Progesterone binding in the bovine corpus luteum

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I declare that the work reported herein and this thesis are my own, and have not previously been submitted, nor are concurrently being submitted, for any degree other than that of the degree of Doctor of Philosophy of The University of Edinburgh.

Michael T. Rae

Abstract

In the ovary, following the preovulatory surge of gonadotrophins, but before ovulation, follicular cells begin the changes associated with luteinization which lead to the formation of the corpus luteum. Cells from the preovulatory follicle differentiate into small and large luteal cells. The corpus luteum is a transient endocrine organ which has a finite lifespan, and is essential for the establishment and maintenance of early pregnancy in all mammalian species, however, if no pregnancy occurs then it regresses. The major secretory product of the corpus luteum is the steroid hormone progesterone. Whilst the pattern of progesterone secretion during the oestrous cycle is well established, the mechanism by which progesterone leaves luteal cells in which it is synthesized and the regulation of this synthesis is less well understood.

This project was designed to examine the intracellular location of progesterone in bovine luteal cells. These experiments demonstrated the existence of a particulate membrane fraction of luteal cells where much of the endogenous progesterone was located. Results suggest an association between this fraction and the plasma membrane. Moreover, it was shown that these membranes were able to bind exogenous radiolabelled progesterone in a highly specific manner. Other steroids, precursors etc. were bound poorly. Thus, the experiments herein describe the characterisation of this novel progesterone binding site, its distribution in the cells of the bovine corpus luteum and preovulatory follicle, and attempts to purify and identify the progesterone binding protein.

Results from these experiments indicated that the progesterone binding site investigated was distinct from classical genomic progesterone receptors. This non-classical progesterone binding protein (NCP_4-BP) was found in both large and small luteal cells of the corpus luteum, though levels were greater in large cells. NCP_4-BP was also found in the theca and granulosa cells of the preovulatory follicle. Binding characteristics of the NCP_4-BP were determined, and partial purification achieved. Results demonstrated that progesterone binding was not due to (i) steroid metabolizing enzymes, (ii) non-specific intercalation of steroid into bi-layer membranes or (iii) the

genomic progesterone receptor. Studies suggest that, given the pivotal role of progesterone in the bovine oestrous cycle, and progesterone's potential autocrine control over its own synthesis, that the membrane located progesterone binding protein described herein is likely to be involved in the regulation of luteal function in cows, and thus the regulation of the bovine oestrous cycle.

Acknowledgements

In carrying out the work that is described in this thesis, and in the preparation of the manuscript, there have been a number of people whose invaluble aid I wish to acknowledge. Indeed, to thank the following people only from an academic, learning point of view would be unjust and ungrateful; they have all displayed great friendship towards me in and out of the lab, and it is for this above anything else that I would like to thank them.

Firstly, my supervisor, Dr. Tony Bramley. His help with techniques and in understanding and learning how to seek out the relevant has been invaluable. It is thanks to Tony's guidance that I have developed a well rounded and I hope well structured view of the discipline of science. Garry Menzies has also proved an invaluble source of help over the years, in teaching me techniques, providing helpful suggestions of how to 'make it work', and emphasising the importance of a sense of humour. I am grateful to both Tony and Garry for not only their patience, time and interest in my work, but just as importantly, for their friendship over the course of my project, and I hope in the years to come. Dr. Maggie Paterson also deserves a special mention, as without her patient demonstration of SDS-PAGE and Western blotting, this section of work would have taken much longer. Thanks are also due to Harry Boyle for his assistance with experiments involving HPLC analysis (and for a few pints here and there). To my second supervisor Professor Alan McNeilly - thanks for encouraging and feeding my interest in reproductive biology all those years ago when I was completing my honours degree; and thanks for the help and friendship given to me since then.

When I began my project I was fortunate enough to overlap with Dr. Matt Turner who was completing his thesis. Matt was very willing to explain some of the reproductive biology basics which are often taken for granted by texts. First and foremost however Matt was simply a good friend to have in and out of the lab, and it is with sadness that he is referred to in the past tense, having tragically died in a mountaineering accident. I would dedicate

this thesis to his memory if I did not know that he would turn in his grave at the thought of it.

My acknowledgements would be far from complete if I did not mention my friends from the smokers tea-room - thanks for all the discussions, laughs and cigarettes! Also, thanks are due to Tom McFetters and Ted Pinner for their assistance with computer operation and for the preparation of the photographs herein.

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Finally, I wish to thank my parents, Dorothy and George Rae, without whose encouragement to follow my own interests, neither this thesis nor my undergraduate degree would have materialized. It is therefore fitting that I dedicate this thesis to them.

Chapter 1

Literature Review

1.1 Endocrinology of the bovine oestrous cycle

The following is a summary of the secretory patterns of the hormones controlling the oestrous cycle in cows. These hormonal secretion changes and the effects they have are discussed further in following sections. Where hormone secretory profiles are shown, it should be noted that the diagrams are composite profiles from a number of studies to give an average, representative secretory pattern, not absolute concentrations or times.

1.1.1 Luteinizing hormone (LH)

LH, secreted by the pituitary, follows the secretory pattern shown in Fig. 1.1. From ovulation to day 5 basal LH secretion values remain constant. A small increase in LH secretion has been observed between days 6 and 10; levels then return to basal before a second increase which begins on day 17 and culminates in the LH surge on day 20 (Schams *et al*, 1977). Basal LH secretion values have been found to be in the range of 0.5 - 2.5 ng/ml and LH surge values in the range of 20 - 40 ng/ml (Schams *et al*, 1977; Dobson, 1978). Following luteolysis, increases in LH secretion are thought to be due to the increases in oestradiol secretion from developing follicles (Schams *et al*, 1977). For simplicity, the diagram below does not show the pulsatility of LH secretion. Experimental evidence indicates that pulsatility of LH secretion is of more importance than basal circulating concentrations. LH pulse frequency increases during the follicular phase of the oestrous cycle, due to lack of progesterone suppression of its release (Wallace *et al*, 1988).

Prior to the preovulatory LH surge, pulse frequency continues to increase, doubling 24 hours after progesterone secretion has declined (due to regression of the corpus luteum) (Baird & McNeilly, 1981). LH pulses are closely followed by pulses of oestradiol and androstenedione from the follicle (Baird*et al*, 1976). Elevation of oestradiol levels at this time decrease the pulse amplitude of pituitary secreted LH (Baird & McNeilly, 1981). It has been suggested that follicular responsiveness to FSH may be modulated by LH pulsatility; indicating a role of LH in conjunction with falling FSH secretion in the induction of atresia in FSH deprived follicles (McNeilly *et al*, 1991). During the luteal phase in sheep, pulse frequency of LH is depressed from ca. 1/hour to ca. 1/ 4-6 hours (Baird, 1978). From experiments performed in cattle, it is suggested that progesterone exerts negative feedback on the pulse frequency of LH secretion, thereby altering follicular growth (Stock & Fortune, 1993). Control of oestradiol-17β secretion from the dominant follicle is strongly associated with LH pulse frequency during the follicular phase in cows. Small increases in pulse frequency are thought to play a role in the maintenance of a dominant follicle; decreases in LH pulse frequency are associated with a decline in follicular oestradiol production and atresia of the dominant follicle (Stock & Fortune, 1993). Relationships between hormones during the oestrous cycle are discussed further in following sections.

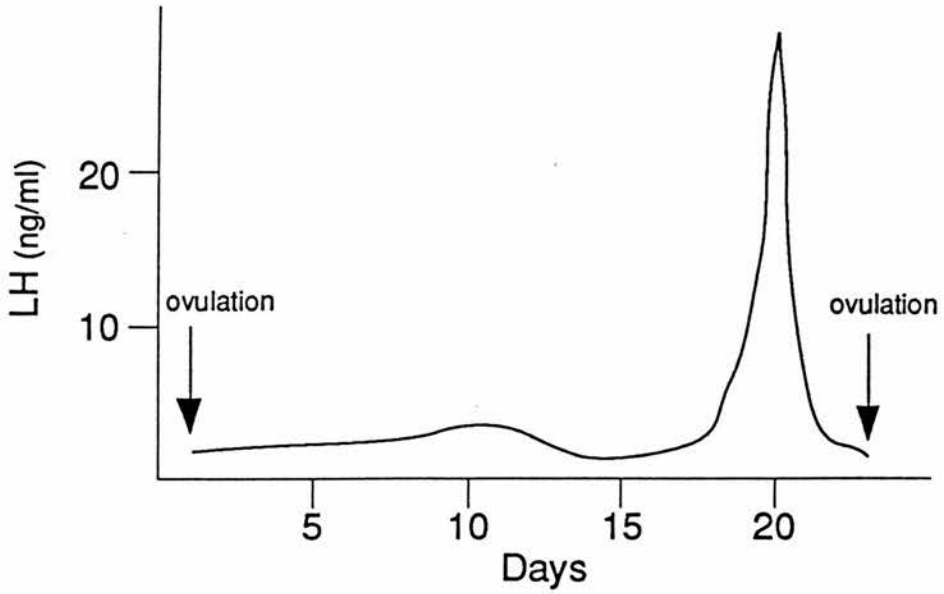


Figure 1.1 Peripheral plasma concentrations of LH during the bovine oestrous cycle (data from Dobson & Kamonpatana, 1986; Dobson, 1978 and Schams *et al*, 1977)

It has been reported that the LH surge occurs concomitantly with the peak of oestradiol secretion (Schams *et al*, 1977), though measurements were of urinary oestradiol and not plasma oestradiol. However, by assaying plasma oestradiol it has been confirmed that both these hormones peak at approximately the same time (Dobson, 1978). Oestrus occurs concomittantly with the peak of oestradiol, and can be measured by decreases in the electrical resistance of vaginal mucus, which reaches its lowest point at the time of the LH peak (Schams *et al*, 1977). Administration of oestradiol to ovariectomised cattle caused a small increase in the concentrations of acidic LH isoforms, which have less bioactivity than basic LH isoforms. However, differences in pituitary isoforms of LH do not differ from those found in intact animals during the follicular phase, thus there would not appear to be a significant effect of oestradiol on the ratios of LH isoforms found in the bovine pituitary (Kojima *et al*, 1995).

1.1.2 Follicle stimulating Hormone (FSH)

Secretion of FSH follows a pattern of small peaks leading to a main peak at the same time as the LH surge (Schams *et al*, 1977) (Figure 1.2).

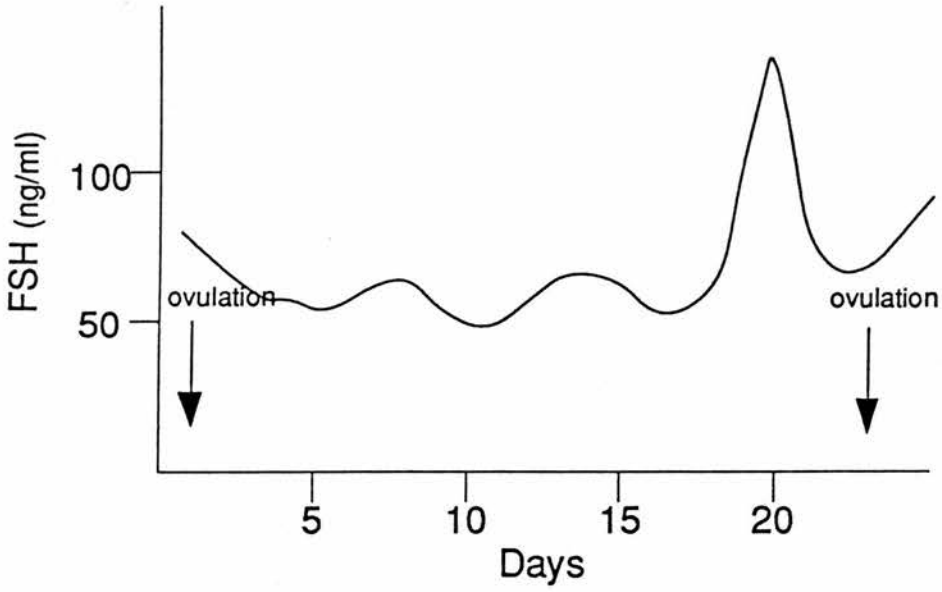


Figure 1.2. Peripheral plasma concentrations of FSH throughout the bovine oestrous cycle (Data from Schams *et al*, 1977; Dobson, 1978).

Studies have demonstrated that follicular growth correlates with increases in FSH secretion (Schams *et al*, 1977). Apart from the preovulatory peaks, there was little correlation between LH and FSH secretion in experiments where measurements of both hormones have been performed (Schams *et al*, 1977; Dobson, 1978). Thus there may be a separate mechanism of release, or, as has been recently suggested, the secretion of FSH may be controlled by inhibin in cows, which does not affect LH secretion (Turzillo & Fortune, 1990). A second peak of FSH has been reported (Dobson, 1978). This secondary peak of FSH occurs approximately 24 hours after the preovulatory surge, at the time of ovulation (Schams & Karg, 1969). The functional significance of this second peak is uncertain, though it is suggested to have

importance in the re-initiation of follicular growth after ovulation (Turzillo & Fortune, 1990). The DNA sequence of the bovine FSH receptor has recently been cloned, and the receptor shown to consist of 678 amino acids (Houde *et al*, 1994). It was also demonstrated that in bovine granulosa cells, down-regulation of the FSH receptor occurs at the mRNA level when exposed to constant FSH levels (Houde *et al*, 1994). A number of isoforms of FSH exist in the bovine pituitary. Studies have demonstrated that relative amounts of these isoforms are not altered in response to oestradiol, and thus are unlikely to be influenced during the follicular phase of the oestrous cycle (Kojima *et al*, 1995).

1.1.3 Progesterone

On the two days preceding ovulation, and for a further three days post-ovulation, progesterone levels are maintained at the lowest levels observed throughout the oestrous cycle (approximately 0.2 - 0.6 ng/ml) (Schams *et al*, 1977). The low levels of progesterone secretion observed at this time are a reflection of the cessation of progesterone secretory activity by the corpus luteum following its regression. Progesterone secretion only increases from day 4 onwards to approximately the tenth day after ovulation, with circulating plasma levels of 6 - 8 ng/ml (Schams *et al*, 1977) (Figure 1.3). Secretion of progesterone then decreases rapidly 5 days before the next ovulation (Schams *et al*, 1977).

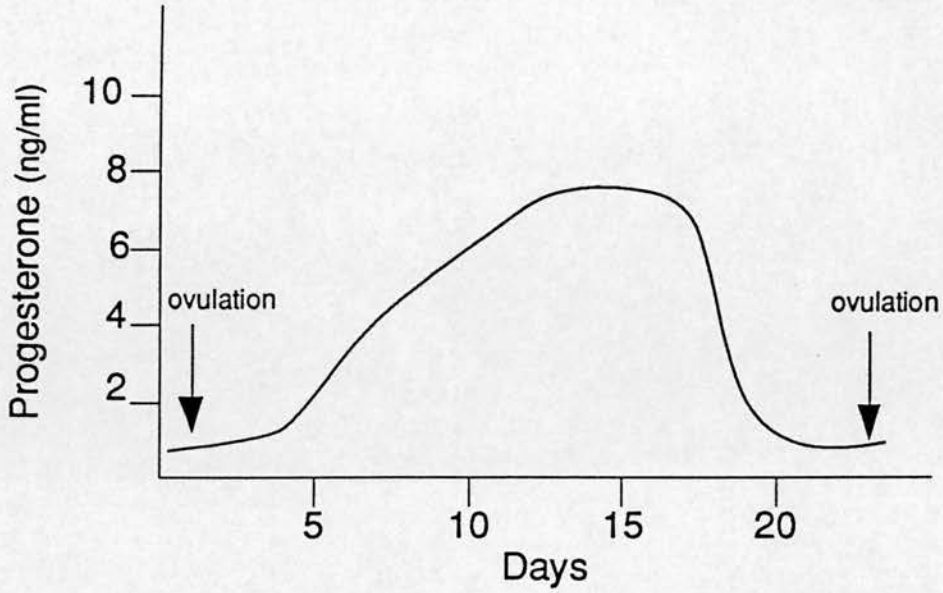


Figure 1.3 Peripheral plasma secretory pattern of progesterone over the bovine oestrous cycle. (Data from Dobson & Kamonpatana, 1986; Schams *et al*, 1977)

There is some apparent disagreement over whether or not there is a preovulatory rise in progesterone synthesis and secretion just prior to ovulation. In some studies no preovulatory rise in progesterone secretion was observed (Schams *et al*, 1977). However, *in vitro* experiments designed to study the progesterone secreting ability of the bovine preovulatory follicle, in which follicles were removed at various stages before and after the preovulatory LH surge, have indicated that after the LH surge, but before ovulation occurs, there is an increase in the progesterone synthesising ability of follicular cells (Dieleman & Blankenstein, 1985). Up until the LH surge, follicular steroidogenesis is mainly concerned with oestrogen production, but after the LH surge, moderate levels of progesterone are produced by follicular cells cultured *in vitro* (Dieleman & Blankenstein, 1985). 20 hours after the LH surge, and continuing through to ovulation, there is a sharp 4-fold increase in the amount of pregnenolone converted to progesterone in

follicular cell cultures (Dieleman & Blankenstein, 1985), in agreement with measurements of progesterone concentrations in follicular fluid of preovulatory follicles removed 20 hours post-LH surge (Dieleman *et al* 1983). It was suggested that this sharp increase in progesterone synthesis and secretion, and its correlation with the morphological luteinization of granulosa cell layer reflects granulosa-derived progesterone, and that the smaller amounts of progesterone secreted prior to this time (20 hours post-LH surge) are theca-cell derived (Dieleman *et al,* 1983b). However, though thecal progesterone secretion *in vitro* increases after the LH surge, the granulosa cells continue to contribute a major amount of total follicular progesterone (Fortune & Hansel, 1979). Progesterone secretion during the luteal phase is discussed in more detail in following sections.

1.1.4 Oestradiol 17β

The pattern of oestradiol-17β secretion is shown in Figure 1.4. This pattern was determined in many studies by direct radioimmunoassay on bovine plasma (Dobson & Dean, 1974). Other studies using measurement of urinary oestradiol with correction of results for creatinine clearance have shown a similar pattern (Schams *et al*, 1977).

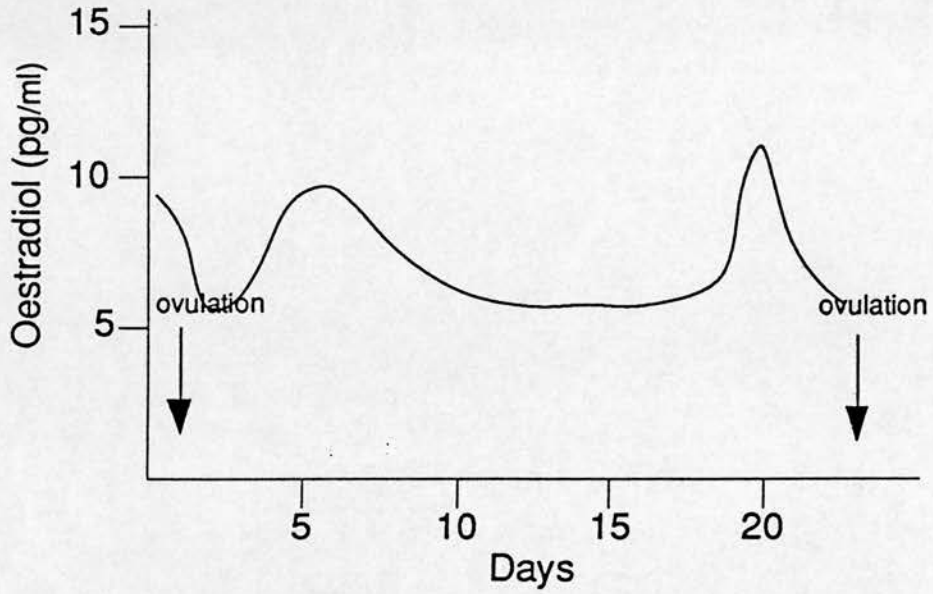


Figure 1.4. Plasma oestradiol secretory pattern over the bovine oestrous cycle. (Data from Dobson & Kamonpatana, 1986; Dobson & Dean, 1974)

Increases in oestradiol-17β over the oestrous cycle are considered to be reflections of follicular growth (Dobson & Dean, 1974). Some studies have demonstrated that of the total oestrogens secreted, oestradiol 17α is present in the largest proportion, in significantly greater quantities than oestradiol 17β (Dobson & Dean, 1974), though the significance of the proportions of each epimer of oestradiol secreted is unclear. Concentrations of oestradiol were observed to increase 4-5 days before oestrus, peaking at the time of

oestrus (Dobson, 1978), with base values of approximately 1 - 6 pg/ml and elevated peak values of approximately 10 pg/ml (Dobson & Dean, 1974).

1.2 Oestrus

The behavioural characteristics associated with the oestrous cycle, termed oestrus behaviour, lasts between 12.5 - 27.5 hours in cows (Dobson & Kamponpatana, 1986). For the effective use of artificial insemination in cows, it is important to be able to recognise the symptoms of oestrus so that the insemination can be timed correctly. This behaviour is characterised by cows standing to be mounted, indeed, this is used by many dairy herd owners to gauge the reproductive cycle stage that animals are at (Williamson *et al*, 1972). Other signs include reddening and swelling of the vulva, and the secretion of mucus from the vulva during oestrus.

The day of oestrus behaviour is termed day 0 of the bovine oestrous cycle. In cows, this cycle lasts approximately 21 days. It has been reported that 85% of cows display oestrus behaviour between days 18 - 22 (Asdell, 1964), with ovulation occurring approximately 10 hours after the end of the display of oestrous behaviour (Asdell, 1964). Though daylength has been shown to influence reproductive timing (Bullman & Lamming, 1978) as has environmental temperature and rainfall (Rahka & Igboeli, 1971), these factors only appear to affect animals whose environment may show extremes, such as domestic buffaloes found in Egypt and India (El-Fouly *et al*, 1976), and have not been of major importance in temperate countries such as Britain, in which dairy and beef cow production does not fall under full control of these factors.

1.3 Temporal endocrine relationships during the bovine oestrous cycle

It has been demonstrated that the increased secretion of oestradiol prior to the preovulatory surge of LH (Schams *et al*, 1977) is made up of high frequency, high amplitude pulses of oestradiol (Walters & Schallenberger, 1984). In ewes, it has been observed that such pulses of oestradiol are a direct consequence of LH pulses stimulating the follicle (McNeilly *et al*, 1982). Experimental data from studies in cows have also demonstrated a close similarity between LH pulse freqency and oestradiol pulse frequency (Walters & Schallenberger, 1984). Whereas pulse frequency remained similar for the two hormones, indicating a link between oestradiol and LH secretion, pulse amplitude of oestradiol increased prior to the LH surge (Walters & Schallenberger, 1984), indicating that the ovarian responsiveness to LH may have increased (Staigmiller *et al*, 1982).

Prior to the LH surge, high oestradiol concentrations, secreted from the follicle, appear to exert two types of feedback, positive and negative (Walters & Schallenberger, 1984). Positive feedback occurs at the pituitary, by sensitising the pituitary to pulses of GnRH from the hypothalamus (Kesner *et al*, 1981), resulting in increased LH secretion in response to GnRH. Using oestradiol treated ovariectomised cows and castrated steers as models (since an oestradiol-induced LH surge occurs in oestradiol treated ovariectomised cows but not in castrate steers), it has been observed that oestradiol increased pituitary responsiveness to GnRH (Kesner *et al*, 1981). In both animal models, treatment with exogenous GnRH after oestradiol treatment induced an LH surge, though GnRH alone was incapable of this action (Kesner *et al*, 1981). Moreover, results from these studies indicated that maximal pituitary responsiveness to GnRH occurs some time before the

oestradiol-induced LH surge occurs, suggesting that oestradiol suppresses GnRH secretion at this time; it appears that this suppression is lifted some time after oestradiol exposure (c. 12 hours), eliciting an LH surge (Kesner *et al*, 1981). Though secretion of LH has been shown to increase in response to high oestradiol concentrations (Walters & Schallenberger, 1984), pulse amplitude of LH does not increase (Schallenberger *et al*, 1984). In rats it has been demonstrated that oestradiol can reduce GnRH pulse amplitude but not pulse frequency (Sarkar & Fink, 1980), and in ewes it has been observed that LH pulse amplitude is closely related to GnRH pulse amplitude (Clarke & Cummins, 1982), thus it has been suggested that in cows high oestradiol concentrations, whilst increasing pituitary responsiveness to GnRH, decrease the pulse amplitude of GnRH by negative feedback on the hypothalamus, thereby decreasing the pulse amplitude of LH pulses from the pituitary (Walters & Schallenberger, 1984). Ovariectomy in cows has demonstrated enhanced pituitary levels of mRNA encoding for LHβ, FSHβ and the common gonadotrophin α subunit (Cupp *et al*, 1995). Addition of exogenous oestradiol at levels found during the bovine follicular phase to these ovariectomised animals depressed levels of all gonadotrophin subunits However, secretion of LH was enhanced (Cupp *et al*, 1995). Thus, though secretion of LH during the bovine follicular phase is enhanced, the expression of subunits for gonadotrophins is not. Though overall secretion of FSH was unchanged by this treatment, secretion frequency of FSH was enhanced, suggesting that oestradiol regulates FSH and LH secretion at the levels of the gonadotroph (Cupp *et al*, 1995). However, the change observed in pulsatility of FSH secretion with oestradiol treatment does not take into account the action of ovarian factors such as inhibin (Cupp *et al*, 1995) which is known to suppress FSH secretion in ewes (Ying, 1988). However, these

studies indicated that oestradiol plays a role in modulation of bovine FSHβ expression which does not appear to be dependent on any other factors (Cupp *et al*, 1995).

The trigger for the LH surge remains enigmatic. It does not appear that increased pituitary sensitivity to GnRH is the sole cause of the surge (Kesner *et al*, 1981). In both sheep (Baird & McNeilly, 1981) and cows (Walters & Schallenberger, 1984), oestradiol secretion decreases immediately prior to the LH surge, then increases as the surge commences. It has been postulated that this decrease in oestradiol immediately prior to the preovulatory surge of LH may be the trigger for the surge, since a decrease of circulating oestradiol at this time could free the hypothalamus from the negative feedback imposed on it by elevated oestradiol levels (Walters & Schallenberger, 1984). Thus the amplitude of GnRH pulses could increase, stimulating the pituitary, which, after oestradiol priming is maximally responsive to GnRH at this time (Kesner *et al*, 1981), resulting in a maximal pituitary response of high amplitude, high frequency pulses of LH, characteristic of the preovulatory LH surge (Walters & Schallenberger, 1984). Thus it appears that both increased pituitary responsiveness to GnRH and increased secretion of GnRH are necessary to elicit the oestradiol-induced preovulatory surge of LH (Kesner *et al*, 1981).

