats and, as no attempt was made to establish whether or not these heats were preceded by one or more silent heats, they were not directly comparable to the results of this study. Within the group of animals, excluding false heats, in this thesis, 7/9 of the first post-partum heats were silent. The majority of second cycles (7/8) and all third cycles (3/3) were preceded by overt heat. In contrast to the findings of Edgerton and Hafs (1973) therefore, no apparent difference existed between the pattern or amounts of progesterone secreted after the first and second overt heats, represented by the second and third cycles in this thesis.

The luteotrophic and luteolytic factors regulating corpus luteum function in the normal cycling animal have been extensively studied (see Chapter One). It may be that reduced amounts of

progesterone secreted during the first post-partum oestrous cycle reflected a defective trophic stimulus at this time. The evidence implicating pituitary LH as the luteotrophin in the cow has previously been reviewed (see Chapter One). Saiduddin (1958) found that the pituitaries of post-partum cows from day 30 onwards contained high levels of LH suggesting no deficiency of synthesis of this hormone by this time. Obviously in the case of the first as well as subsequent cycles, sufficient LH was released to bring about ovulation. It has been established that the pre-ovulatory release of LH represents the maximum amount of this hormone released at any time throughout the cycle (Snook et al., 1971). In addition to triggering the process of ovulation, it has been found that this pre-ovulatory surge of LH programmes the cells which will form the corpus luteum for eventual secretion of progesterone. The release only of very small amounts of pituitary LH seems necessary to maintain luteal function in the cow (Hansel and Snook, 1970). It was therefore considered unlikely that the reduced pattern of progesterone secretion recorded during the first post-partum cycles in the cows in this study resulted from a defective trophic stimulus. Additional evidence to substantiate this suggestion is found in the above mentioned studies of Edgerton and Hafs (1973). These workers reported that following the first overt heat the reduced progesterone levels found were associated with high levels of LH.

Many factors including post-partum infections of the uterus (Gier, Singh and Marion, 1962), dilation of the uterus (Ginther, Woody, Janakiraman and Casida, 1966) and the infusion of inter-uterine saline (Gripper and Littlewood, 1969) have been observed to decrease the

period of luteal function by inducing morphological as well as functional regression of the corpus luteum. Similarly the administration of oxytocin during metoestrus has been shown to shorten the life span of the corpus luteum (Armstrong and Hansel, 1965; Donaldson et al., 1970). Although this could be overcome with LH and HCG (Hansel and Snook, 1970), evidence was obtained by studies on hysterectomised animals that the induced luteolysis involved factors other than interference with the release of luteotrophin. Complete luteal regression could only be induced by oxytocin in intact animals, suggesting that oxytocin acts primarily through the release of a uterine luteolytic factor. Following administration of oxytocin on days 2 - 4 after oestrus, the pattern of secretion of plasma progesterone was unaltered, as compared to untreated animals, up to day 5. In 'normal' cycles plasma progesterone remains elevated after day 5. In the animals treated with oxytocin alone the secretion of progesterone fell from day 5 and resulted in a reduced oestrous-oestrous interval (Donaldson et al., 1970). It is suggested that oxytocin operates through the release of uterine luteolysin (prostaglandin) to modify luteal function. The independent studies demonstrating that prostaglandin F2α was ineffective in inducing luteolysis before days 4 - 5 of the cycle (Rowson, Tervit and Brand, 1972) possibly explains the observation that only after this time does oxytocin have an apparent effect on luteal function. The plasma progesterone levels during the first cycles of the animals in this thesis when examined on a day to day basis, as opposed to a percentage of the cycle, show that in general plasma progesterone began to rise from two to four days

after oestrus, and fell from two to three days before the next oestrus (Fig 68). This pattern of increase and decrease on a daily basis was similar to that reported in the normal cycling cow (see Chapter Two) and in subsequent cycles in this study. This indicated that the markedly shortened first oestrous-oestrous interval comprised a period of progesterone secretion similar to the normal, but that the secretion was prematurely terminated before the elevated levels typifying the latter part of dioestrus in the normal cycle could be reached. These observations could suggest that at least part of the explanation for the pattern of first cycle progesterone secretion involves the release of prostaglandin either in abnormal amounts or at an abnormal time, in a manner similar to that artificially produced by oxytocin administration. It would be possible to establish the importance of this suggested effect by hysterectomising cows immediately after their first post-partum ovulation and determining if this resulted in a period of 'normal' luteal function.

Studies on uterine involution after calving have shown that in terms of the diameter of the uterine horns they have fully contracted within 3 - 4 weeks of calving (Morrow et al., 1969). In terms of this criteria, all the animals in this thesis were normal in that their uterine horn diameters had all ceased markedly to decrease by 3 - 4 weeks post-partum. Although the uterine epithelial mucosa of post-partum cows would appear restored to the non-pregnant state 20 - 30 days after calving (Rasbech, 1950; Riesen, 1968; Wagner and Hansel, 1969), histological changes taken to indicate continuing

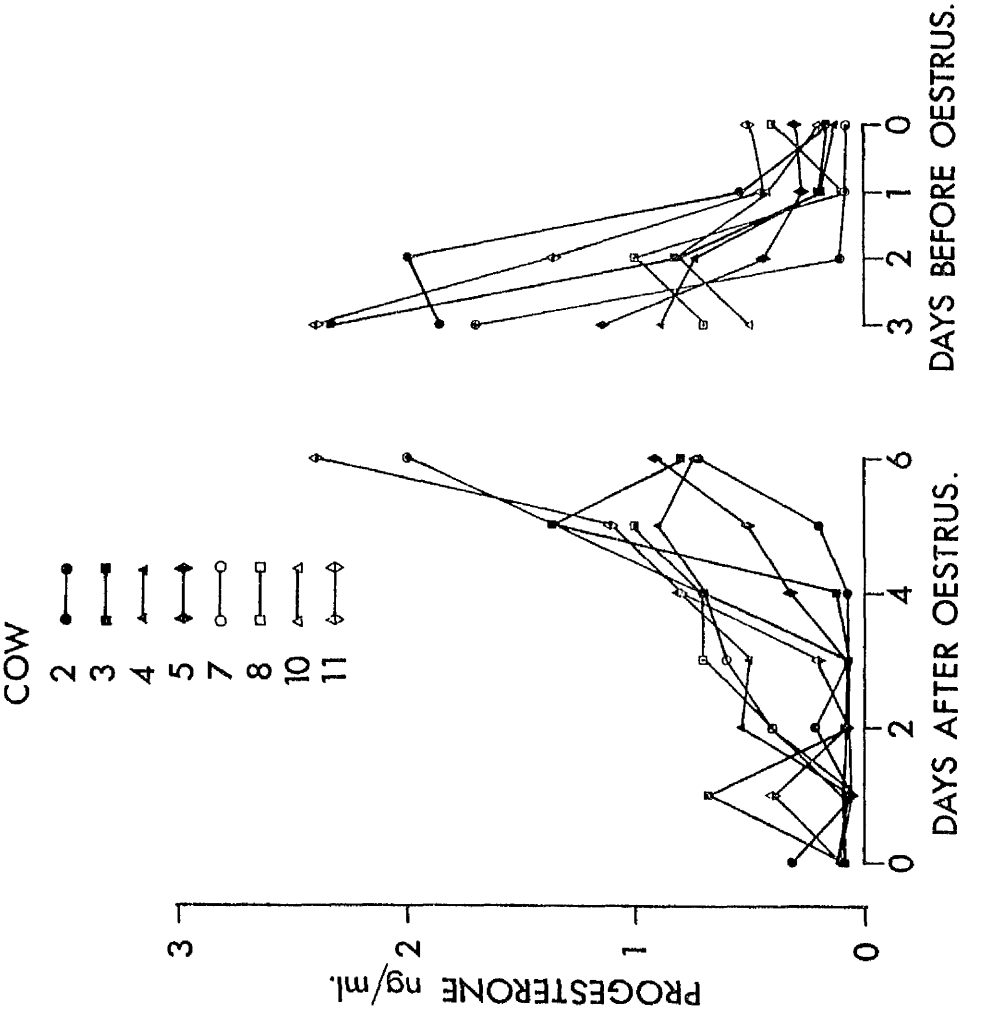


FIG 68 PROGESTERONE LEVELS DURING THE FIRST OESTROUS CYCLES AFTER CALVING

involution at the cellular level have been described as still occurring after 50 - 60 days (Anderson et al., 1969).

The difficulty of establishing pregnancy in the early post-partum period could be related to the state of the uterus and 'abnormal' first cycle luteal function.

The majority of first ovulations after calving in this and in other studies have been associated with the absence of oestrous behaviour. It may be that this represents a mechanism whereby the cow prevents the possibility of conception taking place at this time and therefore avoids presenting the conceptus with a uterine environment unsuitable for its subsequent development. The unsuitable uterine environment in turn manifests itself by modifying luteal function to a level where the amount of progesterone secreted would be insufficient to maintain the conceptus. However, progesterone was secreted, albeit in reduced amounts, during the first cycle and this, as previously noted, was possibly involved in ensuring that subsequent ovulations were accompanied by overt heats, allowing mating to take place. It therefore appears that just as oestrous behaviour and ovulation follow a pattern of development during the post-partum period, so also does luteal function and that these components of the oestrous cycle are integrated in a manner which first prevents, then permits not just conception, but subsequent development of the foetus. It is likely that this process of development continues after the first overt heat and 'normal' ovulation and luteinisation explaining how much easier it is to get cows pregnant over increasing numbers of cycles after

parturition (Thatcher and Wilcox, 1973), but no evidence in terms of length of the cycle or the amounts of progesterone secreted could be found to explain this in the present study. However, it may be that progesterone has an effect on the return to normal of the post-partum uterus, and that this necessitates the effect of progesterone produced over several cycles in some cows to return the endometrium to normal.

In some animals (Cows 1 and 11 in this study) the above does not apply and the first post-partum ovulation was accompanied by overt heat. Reasons for this difference were not apparent but it is of interest to note that there is a significant difference ($P < 0.02$) when the first overt heat dioestrous progesterone levels were compared to the first silent heat dioestrous progesterone levels (Table 40). Acknowledging the limited amount of data available for carrying out this analysis, these results suggest that in some cows the development of more 'normal' luteal function need not be preceded by a period of reduced luteal function and that in these individuals the first ovulation can therefore be accompanied by overt heat.

The circulating progesterone levels recorded over the complete cycle of all heats other than the first, not associated with pregnancy, were similar to the results from normal cycling cows reported by other workers (Fig 69) (see Chapter Two). Following the low levels around oestrus, increasing plasma progesterone concentrations were found from around 10 - 20% of the cycle to 50 - 65% of the cycle. This pattern of increasing progesterone was similar to that reported by Donaldson et al. (1970) but the levels recorded by these workers on day 5 (which

TABLE 40 : COMPARISON OF PLASMA PROGESTERONE LEVELS DURING DIOESTRUS PERIOD* OF THE FIRST SILENT AND FIRST OVERT HEATS POST-PARTUM.

ASSOCIATED OESTRUS	NUMBER HEATS	PLASMA PROGESTERONE ng/ml $\bar{x} \pm 1$ S.D.
SILENT	7	0.82 ± 0.67
OVERT	2	1.55 ± 1.21

*Dioestrus taken as 41 - 80% of the inter-oestrous interval.

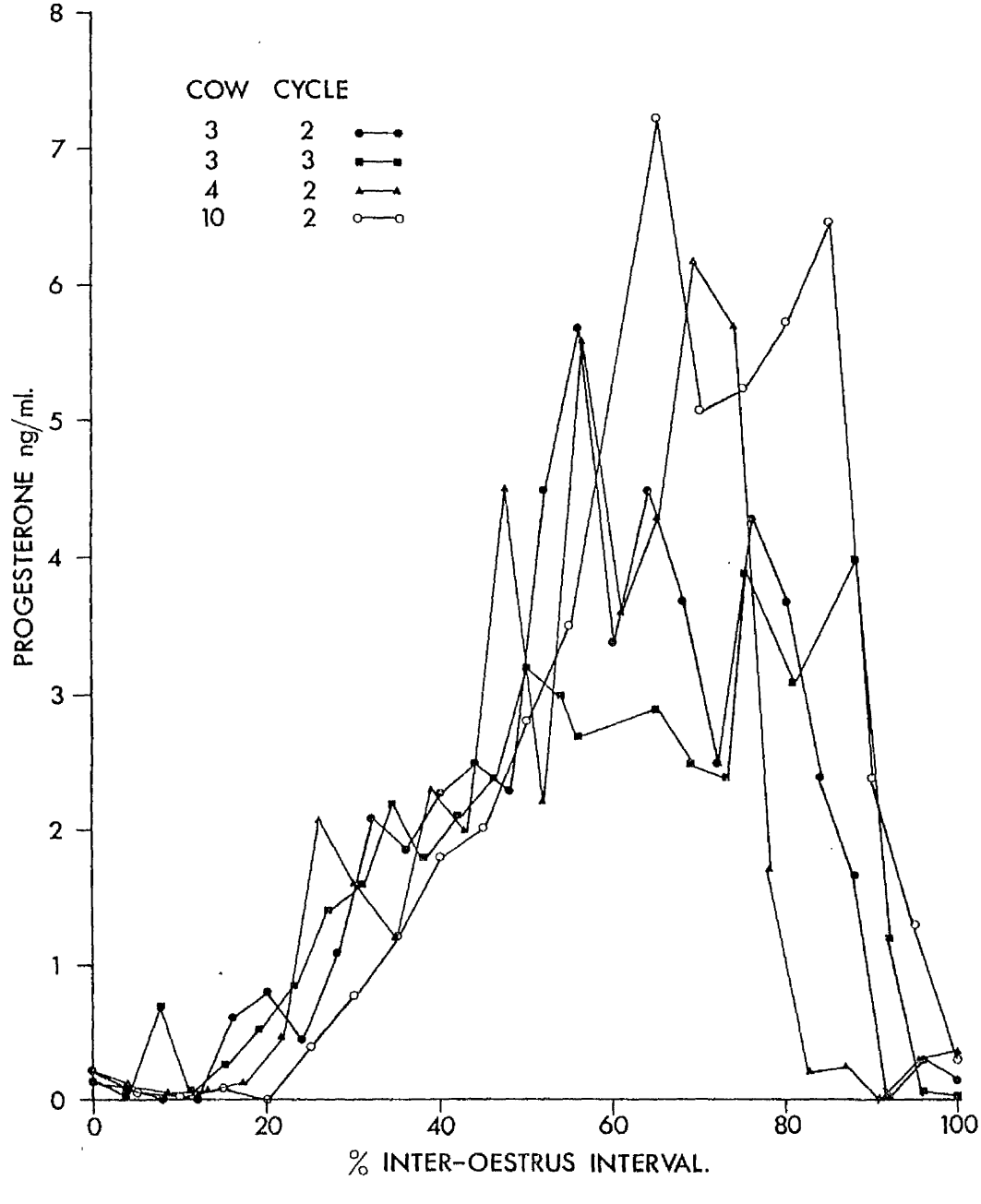


FIG 69 PLASMA PROGESTERONE LEVELS DURING NON-PREGNANT OESTROUS CYCLES (OTHER THAN THE FIRST CYCLES AFTER CALVING)

would be equivalent to around 20% of the cycle in this study) were higher. Relative to milked dairy cows, little information is available on plasma progesterone levels in suckled cows. A pattern similar to the results recorded in this thesis has been reported by Stabenfeldt et al. (1969), Pope et al. (1969) and Henricks et al. (1970).