Concomitant with the preovulatory surge of LH, a surge of FSH occurs (Walters & Schallenberger, 1984), though, whereas the LH surge may be up to 20-fold greater than basal secretion, the surge of FSH has been observed to be only 2-3 fold greater than basal secretion (Walters & Schallenberger, 1984). Pulses of these two gonadotrophins have been observed to occur

simultaneously, indicating that the releasing hormone GnRH appears likely to effect the release of both LH and FSH from the pituitary (Walters & Schallenberger, 1984). However, as mentioned above, a second FSH peak occurs some 4 - 12 hours post-preovulatory surge in cows (Dobson, 1978). The mechanism controlling the secondary FSH surge is unclear. In sheep it has been suggested that a secondary FSH surge is the result of decreased inhibition of FSH release because of low oestradiol concentrations, whereas in humans low oestradiol concentrations have been implicated with inhibition of gonadotrophin release (Johnson & Everitt, 1988). Experiments in rats have demonstrated that exogenous administration of oestradiol has little effect on the secondary FSH surge (Chappel & Barraclough, 1977). Thus it has been postulated that in rats the secondary FSH surge is a result of lowered inhibin concentrations allowing FSH secretion to increase above basal values (Elias *et al*, 1982; Depaulo *et al*, 1979). However, in cows, the secondary FSH surge has been demonstrated to be made up of high amplitude, high frequency pulses of FSH, suggesting the involvement of GnRH, as in the preovulatory surge of FSH (Walters & Schallenberger, 1984). This has led to the suggestion that post-preovulatory surge alterations in pituitary responsiveness to GnRH occur, allowing a selective response to low GnRH concentrations and resulting in the secondary, post LH/FSH preovulatory surge, surge of FSH (Walters & Schallenberger, 1984). In ovariectomised ewes it has been observed that treatment with oestradiol to levels comparable to late follicular levels in intact, cycling animals induces an LH surge accompanied by a GnRH surge that lasts longer than the LH surge (Moenter *et al*, 1990). This extended duration of the GnRH surge has also been observed in cattle (Kesner *et al*, 1981). It has been suggested that this may be the mechanism by which the LH surge is terminated ie. by

down- regulation of GnRH receptors due to continuous exposure to elevated GnRH levels (Moenter *et al*, 1990), since it has been demonstrated that continuous exposure of ewes to exogenous GnRH decreases the number of pituitary receptors for GnRH (Nett *et al*, 1981).

Progesterone secretion has also been demonstrated to increase at approximately the same time as oestradiol secretion increases (Walters & Schallenberger, 1984). This may be a result of functional luteinization occurring in follicular cells before ovulation (Dieleman *et al*, 1983). Oxytocin secretion has been observed to be low in the absence of a functional corpus luteum (Walters & Schallenberger, 1984), even though high concentrations of oxytocin have been found in the follicular fluid of bovine follicles prior to ovulation (Schams *et al*, 1983).

1.4 Bovine follicular dynamics

Follicle development can be viewed as two main processes, follicle recruitment and follicle selection. Follicle recruitment involves the development of a number of follicles, recruited in waves. Follicle selection is the subsequent development of a dominant follicle from the pool of follicles which have undergone the initial stages of development. Initially, these processes were examined by morphologic and histological examination of ovaries removed post-mortem from cows at various stages of the oestrous cycle. The development of techniques such as ultrasound imaging of cattle ovaries have contributed much information in recent years, since this technique allows the ovaries of a normally cycling animal to be examined intact, via an intrarectal ultrasound probe.

Follicle growth rates and developmental stages have been elucidated in cows by a number of studies. These studies have demonstrated that early follicular growth is due to proliferation of granulosa cells (Lussier *et al*, 1987). Antrum formation is first detectable in follicles with a diameter of 0.14 - 0.28 mm (Lussier *et al*, 1987). Growth of follicles additional to the growth observed due to granulosa cell proliferation appears to be a result of antrum development (Lussier *et al*, 1987). It is postulated that two complete oestrous cycles are required for a follicle to grow from antral size to preovulatory size (Lussier *et al*, 1987). Antral follicles display slower growth than follicles in the size range 0.68 - 3.67 mm; the increase in growth rate is thought to be a reflection of an increase in mitotic index and a decrease in the duration of mitosis (Lussier *et al*, 1987). Treatment of heifers with bovine somatotrophin (BST) has been shown to increase the numbers of 2-5 mm antral follicles (Gong *et al*, 1991). This suggests that growth hormone (GH) may be implicated in folliculogenesis (Gong *et al*, 1991). Subsequent studies have indicated that increased numbers of small antral follicles in response to BST treatment may be via increased levels of IGF-1 or insulin (Gong *et al*, 1993 a;b). Moreover, increased small antral follicle numbers by BST treatment did not affect the inhibitory influence of the dominant follicle on subordinate follicle growth (Gong *et al*, 1993 a).

In individual cattle, the number of waves of follicles that are recruited in the oestrous cycle has been shown to vary. Waves of follicle development were first reported by Rajakoski (1960), who observed two waves in each oestrous cycle, the first occurring between days 3 - 12 and the second between day 12 and the onset of oestrus. More recently it has been shown that each wave consists of the development of 3-6 follicles > 5 mm (Fortune *et al*, 1988), one

of which will develop to become the dominant follicle, whilst the others regress. From studies in heifers, it has been observed that the oestrous cycle has 2 - 3 waves of follicle development, three waves being the most common (Savio *et al*, 1988; Gong *et al*, 1993a). Other studies have indicated that two waves of follicle development may be more common than three (Ginther *et al*, 1989a). Breed and parity are unlikely to be factors influencing the number of waves an animal displays over the course of the oestrous cycle. Genetic and/or environmental factors controlling the number of follicular waves are unknown (Ginther *et al*, 1989a). The dominant follicle of the first wave reaches its maximum size by day 6 (Savio *et al*, 1988). Follicles of the second and third waves may grow either on the ovary that possessed the first wave, or on the contralateral ovary (Savio *et al*, 1988). However, the morphologically dominant follicle, (that is, the largest follicle observable by ultrasound), may not be the functionally dominant follicle (Lavoir & Fortune, 1990). Evidence for this comes from studies in which the ability of follicles to ovulate in response to administration of a luteolytic dose of $PGF_{2\alpha}$ was examined. These results suggested that morphological dominance and functional dominance were congruent during the development of the dominant follicle, but when growth slowed and the development of the dominant follicle had plateaued, the morphologically dominant follicle may have lost functional dominance, since ovulation could occur in a follicle from the second wave of follicular recruitment (Lavoir & Fortune, 1990). Thus the use of follicle size alone as a criterion for assessment of dominance is unsuitable in cattle (Fortune *et al*, 1991). In cycles where there are three waves of follicle development, the morphologically dominant follicle of each wave has been shown to be present on days 6 (first wave), 16 (second wave) and 21 (third wave) (Savio *et al*, 1988). The period of detection (from follicle

detection to atresia) was longer in dominant follicles from the first wave than in subsequent waves. It has been suggested that the first dominant follicle may be able to inhibit growth of other follicles, though the mechanism by which it exerts this suppression of the second wave is unknown (Savio *et al*, 1988; Lazar & Maracek, 1994). Conversely, it has been observed that the presence of a large oestrogenic dominant follicle actually promoted the appearance of 5 - 10 mm diameter follicles at all stages of the oestrous cycle (Lazar & Maracek, 1994). Ginther *et al* (1989a) noted that dominant follicles did not begin to regress until after the next successive wave of follicles appeared. Moreover, it was observed that the time interval (approximately 3 days) between the emergence of the next follicular wave and the commencement of regression of the existing dominant follicle was similar to the interval observed between the time at which a follicle displayed morphological dominance and the point at which inhibition of the subordinate follicles of the same wave occurred (Ginther *et al*, 1989a). In cycles with only two waves of follicular development, growth of secondary follicles was similar to the growth of secondary follicles in three wave cycles (Savio *et al*, 1988). Whereas the secondary follicle in two wave cycles continues to grow and then ovulate, in 3 wave cycles the secondary dominant follicle regresses (Savio *et al*, 1988). A third dominant follicle, presumably inhibited by the secondary dominant follicle, then develops and grows at a comparable rate to that of the final phase of growth of a secondary dominant follicle from a two wave cycle (Savio *et al*, 1988). Growth rates of dominant follicles from the first and third waves are fastest in the latter stages of follicle growth, suggesting that growth of the secondary dominant follicle is slowed by the high levels of progesterone secreted from the corpus luteum during the luteal phase, presumably by

inhibition of gonadotrophin secretion from the pituitary (Savio *et al*, 1988). Thus, depending on the time of luteal regression, the secondary dominant follicle may either ovulate, or (if the luteal phase continues), become atretic, in which case the third dominant follicle will ovulate (Savio *et al*, 1988). Ovulatory follicles of three wave cycles tend to be smaller in diameter than the ovulatory follicles of two wave cycles since the time between emergence and ovulation may be up to 4 days shorter in cycles in which the tertiary dominant follicle is the ovulatory follicle (Ginther *et al*, 1989). Three wave cycles were also observed to be longer than two wave cycles by up to two days (Ginther *et al*, 1989).

It has been suggested that a follicular wave in cattle is produced approximately every seven days in response to basal gonadotrophin concentrations (Fortune *et al*, 1991). Evidence to support this hypothesis comes from the observation that animals which only exhibit two waves of follicular development (ie. in which the second dominant follicle is destined to become the ovulatory follicle) have both shorter cycles overall and shorter luteal phases. Thus at the time of luteal regression, the second dominant follicle remains the morphological and functionally dominant follicle and ovulates (Fortune & Sirois, 1989). Moreover, in two wave animals, it was observed that the first dominant follicle grew larger than that in three wave animals; this existence of the larger first dominant follicle appears to delay the onset of the second follicular wave (Fortune & Sirois, 1989).

The effect of cycle length on the number of follicular waves has been studied in detail by prolonging the oestrous cycle to 30 days by administration of exogenous progesterone at levels found during the luteal phase. Though

some animals showed 4 - 5 waves of follicular development in this extended cycle, others showed only the normal three waves (Sirois & Fortune, 1990). Animals which were treated with lower doses of progesterone (1.5 ng/ml), that is, sub-luteal phase concentrations, also displayed only three follicular waves, and in some cases only two follicular waves, during the extended cycle (Sirois & Fortune, 1990). These animals displayed prolonged final waves associated with complete suppression of growth of other follicles, indicating that subtle changes in progesterone levels, either directly or indirectly, may have the ability to alter the timing of follicular waves and the length of dominance displayed by dominant follicles (Sirios & Fortune, 1990). The regularity of follicular waves in cattle ovaries subjected to basal concentrations of gonadotrophins is further illustrated by the observation that follicular waves continue during pregnancy in the cow (Ginther *et al*, 1989b). The preovulatory surge of gonadotrophins disturbs this balance of repeated waves of follicular development by causing ovulation of the follicle that is functionally dominant at the time of the surge (Fortune *et al*, 1991). After the ovulatory surge of LH and FSH, there is a secondary surge of FSH approximately one day later (Dobson, 1978; Schams & Karg, 1969). Inhibition of this secondary surge of FSH (by treating heifers with bovine follicular fluid (bFF) containing inhibin which inhibits the secretion of FSH, but not that of LH or progesterone) delays the onset of the first wave of follicular development after ovulation by approximately two days (Turzillo & Fortune, 1990). The early luteal phase increase in circulating oestradiol was delayed for a similar time, as was the appearance of the second follicular wave, leading to ovulation of the second dominant follicle in most of the bFF treated animals (Turzillo & Fortune, 1990). These results suggest that the secondary peak of FSH may be important in the initiation of follicular

growth after ovulation, though a direct effect of the bFF on the ovary could not be ruled out (Turzillo & Fortune, 1990).

1.5 Ovulation

Ovulation occurs in cows approximately 10 hours after the end of oestrus (Asdell, 1964). The exact mechanism by which the ovulatory follicle ruptures is unclear, but would not appear to be simply due to increased intrafollicular hydrostatic pressure (Johnson & Everitt, 1988). As in other species such as sheep (Murdoch & Dunn, 1983), prostaglandins are implicated in the ovulatory process of cows (De Silva & Reeves, 1985). Indomethacin, a substance known to block prostaglandin synthesis, has been shown to prevent ovulation in cows when infused into the ovary below the preovulatory follicle, but not when administered by intramuscular or intrauterine injection, suggesting that local prostaglandin synthesis in the ovary may play an important role in ovulation (De Silva & Reeves, 1985). Additional support for the involvement of ovarian produced prostaglandin in ovulation comes from *in vitro* studies in which synthesis of prostaglandins by bovine follicular tissue has been demonstrated; moreover, this prostaglandin synthesis was shown to be enhanced by LH (Shemesh & Hansel, 1975a).

1.6 Luteinization

After the preovulatory gonadotrophin surge, but before ovulation, the follicular cells of the ovulated follicle begin the changes associated with luteinization (Niswender & Nett, 1988). These changes eventually lead to the formation of the corpus luteum. Formation of this transient organ is a

complex process, likened to that of wound healing and tumour formation (Smith *et al*, 1994). Cells from the preovulatory follicle differentiate into small and large luteal cells. Implicated in this process is an increase in the diameter of the steroidogenic cell types (Farin *et al*, 1986). The morphology, origins, functions and biochemical properties of luteal cells are discussed in detail in following sections.

1.7 Morphology of luteal cells

In the bovine corpus luteum, at least five different cell types have been described. The early descriptions were based on physical properties of the cells examined such as size, morphology and staining (Foley & Greenstein 1958). It is now accepted that the corpus luteum of domestic ruminants contains two steroidogenically active cell types; theca lutein cells (TLC) derived from the theca cells of the preovulatory follicle, and granulosa lutein cells (GLC) , derived from the granulosa layer of the preovulatory follicle (Sinha *et al*, 1971; Mossman & Duke, 1973). These cell types are often termed small (TLCs) and large (GLCs) because of their distinct size differences, though recent developments in the study of the luteal cell types has indicated that these terms may be somewhat misleading because of the heterogeneity in size of each cell type and the controversy over the origins of each cell type (Donaldson & Hansel, 1965; Alila & Hansel, 1984). Indeed, it has recently been suggested that there may be sub-types of small luteal cells from ovine corpora lutea, though no functional differences were noted (Brannian *et al*, 1993). Nonetheless, in general TLCs are approximately 10-22 μm in diameter, and GLCs are considered to be >25 μm in diameter in cows (Alila & Dowd, 1991). Approximately 40% of total luteal tissue is made up of GLCs and 30% is TLC derived in the ovine corpus luteum (Rodgers *et al*,

1984). Endothelial cells, fibroblasts and other vascular system associated cells in conjunction with connective tissue make up the remainder of the CL. What were previously described as Type 5 cells and thought to be microvascular endothelial cells are now thought to be similar to immature granulosa cells, and are suggested to play a role in the renewal of luteal cells, though the origin of these cells and their importance in the lifespan of the corpus luteum is at present unknown (Spanelborowski *et al*, 1994). Though GLCs account for a larger percentage of the total cellular volume of the bovine CL they are present in much lower numbers than small (TLC) cells. A number of different studies have observed that there are 10-20 times more small cells than large cells (O'Shea *et al*, 1986, 1989; Hansel *et al*, 1987). However, tissue dispersion methods vary; separate studies have indicated that bovine GLCs are more sensitive than TLCs to tissue dispersion (O'Shea *et al*, 1989; Broadley *et al*, 1994) and thus may be numerically underestimated in cell counts of dispersed luteal tissue.

Morphological features found in other steroidogenic cells are present in both luteal cell types (Christensen & Gillim, 1969; Enders, 1973). Both cell types contain numerous lipid droplets (presumably containing esterified cholesterol stores for utilisation by steroidogenic enzymes), numerous mitochondria (fewer in small luteal cells) and well-developed smooth endoplasmic reticulum and Golgi. The nuclei of the two cell types are morphologically distinct; large luteal cells possess spherical nuclei whereas the nuclei of small luteal cells are irregular in shape (Koos & Hansel, 1981). Large luteal cells display an ultrastructure consistent with not only steroidogenic cells but also that normally found in cells whose function is the secretion of protein (Fawcett *et al*, 1969), namely well developed Golgi and rough endoplasmic reticulum. Large luteal cells also possess a more

prominent surrounding basal lamina than small cells and have numerous cell surface microvillous foldings (Enders, 1973). It has been observed that large luteal cells contain many electron dense granules in their cytoplasm, of approximately 0.2-0.4 μm in diameter. These granules have been demonstrated to be distinct from lysosomes and microperoxisomes due to their lack of acid phosphatase and catalase activity (Gemmell *et al* ,1974; Gemmell & Stacy, 1979). Indeed, initial experiments suggested that these granules were the 'missing link' in progesterone secretion, that is, they were initially thought to contain progesterone in a protein-bound form and were believed to transport progesterone for active secretion by luteal cells (Gemmell *et al*, 1974; Stacy *et al*, 1976; Sawyer *et al* , 1979). At times of elevated progesterone secretion numbers of these granules increased and exocytosis of these granules could be observed. However, if this was the mechanism of secretion of progesterone from luteal cells then it raises a number of questions, not least that since small luteal cells contain no secretory granules, then the mechanism of progesterone secretion must be functionally different in the two cell types. Also, binding of exogenous progesterone by these granules could not be demonstrated (Sernia *et al*, 1982; Rice *et al*, 1986). Indeed, later studies in both sheep (Rice *et al* , 1986) and cows (Guldenaar *et al*, 1984) demonstrated that these secretory granules contained oxytocin and neurophysin, not progesterone. Subsequent studies have demonstrated that the secretion of progesterone and oxytocin are independent of each other in bovine luteal cells cultured *in vitro* (Luck, 1988). The accepted theory of the mechanism whereby progesterone is secreted by the luteal cell is that progesterone simply diffuses out of its cell of origin down the concentration gradient set up between the progesterone producing cell and the circulation (Enders, 1973; Carlson *et al*, 1983a). In the latter study,

it was shown that progesterone intercalates into phospholipid bi-layers which contain cholesterol, suggesting a simple diffusion mechanism for progesterone release from the luteal cell across the plasma membrane (Carlson *et al*, 1983a). Though this theory is still widely accepted, it has not gone unchallenged, and the actual mechanism of progesterone secretion, if indeed different to that proposed by Carlson, remains enigmatic.

1.8 Origin of luteal cells

Early studies in the porcine and guinea pig CL indicated that the cell types of the corpus luteum were derived from both the thecal and granulosa cells of the preovulatory follicle (Loeb, 1906; Corner, 1919). This theory of the origins of luteal cells is still accepted today, and has been described in a number of species including the cow (Donaldson & Hansel, 1965; Priedkalns *et al*, 1968), the sheep (McClellan *et al*, 1975; O'Shea *et al*, 1980), the pig (Corner, 1919) and human (Guraya, 1971), though more recent studies have indicated that the divisions between the two cell types may not be as clear as was previously thought. Since the initial suggestion in 1965 that small luteal cells can transform to become large luteal cells (Donaldson & Hansel, 1965), a wealth of studies have been performed to investigate the origins of luteal cells in newly-formed and fully developed corpora lutea. Direct evidence to support this theory of small to large cell conversion arose from studies in which monoclonal antibodies specific to cell surface antigens for each follicular cell type were used to probe luteal tissue from various stages of the bovine luteal phase (Alila & Hansel, 1984). These studies found that in luteal tissue from the early luteal phase, 70-80% of large luteal cells bound an antibody raised to antigens on the granulosa cell surface whereas

approximately 70% of small cells bound only the anti-theca cell surface antigen antibody. However, in later luteal phase corpora lutea, approximately 50% of the large luteal cells bound only the antibody directed against the theca cell surface antigens (Alila & Hansel, 1984). The conclusion reached from this was that two sub-populations of large luteal cells exist - those derived initially from the granulosa cells of the preovulatory follicle and others which develop from the small, theca cell-derived luteal cells as the corpus luteum matures (Alila & Hansel, 1984). However, as mentioned previously, tissue dispersion by enzymes does appear to preferentially damage large luteal cells. Thus antigenicity of large luteal cells may be lost during tissue disruption, and large luteal cells may be numerically underrepresented in disrupted tissue cell subpopulations (Farin *et al*, 1986; Braden *et al*, 1988; O'Shea *et al*, 1989). The possibility of the large luteal cells which bind the antibody directed against small luteal cells actually being "larger" small luteal cells cannot yet be completely discounted. Earlier evidence for a small to large cell conversion was derived from studies in which alkaline phosphatase (a commonly used plasma membrane marker) was found, in early luteal tissue, to be confined to the plasma membrane of the theca lutein (small) cells. In the preovulatory follicle it has been observed that this enzyme is only found in theca interna cells, and is absent in the granulosa cells of sheep, pigs and cows (Corner, 1948). Up to day 9 of the luteal phase in cattle (Lobel & Levy, 1968) and day 8 in sheep (O'Shea *et al*, 1980) alkaline phosphatase activity was found only in small luteal cells, as defined morphologically. After this time, alkaline phosphatase activity was found in both small and large luteal cells, supporting the small to large cell conversion theory. Indeed, more recent studies have indicated that, in

sheep, LH has the ability to stimulate the conversion of small to large luteal cells (Niswender *et al*, 1985a; Farin *et al* 1988).

One other theory of the origins of luteal cells exists. Whilst not in complete agreement with those discussed, it does highlight the uncertainty involved in pinning down exact cellular origins of luteal cells throughout the luteal phase. From detailed morphometric analyses using cell ultrastructure rather than cell size as the criterion to identify cell types of the ovine corpus luteum, it has been demonstrated that the volume of the corpus luteum occupied by each cell type remains relatively constant, though the ratio of the numbers of small : large cells increases throughout the luteal phase, (Farin *et al*, 1986). It was observed that whilst small luteal cells expanded in numbers, their size remained constant; large luteal cells appeared to expand in size whilst the numbers of large luteal cells did not appear to increase significantly. Since it is difficult to differentiate between small and large luteal cells by their respective sizes because of an apparent size similarity at that time in the early sheep corpus luteum (Farin *et al*, 1986), it has been suggested that the "small" luteal cells that appear to grow to become large luteal cells were indeed always true "large" cells, but, in the early luteal phase appeared small in size (Farin *et al*, 1986). Though compelling, this data has not achieved the same acceptance as data derived from studies using cell-type-specific antibodies. Figure 1.5 is a diagrammatic representation of the current theories of luteal cell origins.

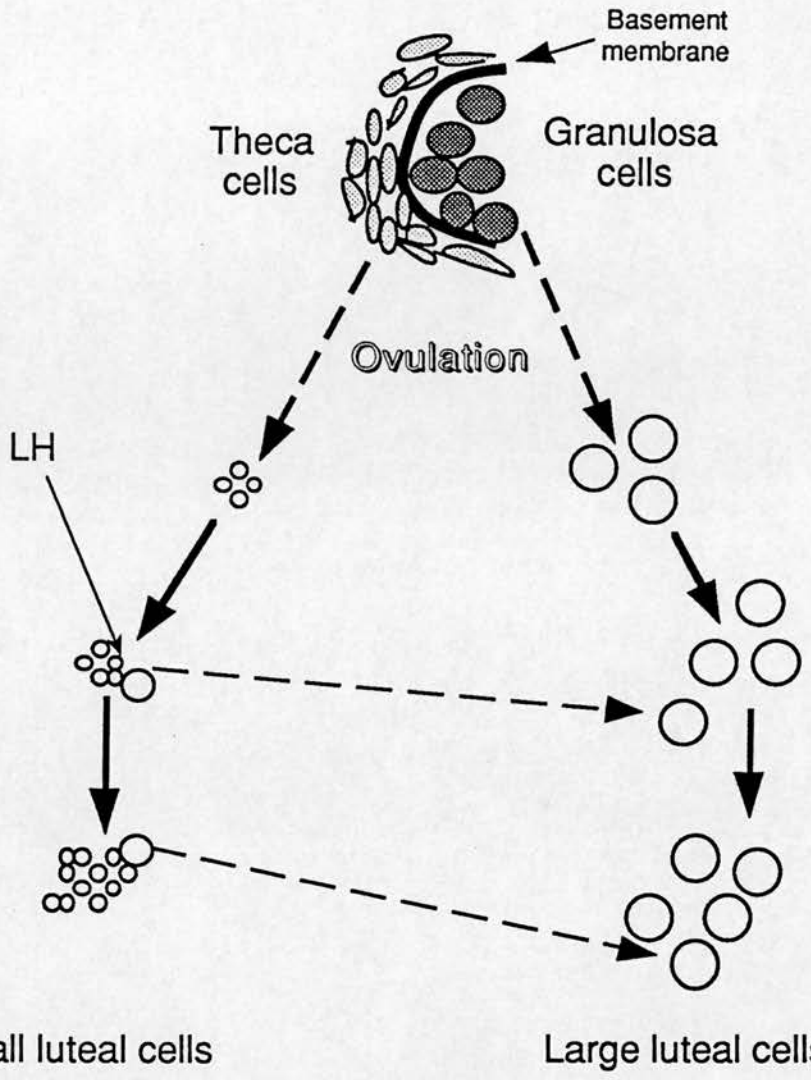


Figure 1.5 Diagrammatic representation of the origin of the two cell types of the ruminant corpus luteum from the preovulatory follicle.

1.9 Functional differences between small and large luteal cells

The observation that the corpus luteum is made up of two morphologically distinct luteal cell types raises the question of whether or not the two steroidogenic cell types are also functionally distinct. With the advent of cell separation techniques such as flow cytometry and centrifugal elutriation, studies on isolated large and small luteal cell populations have begun to clarify the differences in the functions of the two cell types.

1.9.1 Large and small cell responses to luteinizing hormone (LH)

A very striking difference between the two cell types is found in the receptor populations expressed by each type. Numbers of LH receptors found on small cells are much higher than numbers localized to large cells, highlighting an important functional difference (Fitz *et al*, 1982). The control of luteal steroidogenesis is exerted by both intra-ovarian paracrine factors and endocrine factors produced elsewhere in the body. Probably the most important endocrine factor is LH, synthesised and secreted by the pituitary. LH is considered to be the single most important factor involved in the support and function of the corpus luteum in cattle. Classic experiments that demonstrated LH to be the major luteotrophin were performed some years ago. It was shown that exogenous LH can prolong the lifespan of the bovine corpus luteum (Hansel, 1967; Hansel *et al*, 1973), that LH stimulates progesterone production *in vivo* (Schomberg *et al*, 1967; Niswender *et al*, 1976) and *in vitro* (Armstrong & Black, 1966; Hansel, 1971) and that blockade of the action of LH by a specific antibody to LH reduces progesterone secretion and luteal mass in heifers (Snook *et al*, 1969). Radioligand binding allowed the presence of the LH receptor to be demonstrated in specific tissues and cell types. From studies in the rat corpus luteum, the LH receptor was localized to the plasma membrane of luteal cells. In the rat luteal cell, the LH receptors were found predominantly in regions characterised by microvillous folds. In contrast, very few receptors were found on the basolateral surfaces of the cells (Anderson *et al*, 1979). This has since been demonstrated in a number of species. Indeed, LH receptor binding of radiolabelled LH/hCG is widely utilised as a marker for the

plasma membrane of luteal cells (Bramley & Menzies, 1988a; 1988b; 1993; 1994; Menzies & Bramley, 1994). The mechanism of action of LH (and hence the mechanism of signal transduction from receptor to effector) has been the subject of intensive experimental investigation. The LH receptor is a member of the large family of seven-transmembrane guanosine nucleotide-binding proteins (G-proteins) linked receptors, and displays sequence similarity to other receptors involved in G-protein activation (Niswender & Nett, 1994). The LH receptor structure comprises a 26 or 27 amino acid signal peptide with a large N-terminal extracellular domain (333 to 341 amino acids) containing the hormone binding site(s) and six putative glycosylation points (Loosfelt et al, 1989), seven transmembrane domains and a seventy amino acid cytoplasmic C-terminus tail, which is involved in G-protein activation (Niswender & Nett, 1994). The COOH terminal intracellular tail has been shown to possess putative protein kinase C phosphorylation sites, but is not thought to possess the sites required for phosphorylation by protein kinase A (Loosfelt *et al*, 1989; Koo *et al*, 1991).