Within the period up to 50% of the cycle both temporary peaks (Donaldson et al., 1970) and declines (Plotka et al., 1967) have been recorded in the secretion of plasma progesterone. In only one of the non-pregnant cycles in this study (Cow 10, cycle 2) was a consistently increasing pattern of plasma progesterone observed over the period to 50% of the cycle. Of the remaining three cycles, both temporary increases and declines were recorded within this period. Due to the variability in levels within the overall period of progesterone secretion found in this study, and by other workers, it is unlikely that they were of functional importance but simply reflected transient alterations in luteal activity around the time the blood samples were taken. The finding by Dobrowolski et al. (1968) that ovarian venous blood progesterone levels fell between days 9 and 13 days of the cycle, was not substantiated by the peripheral plasma hormone levels in this thesis. The results in this thesis agree with the observations of Christensen et al. (1974) that maximum secretion of progesterone occurred in late dioestrus in the cow. The observations on luteal structure and function in suckled animals in this thesis therefore indicated that suckling did not interfere with the development of the corpus luteum, maximum secretion by this tissue, or its period of functional persistence.

CHAPTER FIVE

PREGNANCY

5.1. Introduction

After calving, a period of anoestrus is under normal circumstances, eventually followed by a resumption of cyclical ovarian activity and associated behavioural changes. At this point, due to the occurrence of standing oestrus, mating can take place. However, it is well recognised that mating at the time of overt heat offers no guarantee of pregnancy. Even if ovulation and conception occur, the ovum must continue to develop for a period before pregnancy can be said to have become established. Some endocrine aspects of the transition from the non-pregnant to the pregnant state were studied.

5.2. Experimental design

Certain of the cows within this study, after having been inseminated at the time of overt heat, were subsequently diagnosed, by rectal palpation of their uteri and contents, as having conceived and developed an embryo. By monitoring the same parameters as those studied before conception, morphological and functional changes could be integrated with respect to early pregnancy, and compared with the observations made during the non-pregnant period.

5.3. Results

The days of heat at which the animals became pregnant are given on the individual cow results in Figs 34 to 63. The plasma progesterone, oestrogens and androgens of these cows are also given in Figs 34 to 63. Rectal palpation of follicles, corpora lutea, cystic structures and

uterine tone, within the period of early pregnancy, are, as for the non-pregnant period, illustrated in these figures.

5.4. Discussion

For convenience, the calving to conception intervals of the animals that became pregnant are summarised in Table 41. It is apparent that by adding a gestation length of 280 days to the mean conception interval of 78 days that these cows had a potential calving interval of less than one year. It has been established that the average calving interval of suckled beef cows, in recorded herds, was 370 days with the upper point of the range being around 400 days (Meat and Livestock Commission, 1973). In dairy cattle for maximum economic milk production a calving interval of 365 days is desirable although in practice the optimal time is frequently not achieved (Esselmont and Ellis, 1974). In terms of the overall interval from calving to re-establishment of pregnancy, the animals in this thesis compared favourably with the better suckled and milked herds. Although the calving to conception interval is obviously influenced by many factors, such as the onset of cyclical ovarian activity and the occurrence of standing heat, it is also dependent in cycling cows on the number of matings required to achieve conception. The satisfactory calving interval of the cows in this study reflects the fact that with one exception (Cow 3) they became pregnant to one insemination, in contrast to the average 1.5 services/conception, reported in other investigations in commercial herds (Boyd, 1976). Due to the limited size of the current study, no conclusion can be offered to explain the high conception rate. However, it may be related to the fact that

TABLE 41. CALVING TO CONCEPTION INTERVAL

Cow No.	Calving to conception interval - days	No insemination to conception
2	78	1
3	96	2
4	78	1
5	70	1
7	64	1
8	89	1
10	72	1
11	78	1

insemination was only performed on animals exhibiting standing oestrus to a bull, rather than serving cows on the basis of some of the other signs associated with oestrus, or even perioestrous behaviour.

The plasma progesterone levels for seven days after the overt heats at which these cows conceived, illustrated in Figs 34 to 61, are given together in Fig 70. It can be seen that in all animals a similar pattern of increasing plasma progesterone levels was recorded during this period. Following oestrus, a significant increase in the concentration of this hormone was first found, on average, between 3 and 4 days after mating. Thereafter insignificant elevations were found with successive days.

For comparison with the progesterone levels recorded during metoestrus after conception, the plasma progesterone concentrations over the same period in the previously studied non-pregnant cycles, other than the first, are given in Fig 70. It was apparent from these results that although the progesterone levels in the two groups were not distinguishable on days 0, 1 and 2, a highly significant difference ($P < 0.05$) was recorded on days 3 to 7.

Although it is generally accepted that the corpus luteum during metoestrus is a similar structure in both pregnant and non-pregnant animals, the results in this thesis suggest that a functional difference may exist between the two groups. A similar tentative suggestion has been made by Henricks, Dickey and Niswender (1970) who found that in pregnant cows the rate of increase in plasma progesterone concentrations was greater than in non-pregnant animals from day 0 to

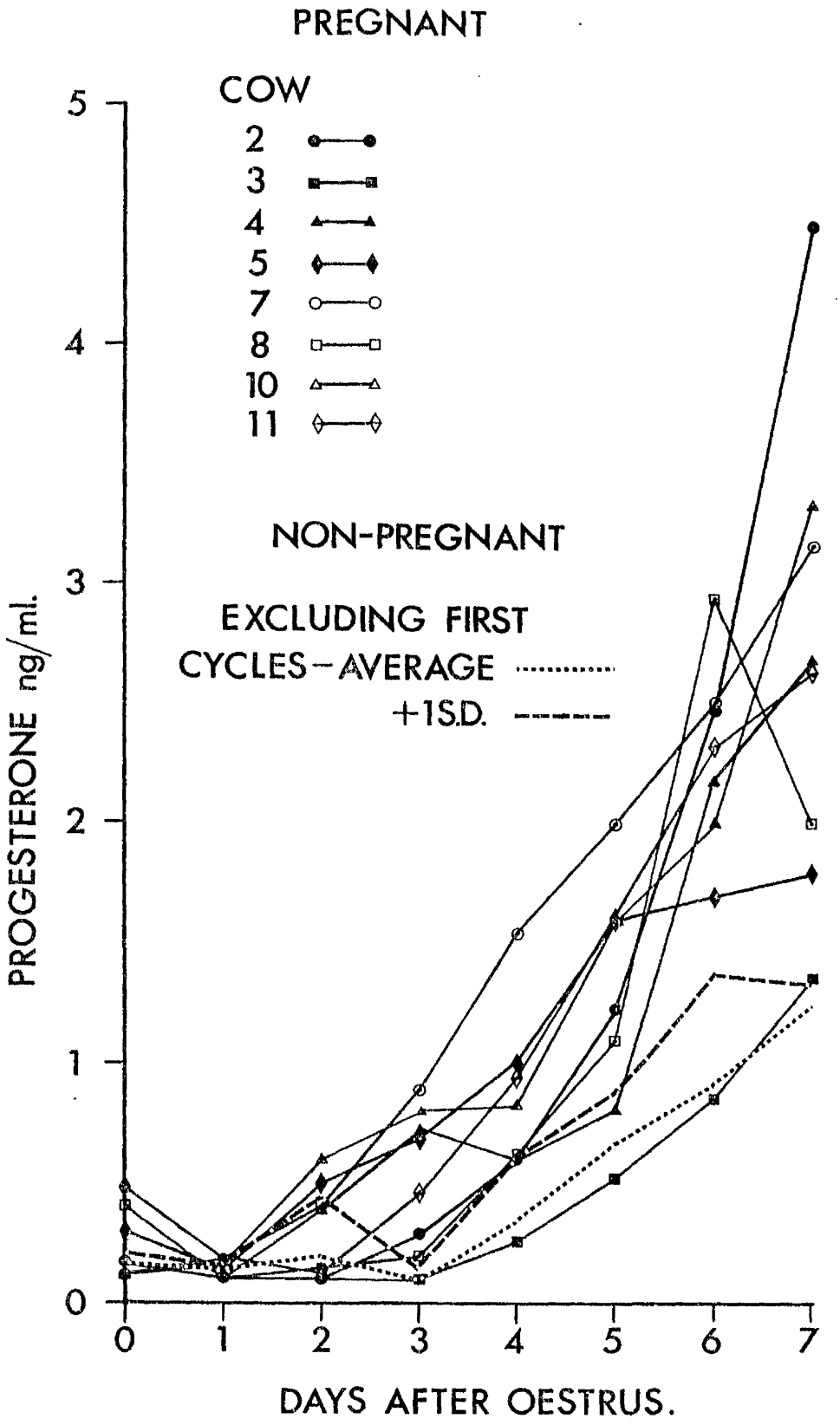


FIG 70 PLASMA PROGESTERONE AFTER THE HEATS AT WHICH THE ANIMALS CONCEIVED, AND THE LEVELS OVER A SIMILAR PERIOD OF NON-PREGNANT CYCLES

day 6 after oestrus. Obviously further studies will be required to determine if these apparent differences either in the rate of secretion during metoestrus or in the plasma progesterone levels within part of the period represent a positive luteotrophic effect dependent on the presence of a conceptus. Should it be possible to differentiate pregnant from non-pregnant cows within the period of metoestrus, by means of progesterone assays, significant shortening of the calving to conception interval becomes possible. By treating animals that have not conceived with prostaglandins, shown to be effective from day 4 of the cycle onwards (Rowson, Tervit and Brandt, 1972; Dobson, Cooper and Furr, 1974) dioestrus could be prematurely terminated and these cows re-inseminated.

It has previously been suggested (Plotka et al., 1967) that an embryonic luteotrophin stimulates luteal function 10 days after oestrus. Comparison of the plasma progesterone levels in these pregnant cows with the non-pregnant cycle results previously presented, fails to substantiate this hypothesis due to the non-significant difference in circulating amounts of this hormone between days 10 and 16 (Fig 71). Assuming that if these animals had returned to heat at this time this heat would again have been followed by an inter-oestrous interval of 21 days, the levels of plasma progesterone within a period of this second pregnancy 'cycle' could be calculated on a basis of the inter-oestrous interval and compared with the levels during the first 'cycle' after conception, and with non-pregnant cycles. It was apparent (Table 42) that the average progesterone level during 40 - 80% of this second pregnancy 'cycle' was not significantly different from

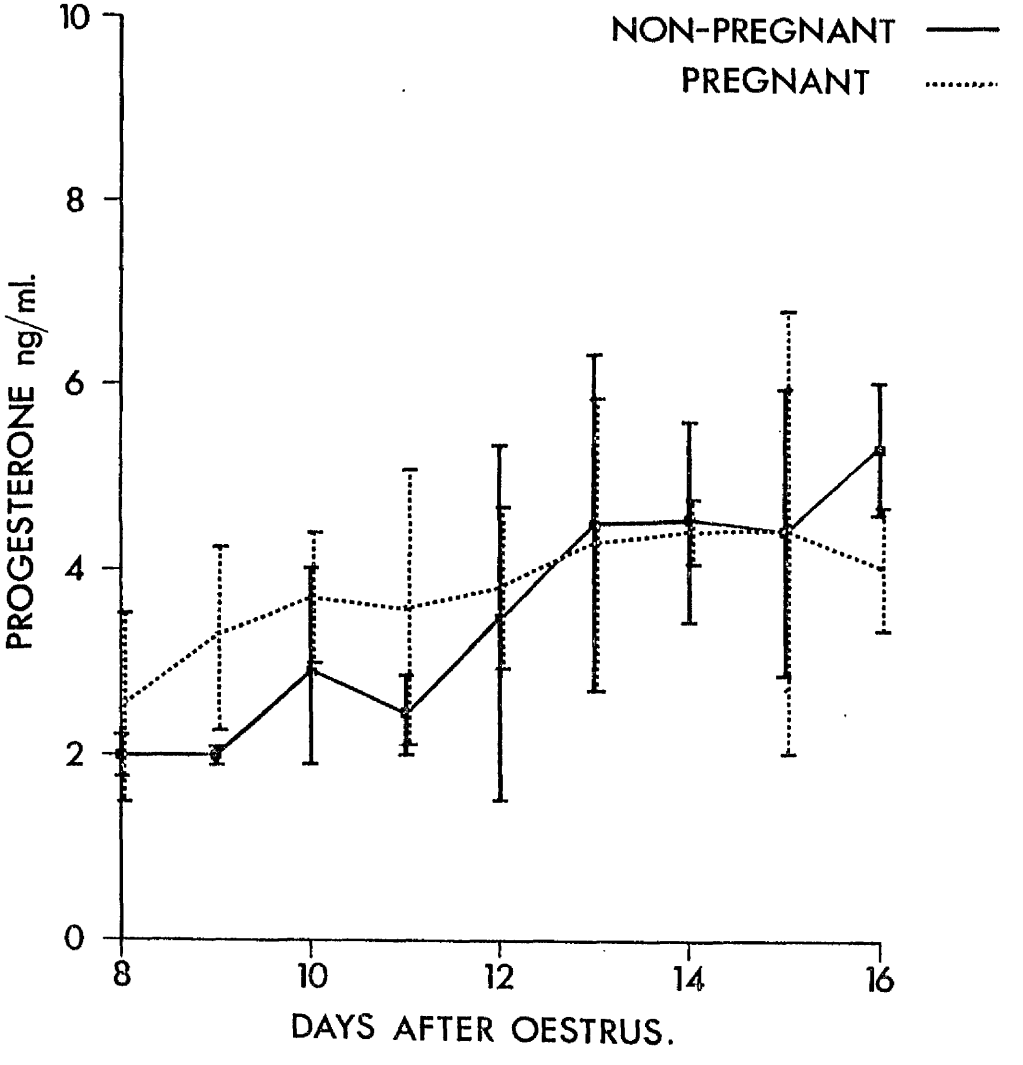


FIG 71 PLASMA PROGESTERONE LEVELS BETWEEN 10 AND 16 DAYS AFTER HEAT IN NON-PREGNANT AND PREGNANT CYCLES

TABLE 42 PLASMA PROGESTERONE LEVELS DURING PREGNANCY COMPARED TO THOSE DURING NON-PREGNANT CYCLES (EXCLUDING THE FIRST POST-PARTUM CYCLES)

	No. cycles	Progesterone** ng/ml $\bar{x}$ ± 1 S.D.
Non-pregnant	5	3.2 ± 1.6
Pregnant - 'first cycle*'	8	4.0 ± 1.3
Pregnant - 'second cycle*'	5	5.8 ± 2.1

* The first and second cycles after conception.

** Levels calculated from 41 - 80% of the actual or estimated oestrous cycle.

that over a comparable period of the first pregnancy 'cycle.' However it could be seen that progesterone secretion was significantly greater over this period in the second pregnancy 'cycle' than it was in non-pregnant cycles. Although Estergreen, Frost, Erb and Bullard (1965) considered that on the basis of progesterone concentration in luteal tissue the corpus luteum exhibited reduced function from day 30 of pregnancy these results are not at variance with the above suggestion of elevated levels during the second pregnancy cycle (i.e. from days 29 - 38). Estergreen et al. (1965) based their suggestion on a comparison of luteal tissue progesterone concentrations from animals between 15 and 30 days pregnant with the levels in animals from 37 - 95 days of gestation. It is possible, therefore, that the levels recorded in this study were followed by a later period of reduced secretion.

The existence of an embryo may therefore influence luteal function at three times during early pregnancy -

(a) during the early period when the corpus luteum is acquiring function

(b) around day 17 when it is considered to prevent release of the uterine luteolysin (Moor and Rowson, 1964)

(c) about 29 days after mating when again a positive luteotrophic effect is tentatively suggested.

It is apparent from the average $\pm$ 1 S.D. progesterone levels for the pregnant cows in this study that from day 8 onwards the concentration of this hormone in the plasma remained in excess of

2.0 ng/ml (Fig 72). This, however, gives no indication of the amounts of this hormone necessary to maintain the conceptus. Raeside and Turner (1952) and Uren and Raeside (1951) found that daily intramuscular injections of between 25 and 75 mg progesterone in oil failed to maintain pregnancy in animals where the corpus luteum had been removed between day 36 and 67. McDonald et al. (1958) surgically excised the corpora lutea from 60 day pregnant cows and were able to prevent termination of pregnancy by the intramuscular injection of 100 mg progesterone in oil daily. Although McDonald et al. (1952) worked at a later stage of pregnancy than the animals in this thesis, it is tentatively suggested from the results of the individual cows in this thesis that a level of at least 2.0 ng/ml of progesterone is required to maintain the conceptus in early pregnancy. It is apparent that in many cases the plasma concentrations of progesterone in the individual cows was considerably in excess of this amount suggesting, as has previously been implied from studies on excision of the corpus luteum, that this structure can produce greater amounts of progesterone than those required to maintain a pregnancy (Tanabe, 1966).