1.9.2 Action of LH receptor

The LH receptor is coupled to adenylate cyclase; binding of ligand (LH/CG) to the receptor results in activation of this enzyme in the corpus luteum (Marsh, 1975) by interaction with G-proteins . The G-protein complex which activates (stimulates) adenylate cyclase is termed G_S (Abramowitz & Birnbaumer, 1982; Gillman, 1984). G-protein complexes are heteromeric proteins composed of three subunits designated α, β and γ (Johnson & Dhanasekaran, 1989). It is the α-subunit that possesses the ability to bind guanine nucleotides; in the inactivated state (ie. no ligand bound to the receptor) this binding site is occupied by GDP. Receptor activation of the G-

protein complex occurs by catalysis of the exchange of bound GDP for GTP. The binding of GTP to the α-subunit, which is a magnesium-dependent process, brings about the dissociation of the trimeric complex subunits (which in the inactivated state, form an α–β–γ complex) to give free α-GTP and β–γ complex. It is the dissociated α-GTP complex that regulates the activation of adenylate cyclase (Johnson & Dhanasekaran, 1989). The activated α-GTP complex stimulates the catalytic subunit of adenylate cyclase (Savard, 1973; Marsh, 1975), which in turn stimulates the conversion of Mg·ATP to cAMP. The cAMP produced then binds to the regulatory subunit of protein kinase A, which causes the dissociation of the catalytic subunit of the protein kinase A. The catalytic subunit of protein kinase A phosphorylates a number of luteal proteins, including some of the enzymes involved in steroidogenesis and other related processes such as cholesterol synthesis and LDL uptake. This response can be deactivated by the action of phosphoprotein phosphatases which dephosphorylate the enzymes phosphorylated by the catalytic subunit of the protein kinase A (Krebs & Beavo, 1979). The intrinsic GTPase activity of the α-subunit ensures hydrolysis of the GTP to GDP after a period of approximately 3-5 minutes. Thus the α–β–γ–complex is reformed until restimulation of the coupled receptor occurs (Johnson & Dhanasekaran, 1989). Although there is some uncertainty about the precise proteins phosphorylated by protein kinase A, it has been suggested that in the luteal cell protein kinase A effects include cholesterol esterase activation (Caffrey *et al*, 1979b), stimulation of the cytochrome P_{450} cholesterol side chain cleavage complex (Caron *et al*, 1975) and stimulation of the transport of cholesterol across the mitochondrial wall (Hall, 1985) to the inner mitochondrial membrane where the side chain cleavage system is localised.

It is now apparent that activation of the LH receptor may activate other second messenger systems in luteal cells. LH has been shown to stimulate the Ca^{2+} polyphosphatidylinositol-protein kinase C pathway. Receptor binding of LH activates phospholipase C. This in turn catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate (InsP3) and diacylglycerol (DAG). InsP3 acts on specific intracellular receptors in the endoplasmic reticulum, resulting in the mobilization of Ca^{2+} from intracellular stores and a rise in intracellular Ca^{2+} concentration. DAG binds to protein kinase C (Nishizuka, 1984; Berridge, 1984), which then phosphorylates a number of different luteal proteins. Both the rise in intracellular Ca^{2+} and protein kinase C (PKC) activation are required to elicit a full biological response (Alila & Dowd, 1991). The bovine corpus luteum has been shown to contain only the alpha and epsilon isoforms of PKC, associated with the cytosol and plasma membrane respectively (Orwig *et al*, 1994).

Further studies to elucidate the Ca^{2+}-polyphosphatidylinositol-protein kinase C pathway have demonstrated that calcium is required for the full LH response of luteal cells in culture. When calcium was removed, the LH-stimulated increase in progesterone secretion from small luteal cells was reduced (Alila *et al*, 1990), in agreement with earlier studies which demonstrated the ability of LH to mobilise intracellular stores of calcium in these cells (Alila *et al*, 1989; Davis *et al*, 1987). This reduction of progesterone secretion in the absence of calcium was only observed in small luteal cells in response to LH stimulation; calcium removal had no effect on small cell basal progesterone production (Alila *et al*, 1990). Moreover, in small cells, the effect of calcium removal could not be negated by addition of 8-bromo-cyclic 3',5' adenosine monophosphate (8-bromo-cAMP) suggesting that the

effects of calcium on progesterone production stimulated by LH in small cells were mediated after cAMP generation. Indeed, it was shown that lowered calcium had no effect on cAMP production in response to LH or PGE2 (Alila *et al*, 1990). Calcium was also demonstrated to be involved in the basal secretion of progesterone from large luteal cells. Basal, LH- and forskolin- stimulated progesterone secretion was reduced in the presence of lowered calcium concentrations in the culture media. However, since lowered calcium had little effect on large cell progesterone secretion in the presence of 8-bromo-cAMP, the effect of calcium on large cell luteal progesterone secretion was concluded to be exerted prior to the generation of cAMP in these cells (Alila *et al*, 1990). It has been suggested from experimental studies performed on sheep luteal cells that each luteal cell type has distinct calcium influx regulatory mechanisms, and that calcium regulation of progesterone secretion plays a more critical role in large cells than in small luteal cells (Martinezzaguilan *et al*, 1994).

Recent studies have also demonstrated the likelihood of the involvement of protein kinase C in transmembrane signalling in the bovine corpus luteum (Davis, 1992). These studies have shown that although basal progesterone secretion in mixed luteal cell populations was enhanced by protein kinase C activators such as the tumour promoting phorbol ester, 12-O-tetradecanolphorbol 13-acetate (TPA) (Brunswig *et al*, 1986; Hansel *et al*, 1987), synthetic diacylglycerol analogues (Brunswig *et al*, 1986) and exogenous phospholipase C (Alila *et al*, 1988a; Benhaim *et al*, 1990), LH-stimulated calcium mobilisation was inhibited, as was accumulation of inositol mono-, bis- and tri-phosphates (Davis, 1992). No effects on progesterone secretion were observed in control or LH-stimulated cells.

Thus it was concluded that this mechanism of signal transduction from the LH receptor was not required for acute LH-stimulated steroidogenesis (Davis, 1992). It has been reported that concentrations of the phorbol ester phorbol 12-myristate-13-acetate (PMA) in the region of 10 nM are stimulatory to progesterone production, but that higher concentrations inhibit steroidogenesis (Brunswick *et al,* 1986). Other studies involving longer culture times of up to 4 hours have demonstrated that though cAMP levels remain elevated, progesterone production is reduced in response to chronic exposure to phorbol esters such as TPA, suggesting that for a maximal steroidogenic response to gonadotrophin stimulation the phospholipase C second messenger pathway may be of importance (Brunswick *et al,* 1986; Budnick & Muckhopadhyay, 1987; Benhaim, 1990). It was suggested that phorbol esters act on the coupling between the LH receptor and the inositol-phospholipid-phospholipase C (Davis, 1992). In small bovine luteal cells, activation of protein kinase C with the phorbol ester PMA potentiated LH-induced increases in cAMP at a range of doses of LH, though progesterone production was increased by PKC activation only at low doses of LH. At higher doses of LH PKC activation was inhibitory to progesterone production (Benhaim *et al,* 1987). It has been suggested that a control system may operate to control the stimulatory response of small luteal cells to LH. Thus, high doses of LH, in addition to stimulating adenylate cyclase, may also activate PKC to control the stimulatory effects of high LH concentrations (Benhaim et al, 1987).

1.10 Ovarian steroidogenesis

The following is a brief review of ovarian steroidogenesis (see review by Strauss and Miller, 1991). There is a great deal of interspecies variation in not only the control of the enzymes involved in steroidogenesis, but also in the actual steroidogenic enzymes present in the corpus luteum. Figure 1.6 shows ovarian steroidogenesis from cholesterol through to the production of oestrogen (cholesterol acquisition and its control by LH is discussed in Section 1.12 - LH mediated effects on steroidogenesis). However, as stated in Section 1.12, in the bovine corpus luteum, the major product of the steroidogenic process is progesterone since the aromatase enzyme and P_{450} C17α hydroxylase/c17,20 lyase enzyme are absent.

Cytochrome P_{450}s are so called because of the Soret absorbance band shift from 420nm to 450nm when reduced in the presence of carbon monoxide (Hall, 1986). All cytochrome P_{450}s contain ca. 500 amino acids and possess a haem binding region close to the carboxy (C) terminus (Strauss & Miller, 1991). The main requirements of these enzymes are reducing factors and oxygen. Reducing agents are donated from NADPH, whose electrons are transferred to the cytochrome P_{450} by a microsomal or a mitochondrial electron transport chain which utilises a flavoprotein constituent (Strauss & Miller, 1991).

Cholesterol side chain cleavage (Cytochrome P_{450}scc)

This is the enzyme that catalyses the first committed step of steroidogenesis, the cleavage of the side chain of cholesterol, the net result being the removal of 6 carbon atoms from the side chain (Strauss & Miller, 1991). This reaction occurs in the inner mitochondrial membrane and yields pregnenolone; this is

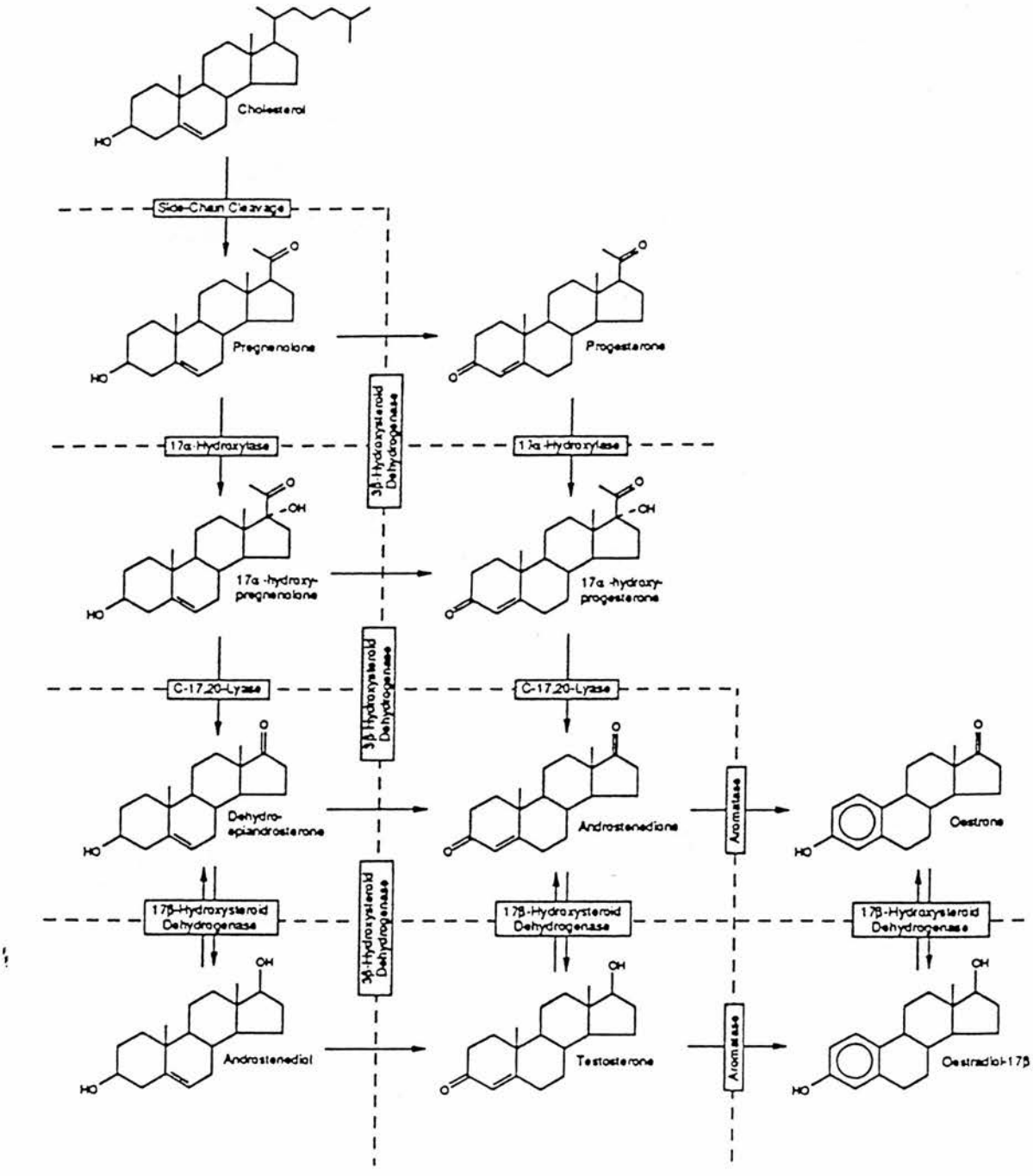


Figure 1.6 Possible routes of ovarian steroidogenesis (reproduced from Turner, 1992)

also the major rate limiting step of steroidogenesis (Strauss & Miller, 1991). The overall equation for the reaction is:

Cholesterol + 3NADPH + $3H^+$ + $3O_2$ ------> Pregnenolone + isocapraldehyde + $4H_2O$ + $3NADP^+$

The associated electron transport system consists of adrenodoxin reductase and the iron-sulphur shuttle protein adrenodoxin (Miller, 1988). The slowest event, and thus rate limiting step, of steroidogenesis is the binding of cholesterol to the haem pocket of P_{450}scc (Strauss & Miller, 1991). The reaction that proceeds entails 2 hydroxylations at C20 and C22, followed by cleavage of the bonds between these carbons. Hence 3 pairs of electrons are required, and 3 cycles of reduction/oxidation of adrenodoxin occur (Strauss & Miller, 1991). Reducing agents are donated to the P_{450}scc by adrenodoxin by cycles of reduction and oxidation. A 1:1 adrenodoxin : adrenodoxin reductase complex is formed, resulting in catalytic reduction of adrenodoxin$^+$ to adrenodoxin$^-$. The reduced adrenodoxin then disassociates from the adrenodoxin reductase and forms a new 1:1 complex with P_{450}scc. Adrenodoxin becomes oxidised, donates the electron from this oxidation to the P_{450}scc, then recomplexes with the adrenodoxin reductase and is once more reduced (Strauss & Miller, 1991). It appears that LH and cAMP are responsible for the upregulation of P_{450}scc and adrenodoxin which occurs as luteal development proceeds (Rodgers *et al*, 1986a). In cows it has been shown that increasing levels of LDL can increase the synthesis of P_{450}scc in cultured granulosa cells (Funkenstein *et al*, 1984). P_{450}scc is detectable in both granulosa and theca cells in the bovine ovary, and, after formation of the corpus luteum, both luteal cells types express this enzyme in increasing amounts as the luteal phase proceeds (Rodgers *et al*, 1986a; b; c).

Cytochrome P_{450} C17 (17α hydroxylase/C17,20 lyase)

As suggested by its names, this enzyme has two functions - (i) hydroxylation of progesterone and pregnenolone at C17, to yield 17α hydroxyprogesterone and 17α hydroxypregnenolone, and (ii) conversion of the 17α hydroxylated C21 steroids into the C19 androgens, androstenedione and dehydroepiandrosterone (DHA). In bovine preovulatory follicles the major androgen produced is androstenedione (Lacroix *et al*, 1974). However, the major products of enzyme catalysis are dependent on various factors, eg. in the presence of low substrate concentrations the C17 hydroxylation reaction predominates (Zuber *et al*, 1986). A similar electron donor system to that which operates with the side chain cleavage system is utilised by cytochrome P_{450} C17 (Strauss & Miller, 1991). The main difference is that P_{450} reductase is the flavoprotein utilised, rather than adrenodoxin reductase (Strauss & Miller, 1991). Two electrons from NADPH are required. The first is transferred from P_{450} reductase, the second from cytochrome b_5 (Miller, 1988).

In the ovary, Cytochrome P_{450} C17 is found only in the endoplasmic reticulum of thecal cells, as demonstrated by immunocytochemistry (Rodgers *et al*, 1986 a). This is integral to the two cell - two gonadotrophin hypothesis of steroid synthesis. Prior to ovulation in cows, follicular content of this enzyme increases with follicular growth (Rodgers *et al*, 1986a). However, after ovulation levels of Cytochrome P_{450} C17 are dramatically reduced. Indeed, no detection of this enzyme in the early bovine corpus luteum was evident (Rodgers *et al*, 1986c).

The hydroxysteroid dehydrogenase family

These enzymes are located in the cytosol or microsomes, and require NADP(H) and NAD(H) as cofactors (Strauss & Miller, 1991).

(i) 3ß-Hydroxysteroid dehydrogenase (3ß-HSD)

NAD^+ is the cofactor used by this enzyme (Miller, 1988). 3ß-HSD catalyses the conversion of pregnenolone to progesterone, 17α hydroxypregnenolone to 17α hydroxyprogesterone, and DHA to androstenedione. There may be a number of isoforms of this enzyme with individual specificities for different substrates; however, identification of these isoforms in the ovary is as yet incomplete (Strauss & Miller, 1991). This enzyme is not thought to be rate limiting for steroidogenesis, and indeed its activity is in excess in the bovine corpus luteum (Caffrey *et al*, 1979a). However, it has recently been demonstrated that levels of 3ß-HSD do come under control of non-ovarian hormones, since it has been demonstrated that $PGF_{2\alpha}$ causes a rapid, large decrease in levels of 3ßHSD mRNA in the ovine corpus luteum (Hawkins *et al*, 1993), which was suggested to be due to decreased synthesis and increased degradation of the 3βHSD (Hawkins *et al*, 1993). Other studies have demonstrated upregulation of 3βHSD transcription in response to hCG stimulation in luteinised porcine granulosa cells (Chedrese *et al*, 1990). Ovine 3βHSD is reported to be found in greater amounts in large luteal cells than in small luteal cells. This is reflected in the progesterone output of these two cell types (Hawkins *et al*, 1993). Thus, though perhaps not rate-limiting in the classical sense, regulation of this enzyme by luteotrophic/luteolytic factors does impose a form of regulation of progesterone secretion.

(ii) 17ß-Hydroxysteroid dehydrogenase (17ß-HSD)

This enzyme, located in the endoplasmic reticulum, is the catalyst for the reversible conversion of oestrone to oestradiol-17ß, androstenedione to

testosterone, and DHA to androstenediol. NADH and NADPH are utilized as cofactors (Strauss & Miller, 1991).

(iii) 20α-Hydroxysteroid dehydrogenase (20αHSD)

This NADP(H) dependent enzyme appears to act catabolically in the ovary, converting progesterone to the biologically inactive derivative 20α-progesterone (Strauss & Miller, 1991).

Aromatase (Cytochrome $P_{450aromatase}$)

This enzyme has been demonstrated to be specific to the granulosa layer of the preovulatory follicle (endoplasmic reticulum), and, as in the case of C17, is absent from the bovine corpus luteum (Savard, 1973). It is the aromatase enzyme which catalyses the conversion (aromatization) of theca-derived androgen to oestrogen (Miller, 1988). This process involves the loss of the C19 methyl group via 2 hydroxylations, and a further hydroxylation of the 2β-hydroxyl group (Miller, 1988). In the same manner that Cytochrome P_{450} C17 utilises a P_{450} reductase electron transport, aromatase receives 3 pairs of electrons from 3NADPH and 3 molecules of oxygen (Thompson & Siiteri, 1974a; b).

5 α reductase

NADPH is utilised as the cofactor for this enzyme. In the ovary, 5α reductase acts on C19 steroids to produce the 5α reduced androgens, and on C21 steroids to produce steroids whose function is currently unknown (Strauss & Miller, 1991).

1.11 The two-cell-two-gonadotrophin hypothesis

The two-cell theory of follicular oestrogen secretion was proposed by Short in 1962. Since then, the theory has been expanded and reworked to give the two-cell-two-gonadotrophin hypothesis, which has now gained almost universal acceptance. The theory proposes that the granulosa cells synthesise oestrogen from androgen supplied by the thecal cells. Indeed, immunocytochemical studies have demonstrated that the enzyme responsible for the production of androgen (17α hydroxylase/C17,20 lyase) is found exclusively in the theca cells of bovine preovulatory follicles (Rodgers *et al*, 1986a). Synthesis of androgen in the theca is under the control of LH at all follicular stages, principally through cAMP production (Henderson *et al*, 1984). The preferred route in cows for the conversion of cholesterol to androgens is the 5-ene-3β hydroxysteroid pathway (Lacroix *et al*, 1974). DHEA produced by this pathway is then converted to androstenedione (Gore-Langton & Armstrong, 1988). It has been demonstrated that the theca cells of cows can contribute to the overall ovarian production of oestrogens (Lacroix *et al*, 1974), however, the extent to which this occurs is minor as compared to oestrogen production by the granulosa. Androgens derived from theca cells diffuse accross the basement membrane to the granulosa cells where they act as substrate for the aromatase enzyme. Control of aromatization in the granulosa cell is exerted by FSH (Dorrington *et al*, 1975). It is now accepted that during granulosa cell maturation LH receptors develop through a priming effect of FSH (Gore-Langton & Armstrong, 1988). Thus LH also exerts some control over oestrogen synthesis, but only in the preovulatory follicle. The other main product of granulosa cell steroidogenesis is progesterone and its C21 metabolites, which are produced under the control of LH (Gore-Langton &

Armstrong, 1988). *In vitro,* increased synthesis of androgen by cultured theca cells is associated with increased expression and activity of c17,20 lyase. Both cell types express side chain cleavage and synthesise progesterone and pregnenolone in cows (Lacroix *et al,* 1974).

1.12 LH mediated effects on steroidogenesis

There is much interspecies variation in almost all aspects of the function and regulation of corpora lutea, and the steroidogenic capacity of corpora lutea varies dramatically from species to species. For example, the bovine corpus luteum secretes no oestrogens, unlike that of the human, secreting only the simplest steroidal products (in terms of the number of enzymatic reactions involved), progesterone, pregnenolone and 20β-hydroxy-4-pregnen-3-one, with progesterone being by far the major secretory product (Mason *et al,* 1962). The bovine corpus luteum appears to lack the 17α-hydroxylase/C17, 20 lyase enzyme responsible for the formation of C19 androgens, and thus secretion of these steroids is absent (Savard, 1973). Indeed, gene expression of this enzyme is absent in the bovine corpus luteum (Lauber *et al,* 1991). The bovine corpus luteum also lacks the ability to synthesise and secrete oestrogenic steroids due to the absence of the aromatase enzyme (Savard & Telegdy, 1965). The luteal cell can be influenced by LH through the scheme of the events triggered by LH binding to its receptor as detailed above. The increase of cAMP dependent protein kinase is thought to have a number of effects on steroidogenesis, though many of the proposed effects remain putative. One of the major effects of cAMP dependent protein kinase increases is the increase in protein synthesis by luteal cells (Niswender & Nett, 1994). This is suggested to be due to an increase in the requirement of carrier proteins to shuttle the various intermediates of steroidogenesis to the

particular sites of the steroidogenic enzymes in the cell (Niswender & Nett, 1994), since sterols and steroids alone are insoluble in aqueous media. *In vitro*, SCP2 (sterol carrier protein 2), a protein whose function is thought to be the intracellular transport of cholesterol to the inner mitochondrial membrane where it undergoes side chain cleavage to form pregnenolone (Steinschneider *et al*, 1989) is a substrate for protein kinase C. Thus the secondary Ca^{2+}/ InsP3/DAG signalling pathway from the LH receptor may exert some of its action via phosphorylation of SCP2. In the rat corpus luteum, oestradiol has been shown to increase levels of SCP2 (McLean *et al*, 1989). Cholesterol utilised in the steroidogenic process has three main origins, (i) *de novo* synthesis, (ii) LDL/HDL mediated uptake and (iii) utilisation from intracellular pools of cholesterol ester. Cholesterol ester hydrolase, (the enzyme which hydrolyses cholesterol esters to free cholesterol for utilisation in steroidogenesis) has been demonstrated to be activated through phosphorylation by cAMP dependent protein kinase (Caffrey *et al*, 1979b), and thus is likely to be under regulation of LH (Niswender & Nett, 1994). LDL receptor expression has been shown to be increased by gonadotrophin stimulation in ovarian tissue (Strauss & Miller, 1991), presumably through increased transcription of the LDL receptor mRNA (Golos & Strauss, 1987).

HMG-CoA reductase, the enzyme that catalyses the rate limiting step of *de novo* cholesterol biosynthesis, is increased in cells stimulated by LH, at least in part through increased transcription of the mRNA encoding for this enzyme (Golos & Strauss, 1988). HMG-CoA reductase is also regulated by negative feedback exerted by local factors. Hydroxysteroids (eg. 25-hydroxy cholesterol) inhibit cholesterol and LDL receptor synthesis. In the rat ovary, 25 and 26-hydroxycholesterol are both potent inhibitors of the *de novo*

cholesterol synthesis pathway. 26-cholesterol hydroxylase is inhibited by progesterone and pregnenolone. This in turn reduces production of 26-hydroxycholesterol allowing *de novo* cholesterol synthesis and LDL receptor expression to be maintained (Strauss & Miller, 1991). It appears that the regulation of expression of LDL receptor mRNA and HMG-CoA reductase mRNA in response to negative feedback of sterols is through two different routes, though as yet these have not been elucidated (Strauss & Miller, 1991).

The next point of LH regulation of steroidogenesis is the cholesterol side chain cleavage enzyme, located in the inner mitochondrial membrane. Levels of P450 scc and its associated electron transport system consisting of adrenodoxin and adrenodoxin reductase are increased in response to gonadotrophin stimulation. It has been observed that cAMP analogues act via cAMP dependent protein kinase to increase levels of synthesis and accumulation of the respective mRNAs for the components of the cholesterol side chain cleavage system (Goldring *et al*, 1987). In addition to the levels of these enzymes, their actual enzymatic activities may also be modulated by activation of the LH receptor. Cytochrome P_{450} scc can be phosphorylated by calcium dependent protein kinase C, and adrenodoxin by cAMP dependent protein kinase, though the significance of these processes in intact cells remains unknown (Strauss & Miller, 1991). Gonadotrophin stimulation of large luteal cells is not essential for the continued expression of the constituent parts of the cholesterol side chain cleavage system, unlike small luteal cells where a large decrease in the activity of this enzyme is observed when cAMP stimulation is removed (Aflalo & Meidan, 1993). However, these experiments used bovine preovulatory follicle cells luteinized in culture (Aflalo & Meidan, 1993). Whether the same regulatory mechanisms exist in cells which luteinize *in vitro* or *in vivo* remains unknown. Finally,

availability of cholesterol to the side chain cleavage system located in the inner mitochondrial membrane also appears to be under some control of LH. The intracellular transport of cholesterol is mediated, at least in part, by the cytoskeleton. Substances which inhibit microfilament (but not microtubule) formation have been shown to cause a decrease in steroidogenesis in cultured bovine luteal tissue (Gwynne & Condon, 1982), and microtubules are implicated in regulation of steroidogenesis by rat granulosa cells (Carnegie *et al*, 1987). However, the exact processes by which components of the cytoskeleton facilitate cholesterol movement remain to be elucidated (Strauss & Miller, 1991). Carrier proteins such as SCP2 (see above) and fatty acid binding protein may also play a part in the regulation of the bioavailability of cholesterol to the inner mitochondrial membrane, though again, these processes are poorly understood (Strauss & Miller, 1991).