The plasma progesterone levels recorded at the time corpora lutea were palpated in both the pregnant and non-pregnant periods of all the cows in this study, are presented in Fig 73. It is apparent that within corpora lutea of similar size, wide fluctuations in plasma progesterone levels occurred. Although, in part, factors such as pregnancy may influence the amount of this hormone produced by luteal tissue, other effects, some of which have previously been mentioned, undoubtedly also apply.

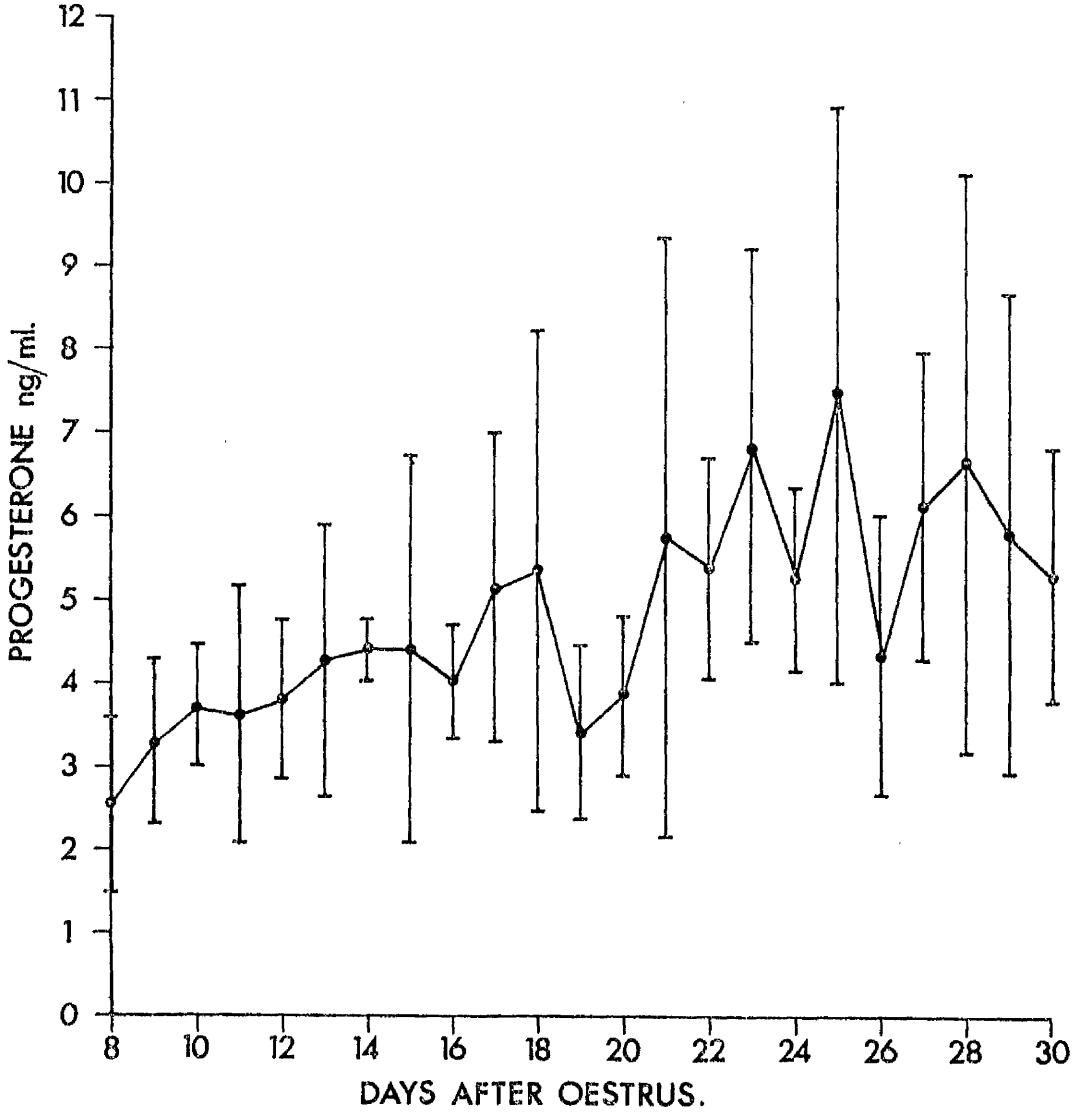


FIG 72 AVERAGE $\pm$ 1 S.D. PROGESTERONE LEVELS FROM DAY 8 OF PREGNANCY

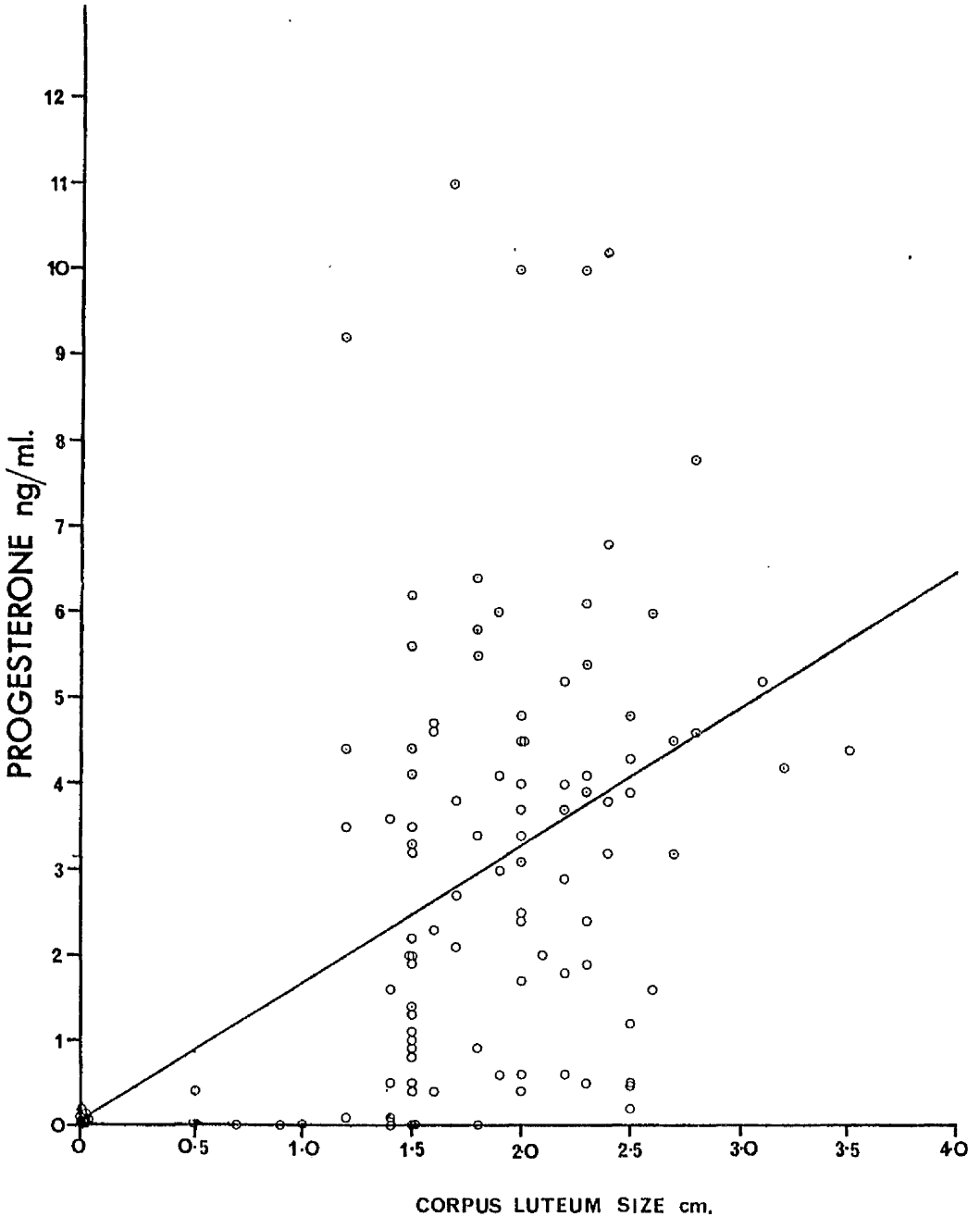


FIG 73 RECTAL PALPATION OF CORPORA LUTEA AND THE CORRESPONDING PLASMA PROGESTERONE LEVELS AT THE TIME OF PALPATION

Based on chemical and biological assays for androgens, Gassner (1952) suggested that androgens in the female cow were derived from progesterone and that they were therefore present at elevated levels in the presence of a corpus luteum. The peripheral plasma androgen levels in the individual animals fail to substantiate this hypothesis. Although elevated levels of androgens were found in Cows 2 and 4 when a functional corpus luteum was present in the ovaries in the other animals, levels at least as great as those in dioestrus were found around the time of ovulation. It has previously been suggested (see Chapter Three) that androgens are secreted by follicular development. It may be that in animals where elevated levels of androgens were detected during the period of luteal function these were associated not with this tissue, but with the concurrently present follicular growth.

During early pregnancy a variable pattern of total follicular surface diameters was recorded. Although in one animal (Cow 8) no follicles were palpated in the ovaries for a time after conception, in others (Cows 4, 5 and 11) follicles with a total surface diameter in excess of those recorded in the non-pregnant period were found. In addition, peaks of plasma oestrogens of comparable magnitude to those recorded in the non-pregnant period in association with ovulation and oestrus were detected (e.g. Cow 11, day 100; Cow 10, day 96). It appears, therefore, that follicular development, and function, contrary to the view expressed by Roberts (1971), were not suppressed in the presence of the corpora lutea found during early pregnancy.

It was apparent from the results of the individual cows that

during early pregnancy, cystic structures were present in the ovaries of Cows 2, 3, 4, 7, 10 and 11. These structures were similar in character to those found in the ovaries of Cows 3 and 4 during dioestrus, when these latter animals were not pregnant. Although these structures were classified as cysts on the basis of their size, period of persistence, their smooth surface, and their soft fluctuating consistency, they were not considered to represent examples of the typical clinical cystic ovarian disease syndrome as described by Garm (1944) and Dawson (1958). Within the description of this syndrome both cystic follicles and cystic corpora lutea have been included (Roberts, 1955; McEntee, 1958). In the animals in this thesis the cysts were not considered to represent abnormal corpora lutea, as a normal corpus luteum could be palpated in all animals and differentiated from, on at least some occasions, the cyst. The fact that in animals where only a cyst could be palpated a corpus luteum was found before, after, and on occasions within the period when only the cyst appeared present, coupled with the finding that the corpus lutea in all cases was present at the same position in the ovaries, was taken to indicate that the corpus luteum persisted over the period but that its presence was obscured by the cyst. Examination of the peripheral plasma hormone levels over the periods when cysts were present in the ovaries of the cows (summarised in Table 43), shows that their presence was not associated with reduced progesterone secretion compared to that found when corpora lutea were present with no cyst in the ovaries. On the occasions corpora lutea could be distinguished from the cysts, no differences in the size of these former structures, compared to those found when cysts were not present, could be appreciated.

TABLE 43 PLASMA HORMONE LEVELS IN THE PRESENCE OF CYSTIC STRUCTURES IN THE OVARIES

	Cow	Cycle	Days post-partum	Days after Oestrus	C.L. diameter cm. $\bar{x}$	Cyst diameter cm. $\bar{x}$	Progesterone ng/ml $\bar{x} \pm 1$ S.D.	Oestrogens pg/ml $\bar{x} \pm 1$ S.D.