The enzyme which catalyses the final conversion in bovine luteal steroidogenesis of pregnenolone to progesterone is the 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3ß-HSD) enzyme. Present in excess in ovarian cells, it has been suggested that this enzyme does not respond to LH stimulation in the acute fashion of previously discussed responses, but instead, 3β-HSD synthesis may reflect part of a longer term regulation of steroidogenesis by LH in the corpus luteum (Niswender & Nett, 1994). End product inhibition occurs with all of the enzymes discussed above (Niswender & Nett, 1994). The affinity of 3β-HSD for progesterone, its product, is approximately six times greater than that of its substrate, pregnenolone (Caffrey *et al*, 1979a). Oestradiol and testosterone have similar high affinities for this enzyme (Caffrey *et al*, 1979a). Thus the question of how progesterone synthesis can proceed without the products formed inhibiting synthesis is raised. It is suggested that binding proteins sequester

progesterone and thus prevent negative feedback effects on its own production (Caffrey *et al*, 1979a; Rothchild, 1981). Secondarily, the high blood flow to the corpus luteum may also help to keep levels of steroid down (Caffrey *et al*, 1979a) by efficient removal into the blood.

1.13 Progesterone secretion

In general, the cAMP pathway of second messenger generation from the LH receptor is considered to be the primary pathway in luteal cells, with the Ca^{2+}-InsP3/DAG pathway of secondary importance (Alila & Dowd, 1991). The mechanisms of LH stimulated progesterone secretion are still not fully understood. In large luteal cells from the bovine corpus luteum, basal progesterone secretion is 5-10 fold greater than the basal secretion of progesterone from small luteal cells (Koos & Hansel, 1981). Bovine large cells cultured *in vitro* show little or no response to LH concentrations that maximally stimulate progesterone production in small luteal cells (1-5 ng/ml) however, and concentrations of up to 100 ng/ml LH are required to elicit an increase in progesterone production (Alila *et al*, 1988a). Thus it appears likely that *in vivo*, large cells do not respond to LH stimulation, since the levels of LH found in the circulation of ruminants are only of the order of 5-10 ng/ml (Alila & Dowd, 1991). In sheep large luteal cells, which possess LH receptors, little or no response to LH is observed (Harrison *et al*, 1987). Since other trophic substances such as PGE_2 and PGI_2 can mediate an increase in progesterone secretion through the cAMP second messenger system in bovine large cells (Alila *et al*, 1988b), as indeed do exogenous cAMP analogues (Fitz *et al*, 1984; Silvia *et al*, 1984; Alila *et al*, 1988a), it appears that the LH receptors of large luteal cells are uncoupled from the

cAMP second messenger system (Hoyer *et al*, 1984; Hoyer & Niswender, 1986; Harrison *et al*, 1987; Davis *et al*, 1989). The mechanism of this apparent uncoupling is as yet unknown (Alila & Dowd, 1991). It is also possible that an apparent uncoupling may be simply an artifact produced by the process of tissue disruption.

Intercellular communication between large and small luteal cells is also implicated in the control of progesterone secretion. In sequential perifusion systems of separated bovine luteal cell types, small luteal cell secretions were observed to inhibit large luteal cell progesterone production, whilst large luteal cell to large luteal cell perfusions produced the most progesterone (Del Vecchio *et al*, 1994). Arachidonic acid was implicated in these effects since the inhibitory effects of small luteal cells on large luteal cell progesterone production could be negated by it, suggesting that exogenous arachidonic acid increases the production of a small cell-derived luteotrophic eicosanoid or inhibits production of a small cell-derived luteolytic eicosanoid (Del Vecchio *et al*, 1994). Arachidonic acid added to a large to small luteal cells perfusion arrangement inhibited progesterone secretion in small luteal cells, perhaps through stimulation of oxytocin release (Del Vecchio *et al*, 1994). Contact dependant communication (via gap junctions) between bovine luteal cells has recently been reported (Redmer *et al*, 1991). Small cell to small cell and small cell to large cell contact dependent communication was evident, however, this type of communication was not observed between large luteal cells (Redmer *et al*, 1991). Other studies have shown stimulatory effects of oxytocin on luteal cell progesterone production which appear to be dependent on cell contact-mediated communication (Miyamoto & Schams, 1991). Recent reports indicated that oxytocin had little effect on progesterone production in bovine luteal cells in contact with each other (Del

Vecchio *et al*, 1995). However, these studies excluded non-luteal cells from the culture systems and thus the effects of oxytocin may be dependent on luteal cell contact with non-luteal cells of the corpus luteum Contact dependent communication between bovine large and small luteal cells occurs at all stages of the luteal phase (Del Vecchio *et al*, 1995). Large and small luteal cells in contact with each other secrete significantly more progesterone in response to stimulation with LH than do separated large and small cells, (Del Vecchio *et al*, 1995), underlining the importance of intercellular contact between bovine luteal cells in luteal cell responses to LH.

Basal bovine luteal progesterone secretion has been shown to be pulsatile (Rossmanith *et al*, 1991). Pulse frequency was unaffected by hCG stimulation of tissue slices of bovine corpora lutea perfused *in vitro*. However, the amplitude and release rates of progesterone were increased (Rossmanith *et al*, 1991; 1992). This pulsatile progesterone release was suggested to be due to large luteal cells since it appeared to be independent of LH stimulation. Furthermore, the antiprogesterone ZK 96.734 increased the amplitude and frequency of the progesterone pulses, suggesting that progesterone exerted inhibitory effects on its own release by short, local inhibitory progesterone feedback in the bovine corpus luteum (Rossmanith *et al*, 1991). In sheep, it has been demonstrated that secretion of progesterone from the corpus luteum *in vivo* is episodic during the early and late luteal phase (McNeilly *et al*, 1992). Suppression of LH release had no effect on pulsatile progesterone secretion, indicating that pulsatile progesterone secretion was not LH dependent (McNeilly *et al*, 1992). It has been suggested that the intrinsic pulsatile release of progesterone is maintained, and progesterone secretion optimised by hCG stimulation of the bovine corpus luteum *in vitro*

(Rossmanith *et al*, 1992). LH support of the corpus luteum is required during its formation and development in cows (Peters *et al*, 1994), and insufficient LH support during the early stages of luteal development reduced large luteal cell populations in the corpus luteum, suggesting that LH induced differentiation of small luteal cells to large luteal cells (Peters *et al*, 1994).

Recently, steroids have been implicated in the control of the cells from which they are produced. The regulation of steroidogenic cells by steroids was proposed by Rothchild in 1981, who suggested that progesterone may have the ability to stimulate its own synthesis and secretion. Evidence for this arose from observations that (i) a direct relationship existed between intraluteal progesterone concentrations and the rate of progesterone secretion, and, as discussed above, (ii) a lack of correlation between progesterone secretion and the luteotrophin (LH) thought to drive progesterone secretion (Rothchild, 1981). Also, progesterone is known to activate cholesterol esterases (Caffrey *et al*, 1979b), thereby increasing substrate availability for its continued synthesis. Recently, steroid receptors have been described in the corpus luteum of a number of species, principally the primate corpus luteum. Primate luteal tissue has been shown to possess classical receptors for androgens (Hild-Petito *et al*, 1991; Zelinski-Wooten *et al*, 1994) and progesterone receptors (Aladin Chandrasekher *et al*, 1994), but not oestrogen receptors (Aladin Chandrasekher *et al*, 1994). It has been suggested that androgens may be inhibitory to progesterone production in the primate corpus luteum (Hild-Petito *et al*, 1991). There is disagreement over the numbers of progesterone receptors present in the corpus luteum throughout the luteal phase. Some workers report changes in levels throughout the luteal phase of primates (Hild-Petito *et al*, 1988;) and humans (Horie *et al*, 1992), with peak numbers present at mid-luteal phase which

gradually decline towards the end of the luteal phase, and, low levels of progesterone receptor mRNA in early primate luteal tissue which increase by the mid-luteal phase and are then maintained throughout the luteal phase (Duffy & Stouffer, 1995). In contrast, others report no changes in luteal progesterone receptor content throughout the human menstrual cycle (Duncan *et al*, 1994). Studies have indicated that LH, in addition to promoting progesterone secretion during luteinization, also promotes progesterone receptor expression (Hild-Petito *et al*, 1988; Aladin Chandrasekher *et al*, 1994). The intracrine/paracrine actions of steroids on the corpus luteum are as yet poorly understood, though this work is at present still in a period of relative infancy, and current investigations may throw light on the mechanism whereby progesterone controls its own synthesis and secretion from luteal cells.

Though large luteal cells are thought to have a much higher basal output of progesterone, small luteal cells (by virtue of their greater numerical representation in the corpus luteum and their responsiveness to LH) are thought to make a significant contribution to the total progesterone output of the bovine corpus luteum (Koos & Hansel, 1981). In this study (Koos & Hansel, 1981), contamination of large luteal cell populations with clumps of small luteal cells was taken into account and results of progesterone output measurements corrected accordingly, enabling effects of LH and $PGF_{2\alpha}$ on large and small luteal cell populations to be studied. In the absence of LH, the progesterone output of large luteal cells was approximately twenty fold greater than that of small luteal cells, however in response to LH stimulation the output of progesterone from large luteal cells increased only slightly, whereas that of the small luteal cells increased eleven-fold in the first hour of LH stimulation, and continued to increase over the three hour culture period

(Koos & Hansel, 1981). At low concentrations of LH, small cells continued to respond, but no increased progesterone secretion was observed from the large luteal cell culture. Neither cell type responded to PGF2α, either alone or in conjunction with LH. Moreover, neither cell type appeared to have the ability to convert exogenously added testosterone to oestradiol (Koos & Hansel, 1981), agreeing with the generally accepted theory that the bovine corpus luteum contains no aromatase enzyme and hence is incapable of oestradiol synthesis. (Savard, 1973). However, other studies have shown that both large and small cells from bovine corpora lutea respond to $PGF_{2\alpha}$ stimulation by increasing progesterone production (Alila *et al*, 1988a). These experiments demonstrated that basal progesterone secretion from small luteal cells was stimulated by $PGF_{2\alpha}$ (via PKC activation), but whereas $PGF_{2\alpha}$ potentiated LH-stimulated progesterone secretion in small cells, this effect was reversed in large luteal cells (Alila *et al*, 1988a; b). No effect of $PGF_{2\alpha}$ on large luteal cell basal progesterone secretion was observed (Alila *et al*, 1988a). Given the low response to LH observed in large luteal cells, it would appear that small luteal cells are the luteal cell type which respond to the stimulatory effects of $PGF_{2\alpha}$ (Alila *et al*, 1988a). PGI_2 and PGE_2 have both been demonstrated to stimulate progesterone production in small and to a lesser extent, large luteal cells, and thus may play an important paracrine regulatory role, however, in large luteal cells these stimulatory effects can be abolished by $PGF_{2\alpha}$, suggesting that large luteal cells are the target for the luteolytic action of $PGF_{2\alpha}$ (Alila *et al*, 1988b).

The number of LH receptors present in the corpus luteum is in a state of dynamic flux, and varies with the stage of the luteal phase. Numbers of luteal LH receptors correlate with the weight of the corpus luteum and its levels of progesterone secretion (Diekman *et al*, 1978a). The percentage of

occupied LH receptors at the time of maximal progesterone secretion in the ewe corpus luteum is approximately 0.6% (Diekman *et al*, 1978a). Similarly, during the mid-luteal phase of the cow maximum progesterone secretion occurs and maximum numbers of LH receptors are found (Rao *et al*, 1976). However, the luteal expression of LH receptor was more highly correlated with progesterone secretion than with circulating levels of LH (Niswender *et al*, 1985a). Indeed, in *in vitro* studies of early bovine corpora lutea, exogenous progesterone increased numbers of LH receptors, as did forskolin treatment, perhaps via increased progesterone production (Jones *et al*, 1992). Presumably, increases in receptor numbers in the presence of low circulating levels of LH may enable the corpus luteum to maximise its response to LH by ensuring a large supply of unoccupied receptors (Niswender & Nett, 1994). LH also controls the numbers of LH receptors found in the corpus luteum. Indeed, increased concentrations of LH initially 'up-regulate' the number of LH receptors, followed by a reduction in the numbers of LH receptors, which appears to indicate a correlation between the numbers of occupied receptors and the numbers of receptors lost (Niswender *et al*, 1985a). Though the mechanism for up-regulation of LH receptors is unclear, down-regulation (receptor loss) occurs through the process of receptor-ligand internalisation (Niswender *et al*, 1985a). It is thought that the LH receptor : LH complex is internalised by the cell, and subsequently degraded by lysosomal enzymes, leaving the free receptor which can then be recycled back to the luteal cell surface (Niswender & Nett, 1994).

1.14 Oxytocin

Though progesterone is considered to be the single most important product of the bovine corpus luteum, other hormones are also synthesised and

secreted in significant amounts. In the preovulatory follicle of cows, oxytocin is synthesised and secreted predominantly by the granulosa cells (Voss & Fortune, 1991). The ability to secrete oxytocin is greatly enhanced in the late follicular phase, after the LH surge (Voss & Fortune, 1991). Oxytocin was demonstrated to increase progesterone production by granulosa cells, suggesting a possible autocrine role of oxytocin in the control of granulosa cell steroidogenesis (Aladin Chandrasekher & Fortune, 1990). The corpus luteum of the cow contains considerable amounts (0.4 - 1.8 μg/g) of oxytocin (Alila & Dowd, 1991). Bovine large luteal cells synthesise and secrete oxytocin (Ivell & Richter, 1984). Oxytocin is packaged in secretory granules (Rice & Thorburn, 1985), and is released from the corpus luteum concomitantly with its carrier protein, neurophysin (Sawyer *et al*, 1986). Levels of mRNA encoding oxytocin are minimal before ovulation, however, the mRNA coding for oxytocin is detectable in luteal tissue from the early luteal phase (Fehr *et al*, 1987), and the proportion of mRNA to total mRNA increases dramatically at the time of ovulation (Ivell *et al*, 1985). Secretion of oxytocin is maximal during the mid-luteal phase, and decreases back to preovulatory levels as the corpus luteum regresses (Alila & Dowd, 1991). The factors regulating the synthesis and secretion of oxytocin are poorly understood. In cows, injection of exogenous PGF2α stimulates increased secretion of oxytocin (Schallenberger *et al*, 1984). This effect was not seen in *in vitro* experiments (Hirst *et al*, 1986). Stimulation of oxytocin secretion *in vitro* was observed when ovine luteal cells were stimulated with PGF2α; however, this effect was limited to tissue collected on day 6 of the ovine oestrous cycle, and tissue from day 12 showed no response (Cooke & Ahmad, 1994a). It was suggested that the effects of PGF2α on the secretion of oxytocin *in vivo* may be due to the changes observed in the blood supply to

the corpus luteum observed at the onset of luteal regression which are attributable to PGF2α (Hirst *et al*, 1986). *In vitro*, a number of different factors have been shown to stimulate oxytocin secretion, including the secretagogues CuATP and MgATP (Rice *et al*, 1986), arachidonic acid and its metabolites (Hirst *et al*, 1988), IGF-1 and insulin (McArdle & Holtorf, 1989; Sauerwein *et al*, 1992). Conversely, administration of exogenous oxytocin has been shown to cause a release of PGF2α into the uterine vein of progesterone primed cattle (Milvae & Hansel, 1980b). However, injection of oxytocin does not induce luteolysis, except on days 2 - 6 of the oestrous cycle (Hansel & Wagner, 1960; Ivel *et al*, 1985). It has been suggested that this impairment of normal luteal function is due to interference with the normal processes of oxytocin synthesis and secretion (Hansel & Dowd, 1986). Administration of oxytocin at this time has been shown to impair the ability of the corpus luteum to synthesise the luteotrophin PGI-2, (see next section) but not the luteolysin PGF2α; though progesterone production was not impared significantly, the luteal cell's ability to respond to LH was diminished (Hansel & Dowd, 1986). The effects of oxytocin are discussed further in Section 1.17, Luteal regression.

1.15 Arachidonic acid and arachidonic acid metabolites

The corpus luteum of the cow has been demonstrated to contain large quantities of arachidonic acid (ca. 3 mg/g) which is mostly esterified in membrane phospholipids (Lukaszewska & Hansel, 1980). The origins of luteal arachidonic acid are unclear; some may be uterine derived (Shemesh & Hansel, 1975b), whereas some may be derived from the action of phospholipases on membrane phospholipids (Alila & Dowd, 1991). Infusion of arachidonic acid directly into the bovine corpus luteum causes a decrease

in the levels of plasma progesterone *in vivo* (Hansel & Fortune, 1978). However, *in vitro* studies have observed increased secretion of oxytocin from large cells and increased progesterone secretion from both luteal cells types when arachidonic acid is added to culture media (Lafrance & Hansel, 1992). *In vitro,* the bovine corpus luteum has the ability to bind significant quantities of arachidonic acid (Shemesh & Hansel, 1975b). However, since the metabolites of arachidonic acid also have known effects on luteal tissue, attributing effects directly to arachidonic acid is difficult (Alila & Dowd, 1991). Important metabolites of arachidonic acid found in the bovine corpus luteum are prostacyclin (PGI-2) (Nothnick & Pate, 1989) and 5-hydroxyeicosatetraenoic acid (5-HETE). PGI-2, a product of the cyclooxygenase pathway of arachidonic acid metabolism, is a potent luteotrophin, causing increased progesterone synthesis and secretion both *in vivo* and *in vitro* (Milvae & Hansel, 1980a). The corpus luteum of the cow has higher levels of PGI-2 synthesis than any other tissue studied (Sun *et al,* 1977). It has been suggested that PGI-2 is required for normal development of the early corpus luteum (Hansel & Dowd, 1986). Infusion of indomethacin, an inhibitor of the cycloxygenase pathway, into the uterus in the early luteal phase, inhibits normal CL formation (Milvae & Hansel, 1985). In the late luteal phase, intrauterine infusion of indomethacin prolongs CL lifespan, due to inhibition of PGF2α synthesis (Lewis & Warren, 1977). 5-HETE, a product of the lipoxygenase pathway of arachidonic acid metabolism, is luteolytic in the bovine corpus luteum (Hansel & Dowd, 1986). It is not known whether or not the bovine corpus luteum can synthesise 5-HETE, but experiments have shown that this compound is present in luteal tissue (Hansel & Dowd, 1986). *In vitro,* products of the 5- and 15-lipoxygenase pathways have the ability to reduce progesterone

secretion and levels of PGI-2, but do not affect PGF2α levels (Milvae *et al*, 1986). It is suggested that 5-HETE may be an important uterine derived luteolysin (Hansel & Dowd, 1986), and 5-lipoxygenase products of arachidonic acid metabolism may play a role in the control of luteal oxytocin release (Cooke & Ahmad, 1994b).

1.16 Other substances implicated in bovine corpus luteum function

In addition to the products of the corpus luteum described above, other substances are known to affect the function of the bovine corpus luteum. Growth factors such as epidermal growth factor (EGF) have been implicated in luteal function in the ewe, and the EGF receptor has been found in ovine luteal tissue (Niswender *et al*, 1985b). However, no effects of EGF on the production of progesterone *in vitro* have been observed (Battista *et al*, 1989a). Transforming growth factor-alpha (TGFα) is expressed in the corpus luteum, and has been immunolocalised predominately to large luteal cells; in the preovulatory follicle it is known to have mitogenic and steroidogenic actions, though its function in the corpus luteum remains under investigation (Lobb & Dorrington, 1993). Insulin-like growth factor-I (IGF-1) immunoreactivity has been localised to bovine large and small luteal cells, and insulin-like growth factor-II (IGF-II) is localised to non-steroidogenic luteal cells, in particular to the pericytes of capillaries and the perivascular fibroblasts of luteal arterioles and venules, where it is suggested to play a role as an autocrine growth factor (Amselgruber *et al*, 1994). In the rabbit corpus luteum IGF-1 has been shown to be capable of maintaining progesterone secretion to the same extent as LH during culture, suggesting a possible role in the regulation of luteal steroidogenesis (Constantino *et al*, 1991). The ruminant corpus luteum also contains amines such as adrenaline, noradrenaline, seretonin and dopamine which stimulate progesterone

production *in vivo* (Battista *et al*, 1987) and *in vitro* (Battista *et al*, 1989b). However the mechanism of stimulation, the physiological roles and the origins of these amines remains unclear (Alila & Dowd, 1991). In sheep, conceptus proteins had no effects on PGF2α binding to large luteal cells, however, they blocked events downstream of PGF2α receptor binding and protein kinase C activation, since antisteroidogenic effects of phorbol ester and calcium ionophores were abolished in the presence of conceptus proteins (Wiltbank *et al*, 1992). Alpha interferon (INFα) has been observed to stimulate progesterone production from the bovine corpus luteum *in vitro* when given in low doses, and a role for conceptus proteins in the maintenance of the bovine corpus luteum during pregnancy has been clearly established (Luck *et al*, 1992). Recombinant bovine INFα I which has a 70% sequence homology with bovine trophoblast protein-1 delays luteolysis (Gaverick *et al*, 1992), and INFα I may act as an anti-luteolysin in sheep (Parkinson *et al*, 1992). INFα may also lengthen the lifespan of the bovine corpus luteum through suppression of luteal oxytocin secretion and consequent interference with oxytocin-stimulated uterine release of PGF2α (Plante *et al*, 1991). However, inseminated heifers given exogenous bovine INFαI at approximately the time when luteal maintenance by the conceptus begins showed that INFα I had no enhancing effects on pregnancy rates (Barros *et al*, 1992a). Rather, treated animals showed a slight reduction in pregnancy rate as compared to controls (Barros *et al*, 1992a). Furthermore, progesterone concentrations in plasma from cows treated with INFα were reduced (Barros *et al*, 1992b). Since INFα suppressed pituitary LH secretion, and since the decrease in circulating progesterone levels was not due to enhanced rates of clearance of steroid, it was suggested that reductions in

progesterone secretion were due to interference with pituitary LH driven luteal synthesis of progesterone (Barros *et al*, 1992b).

Tissue inhibitor of metalloproteinases (TIMP-1) is a major peptide secretory product of the ovine corpus luteum (Smith & Moor, 1991). The presence of mRNA encoding TIMP-1 protein has been demonstrated in large and small ovine luteal cells (Smith *et al*, 1994b). In addition to the inhibitory effects of this protein on metalloproteinase action, TIMP-1 is also known to possess growth factor activity (Hayakawa *et al*, 1992), though its role in luteal function has not yet been elucidated. Beta-carotene, the lipid soluble antioxidant which imparts the orange colour to the bovine corpus luteum, appears to be necessary for normal luteal function in cows, since dietary deficiencies lead to follicular atresia and disrupted luteal regression (Lutwack-Mann, 1962). Moreover, lack of anti-oxidant activity has been implicated in the crosslinking of P450scc to its electron donor adrenodoxin (Young *et al*, 1995).

1.17 Luteal regression

The corpus luteum is a transient organ; if pregnancy does not occur luteal regression takes place. The mechanism of luteal regression (luteolysis) has been the subject of intensive investigation over a number of years. Whilst the mechanism of luteolysis in domestic ruminants is not fully clear, more is understood of the process of luteolysis in these species than in the primate and human.

It has been recognised for some time that luteal regression in cows involves the uterus. Hysterectomy prolongs the luteal phase (Wiltbank & Casida, 1956). Moreover, the uterus exerts its luteolytic effect(s) locally (Ginther *et al*, 1967). Removal of the uterine horn (unilateral hysterectomy) adjacent to the

ovary that posseses the corpus luteum extended the lifespan of that corpus luteum; removal of the uterine horn on the opposite side to the ovary with a functional corpus luteum had no effect and luteolysis proceeded as normal (Ginther *et al*, 1967). Thus, the uterine luteolytic factor does not reach the ovary through the circulatory system of the body, but directly affects the adjacent ovary (Niswender & Nett, 1994). Removal of vascular connections between ovary and uterus prolongs luteal function and lifespan in cows. Although there are no direct connections between the uterine vein and ovarian artery, the two blood vessels are in intimate contact. The ovarian artery is coiled round the uterine vein from the junction between ovary and uterus all the way to the connections with the aorta and vena cava (Del Campo & Ginther, 1973a,b). Where contact between the two blood vessels occurs, thinner walls are found (Del Campo & Ginther, 1974). Although removal of the uterine horn opposite to the functional corpus luteum does not induce luteal regression, in both sheep (Ginther *et al*, 1973; Mapletoft & Ginther, 1975) and cows (Mapletoft *et al*, 1976) surgical joining of the uterine vein with the ovarian artery does induce luteal regression, but only in the ovary adjacent to the uterine horn which was removed. Luteal regression in unilaterally hysterectomised sheep and cows can also be demonstrated if the ovarian artery from the side adjacent to the remaining uterine horn is connected to the uterine vein from the hysterectomised side (Mapletoft *et al*, 1976). Thus it appears that uterine luteolytic factor(s) are transported directly to the ovary by the close contact between ovarian artery and uterine vein. In species where this contact is not present, (eg. horses; Ginther *et al*, 1972), local uterine effects of luteal regression do not occur (Niswender & Nett, 1994).

It was a number of years after the identification of the uterus as the site of production of luteolytic effects that PGF2α was identified as the uterine derived luteolytic factor. Luteolytic effects of PGF2α were initially described in pseudopregnant rats (Pharriss & Wyngarden, 1969) but have been demonstrated in a number of other species, including cows (Lauderdale, 1972; Liehr *et al*, 1972; Rowson *et al*, 1972). Treatment of cattle with indomethacin (see above), which blocks PGF2α synthesis by inhibiting the cyclooxygenase pathway of arachidonic acid metabolism, prevents normal luteal regression (Lewis & Warren, 1977). Moreover, passive immunisation of cattle (Fairclough *et al*, 1981) and sheep (Scaramuzzi & Baird, 1976; Faiclough *et al*, 1976) to PGF2α prevents spontaneous luteal regression. Endometrial concentrations of PGF2α in cows are highest during the time of luteal regression (Shemesh & Hansel, 1975b). Thus, the major luteolysin of the bovine corpus luteum is uterine-produced PGF2α. However, inhibitors of the lipoxygenase pathway have also been shown to prolong the oestrous cycle (Milvae *et al*, 1986). It has been suggested that arachidonic acid itself, derived from the uterus, may also play a role in luteolysis (Alila & Dowd, 1991).

The mechanism by which PGF2α affects luteal cells appears to be exerted in a number of ways. An early suggestion for the action of PGF2α on luteal cells was that it decreased blood flow, leading to luteal tissue hypoxia and luteal regression. However, although luteal blood flow during luteolysis is reduced, it is still much greater than blood flow to the rest of the ovary (Niswender *et al*, 1976). Indeed, the blood supply to the corpus luteum is extremely high. Luteal blood vessels are primarily sinusoidal vessels rather than capillary beds with arteriole supply (Koering & Thor, 1978). The control of luteal blood flow is complex; trophic hormones such as LH

increase ovarian blood flow but do not affect luteal blood supply (Norjaavara *et al*, 1987; Wiltbank *et al*, 1989a). It has been suggested that the lack of vascular smooth muscle in early luteal tissue may be due to formation of sinusoidal vessels from endothelial cells; with insufficient time for growth and differentiation of smooth muscle in these vessels (Wiltbank *et al*, 1990). It is at present unclear whether or not fully developed corpora lutea posses greater development of these structures that permit active regulation of luteal blood flow (Wiltbank *et al*, 1990). PGF2α causes a decrease in luteal blood flow, with a concomitant rapid decrease in progesterone secretion in sheep (Niswender *et al*, 1976) and cows (Heath *et al*, 1983). The decrease in progesterone secretion is a reflection of a decrease of 3βHSD mRNA which occurs very rapidly in large ovine luteal cells in response to PGF2α (Hawkins *et al*, 1993). These events are attributed to increases in intracellular calcium levels and PKC activation in ovine luteal cells. However, as previously discussed, PKC may be stimulatory to progesterone production in the bovine corpus luteum, and the mechanism of action of PGF2α may differ in these two species. Indeed, a number of important differences between the ovine and bovine corpus luteum exist. For example, in sheep PGI2, PGE_2 and $PGF_{2\alpha}$ have no affect on progesterone synthesis in small luteal cells (Fitz *et al*, 1984a), however, all of these substances increase small luteal cell progesterone production in the bovine corpus luteum (Alila *et al*, 1988b). In addition, $PGF_{2\alpha}$ does not effect basal progesterone synthesis in large bovine luteal cells (Alila *et al*, 1988a; b), however, it is directly cytotoxic to large luteal cells from the ovine corpus luteum (Fitz *et al*, 1984b). Nonetheless, $PGF_{2\alpha}$ would appear to be the luteolytic hormone in cows, though its exact mechanism of action has yet to be elucidated in the bovine corpus luteum. It is unclear how the corpus

luteum regresses in response to uterine PGF2α, whilst also synthesising significants amounts of this substance (Alila & Dowd, 1991). It has been suggested that the relative numbers of theca-derived and granulosa-derived luteal cells, and thus the numbers of LH receptor-expressing large luteal cells (small luteal cells which have developed into large luteal cells) may vary between sheep and cows at particular stages of the luteal phase, giving rise to some of the differences between the two species luteal cell responses to prostaglandins. Luteal derived $PGF_{2\alpha}$ may play a regulatory role over bovine luteal progesterone production (Alila et al 1988a), and the effects of prostaglandins are dependent on their concentrations, the proportion of each luteal cell type present and the receptor content of the corpus luteum (Alila et al, 1988b).

Concomitant with decreased progesterone secretion at the onset of luteolysis, release of secretory granules takes place in large bovine luteal cells (Heath *et al*, 1983). It has been suggested that this degranulation may represent PGF2α-driven luteal secretion of oxytocin which may act on the uterus to promote increased secretion of PGF2α (Heath *et al*, 1983). Following the decrease in serum progesterone levels, the numbers of LH receptors in the corpus luteum falls (Diekman *et al*, 1978b). *In vitro*, LH induced increases of cAMP and progesterone secretion were blocked by PGF2α (Fletcher & Niswender, 1982). Since it is the small luteal cells which possess LH receptors, whilst large luteal cells possess receptors to PGF2α, a form of cellular communication has been suggested between the two cell types (Niswender & Nett, 1994; Pate, 1994). Communication between luteal and non-luteal cells in the corpus luteum may also play a role in the transmission of the luteolytic signal, in particular, communication with cells of the immune system, which appear to be actively involved in luteolysis (Pate,

1994). Indicative of this communication between ovine small and large luteal cells is the observation that a luteolytic dose of PGF2α decreases the number of large luteal cells within 12 hours of administration, whereas small cell numbers do not decrease until around 24 hours after PGF2α administration (Braden & Niswender, 1985). Moreover, Redmer *et al* (1991) have demonstrated the formation of gap junctions between luteal cells in culture. They suggest that contact-dependent communication may allow the transmission of signals from cell to cell without the requirement that all cells possess specific receptors for the hormone originally transmitting the signal. Whereas large to small and small to small luteal cell contact-dependent-communication was observed, (Redmer *et al*, 1991), contact-dependent-communication was not observed between large luteal cells (Redmer *et al*, 1991). If luteolytic signals are transmitted by this form of communication, large to large cell communication would not be required since large luteal cells can already respond to PGF2α through specific PGF2α receptors. However, large to small cell and small to small cell communication could then allow the transmission of the luteolytic signal to all cell types of the corpus luteum. Recently however, studies have indicated the binding of LH and PGF2α to both large and small bovine luteal cells (Chegini *et al*, 1991). Indeed, earlier studies have indicated the ability of both bovine luteal cell types to respond to $PGF_{2\alpha}$ and LH (Alila *et al*, 1988a; b) However, PGF2α binding increased in large luteal cells to a much greater extent than in small luteal cells from early to late luteal phase (Chegini *et al*, 1991), suggesting that, functionally, large cells are more sensitive to the luteolytic signal of PGF2α than are small luteal cells. Interestingly, specific binding of LH/hCG to luteal blood vessels was also shown (Chegini *et al*, 1991). Thus the model of luteal cell function in which small cells respond to luteotrophic (LH)

stimuli, whilst large cells respond to PGF2α may require some revision, not least from the observations that small bovine luteal cells appear to be sensitive to $PGF_{2\alpha}$, responding with increased progesterone production (Alila et al, 1988,a; b). Coupled with the apparent response of non-luteal cells to trophic stimuli, the regulation of luteal function appears to be much more complex than was previously thought.

The direct involvement of oxytocin in the process of luteal regression has been suggested. The trigger for the release of PGF2α from the uterus is thought to be the secretion of oxytocin from the corpus luteum (Flint *et al*, 1989) and the acquisition of oxytocin receptors by the endometrium. Early studies demonstrated that treatment of heifers with high doses of oxytocin in the first 7 days of the oestrous cycle impairs the development of the corpus luteum (Armstrong & Hansel, 1959), though no effects were observed if oxytocin was administered at the end of the cycle, before the onset of luteolysis. Active immunization of sheep against oxytocin prolongs the oestrous cycle (Sheldrick *et al*, 1980; Schams *et al*, 1983). In cows, hysterectomy negates the effects of oxytocin on early luteal regression (Armstrong & Hansel, 1959; Ginther *et al*, 1967), thus suggesting involvement between oxytocin and the uterus. Oxytocin may also act directly on luteal cells. Bovine large luteal cells synthesise oxytocin and neurophysin (Ivell & Richter, 1984). It was suggested that oxytocin may mediate large - small cell communication (Schwall *et al*, 1986), since its presence in large luteal cells of the ovine corpus luteum has been demonstrated (Rodgers *et al*, 1983) and the PGF2α analogue cloprostenol has been shown to increase oxytocin secretion (Flint & Sheldrick, 1982). Moreover, hCG stimulation of progesterone secretion by the bovine corpus luteum was inhibited by oxytocin (Tan *et al*, 1982), suggesting that small luteal cells may possess receptors for oxytocin.

Studies in which luteal oxytocin receptors have been demonstrated (Sernia *et al*, 1989) are not without controversy, since luteal cell preparations from pregnant ewes also contained vascular smooth muscle cells, which are known to contain oxytocin receptors (Alila & Dowd, 1991). Other investigators have suggested that the communication mediator may be neurophysin-1 (Schams *et al*, 1985), which is co-secreted with oxytocin. Studies have shown that low levels of oxytocin are stimulatory to basal and hCG-stimulated progesterone production; at higher levels oxytocin is inhibitory to these processes (Tan *et al*, 1982). Luteal cells from fully developed corpora lutea appear to be unresponsive to oxytocin (Hansel & Dowd, 1986), though inhibition by oxytocin of progesterone production from bovine luteal cells was shown in luteal cells recovered between days 4 - 5 of the oestrous cycle (Schams *et al*, 1987). However, studies in the ewe have shown that though hysterectomy caused luteal depletion of oxytocin, progesterone levels were unaltered (Sheldrick & Flint, 1983a). Also, mediation of the effects of PGF2α on luteal cells does not appear to involve oxytocin directly, since the response of hysterectomised ewes to cloprostenol remains the same as that of the intact animal (Sheldrick & Flint, 1983b). It appears that the response of the corpus luteum to oxytocin is dependent on the time of exposure. Thus it has been suggested that the premature loss of luteal function observed in cows when the early corpus luteum is exposed to oxytocin is because the CL is the main tissue responding to oxytocin at that time; later in the luteal phase the uterus is thought to be the main responding tissue to oxytocin (Alila & Dowd, 1991). Indeed, this critical period (days 2 - 6) is the time when expression of the oxytocin gene rises sharply in the bovine ovary (Ivell *et al*, 1985) and the time that administration of exogenous oxytocin in large amounts causes impairment of normal luteal function. It

has been suggested that this impairment is due to interference with the normal processes of oxytocin synthesis and secretion (Hansel & Dowd, 1986). Administration of oxytocin at this time has been shown to impair the ability of the corpus luteum to synthesise the luteotrophin PGI-2, but not PGF2α; though progesterone production was not impaired significantly, luteal cell ability to respond to LH was diminished (Hansel & Dowd, 1986). The major uterine release of PGF2α follows the start of luteolytic changes (Thorburn *et al*, 1973; Flint & Sheldrick, 1983) in the sheep corpus luteum. Thus, it is postulated that initially, the uterus releases a small amount of PGF2α. This initial release triggers a release of oxytocin from large luteal cells with a concomitant decrease in progesterone production in these cells. Decreased progesterone secretion increases the numbers of uterine oxytocin receptors, which respond to the increased levels of oxytocin, causing further uterine release of luteolytic amounts of PGF2α (Auletta & Flint, 1988). However, recent reports of experiments in microdialysed bovine corpora lutea *in vitro* indicate that oxytocin was stimulatory to progesterone production; this effect being dependent on cell-to cell contact (Miyamoto & Schams, 1991).

The cloning of PGF2α receptors from ovine large luteal cells has shown that the ovine luteal PGF2α receptor consists of 362 amino acids (Graves *et al*, 1995). In sheep, PGF2α binds to specific receptors on large luteal cells, and activates protein kinase C (McGuire *et al*, 1994). This in turn reduces secretion of progesterone, and increases intracellular free calcium which appears to be involved in apoptosis (Sawyer *et al*, 1990), and causes a decrease in mRNA encoding for 3-βHSD, but not cholesterol side chain cleavage mRNA (Hawkins *et al*, 1993; McGuire *et al*, 1994) (however, PKC activation with the phorbol ester PMA did reduce cholesterol side chain

cleavage mRNA levels) (McGuire *et al*, 1994). However, protein kinase C has been demonstrated to increase progesterone secretion in bovine luteal cells (Brunswig *et al*, 1986; Hansel *et al*, 1987; Alila *et al*, 1988a; Miyamoto *et al*, 1993). Thus further experimental work is required to resolve the apparent difference between sheep and cows in their response to PGF2α (Niswender & Nett, 1994). Indeed, PGF2α increases progesterone synthesis by dispersed bovine luteal cells (Hansel *et al*, 1973; Hixon & Hansel, 1979; Weston & Hixon, 1980). Perfusion of bovine corpora lutea *in vitro* demonstrated that PGF2α actually stimulated progesterone and oxytoxcin secretion from corpora lutea removed from early and mid-luteal phase animals over a thirty minute perfusion time, though this effect was less evident in late luteal phase corpora lutea (Miyamoto *et al*, 1993). The stimulation of progesterone secretion is greater in small rather than large luteal cells, and PGF2α also potentiates small luteal cell responses to LH (Alila *et al*, 1988a).

PGF2α has been used for synchronizing oestrus in a large number of studies, however, it is ineffective during the early luteal phase in cows, suggesting that the early bovine corpus luteum is unresponsive to PGF2α, and only induces regression of the bovine corpus luteum between day 5 and day 16 after oestrus (Rowson *et al*, 1972; Lauderdale, 1972; Hafs *et al*, 1972). The lack of responsiveness to PGF2α was not due to a lack of luteal PGF2α receptors since these have been demonstrated in the early bovine corpus luteum (Wiltbank *et al*, 1995). It is suggested that the lack of development of the degenerative and blood flow mechanisms in the developing corpus luteum may account for this lack of responsiveness (Wiltbank *et al*, 1995). However, the mechanism involved remains unclear. The action of PGF2α in reducing progesterone secretion may be due in part to interference with the ability of LH receptor activation to promote increased intracellular levels of cAMP. In

cows, the reduction of progesterone secretion occurs prior to interference with LH action on luteal cells (Weston & Hixon, 1980). Membrane related events such as ligand binding to plasma membrane located receptors are affected by the structure of the membrane. PGF2α has been shown to affect plasma membrane structure (Carlson *et al*, 1983b). Both PGF2α-induced and spontaneous luteal regression in rats were associated with increased membrane fluidity in luteal cells and with a reduction in progesterone secretion (Carlson *et al*, 1983b). These changes occurred within one hour of PGF2α treatment, indicating that an early effect of PGF2α may be to cause changes in the plasma membranes of luteal cells (Carlson *et al*, 1983b), modifying plasma membrane receptor functions, such as the integrity of the LH receptor and intracellular signalling.

Scope of this thesis

The work detailed in this thesis was undertaken to examine the subcellular location of progesterone in luteal cells, in an attempt to elucidate the mechanism and regulation of progesterone secretion, leading to a deeper understanding of the regulation of the bovine corpus luteum.

Chapter 2

Intracellular location of endogenous bovine luteal progesterone

2.1 Introduction

The regulation of progesterone secretion (see Niswender & Nett, 1994 for review) and its levels throughout the bovine oestrous cycle (Dobson & Kamonpatana, 1986; Dieleman & Blankenstein, 1985; Schams *et al*, 1977) have been well documented. However, less well understood is the actual mechanism whereby progesterone leaves the luteal cell in which it is synthesised. Suggestions have included packaging into secretory granules (Gemmell *et al*, 1974; Stacy *et al*, 1976; Sawyer *et al* 1979) in a protein-bound form. Indeed, the existence of cytosolic binding proteins with high affinities for progesterone has been described, but the significance of these to progesterone secretion remains unknown (Willcox & Thorburn, 1981; Willcox & Alison, 1982; Willcox, 1983). Later studies demonstrated that the secretory granules contained oxytocin and neurophysin, but not progesterone (Rice *et al* , 1986 - sheep CL; Guldenaar *et al*, 1984 - bovine CL). Subsequent studies have demonstrated that the secretion of progesterone and oxytocin are independent of each other in cultured bovine luteal cells (Luck, 1988).

The accepted theory of the mechanism whereby progesterone is secreted by the luteal cell remains that proposed by Enders (1973), in which progesterone simply diffuses out of its cell of origin down the concentration gradient set up between the progesterone producing cell and the blood. In a later study, it was shown that progesterone intercalates into phospholipid bi-

layers which contain cholesterol, suggesting a simple diffusion mechanism for progesterone release from the luteal cell across the plasma membrane (Carlson *et al*, 1983a). This theory is still widely accepted, but it has not gone unchallenged, though the actual mechanism of progesterone secretion, if indeed different to that proposed by Enders, remains enigmatic.

To study the intracellular location of progesterone, homogenates of bovine luteal tissue were subjected to fractionation on continuous sucrose density gradients. The locations of the major intracellular organelles in sucrose gradient fractions were determined by utilising the activities of enzymes used as markers of each organelle. To this end, assays which have been developed in other species/tissues were characterised and optimised for use on bovine luteal tissue fractions.

Levels of progesterone in sucrose gradient fractions from corpora lutea fractionated at different stages of the luteal phase of the bovine oestrous cycle were determined by radioimmunoassay. To further establish any association between progesterone and intracellular organelles, in some experiments luteal homogenates were incubated with the saponin, digitonin, prior to fractionation. Digitonin complexes with sterols, such as cholesterol, which possess the 3ß, Δ^5 configuration, (Severs & Robeneck, 1983). Commonly used in freeze fracture studies and as a cell membrane perturbant, digitonin has been demonstrated to form unimolecular 1:1 complexes with unesterified cholesterol. Previous studies have demonstrated the ability of digitonin to increase the buoyant density of cellular membranes containing unesterified cholesterol (see Bramley & Ryan, 1979), this increase in buoyant density being proportional to the levels of unesterified cholesterol in the membrane. Since progesterone does not

possess the 3ß, Δ^5 configuration, it does not complex with digitonin (Severs & Robeneck, 1983). Hence, any increase in the buoyant density of progesterone would indicate association of progesterone with a cellular membrane whose cholesterol has been complexed to digitonin, since although interactions may occur between digitonin and other steroids containing the 3ß, Δ^5 configuration, cholesterol is the major cellular steroid affected by digitonin that is integral to cellular membranes.

Attempts were also made to examine the subcellular locations of steroidogenic enzymes. This was accomplished by fractionation of sucrose density gradient fractions on SDS-PAGE gels, followed by Western blotting with polyclonal antisera to cytochrome P_{450} 3ß hydroxysteroid dehydrogenase, cytochrome P_{450} C17α-hydroxylase/C17, 20 lyase and cytochrome P_{450} cholesterol side chain cleavage. The intracellular locations of these enzymes are well established, but it has not gone completely unchallenged (see Lieberman & Prasad, 1990 for review). Thus in this study the aim was to assay sucrose density gradient fractions for the presence of these enzymes and to correlate this with the distribution of marker enzymes for the major intracellular organelles.

2.2 Materials & Methods

2.2.1 General materials

All substrates, fine chemicals, inhibitors, reagents and unlabelled steroids were purchased from Sigma Chemical Co., Poole, Dorset, U.K., or from BDH, Poole, Dorset, U.K. Polyclonal antisera, raised in rabbits against ovine P_{450} side chain cleavage, human P_{450} 3β-hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase (3β-HSD) and bovine P_{450} 17α hydroxylase/c17,20 lyase were the generous gift of Professor I. Mason, Department of Clinical Chemistry, The University of Edinburgh.

2.2.2 Radiolabelled tracers

Radiolabelled steroid used was: [1,2,6,7-^{3}H]-labelled progesterone (100 Ci/mmol), from New England Nuclear Research Products, Du Pont (U.K.) Ltd., Stevenage, Herts, U.K.

2.2.3 Ovarian Tissue

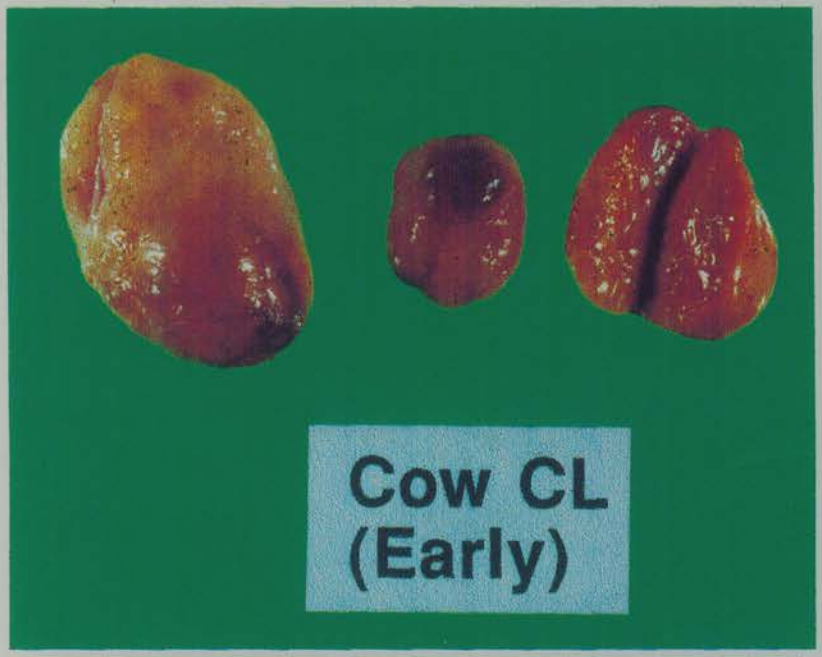
Ovaries were recovered from presumed non-pregnant cows slaughtered at a local abattoir and transported to the laboratory in ice-cold phosphate-buffered saline (PBS; sodium chloride (0.15 mol/l) in sodium phosphate (10mmol/l; pH 7.4) within 1 hour of death. The stage of the luteal phase was assessed by gross morphology (Figure 2.2.1), and mid-luteal phase corpora lutea were dissected free from connective tissue and stroma, minced with scissors and homogenized in ice-cold sucrose (0.3mol/l)/ EDTA (1mmol/l)/Tris-HCl (10 mmol/l), pH 7.4 (SET medium; 200 mg tissue/ml) using a Polytron homogenizer (Kinematica, Lucerne, Switzerland) with two

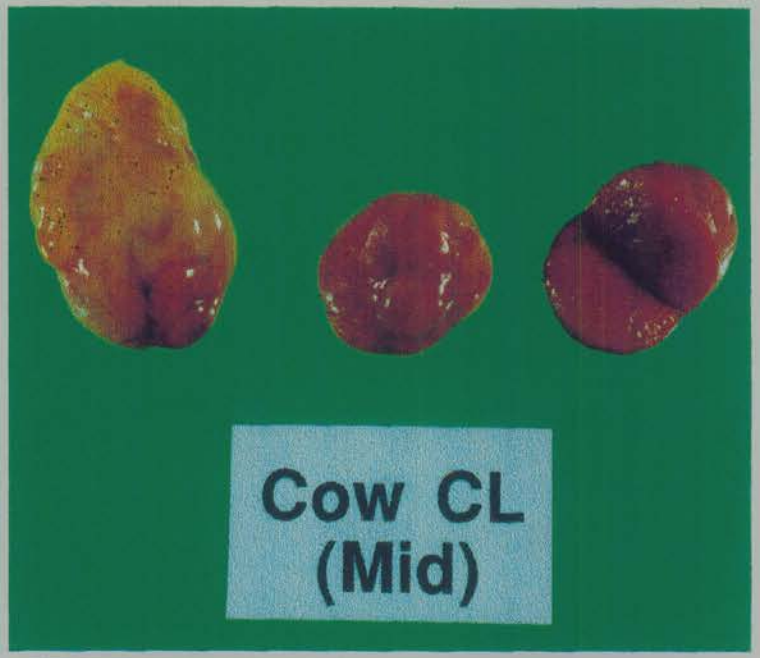
10-s bursts at full speed, interspersed with a 1-min cooling period on ice. Homogenates were then filtered through two layers of cheesecloth.

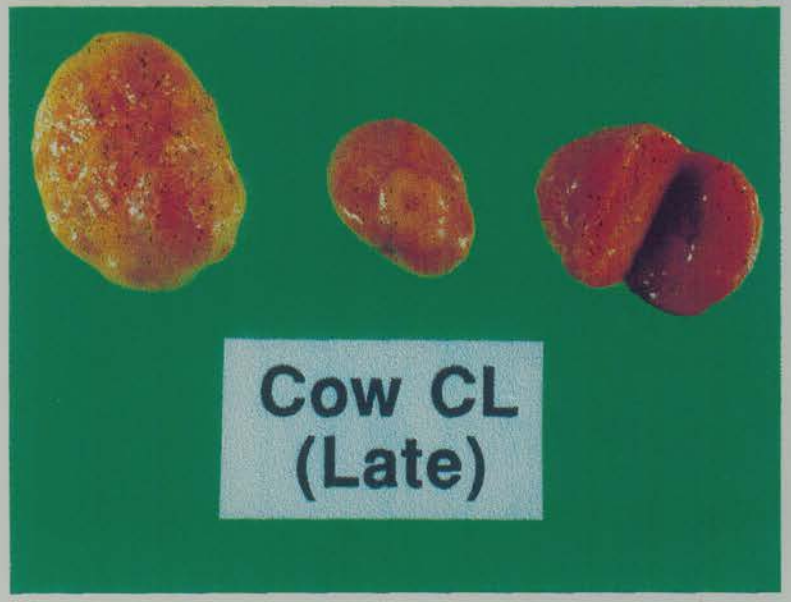
2.2.4 Sucrose density gradient fractionation

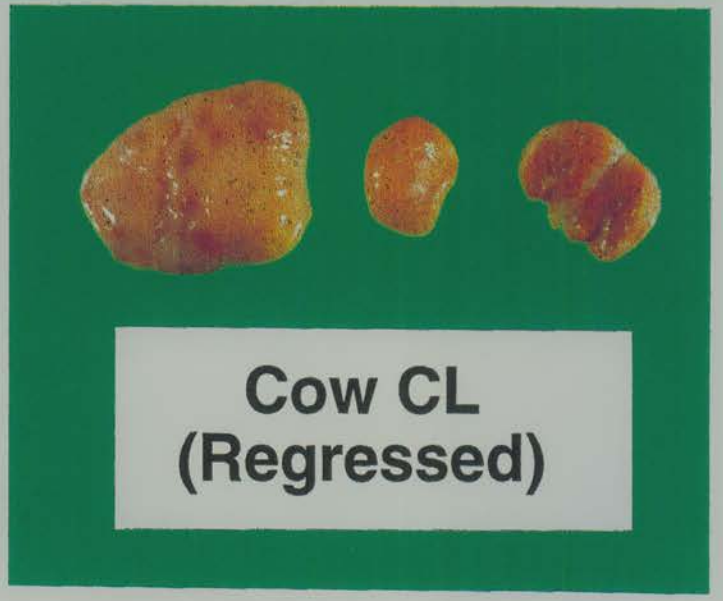
Continuous sucrose density gradients (30 ml; 10-50 % w/w) were prepared by the method of Stone (1974). 15 ml of 10% w/v sucrose (EDTA, Tris pH 7.4) was layered gently onto 15 ml 50% sucrose w/v (EDTA, Tris, pH 7.4) in ultracentrifuge polyallomer tubes. Tubes were capped, then tilted to horizontal and allowed to diffuse for 16 hours at 4°C. Diffused gradients were then returned to vertical, uncapped and stored at 4°C for up to 4 hours before use. Aliquots of mid-luteal phase corpus luteum homogenate (2.5 ml) were mixed with either 0.5 ml SET medium (controls) or 0.5 ml SET medium containing digitonin (BDH) (10 mg/ml). After a 30 min incubation on ice, 2.5 ml aliquots were gently layered over the sucrose density gradients and centrifuged at 30,000 g_{av} for 2h in a Sorvall VTR 50 vertical tube rotor (4°C). Each gradient was then fractionated using a Buchler-Searle Autodensiflo gradient fractionator equipped with a meniscus-sensitive probe, and fractions (1ml) frozen at -20°C until assay. Sucrose gradient fractions were then assayed for endogenous progesterone content and a range of enzyme activities to serve as intracellular organelle markers.

Figure 2.2.1 Bovine corpora lutea staged by gross morphology. From left to right, each plate shows the appearance of a CL from each stage on the whole ovary, the appearance of the CL when dissected free of the ovary, and the appearance of the interior of the CL.


Cow CL
(Early)


Cow CL
(Mid)


Cow CL
(Late)


Cow CL
(Regressed)

2.2.5 Sucrose content and protein content estimations

Measurement of the sucrose content of density gradient fractions was performed using an Abbe refractometer (Atago, Japan). Buoyant densities of enzymatic activities from the sucrose concentration equivalent to the peak of enzyme activity were calculated using tables of standard data. Protein was measured by the method of Lowry *et al*, (1951), using bovine serum albumin (BSA) as a standard.

Conditions were optimised for marker activities specific for the major subcellular organelles and luteal plasma membrane in the bovine corpus luteum based on previously described assays in other species (Bramley & Menzies, 1988a; c). For each enzyme, except where indicated, optimum pH, temperature and duration of incubation, enzyme concentration, co-factor and metal ion concentrations were established. In each case, during optimization of conditions and final measurement of activity levels in sucrose gradient fractions, enzyme assays were performed in duplicate.