Non-pregnant	3	3	76 - 83	8 - 15	2.3	3.2	2.2 ± 0.6	6.3 ± 3.6
	4	3	61 - 71	5 - 15	1.6	3.6	3.2 ± 1.7	4.1 ± 1.9
Pregnant	2	2	85 - 89	9 - 13	2.2	3.8	3.4 ± 0.3	3.5 ± 0.9
	4	4	83 - 86	5 - 8	1.8	3.5	1.9 ± 0.9	3.8 ± 2.0
	5	2	80 - 91	10 - 21	1.7	3.2	3.2 ± 0.8	4.8 ± 3.1
	7	3	76 - 100	11 - 35	1.5	3.5	5.4 ± 2.0	3.4 ± 2.7
	10	3	81 - 102	9 - 30	1.8	3.3	6.2 ± 2.6	6.0 ± 3.6
	11	2	90 - 104	11 - 25	1.5	2.6	6.1 ± 1.7	5.4 ± 3.6
Total Pregnant and Non-Pregnant					$\bar{x} \pm 1$ S.D. = 1.8 ± 0.4	$\bar{x} \pm 1$ S.D. = 3.3 ± 0.6	4.7 ± 2.3	4.8 ± 3.1

Reduced luteal tissue progesterone levels have been reported in cystic corpora lutea by Staples and Hansel (1961). Donaldson and Hansel (1958), however, have suggested that this apparent diminished function was due to the hormones administered to these cows, and the observations should not be extrapolated to represent naturally occurring cystic corpora lutea. As previously noted, some of the cows in this thesis, if examined on some of the occasions cysts were found, would have presented difficulties in determining what structures were present in the ovaries. Only by carrying out repeated rectal palpation of the ovaries in these cases could a corpus luteum eventually be distinguished. Undoubtedly some of these animals with a history of absence of nymphomania and a large cystic structure in the ovaries would have been diagnosed as having either cystic follicles or cystic corpora lutea leading to erroneous interpretations of the hormones produced in association with these structures. Dawson (1975) has demonstrated the degree of inaccuracy of rectal palpation in distinguishing cysts from corpora lutea and normal follicles. Due to the presence of corpora lutea at the same time as the cysts, and the evidence that normal plasma progesterone levels were being produced along with the fact that pregnancy continued in all animals, these cystic structures were considered to have no clinical significance.

Elevated urinary excretion of oestrone and 17α-oestradiol has been reported in cows with cystic ovaries by Lunaas, Refsdal and Garm (1974), the amounts exceeding those found during the follicular phase of the cycle by several times. 17β-oestradiol content of follicular cyst fluid has been found to vary over a wide range (Short, 1962;

Lunaas, 1964; Schjerven, 1971) and the concentration of this hormone in the plasma has been reported as being higher than in cycling cows (Edqvist, Ekman, Gustaffson and Lindell, 1974). The individual, and combined, plasma oestrogen levels of all cows when cysts were present in their ovaries failed to demonstrate increased levels of these hormones compared to that previously noted in association with normal follicular development (see Chapter Two). In contrast to the above observations on cystic follicular structures where a corpus luteum was not present in the ovaries, the result in this thesis demonstrates that similar structures in the presence of functional luteal tissue can produce 'normal' follicular quantities of oestrogens. Garm (1949) suggested that the occurrence of cystic ovaries in cows was associated with a dysfunction of the anterior pituitary causing a deficiency of LH. Histological examination of pituitaries from animals with follicular cysts failed to show any evidence of the normal degranulation of the basophils, containing gonadotrophins, in these animals (McEntee and Jubb, 1957). Further evidence that a failure in the release, rather than the synthesis of LH, was a possible explanation for these persistent follicular structures was provided by the demonstration that a proportion of these cows could be successfully treated by administration of gonadotrophic hormone releasing factor (Edqvist et al., 1974). During dioestrus in the normal cow, the secretion of progesterone is associated with the release of only low levels of LH (Hansel and Snook, 1968). It is apparent from studies in normal cows that follicular development, requiring gonadotrophic stimulation, proceeds throughout dioestrus (Hansel and Wagner, 1969). What is surprising, therefore, is that not all cycling, and pregnant

cows, develop the cystic structures recorded in this thesis. It is apparent that the factors regulating normal follicular atresia during the cycle must be established, as it is probable that it is a failure of these mechanisms that leads to a persistence of follicles and the formation of cysts in animals where a corpus luteum is present in their ovaries.