2.2.6 Determination of the amount of homogenate required for enzyme assay characterisation

Enzyme activities were optimised using filtered luteal homogenate in SET medium, pH 7.4. To determine the concentration of homogenate required to show measurable activities of enzymes, a range of homogenate concentrations were assayed under conditions optimised in other species (Bramley & Menzies, 1988a; c). Homogenate concentrations which were on

the linear portion of the activity curve were then used for the optimisation of other incubation conditions.

2.2.7 Optimum pH determinations

Buffers with pH range from 4 - 10.5 were prepared (0.2M sodium acetate/acetic acid, pH 4 - 6; 0.2M Tris/HCl pH 6.5 - 8; 0.2M Piperazine/HCl pH 8.5 - 10.5). Using the homogenate concentrations determined for each enzyme, enzyme activities were assayed over the pH range 4 - 10.5, at divisions of 0.5 pH units. The pH at which maximal activity of each enzyme were recorded was deemed optimal.

2.2.8 Optimum metal ion concentrations

A range of metal ions were tested for their ability to stimulate marker enzyme activities. The ions tested were :- manganese (manganous chloride), calcium (calcium chloride), EDTA, sodium (sodium chloride), magnesium (magnesium acetate), zinc (zinc sulphate) and potassium (potassium chloride). In the assay of 5'-nucleotidase, the effects of cobalt ions was studied. To avoid any optical interference between these coloured salts and the interpretation of assay results determined spectrophotometrically, enzyme blank tubes (all reagents apart from homogenate) containing the highest concentration of each salt were routinely included.

2.2.9 Intracellular organelle marker enzyme assays

The marker enzyme assays characterised for optimal activity in bovine luteal tissue were cytochrome oxidase (mitochondrial marker) (Gospodarowicz, 1973; Rao *et al*, 1981; Bramley & Menzies, 1986), NADH cytochrome C reductase (endoplasmic reticulum marker) (Bramley & Menzies, 1988a; c;

Greenhalgh, 1990), NADPH cytochrome C reductase (endoplasmic reticulum marker) (Bramley & Menzies, 1988a; c), fluoride-sensitive acid phosphatase (GERL marker) (Bramley & Ryan, 1978a; 1978b; 1979), N-acetyl ß-glucosaminidase (lysosomal marker) (Bramley & Menzies, 1988a), DNA (nuclear marker) (Bramley & Ryan, 1978a) and three plasma membrane markers, alkaline phosphatase (Bramley & Ryan, 1978a; 1979), 5'-nucleotidase (Gospodarowicz, 1973; Menon & Kiburz, 1974; Rao *et al*, 1981; Bramley & Menzies, 1978a; 1986), and the LH/hCG receptor (Gospodarowicz, 1973; Menon & Kiburz, 1974; Rao *et al*, 1981; Bramley & Menzies, 1978a; 1986). Details of the establishment of optimised assays used to determine distribution of marker enzymes in sucrose density gradients of bovine luteal homogenates are given in the following descriptions.

2.2.9 (i) Mitochondrial marker : Cytochrome oxidase

This assay utilises the change in optical density (550 nm) that occurs when dithionite-reduced horse heart cytochrome c is oxidised. Control tubes containing no enzyme (all other reagents present) were routinely monitored for oxidation not due to cytochrome oxidase. Little or no such oxidation was observed to occur. Reaction rates were calculated from the linear slopes recorded using a Phillips flat-bed chart recorder set at 1 cm/min.

Cytochrome oxidase assay

Enzyme (10 µl sucrose gradient fraction) and 100 µl dithionite-reduced horse heart cytochrome C (Sigma) (10 mg/ml in H_2O) were added in quick succession to a 1.3 ml reaction system (500 µl 0.2 M Tris-acetic acid buffer, pH 6.8, 800 µl H_2O). After rapid mixing, this mixture was loaded immediately into a flow-through cuvette system fitted to a Pye Unicam UV

spectrophotometer, and the decrease in optical density at room temperature (20°C), measured for 2 minutes at 550 nm, and recorded on a chart recorder linked to the spectrophotometer. Reaction rates were then calculated from the linear slopes recorded.

2.2.9 (ii) Fluoride-sensitive acid phosphatase (GERL marker)

Acid phosphatase has been shown to catalyse the hydrolysis of p-nitrophenylphosphate, yielding free p-nitrophenol (yellow colour) (Bramley & Ryan, 1978a). Free p-nitrophenol was estimated by absorbance measurements (Pye Unicam UV spectrophotometer) at 420 nm. Each sample was assayed in both the presence and absence of 10 mM sodium fluoride (in duplicate). Subtraction of absorbance values obtained in the presence of sodium fluoride from values measured in the absence of sodium fluoride gave a final estimate of fluoride-sensitive acid phosphatase.

Acid phosphatase/fluoride sensitive acid phosphatase assay

A 1.0 ml phosphatase reaction system (0.1 M sodium acetate-acetic acid buffer, pH 4.7; $\pm$ 10 mM sodium fluoride, 3 mM p-nitrophenylphosphate (pNPP), 0.2% (v/v) Triton-X-100, 25 µl sucrose gradient fraction) was incubated at 37°C for 2 hours. The reaction was stopped by addition of 3 ml 0.5 M NaOH, 0.5 % (w/v) Na_2EDTA (Bramley & Ryan, 1978a), and optical density measured at 420 nm. Fluoride-containing and fluoride-free blanks were used to zero the spectrophotometer.

2.2.9 (iii) Plasma membrane marker I : Alkaline phosphatase

Measurement of liberated p-nitrophenol from the hydrolysis of p-nitrophenylphosphate as described for acid phosphatase formed the basis of this assay.

Alkaline phosphatase assay

A 1ml phosphatase system (0.1 M Piperazine-HCl buffer, pH 10.8, 0.2% Triton-X-100, 5 mM magnesium acetate, 3 mM pNPP, 10 µl sucrose gradient fraction) was incubated at 37°C for 2 hours or until sufficient colour had developed. The reaction was stopped and the reaction rate estimated from the optical density of the sample in the same way as described for acid phosphatase.

2.2.9 (iv) Plasma membrane marker II : LH/hCG receptor

Measurement of levels of binding of ^{125}I-labelled human LH (Chelsea Reagent, specific activity 100 Ci/g) in the presence and absence of excess unlabelled hormone (50 IU/tube hCG - Chorulon, Intervet U.K Ltd, Cambridge) were performed. Each sucrose gradient fraction was assayed in triplicate, with a duplicate set of tubes containing excess Chorulon included for each sample to measure non-specific binding. Specific binding was calculated by subtraction of non-specific binding levels from binding recorded in tubes with no added unlabelled hormone. Positive control tubes containing homogenate of known high specific binding (sheep corpus luteum homogenate) were routinely included to allow comparison of individual assay binding efficiencies.

LH receptor assay

A 1 ml binding system (40 mM Tris-HCl buffer, pH 6.5, 0.5% w/v BSA, ^{125}I-hLH (100,000 cpm) $\pm$ 50 IU hCG, 100 µl sucrose gradient fraction) was incubated overnight at 20°C. To separate bound hormone from free, 0.5 ml ice-cold IgG (0.5% w/v, 40 mM Tris-HCl, pH 7.4) and 1 ml 25% w/v polyethyleneglycol (8000 m.w in 40 mM Tris-HCl, pH 7.4) were added to each tube, vortexed, then centrifuged (2500 rpm, 30 min, 4°C). Supernatants were aspirated, and pellets containing bound LH-receptor complex counted using a Hewlett-Packard 'Crystal' gamma-counter, with a counting efficiency of 75%.

2.2.9 (v) Plasma membrane marker III : 5'- nucleotidase

5'-nucleotidase has been demonstrated to be a marker of the plasma membrane in the rat ovary (Bramley & Ryan, 1978a) and the corpus luteum of sheep and pigs (Bramley & Menzies, 1986; 1988a; c). It catalyses the release of P_i (inorganic phosphate) from adenosine 5'-monophosphate (AMP). Na^+/K^+Tartrate was included in the assay system to prevent interference caused by acid phosphatase activity (Bramley & Menzies, 1986). Activity was estimated from the amount of P_i released.

5'-nucleotidase assay

A 1 ml assay system (40 mM Tris-HCl, pH 6.4, 3 mM cobaltous chloride, 50 mM Na^+/K^+Tartrate, 5 mM AMP, 30 µl sucrose gradient fraction) was incubated for 3 hours at 37°C. The reaction was then terminated by the addition of 1 ml of ice-cold 10% trichloroacetic acid. Tubes were then centrifuged (2500 rpm, 10 min, 4°C). 1 ml aliquots of each de-proteinised sample were then removed, and P_i present in each determined by the

method of Fiske and Subbarow (1925), measured in a Pye Unicam spectrophotometer at OD 660 nm.

2.2.9 (vi) Lysosomal marker : N-acetyl-ß-glucosaminidase

Activity of N-acetyl-ß-glucosaminidase was estimated by measuring the release of free p-nitrophenol from p-nitrophenyl-N-acetyl-ß-D-glucosaminide (Bramley & Menzies, 1986).

N-acetyl-ß-glucosaminidase assay

A 1 ml assay system (0.1 M sodium acetate-acetic acid buffer, pH 5.8, 1 mM magnesium acetate, 2 mM p-nitrophenyl-N-acetyl-ß-D-glucosaminide (Sigma), 20 µl sucrose gradient fraction) was incubated for 2 hours at 37°C. The reaction was stopped by addition of 3 ml 0.5 M NaOH, 0.5% (w/v) Na_2EDTA (Bramley & Ryan, 1978a). Optical density (420 nm) was measured for each sample and the enzyme activity calculated.

2.2.9 (vii) Endoplasmic reticulum markers: NADPH cytochrome C reductase and NADH cytochrome c reductase

The activities of these enzymes were measured in the presence of freshly prepared co-factor (NADPH or NADH). Activity was calculated from the rate of reduction of horse heart cytochrome C at 550 nm recorded on a chart recorder. Potassium cyanide was included in all tubes to prevent cytochrome oxidase re-oxidising the cytochrome C and thus interfering with rates of reduction measured. Reduction rates with no added enzyme or added co-factor were routinely recorded to serve as controls.

NADPH cytochrome C reductase assay

The 1 ml system contained piperazine-HCl buffer over a range of pH values, Triton-X-100 at a range of concentrations, 500 µg cytochrome C, 150 µM NADPH, KCN (0 - 5 mM) and sucrose gradient fractions or whole cow luteal homogenate (5 - 100 µl). Despite varying all reagents and conditions, including testing for stimulation by metal ions, little activity above baseline values was recorded, thus this assay could not be utilised.

NADH cytochrome C reductase assay

A 1.5 ml assay system (0.5 ml Tris acetate-buffer, pH 7.0, 2 µmol KCN, 0.15 µmol NADH) was prepared. To this system, 100 µl (10 mg/ml in H_2O) horse heart cytochrome C and 30 µl sucrose gradient fraction were added in quick succession, immediately prior to measuring the rate of reduction. After rapid mixing, each sample was loaded immediately into a flow-through cuvette system fitted to a Pye Unicam UV spectrophotometer, and the increase in optical density at room temperature, measured for 2 minutes at 550 nm, recorded on a chart recorder linked to the spectrophotometer. Reaction rates were then calculated from the linear slopes recorded.

2.2.10 Progesterone radioimmunoassay

Progesterone content of density gradient fractions was measured by direct radioimmunoassay by a modification of the method developed by McNeilly & Fraser, (1987), or following petroleum ether extraction of fractions. The details of the extraction method were: 100 µl of each sucrose density gradient fraction was extracted with 2 ml petroleum ether (BDH). Also, 20 µl [^{3}H]-progesterone (ca. 1200 c.p.m) was added to each tube for recovery estimation. This mixture was vortexed vigorously for 5 minutes at room temperature, after which the tubes were placed in a freezing bath of dry

ice/ethanol. The ether phase was removed and the aqueous phase (frozen) discarded. The ether residue was then dried down under a stream of nitrogen at 50°C. After reaching total dryness, the residue was reconstituted in 200 µl phosphate-citrate buffer, 1 mg/ml gelatin (progesterone radioimmunoassay buffer). After leaving overnight at room temperature to ensure complete reconstitution and mixing had occurred, a 50 µl aliquot of each sample was removed and mixed with 4 ml scintillant (Ecoscint A, National Diagnostics, Atlanta, Georgia) in scintillation vials, then counted for tritium using an LKB Rackbeta II scintillation counter. Recovery efficiency was calculated by multiplying the counts in each sample by 4 to give an estimation of counts in the total 200 µl of the sample, then dividing this value by the total counts added; the value of this division was multiplied by 100 to give a % recovery. Recoveries were calculated for each sample individually to correct the results of the progesterone concentrations in each sample so that the assay could be compared to the progesterone concentrations obtained by radioimmunoassay of non-extracted samples.

Briefly, the details of the immunoassay were : tubes received either 100 µl progesterone standard (covering the range 5 - 1000 pg) or 50 µl sample at a suitable dilution (1:200). Standards were freshly prepared and assayed for comparison with established in-house standards. Primary antibody (S-361 in-house polyclonal antibody raised in sheep against a progesterone-BSA conjugate) (100 µl) at a concentration of 1:9500 was added. Finally, a 100 µl aliquot of ^{125}I-progesterone in assay buffer (no gelatin added) containing 1 mg/ml 8-anilino-1 naphthalene sulphonic acid (ANS - to block binding to steroid binding globulins) corresponding to 15,000 c.p.m was added, and tubes vortexed. After 3 hours incubation at room temperature 200 µl of

second antibody solution, containing normal sheep serum (1:3200) (SAPU) and donkey-anti-goat IgG (1:64) (SAPU) was added. Tubes were vortexed then incubated overnight at 4°C. Finally, 1 ml of 4% polyethylene glycol, 0.2% Triton-X-100 in 0.9% saline was added to each tube. After mixing, tubes were centrifuged (1000gav., 4°C, 30 min) to separate free steroid from steroid-antibody complex. Supernatants were decanted, tubes dried and pellets counted using a Hewlett-Packard 'Crystal' gamma-counter with a counting efficiency of 75%. B/Bo was calculated using a commercial assay calculation software package ('Assayzap', Biosoft Ltd.). Routinely included controls in the assay were non-specific binding tubes (no primary antibody) and quality controls at the beginning and end of each assay. Within assay variation was calculated to be 10.84 % and the lower detection limit of progesterone immunoassays was 12 pg/100 µl.

2.2.11 DNA content of sucrose gradient fractions

DNA content of bovine corpus luteum sucrose gradient fractions was determined by the method of Burton, (1956), using salmon sperm DNA (Sigma) as a standard.

2.2.12 SDS-PAGE electrophoresis of sucrose gradient fractions

SDS-PAGE was performed by the method of Laemmli, (1970), using a commercially available gel electrophoresis tank (Protean II xi , Bio-Rad). Gel reagents are shown below.

	resolving gel	stacking gel
%Tof Gel	7.5%	3.6%
Reagents		
Acrylamide	10	3.6
1M Tris, pH8.85	15	10 (0.375 M Tris, pH 6.8)
10% SDS	0.4	0.3
ddH_2O	14.6	16
TEMED	0.1	0.1
10% AMPS	0.1	0.1

(All volumes are in ml)

Briefly, resolving gels of approximately 11 cm X 16 cm were formed by pouring between two glass plates on a purpose built casting stand (Bio-Rad). Once poured, resolving gels (7.5%) were overlaid immediately with 1:1 butan-1-ol : H_2O. After polymerization had occurred (ca. 1 hour), the overlay was rinsed off (dH_2O) and a 3.6% stacking gel poured onto the resolving gel. A fifteen-well comb (Bio-Rad) was placed in each stacking gel. After polymerisation of the stacking gel, combs were removed and wells rinsed thoroughly with H_2O. Gels were then clipped to a central water-cooled core. Samples of each fraction (ca. 30 µg protein) from sucrose gradients were denatured by mixing with an equal volume of reducing sample buffer (10% w/v sucrose, 0.2% v/v 2-mercaptoethanol, 0.02% bromophenol blue w/v, 0.2% SDS w/v in 0.375 M Tris-HCl, pH 6.8) in 1.5 ml microcentrifuge tubes. After heating to 100°C for 10 minutes, samples were cooled and applied to the wells of the stacking gel. Low and high molecular weight markers were also applied to each gel (Rainbow markers™, Amersham International plc). Electrophoresis was carried out at 40 mA/gel

during stacking (ca. 1 hour) and 30 mA/gel during separation (ca. 3 hours). After electrophoresis was complete, gels were removed and separated proteins electroblotted on to nitrocellulose membranes (Hybond-C, Amersham International plc) using Bio-Rad electroblotting equipment, in electroblot buffer (0.25 M Tris, 0.6 M glycine, 20% (v/v) methanol) for 15 hours, at 15 V. After blotting was completed, nitrocellulose blots were immediately probed with antiserum against the protein of interest.

2.2.13 Western Blotting

Nitrocellulose blots were initially incubated in 5% (w/v) defatted milk protein (Marvel™, Cadbury Ltd), Tris-buffered saline (TBS) (50 mM Tris/HCl, pH 8.0, 0.85% sodium chloride) for 1 hour to block non-specific binding sites. Blots were then rocked gently in primary antibody at an appropriate (1:200 to 1:1000) dilution in TBS, 0.1 % (v/v) TWEEN 20, 5% (w/v) milk protein for 2 hours. After excess antibody was washed away (TBS, 0.1% v/v TWEEN 20) (1 x 15 min wash, 3 x 5 min washes), blots were incubated with secondary antibody (donkey anti-rabbit, horseradish peroxidase linked IgG) (Amersham International plc) for 1 hour, at 1:4000 dilution in TBS, 0.1% (v/v) TWEEN 20, 5% (w/v) milk protein. After excess secondary antibody was removed by repeated washes (TBS, 0.1 % (v/v) TWEEN 20) (1 x 15 min wash, 3 x 5 min washes), blots were incubated in ECL™ solution (Enhanced chemiluminescence detection kit, Amersham International plc) (10 ml ECL A + 10 ml ECL B) for 60 seconds, wrapped in clingfilm and exposed to autoradiograph (ECL Hyperfilm™, Amersham International plc) film for 15 - 60 seconds, depending on signal intensity. Autoradiographs were then developed by placing in developer (Kodak) for 60 seconds, rinsed with water and placed in fixative (Kodak) for a further 60

seconds. Autoradiographs were then rinsed in water and hung up to dry before being photographed.

2.3 Results

2.3.1 Cytochrome oxidase (mitochondrial marker)

Optimum pH determination

Cytochrome oxidase activity was minimal below pH 3 (Figure 2.3.1). Above pH 7, cytochrome oxidase activity was reduced, compared to levels observed between pH 6.4 - pH 6.8. Activity of cytochrome oxidase was maximal at pH 6.8, thus this was the pH (of incubation) used in further cytochrome oxidase assays.

Metal ion concentration

Of the range of metal ions tested, none apart from calcium showed any stimulatory effect on cytochrome oxidase activity (Figure 2.3.2). Zinc ions at concentrations greater than 0.03 mM inhibited enzyme activity. Stimulation by calcium ions was only observed at very high concentrations (30 mM). Since activity was observed to be high in the absence of metal ions it was decided to omit calcium from further assays because of the high concentrations required, and because stimulation of cytochrome oxidase to these levels made measurement of the initial rate of oxidation (estimated decrease in optical density/min, measured at 550 nm) difficult to measure and prone to error.

Activity of cytochrome oxidase in sucrose gradient fractions of bovine corpus luteum homogenate

Cytochrome oxidase activity was maximal at a buoyant density of 1.16 g/cm^3 (early luteal tissue gradient), 1.15 g/cm^3 (mid-luteal tissue gradient) and 1.12 g/cm^3 (late luteal tissue gradient). In the presence of digitonin, these buoyant densities were increased to 1.17 g/cm^3, 1.16 g/cm^3 and 1.13

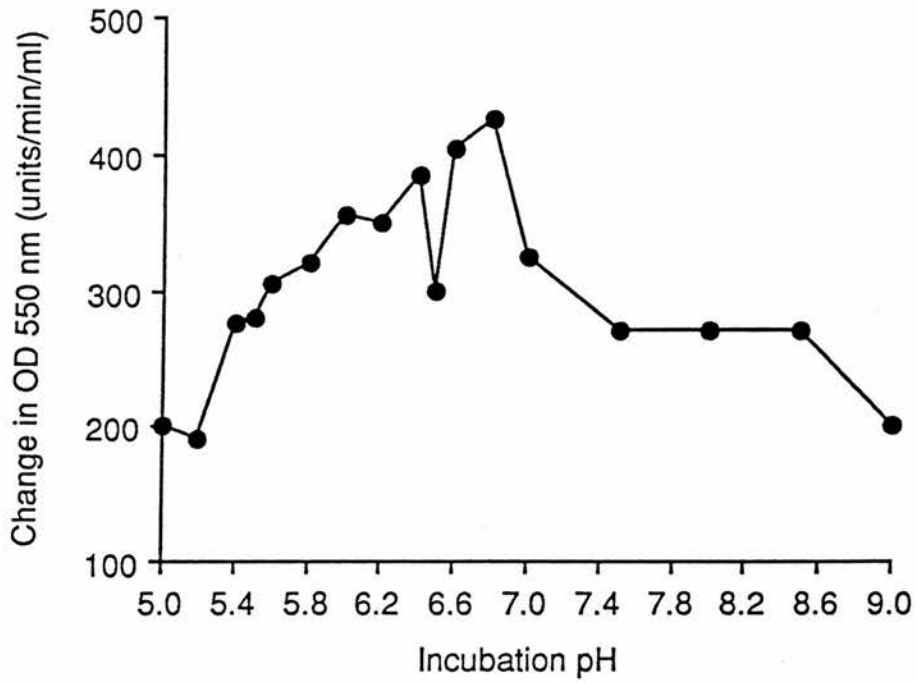


Figure 2.3.1 Determination of optimal pH for bovine luteal cytochrome oxidase activity. Mean cytochrome oxidase (n=3) expressed in change in arbitrary absorbance units per minute per ml of bovine CL homogenate.

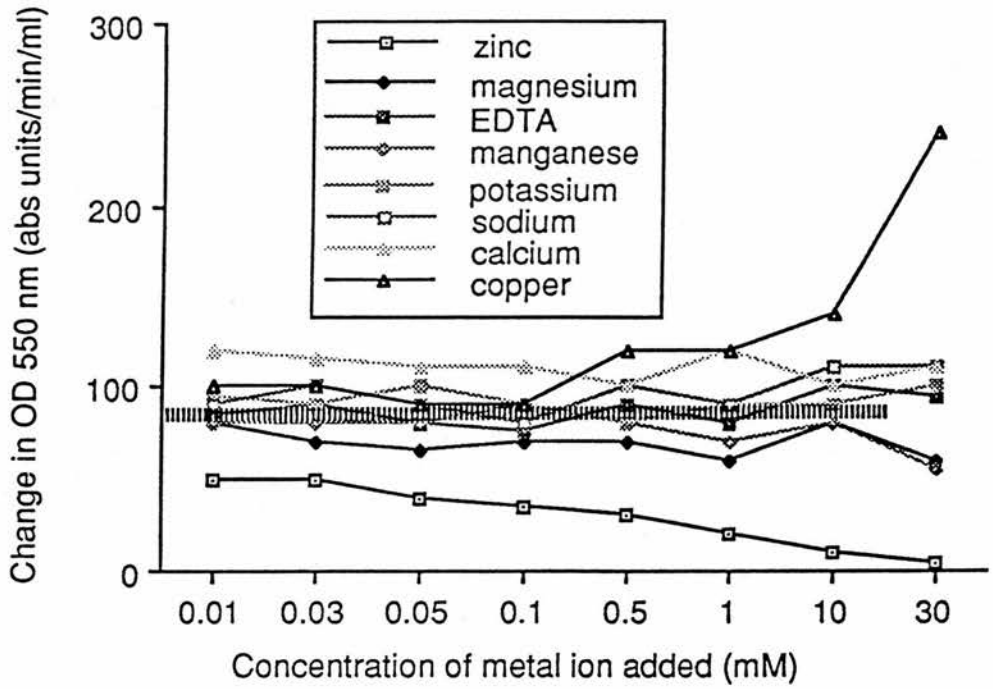


Figure 2.3.2 Effects of incubation with metal ions on cytochrome oxidase activity. Activity in the absence of any added metal ions is indicated by heavy broken line. Experiment was repeated on two different luteal homogenates assayed in duplicate (graph is the mean of these experiments).

g/cm^3 in early, mid- and late luteal tissue sucrose gradients respectively (Figure 2.3.3). Activity was distributed over a wider range of sucrose concentrations in gradients of late luteal tissue.

2.3.2 Fluoride-sensitive acid phosphatase

Optimum pH determination

From Figure 2.3.4, it can be seen that activity of acid phosphatase was maximal between pH 4.4 - 5.0. The optimal pH chosen for further assays was pH 4.7.

Metal ion concentration

None of the ions tested significantly increased acid phophatase activity over values obtained in the absence of added metal ions. Indeed, zinc, manganese and calcium inhibited activity of this enzyme at concentrations greater than 0.01 mM, 0.1 mM and 1 mM respectively (Figure 2.3.5).

Activity of acid phosphatase in sucrose gradient fractions of bovine corpus luteum homogenates

Acid phosphatase activity displayed a buoyant density of 1.15 g/cm^3 (early luteal tissue gradient), 1.16 g/cm^3 (mid-luteal tissue gradient) and 1.17 g/cm^3 (late luteal tissue gradient). In the presence of digitonin, these buoyant densities were increased to 1.17 g/cm^3, 1.18 g/cm^3 and 1.18 g/cm^3 respectively (Figure 2.3.6). Activities were similar at each stage of the luteal phase, as was the magnitude of the buoyant density perturbation induced by digitonin.

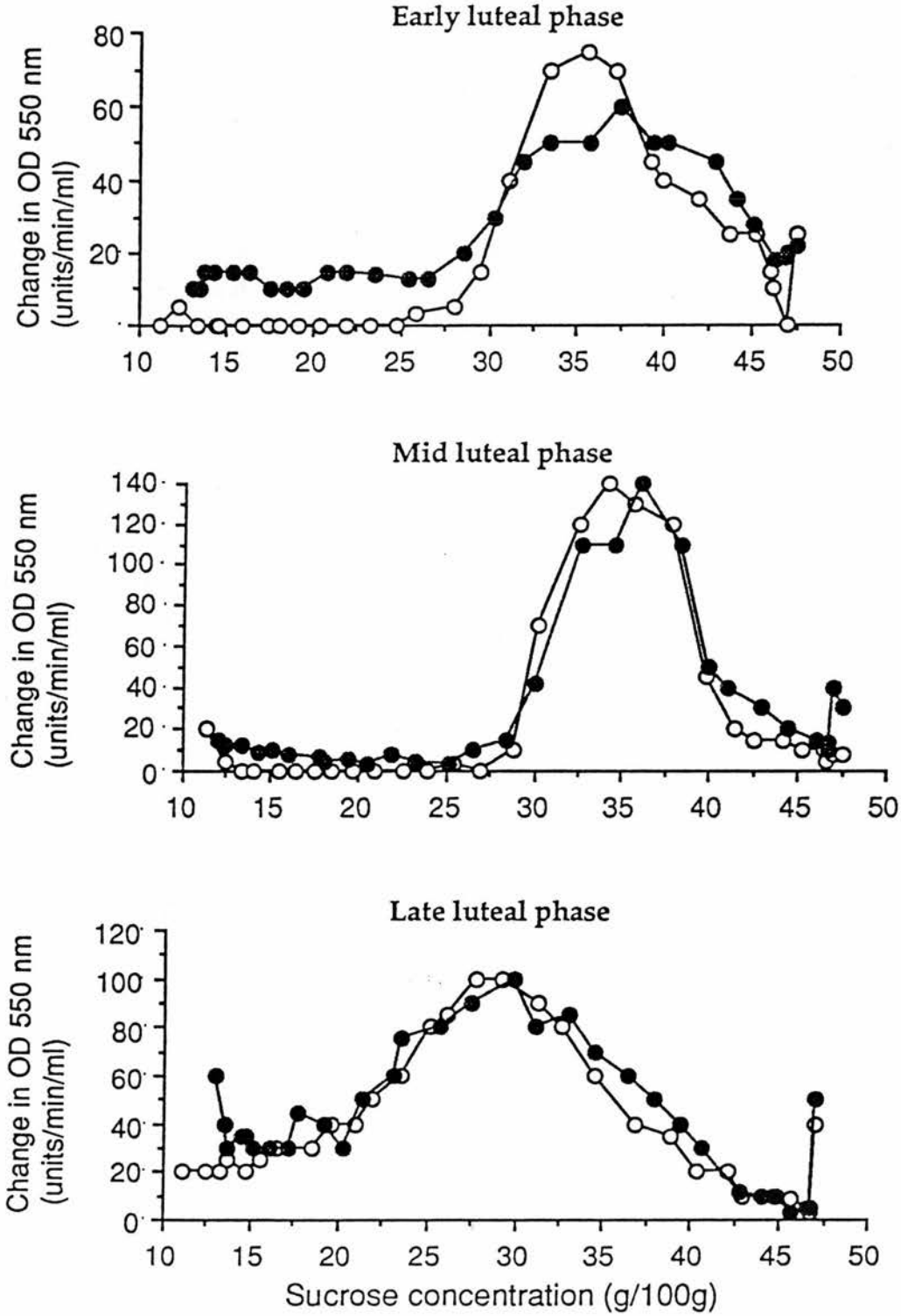


Figure 2.3.3 Distribution of cytochrome oxidase activity in bovine corpus luteum sucrose gradient fractions, with (●) and without (o) digitonin treatment of homogenate prior to fractionation.

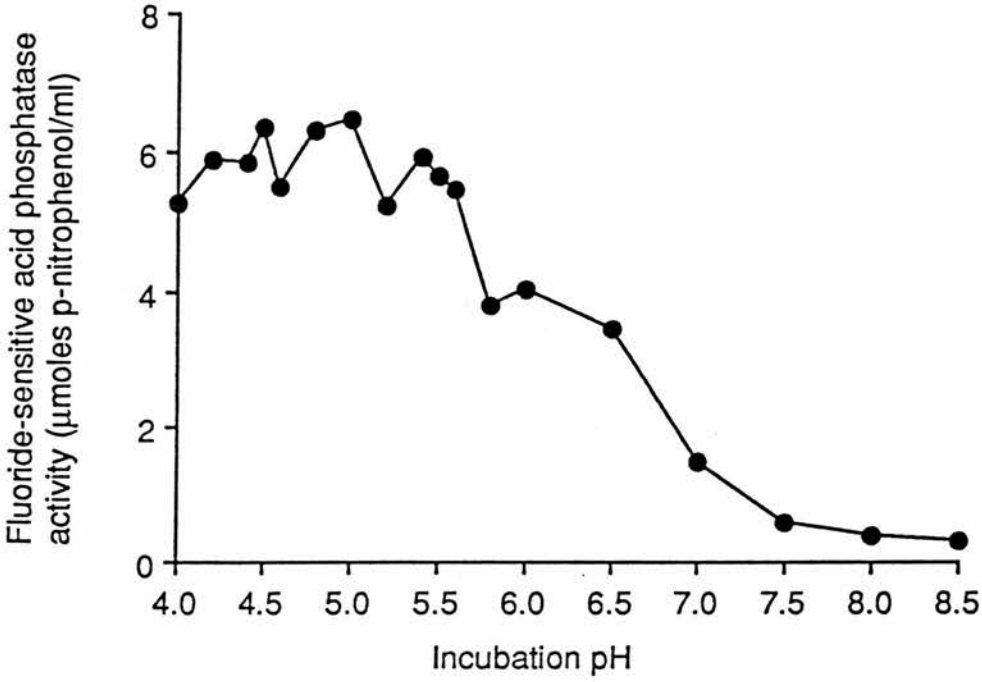


Figure 2.3.4 Determination of optimum incubation pH for fluoride-sensitive acid phosphatase activity. Three bovine CL homogenates were assayed in duplicate, no differences in pH sensitivity were observed between homogenates. Figure is representative of one such experiment.

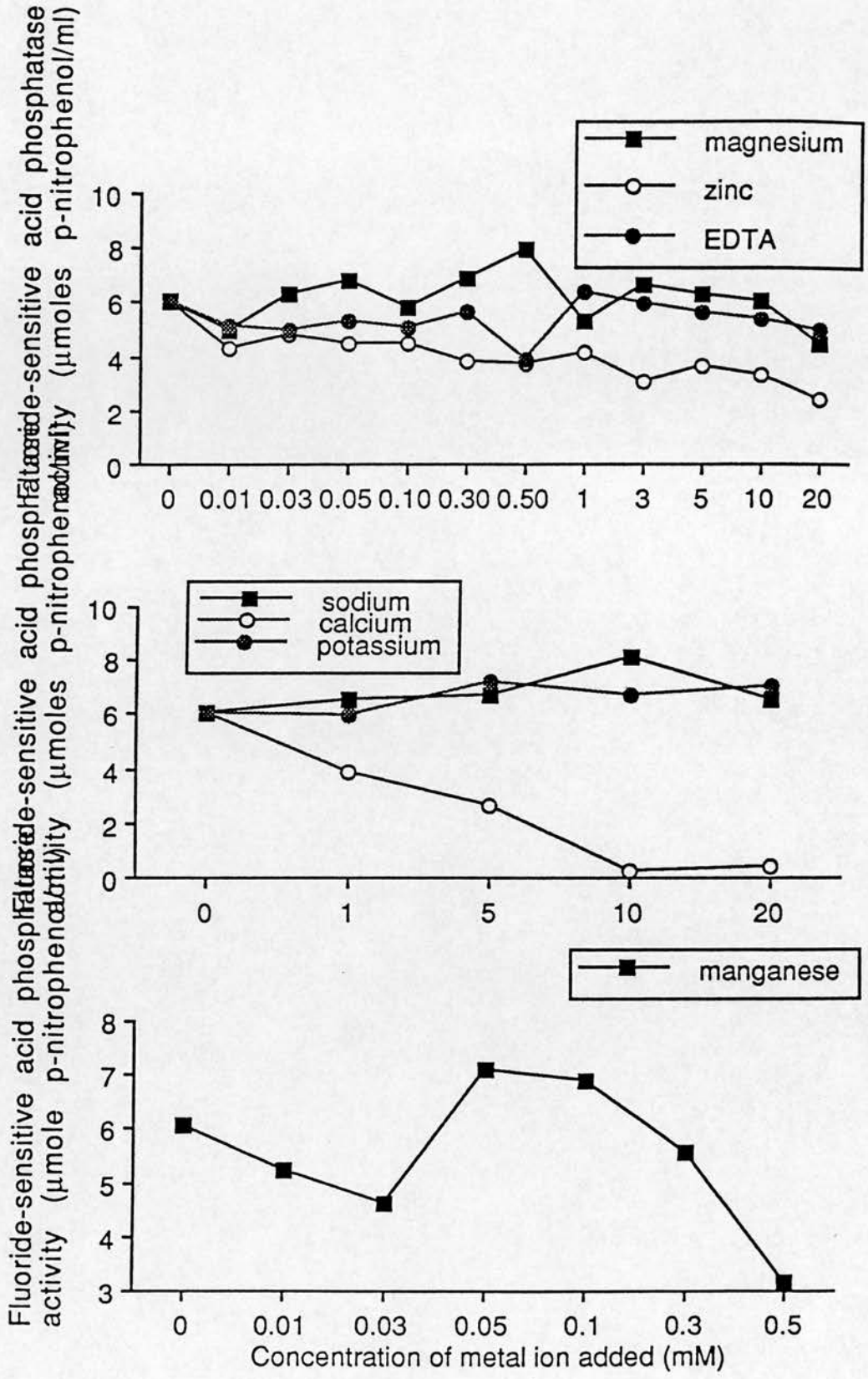
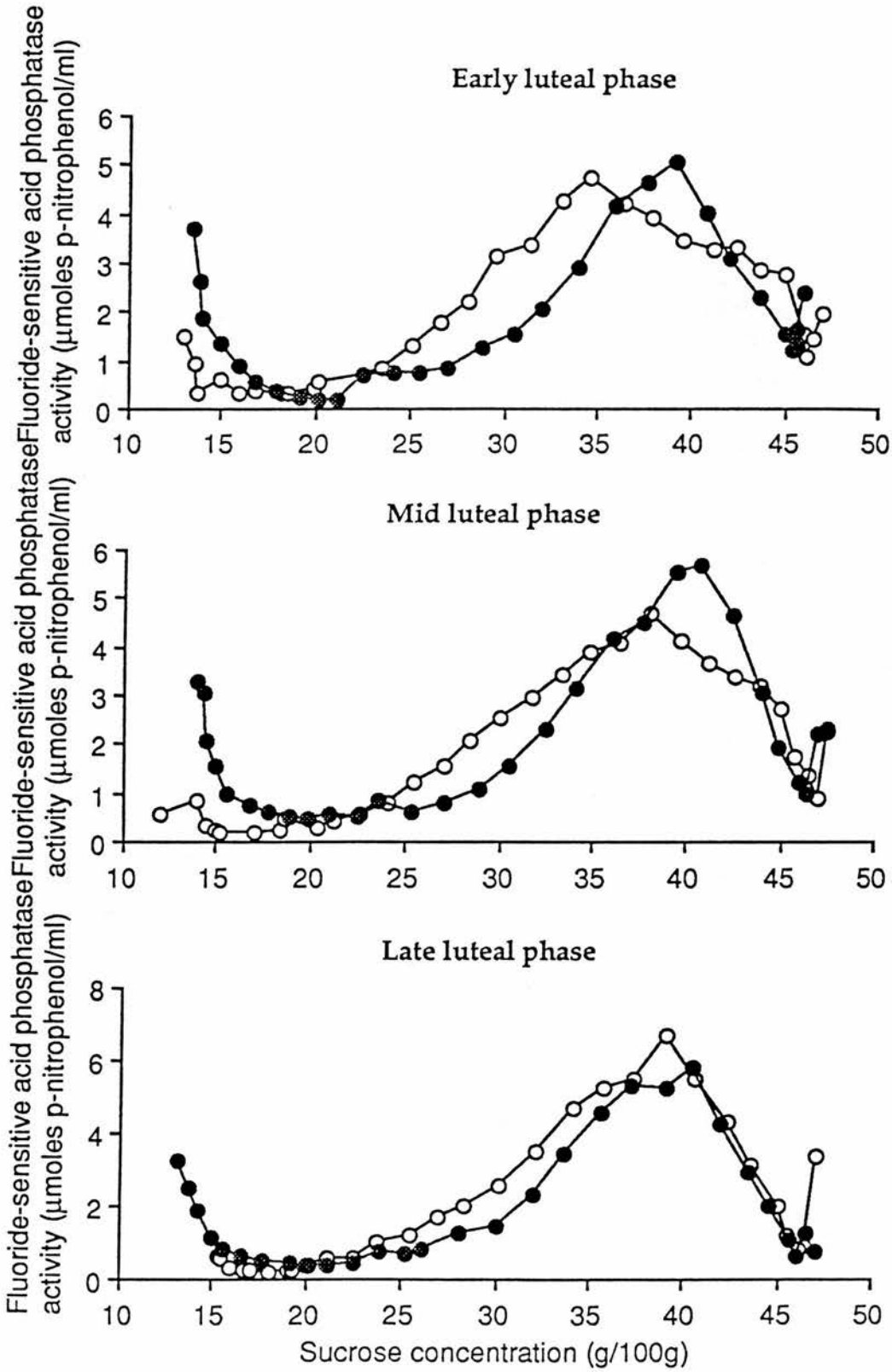


Figure 2.3.5 Effects of incubation with metal ions on bovine luteal fluoride-sensitive acid phosphatase activity. Graphs show the mean of two experiments using two different luteal homogenates assayed in duplicate.

Figure 2.3.6 Distribution of fluoride-sensitive acid phosphatase activity in bovine corpus luteum sucrose gradient fractions, with (●) and without (o) digitonin treatment of homogenate prior to fractionation. Each sucrose gradient fraction was assayed in duplicate, ± fluoride. Two sets of early, mid- and late luteal homogenates were assayed, no differences in the distribution of activity throughout the gradients between different homogenates from the same stage were recorded. Figure is representative of one set of experiments.


Early luteal phase
Mid luteal phase
Late luteal phase
Fluoride-sensitive acid phosphatase activity (µmoles p-nitrophenol/ml)
Sucrose concentration (g/100g)

2.3.3 Alkaline phosphatase (plasma membrane marker)

Optimum pH determination

From Figure 2.3.7 it can be observed that alkaline phosphatase activity was minimal below pH 9. Below pH 7, activity can be accounted for by the presence of acid phosphatase. Alkaline phosphatase was observed to be active at pH 9 and increased to a maximum at pH 10.8. Levels of activity were reduced at a pH greater than pH 11. The pH chosen for use in further assays was pH 10.8.

Metal ion concentration

Zinc was observed to be stimulatory to alkaline phosphatase at 0.03 mM. This stimulation was highly concentration dependent and did not occur outside a very narrow concentration range (Figure 2.3.8). Calcium displayed a broader concentration over which stimulation was evident (maximal stimulation at 5 mM); however, the broadest range of stimulatory concentrations was displayed by magnesium, the stimulatory effect being most pronounced at a concentration of 5 mM. Levels of stimulation by magnesium were greater than the levels of stimulation observed to occur in the presence of calcium ions. Thus in all assays magnesium acetate at a concentration of 5 mM was routinely included, with zinc sulphate at a concentration of 0.03 mM.

Activity of alkaline phosphatase in sucrose gradient fractions of bovine corpus luteum homogenates

Alkaline phosphatase activity displayed a buoyant density of 1.18 g/cm^3 (early luteal tissue gradient), 1.12 g/cm^3 (mid-luteal tissue gradient) and 1.17 g/cm^3 (late luteal tissue gradient). In the presence of digitonin, these buoyant densities were increased to 1.19 g/cm^3 and 1.14 g/cm^3 in early and

mid-luteal tissue sucrose gradients respectively, and decreased to 1.16 g/cm^3 in late luteal tissue sucrose gradients (Figure 2.3.9). Also, in late luteal tissue sucrose gradients it was consistently observed that the bulk of alkaline phosphatase activity failed to enter the sucrose gradients to the same extent that occurred in early and mid-luteal tissue gradients. In gradients from all stages of the luteal phase, alkaline phosphatase activity was enriched in fractions from the lower part of the gradient (45 - 55% sucrose).

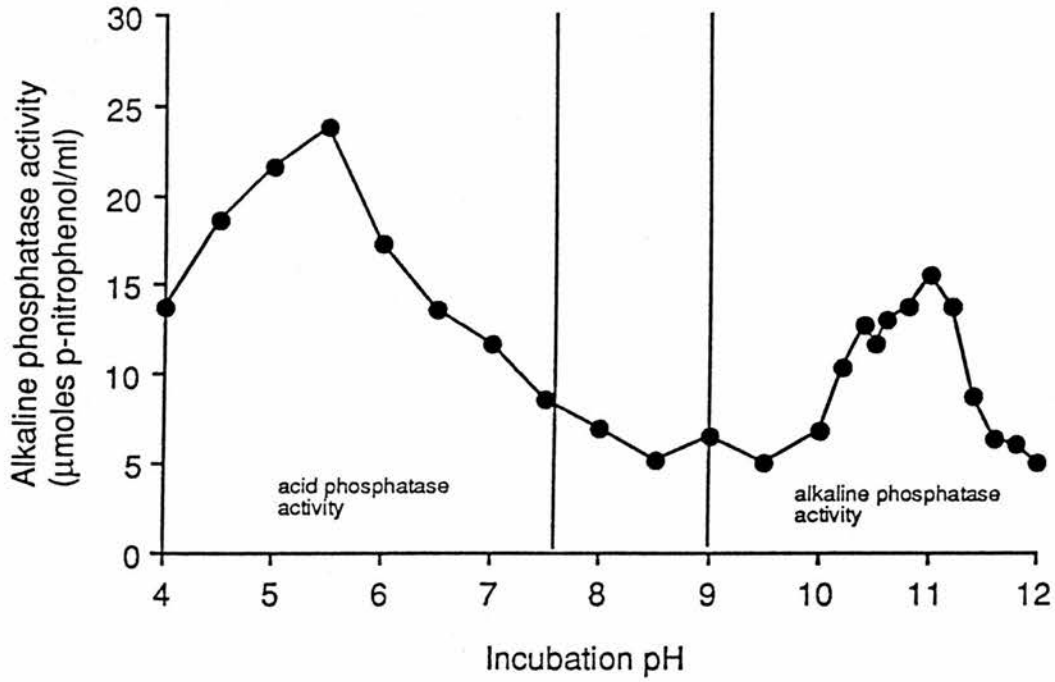


Figure 2.3.7 Optimum pH determination of bovine luteal alkaline phosphatase activity. Activity was measured in duplicate, in the presence of 2 mM magnesium acetate.

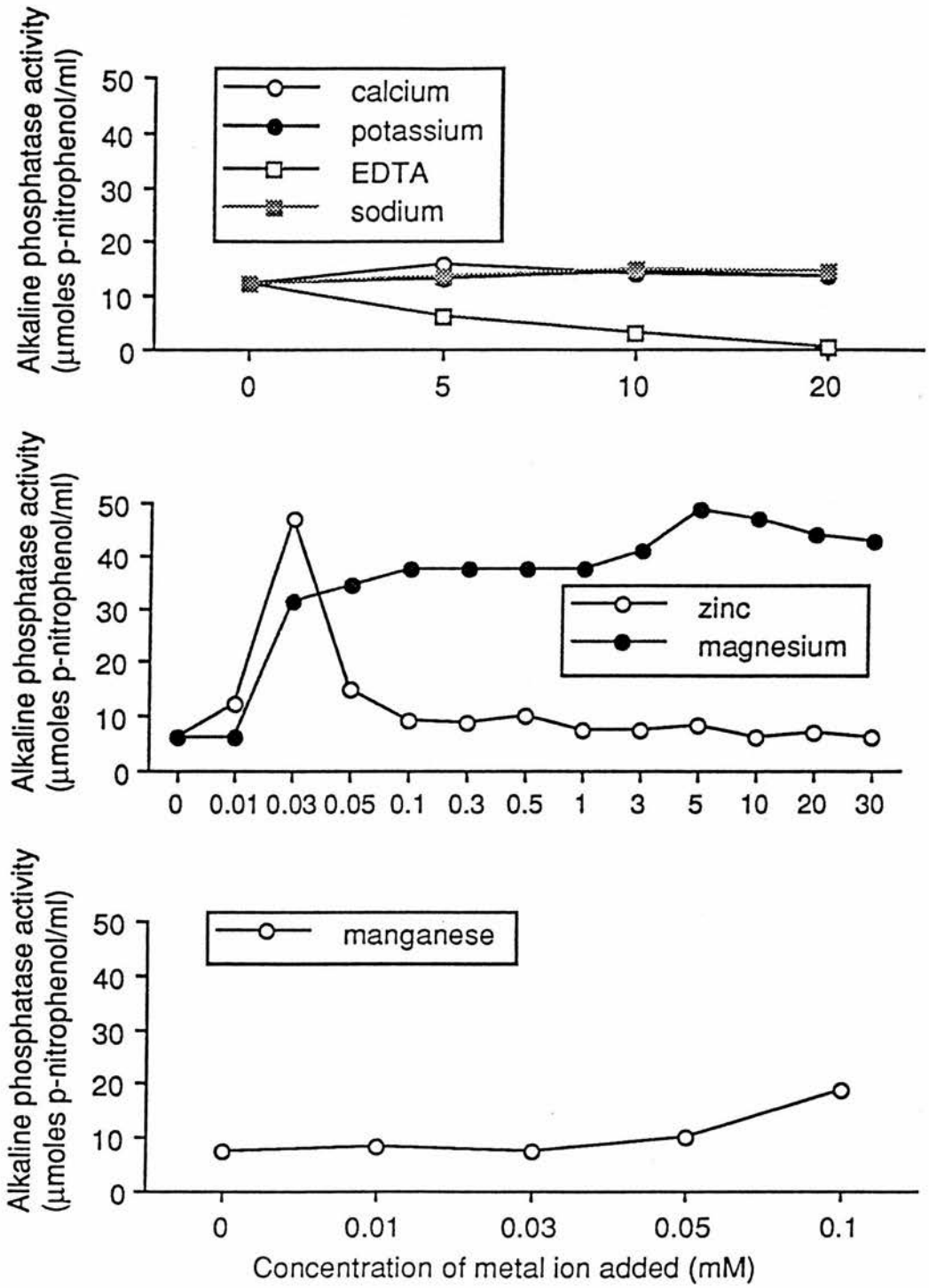
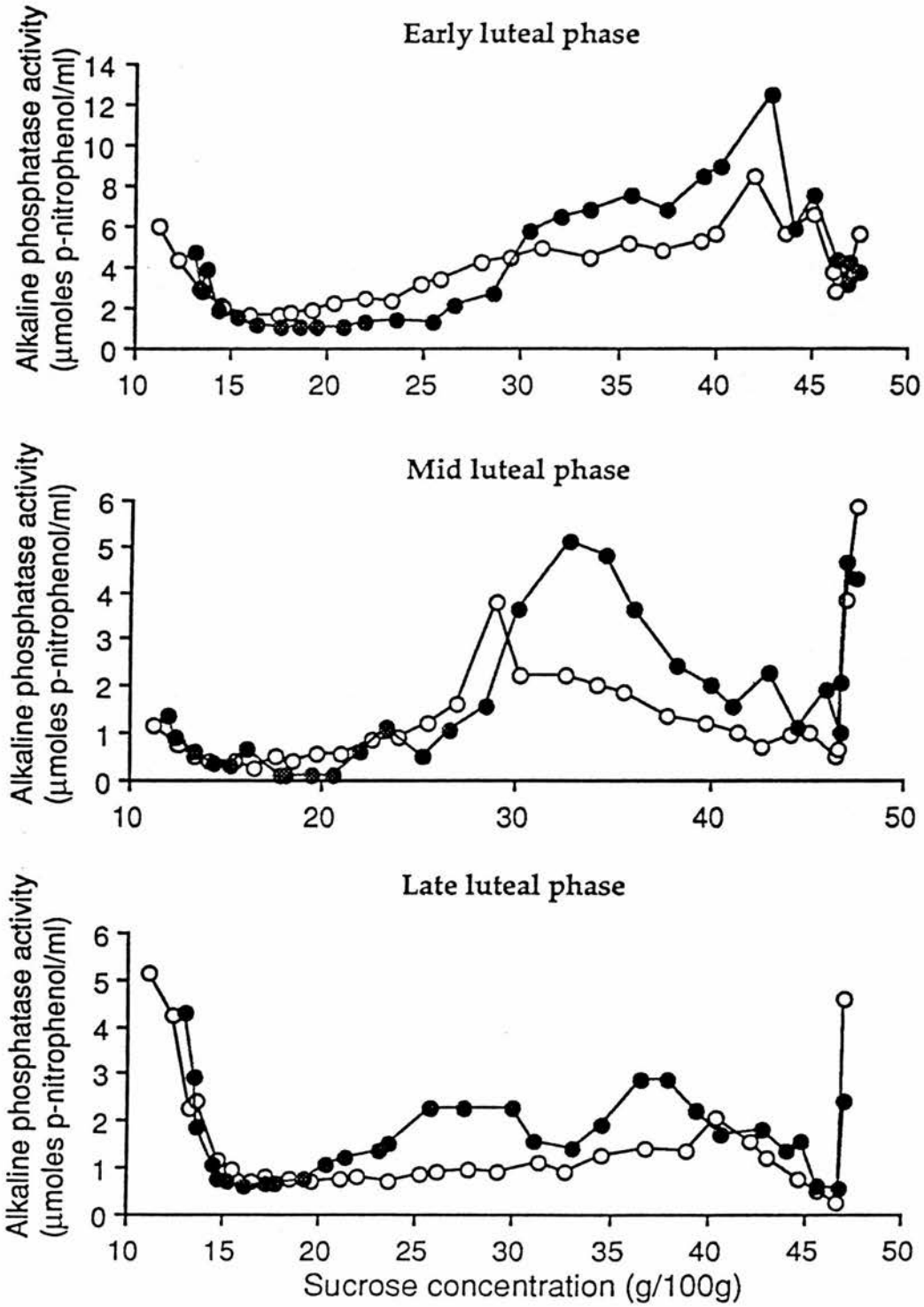


Figure 2.3.8 Effects of incubation with metal ions on alkaline phosphatase activity. Each metal ion concentration was assayed in duplicate. Graph shows the means of two such experiments, using two different luteal homogenates.

Figure 2.3.9 Distribution of alkaline phosphatase activity in bovine corpus luteum sucrose gradient fractions, with (●) and without (o) digitonin treatment of homogenate prior to fractionation. Each fraction was assayed in duplicate. This experiment was performed on two different sets of homogenates, no differences in the distribution of activity in gradients were observed. The above figure is representative of one such experiment.