Following conception, uterine tone was generally reduced from the degree of turgidity noted before this time. In all animals that became pregnant, a similar pattern of uterine tone was observed over the complete period of study, i.e. slight tone at the start of the post-partum period, increased levels especially over the time ovulations were taking place, and a return to reduced levels following conception. However, it was apparent from the results of the individual animals that marked variability existed in the overall degree of uterine turgidity after, as well as before, conception. Some animals (e.g. Cow 5) were recorded as having a degree of turgidity (++) during this period, similar to that found in other animals at the time of oestrus (e.g. Cow 10). Similarly, although a uterus possessing some tone was commonly found during early pregnancy, in some cases a flaccid uterine wall was frequently recorded (e.g. Cow 10). The degree of uterine tone over the complete period of study and its relationship to differing levels of progesterone is illustrated in Fig 74 . It may be that the reduced levels of uterine tone during the pregnancy period was a direct reflection of the pregnant state. It is recognised that the activity of the myometrium is influenced by the ratio of progesterone to oestrogen in the blood (Csapo, 1956). It has already

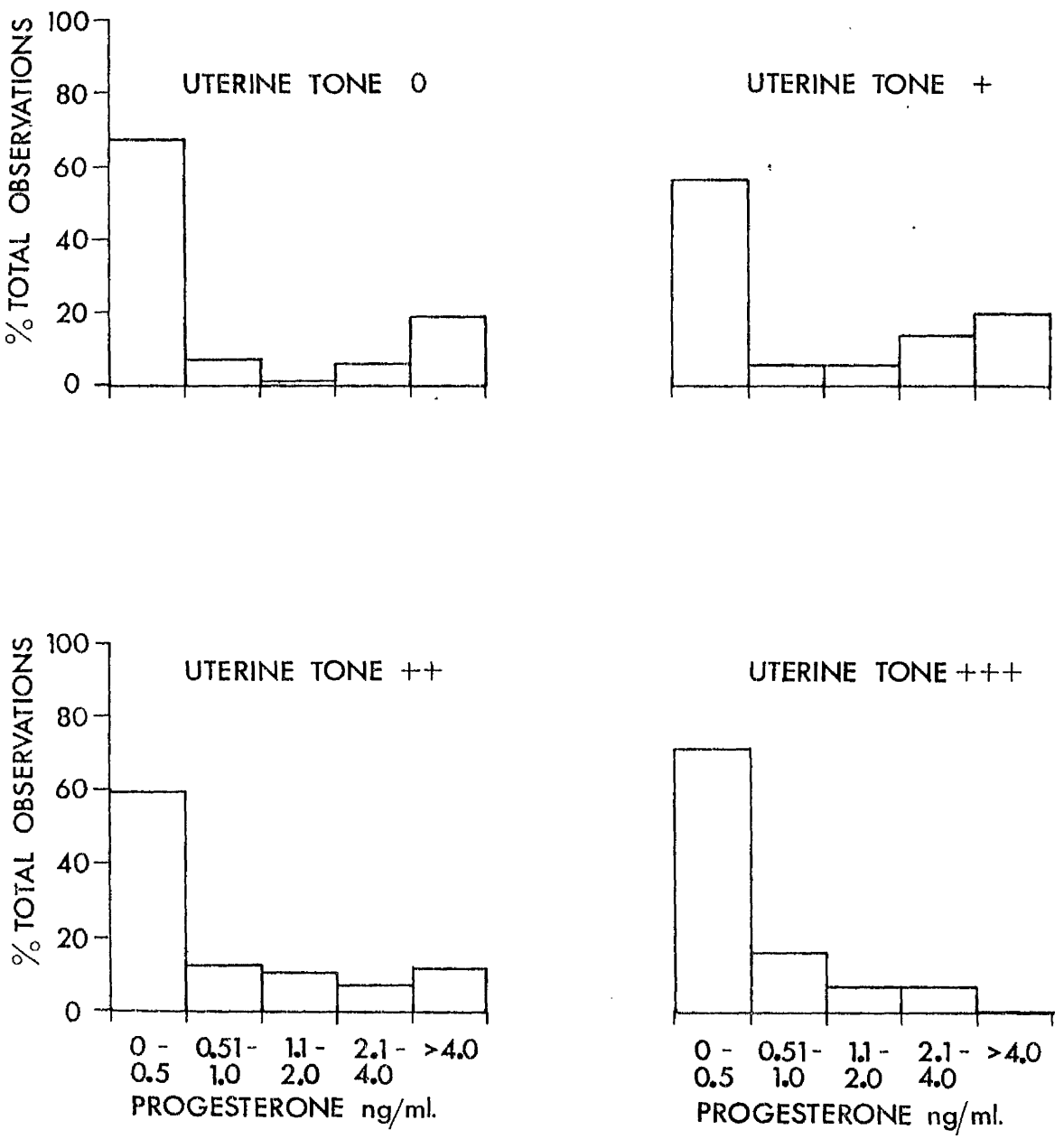


FIG 74 THE RELATIONSHIP BETWEEN UTERINE TONE AND PLASMA PROGESTERONE LEVELS IN ALL COWS OVER THE COMPLETE PERIOD OF STUDY

been noted that the secretion of plasma oestrogens is as great during early pregnancy as before conception. However, it has also been observed that a pattern of increasing progesterone levels was found with increasing ovulations after calving. It may be that this overall decreased tone noted in pregnancy reflected the elevated progesterone levels at this time compared to those present earlier in the post-partum period.

CHAPTER SIX

GENERAL DISCUSSION

Much of the available information on the pre- and post-partum periods of reproduction in the cow have derived from behavioural observations and physical palpation of structures within the ovaries and of the tubular genital tract. In this thesis, besides these accepted criteria of assessing reproductive status, the peripheral plasma progesterone and total oestrogen levels were determined. In addition to providing confirmatory evidence of the results of rectal palpation of ovarian structures, this latter technique having been shown by Dawson (1975) to have a large percentage error, the plasma hormone levels enabled an indication of the functional status of any follicular or luteal tissue development to be made.

Overall the observations on peripheral plasma hormone levels were made possible through the establishment of highly practicable radioimmunoassays for progesterone and oestrogens. Due to the specificity of the anti-progesterone serum, there is little that could be done to improve the method reported, both as regards assay reliability and simplicity of performing the estimations. In the case of the oestrogen assay, although a degree of specificity was sacrificed, in that total oestrogens were determined, the practical advantages gained from the simple extraction, incubation and separation steps coupled with the observations that oestrone is secreted in small quantities by the cow (Glencross et al., 1973) and that oestriol is not secreted (Dorfman and Unger, 1965) were felt not to offer serious criticism of the technique. Both of these methods allowed the routine processing of up to 30 samples in each assay, which could be completed within 24 hours.

Following conception, a corpus luteum forms and secretes progesterone in quantities which at least initially are indistinguishable from that secreted by cycling cows in dioestrus (Tables 40 and 42). Although at a slightly later stage of gestation even more progesterone may be secreted by these corpora lutea (Table 42), by the end of gestation, around 6 to 2 days before birth, the amount of progesterone produced has significantly decreased (Fig 31). Evidence has been presented within this thesis to suggest the prime importance of a sharp decline in progesterone secretion, probably due to intervention of a luteolytic agent, as a pre-requisite to the onset of labour. The above observations on the relative secretion of progesterone by corpora lutea at various stages of gestation suggest that during late pregnancy in the cow an independent mechanism serves to reduce progesterone secretion by this structure. It may be that this initial decline represents the gradual dying off of the corpus luteum as it reaches the end of its natural life span and that this process is then hastened by the superimposition of a lytic mechanism immediately before the onset of birth.

Luteal function has been studied under a variety of circumstances - at term, prior to the first overt heat, in cycling cows and in early pregnancy. Overall comparison of luteal size, determined by assessing corpora lutea diameter on rectal palpation, with the peripheral plasma progesterone levels found in association with these structures at different reproductive stages reveals several facts pertinent both to an understanding of the physiology of dioestrus, and to the practical assessment of the reproductive status of cows.

Within a group of cows where behavioural observations are not available either for physiological or management reasons, the rectal palpation of corpora lutea is often used as evidence of normal cycling. Overall comparison of luteal size with plasma progesterone levels (Fig 73) reveals that within corpora lutea of from 1.5 - 2.5 cm diameter a wide range in plasma progesterone levels was found. Although in part this undoubtedly reflects the fact that functional luteal regression occurs at a more rapid pace than a structural decrease in size, it is apparent that, within this range, size alone cannot be used to assess accurately the level of function. These findings would suggest that if such things as abortion due to progesterone insufficiency do occur, the reason for the abortion will not be able to be appreciated by physical examination alone. Indeed, combining these findings with observations on the inaccuracy of detecting corpora lutea in the first place (Dawson, 1975) would lead one to question rectal palpation of ovarian structures as a valid means of assessing reproductive status. Support to this argument was given by the observations on the occurrence of cystic structures in the ovaries of the cows in this thesis. Although in all instances these structures could, by rectal palpation, be readily recognised as cysts, in no cases were they considered of clinical significance due to the fact that they were always found in association with a functional corpus luteum. However, purely on rectal examination in many instances (e.g. Cow 11, Fig 61) due to the size and position of the cyst a corpus luteum could not be recognised. Thus in the case of the pregnant animals with a history of anoestrus, they could on examination at one instance have been ascribed to the 'cystic ovarian disease' syndrome. Obviously

differentiation between the pathological and 'normal' state could be easily made by assessing the peripheral plasma progesterone levels. It is therefore suggested that at least as regards the presence of corpora lutea plasma progesterone levels should not be thought of as an alternative to physical examination, but should be regarded as the method of choice. As previously stated, it is generally accepted that cystic ovarian structures do not constitute a functional abnormality when they occur in the presence of a corpus luteum in the ovaries. It is, however, possible that if these cysts were associated with increased oestrogen secretion, they may interfere with reproductive function either directly by way of the effects of these hormones on target tissues such as the myometrium, or indirectly both through interference with the normal release of pituitary gonadotrophins or by releasing uterine luteolysin. The results in this thesis suggest that large cystic structures need not necessarily show increased oestrogen production, explaining why in these cows luteal function in the presence of the cysts was indistinguishable from that during normal dioestrus and why pregnancy continued.

Evidence on the level of function associated with corpora lutea has suggested that these structures formed early in the post-partum period may be associated with a progesterone insufficiency as regards the quantities of this hormone required to maintain a pregnancy. However, by the second and subsequent periods of dioestrus after calving, progesterone production was indistinguishable from that in normal cycling, or early pregnant cows. To maximise chances of achieving a calving interval of approximately 365 days demands

that cows be served under commercial herd circumstances from 45 days post-partum onwards. In the light of the above findings on progesterone secretion by early post-partum corpora lutea, it is suggested that for cows to have a chance of becoming pregnant from day 45 onwards they should at least be starting their second oestrous cycle from this time. This in turn would necessitate their first ovulation having occurred around day 24 after calving. Britt et al. (1974) have demonstrated the ability of GnRH to induce ovulation in early post-partum cows. The results in this thesis where animals were subjected to a management system recognised as delaying the occurrence of post-partum ovulations, i.e. suckling, show that even in these cows the ability to respond to GnRH should be present by around day 24. The potential ability to respond to GnRH was judged on the fact that functional follicular development occurred around this time in 9 out of the 10 cows. Combining these observations on follicular and luteal development after calving would suggest a role for GnRH as a routine treatment of post-partum cows with the purpose of assuring that all animals had at least one ovulation and one period of dioestrus by day 45.

It is well recognised that uterine tone increases around the time of oestrus in the cycling cow. The results in this thesis have demonstrated that increased tone can be found outwith heat periods in the post-partum animal. The overall distribution of uterine turgidity as a function of the level of progesterone present in the blood (Fig 65) shows, as would be expected, that maximum tone was associated mainly with low progesterone levels. It was also

apparent, presumably reflecting the fact that uterine tone is not regulated solely by progesterone, that the complete range of turgidity was associated with low levels of this hormone. However, the results of this study demonstrated that increased (++), and on a few occasions (+++) tone could be found when elevated levels of plasma progesterone were present. In general it can be seen, from Fig 65 , that increased (++) or (+++) uterine tone was associated with increased oestrogen levels. However, it was also apparent that a wide variation in the degree of turgidity could be found in association with similar oestrogen levels. Acknowledging that the determination of uterine tone was highly subjective, these findings would indicate that, as with attempted assessment of luteal function by rectal palpation, uterine tone is an imprecise indicator of both cycling activity and the stage of the oestrous cycle.

During pregnancy, it has been suggested (Glencross et al., 1973) that an elevation in plasma oestrogens occurs around the time when if the animal was not pregnant, it would have returned to heat. This would imply that some possibly inherent cyclicity, in terms of follicular development, persists in the ovaries even when final follicular development and ovulation are suppressed by the secretion of progesterone from the corpus luteum. The results in this thesis confirm that increased levels of oestrogens can occur within the period 18 - 25 days after conception (Figs 44, 47, 50, 53, 59, 62). However, it was also apparent that peaks of oestrogens could be detected outwith these times in pregnant cows. These results are interpreted as suggesting that follicular development continues to

occur during pregnancy dioestrus, as during the luteal phase of the cycle in non-pregnant cows, and that rather than this being restricted, by some unknown mechanism, to the period of 18 - 25 days post-conception, the occurrence of peaks of oestrogens within this period was a matter of chance.

One of the most striking observations of this study was the finding that silent heat did not appear to be associated with a deficiency of plasma oestrogens. It has been postulated that this refractory state is due to an elevated threshold to these hormones after calving. It would be of interest to determine if oestrogens administered to post-partum cows could be made to have a more potent oestrous inducing effect if the hypothalamic activity was aroused in these animals in a non-specific manner. In the absence of any obvious reason for the failure of post-partum cows to show overt heat along with early ovulations, the marked endocrinological alterations recorded in late pregnancy have been implicated in having a residual effect on suppression of normal reproductive function after calving. By aborting pregnant cows prior to the terminal stages of gestation, the influence of, for example, a lower level of total oestrogens on the return to ovarian cycling and the expression of oestrous behaviour could be determined.

Within cows showing oestrous or perioestrous behaviour no obvious relationship could be found between the levels of oestrogens at the time of, or immediately preceding, the event and the intensity of oestrous behaviour. Although reference in this thesis has been

made to the heterosexual component of oestrous behaviour in cows, it is well recognised that homosexual behaviour is also normally seen. Furthermore it has been reported that cows in oestrus are more frequent mounters of other cows in heat (Mylrea and Beilharz, 1964). Rather than being a typical part of female sexual behaviour, this could more correctly be considered to represent masculine sexual behaviour. Androgens are well recognised as regulators of male sexual behaviour. It is of interest to note that the level of androgens recorded in the cows in this thesis at no time approached the concentrations of these hormones we have recorded in bulls - typical results for the latter sex ranging from around 0.3 ng/ml to 5.5 ng/ml within the course of a day. It is not known whether androgens in the cow regulate the intensity of mounting other cows. Conversely whether bulls show, or can be induced to show, by injection of the traditional female sex hormones, female as well as male behaviour is not known. These observations would suggest that further studies on the influence of hormones on the regulation of sexual behaviour are required.

Overall, the results of this study into reproduction in the post-partum cow were felt to provide a basis for extension of endocrine investigations into field situations both at a herd and an individual cow level. At the level of the individual cow, their main application would initially be to eliminate deficiencies of, for example, progesterone as a cause of any infertility, and to supplement rectal palpations. It may be that use could be made of the constancy of the pattern of progesterone increase after normal ovulation to study such controversial topics as delayed ovulation. At a herd level routine

sampling for progesterone levels could be incorporated as part of herd fertility control programmes to ensure that all animals were cycling by the required time after calving. These studies could be of obvious economic importance.

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