Early luteal phase
Alkaline phosphatase activity (µmoles p-nitrophenol/ml)
0
2
4
6
8
10
12
14
10
15
20
25
30
35
40
45
50
Mid luteal phase
Alkaline phosphatase activity (µmoles p-nitrophenol/ml)
0
1
2
3
4
5
6
10
20
30
40
50
Late luteal phase
Alkaline phosphatase activity (µmoles p-nitrophenol/ml)
0
1
2
3
4
5
6
10
15
20
25
30
35
40
45
50
Sucrose concentration (g/100g)

2.3.4 LH/hCG receptor binding (plasma membrane marker)

Distribution over sucrose gradient fractions of bovine corpus luteum homogenate (Figure 2.3.10)

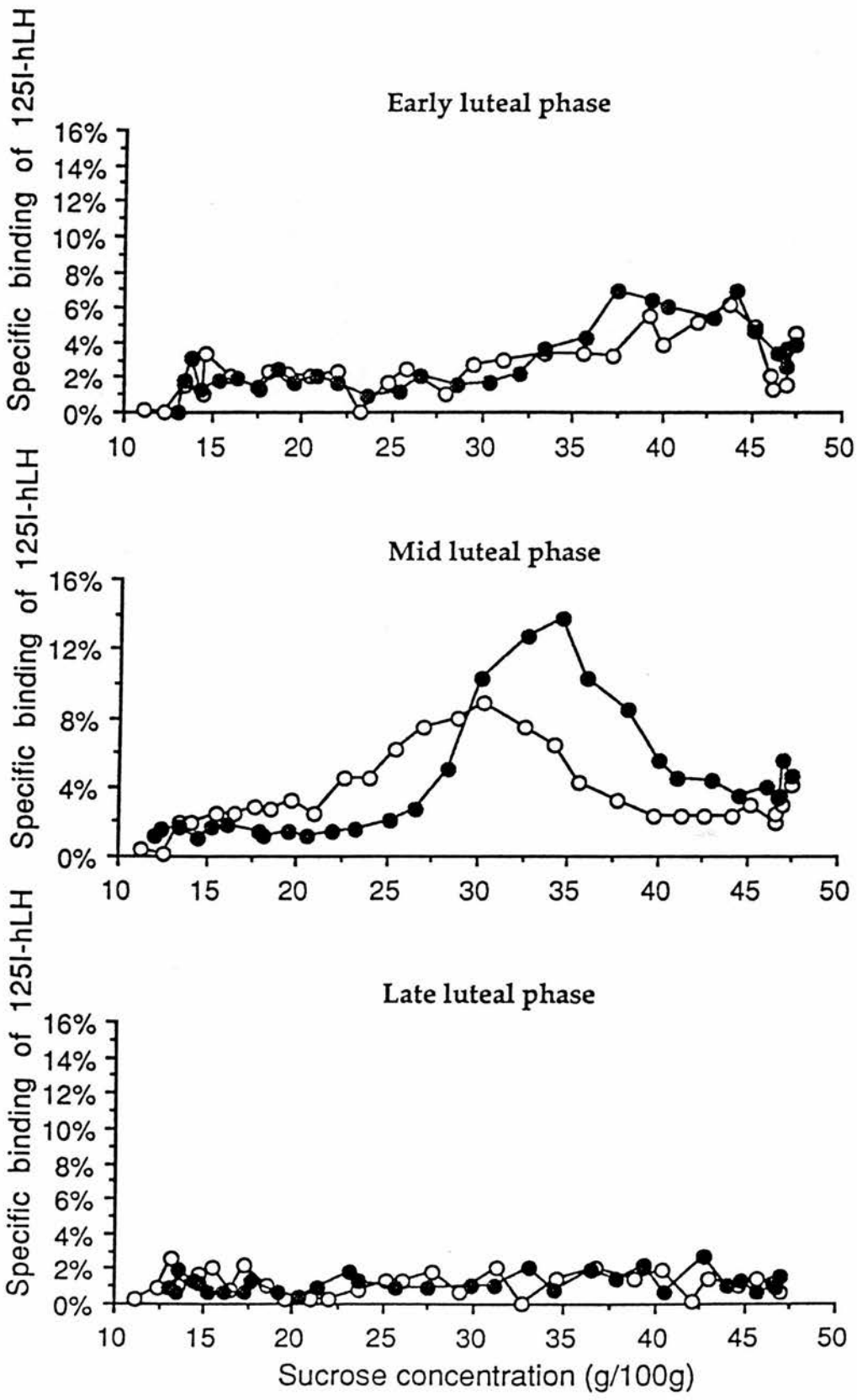
LH/hCG receptor binding equilibrated at a buoyant density of 1.18g/cm^3 (early luteal tissue gradient) and 1.13 g/cm^3 (mid-luteal tissue gradient). In the presence of digitonin, these buoyant densities were increased to 1.19 g/cm^3 and 1.15 g/cm^3 respectively. Late luteal tissue gradients displayed poor binding of iodinated hLH, distributed throughout the sucrose density gradient fractions. Levels of binding were highest in mid-luteal gradients, reaching approximately 2 x that observed in early luteal tissue gradients.

2.3.5 5'-nucleotidase (plasma membrane marker)

Optimum pH determination

Activity of 5'-nucleotidase was maximal at pH 6.4, though activity was observed to be present at pH values 5.8 - 8.0. Thus it appeared that this enzyme had a broad range of pH values at which it was active, and as such does not appear to be acutely pH sensitive (Figure 2.3.11).

Figure 2.3.10 Specific binding of ^{125}I-hLH to sucrose gradient fractions of bovine corpus luteum homogenates from the early, mid- and late luteal phase, with (●) and without (o) digitonin treatment of homogenate prior to fractionation. Assay was performed in triplicate (duplicate NSB for each fraction).


Early luteal phase
Mid luteal phase
Late luteal phase
Specific binding of 125I-hLH
16%
14%
12%
10%
8%
6%
4%
2%
0%
10
15
20
25
30
35
40
45
50
Sucrose concentration (g/100g)

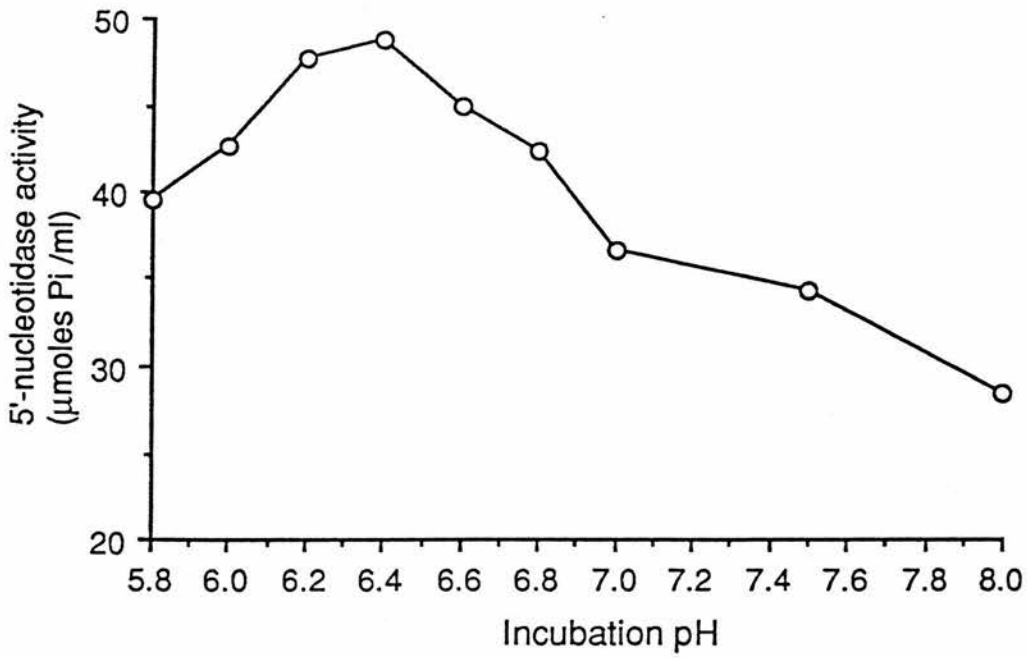


Figure 2.3.11 Determination of optimum incubation pH for bovine luteal 5'-nucleotidase activity. Activity was measured in duplicate in the presence of 3 mM cobaltous chloride. Figure is representative of two such experiments using two different luteal homogenates (no differences in pH response were recorded between homogenates.

Distribution over sucrose gradient fractions of bovine corpus luteum homogenate

In control gradients of mid-luteal tissue, activity of 5'-nucleotidase was found to equilibrate in a broad peak, whose maximum activity equilibrated at 1.13 g/cm^3. In digitonin pretreated gradients, this peak of activity was perturbed to 1.16 g/cm^3, similar to the buoyant densities and digitonin perturbation of the other plasma membrane marker activities assayed (Figure 2.3.12).

2.3.6 N-acetyl glucosaminidase (lysosomal marker)

Optimum pH determination

Below pH 5.2 and above pH 6.0, activity of N-acetyl glucosaminidase was minimal (Figure 2.3.13). Activity increased at pH 5.2, reaching a maximum at pH 5.8. In further assays of N-acetyl glucosaminidase an assay pH of 5.8 was used.

Metal ion concentration

All metal ions tested were inhibitory to N-acetyl glucosaminidase activity at concentrations greater than 0.03 mM, apart from magnesium, whose inhibitory effects were not observed until concentrations in excess of 10 mM. Below this concentration, magnesium was stimulatory to activity, even at concentrations below 0.03 mM (Figure 2.3.14). Greatest stimulation by magnesium ions was observed at 1 mM. Thus this concentration of magnesium acetate was used in future assays of N-acetyl glucosaminidase activity.

Activity of N-acetyl glucosaminidase in sucrose gradient fractions of bovine corpus luteum homogenate

In sucrose gradient fractions from mid-luteal control homogenates, activity of N-acetyl glucosaminidase peaked at a buoyant density of 1.17 g/cm^3. In sucrose gradients of digitonin-pretreated homogenates, activity was found only in the top of the gradient, at a buoyant density of <1.05 g/cm^3 (Figure 2.3.15). This activity was observed to be very strong (readable only when diluted 1:10 in assay buffer) and concentrated into a small number of fractions. Little activity was found in the fractions at the top of the gradients when CL homogenates were not pretreated with digitonin.

2.3.7 NADPH cytochrome C reductase

Activity of NADPH cytochrome C reductase was found to be very low in whole luteal homogenates and sucrose gradient fractions, and in fresh or frozen and thawed fractions. To attempt to increase activity, assays were set up with varying Triton-X-100 concentrations, over a range of NADPH concentrations and EDTA concentrations (since this enzyme is thought to be metal ion independent but may be inhibited by the presence of some metal ions even at low concentrations - Bramley, unpublished data). No increase in activity was recorded. Thus this enzyme was not active enough in bovine luteal tissue to be utilised as an endoplasmic reticulum marker enzyme.

2.3.8 NADH cytochrome C reductase

Optimum pH determination

It was observed that NADH cytochrome C reductase activity was minimal below pH 4 (Figure 2.3.16). Activity increased at pH values greater than 5, reaching maximal levels at pH 7.0. At pH values above 7.0, up to pH 9, activity was relatively unaffected, though above pH 9.5 activity was reduced.

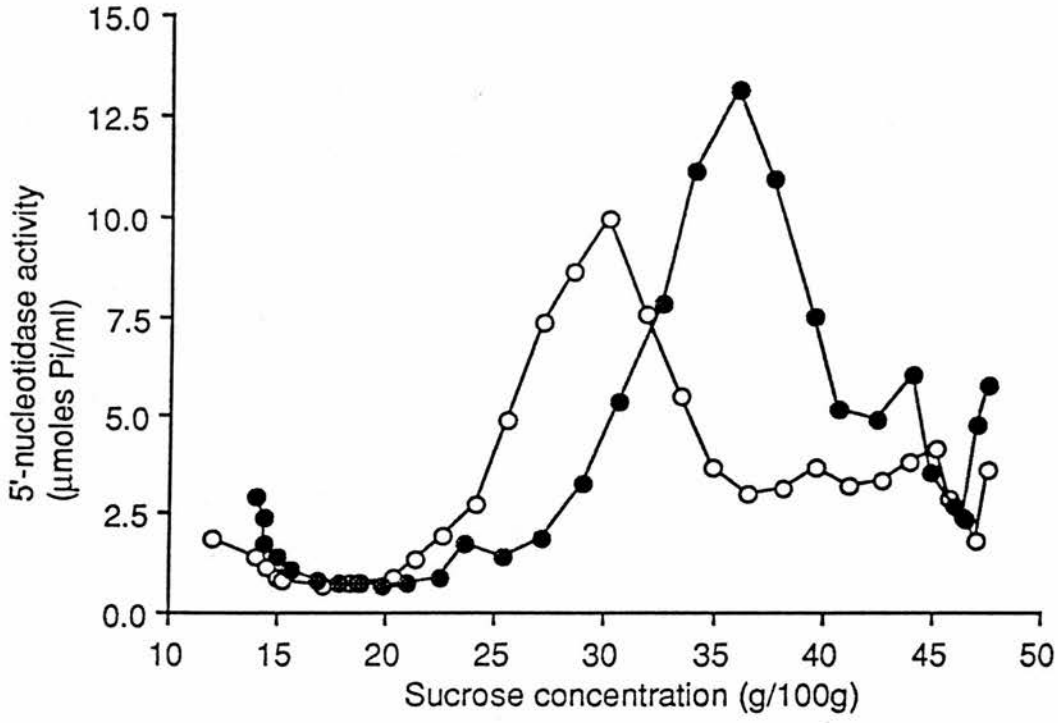


Figure 2.3.12 Distribution of 5'-nucleotidase activity in bovine corpus luteum (mid-luteal phase) sucrose gradient fractions, with (●) and without (o) digitonin treatment of homogenate prior to fractionation.

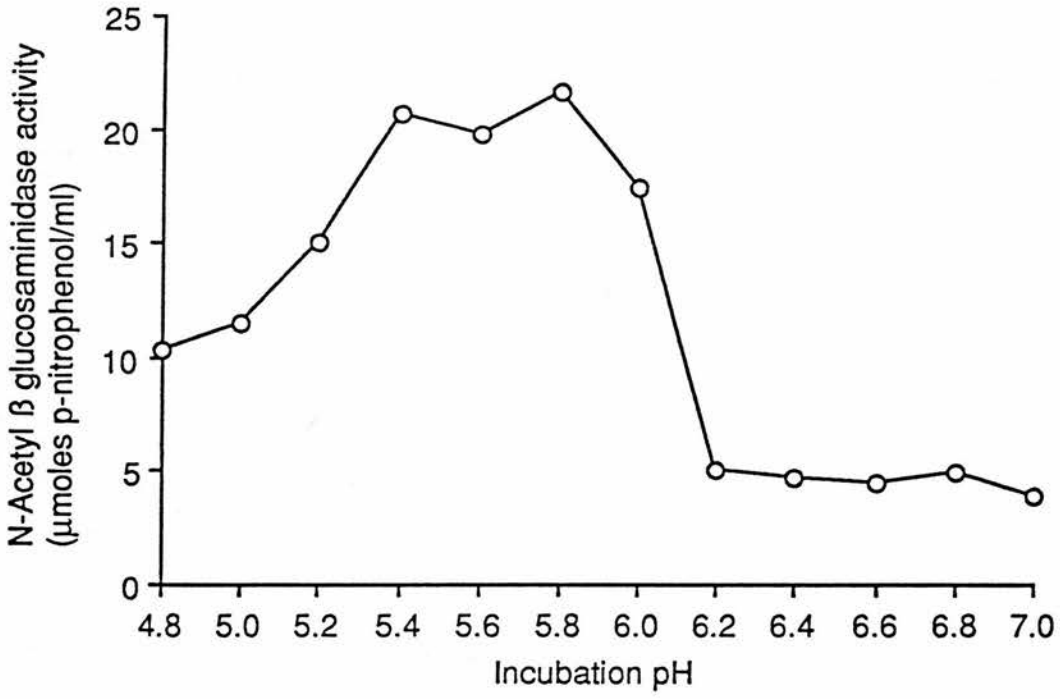


Figure 2.3.13 Determination of optimum pH of N-acetyl-ß-D-glucosaminidase activity in bovine corpus luteum homogenate. Experiment was performed twice in duplicate using two different mid-luteal bovine CL homogenates. Figure is representative data from one such experiment.

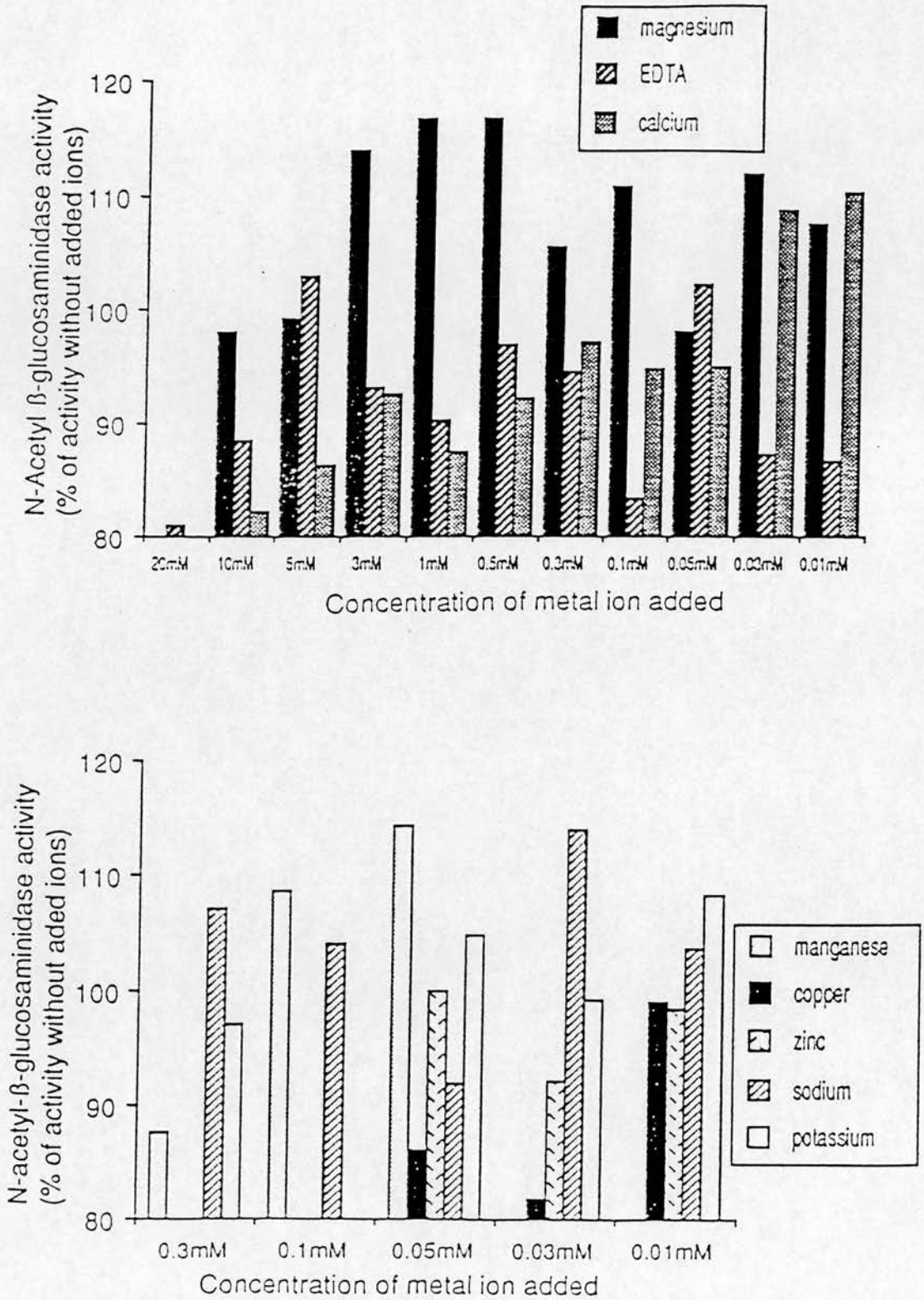


Figure 2.3.14 Effects of incubation with metal ions on bovine luteal N-acetyl-β-D-glucosaminidase activity.

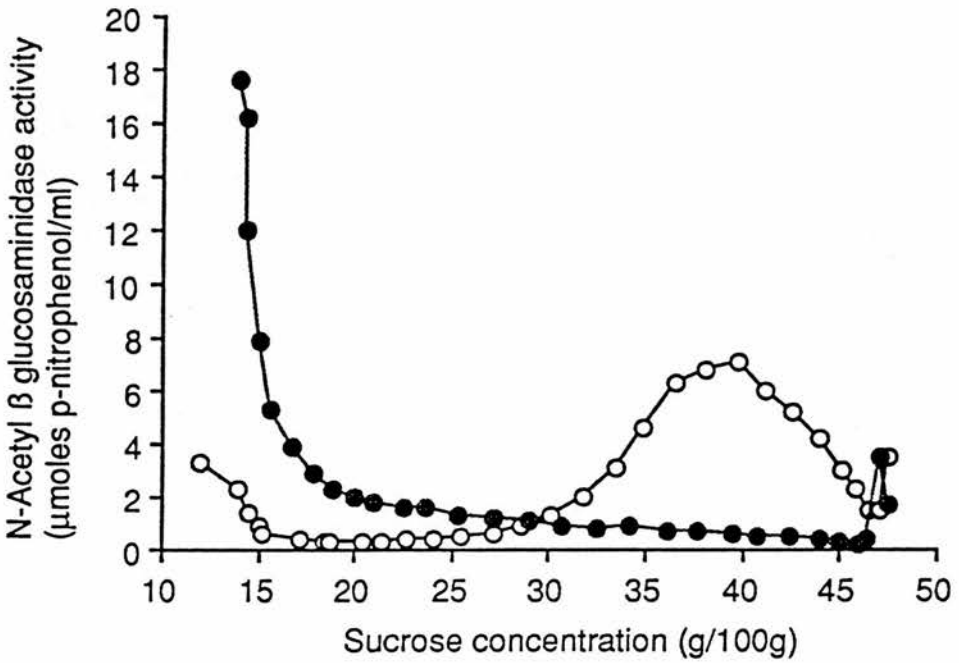


Figure 2.3.15 Distribution of N-acetyl-β-D-glucosaminidase activity in bovine corpus luteum (mid-luteal phase) sucrose gradient fractions, with (●) and without (o) digitonin treatment of homogenate prior to fractionation. Figure is representative of one such experiment, no differences were found using different luteal homogenates (n=2). Assayed in duplicate.

Activity of NADH cytochrome C reductase in sucrose gradient fractions of bovine corpus luteum homogenate

NADH cytochrome C reductase activity displayed a buoyant density of 1.15 g/cm^3 (mid-luteal tissue gradient). In the presence of digitonin, this buoyant density was increased to 1.16 g/cm^3 (Figure 2.3.17). Though these buoyant densities were identical to those displayed by cytochrome oxidase, the pattern over sucrose gradients is different. A much broader peak was displayed by NADH cytochrome C reductase than was displayed by cytochrome oxidase activity, thus NADH cytochrome C reductase does not appear to be co-localized with cytochrome oxidase in the mitochondrion. When the NADH cytochrome C reductase activity across sucrose density gradients is compared to progesterone content on the same sucrose gradients, both displayed similar patterns of distribution and digitonin perturbation, however, the peak of progesterone content was found to be consistently lighter than that of NADH cytochrome C reductase activity in control gradients. Also, in some gradients a much greater digitonin induced increase in the bouyant density of NADH cytochrome C reductase was observed, however, neither progesterone content or cytochrome oxidase followed this pattern.

2.3.9 DNA content of bovine luteal homogenate sucrose density gradient fractions

In both control and digitonin pretreated sucrose gradients, DNA was observed to equilibrate at the bottom of the gradient, in fractions with high sucrose content (Figure 2.3.18) The large amount of DNA measured in the bottom of each gradient may be attributable to purification of nuclei and cell debris.

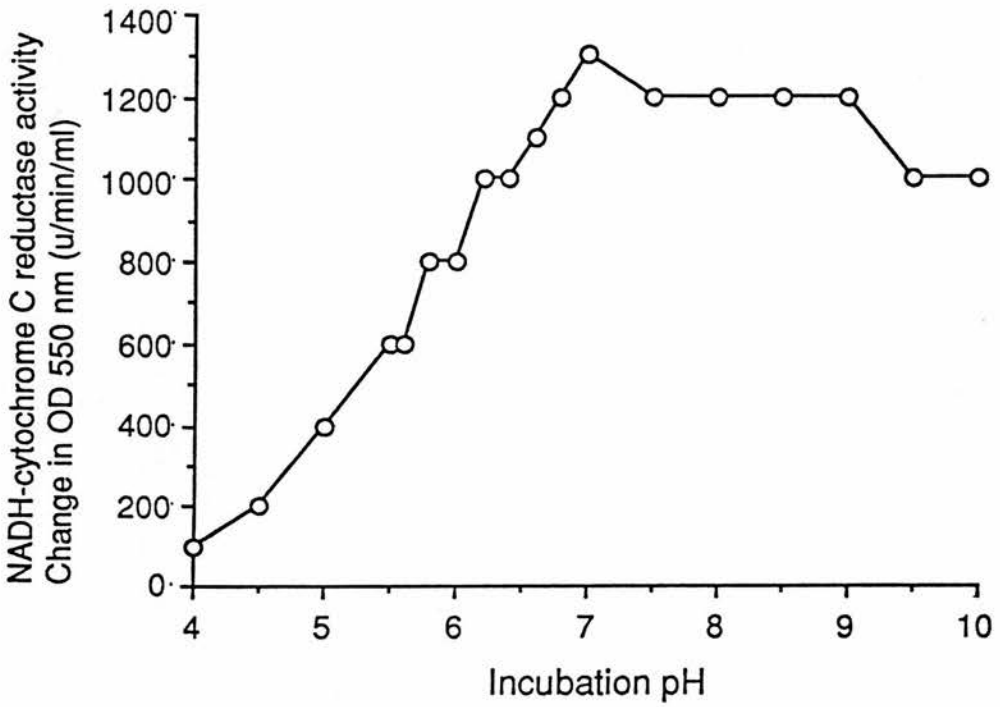


Figure 2.3.16 Optimum pH determination for bovine luteal NADH cytochrome c reductase activity. Figure derived from mean values from two experiments (assayed in duplicate) using two different luteal homogenates.

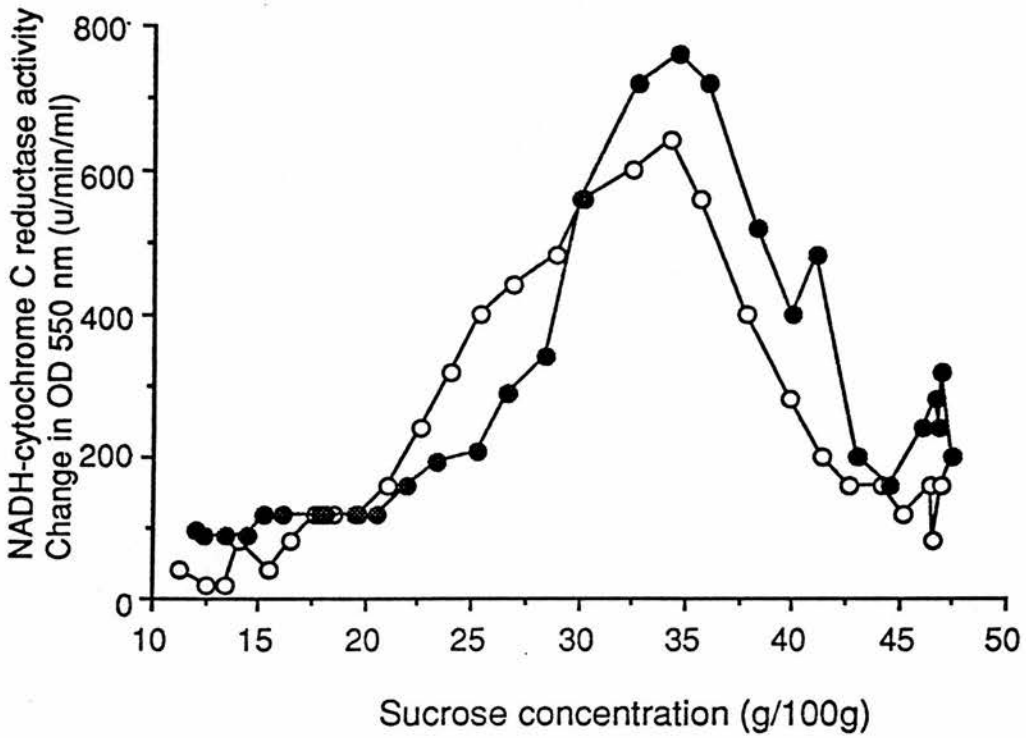


Figure 2.3.17 Distribution of NADH cytochrome c reductase activity in bovine corpus luteum (mid-luteal phase) sucrose gradient fractions, with (●) and without (o) digitonin treatment of homogenate prior to fractionation. Figure is representative of one such experiment, no differences were found in the distribution of activity accross sucrose gradients using different (mid-) luteal preparations (n=3).

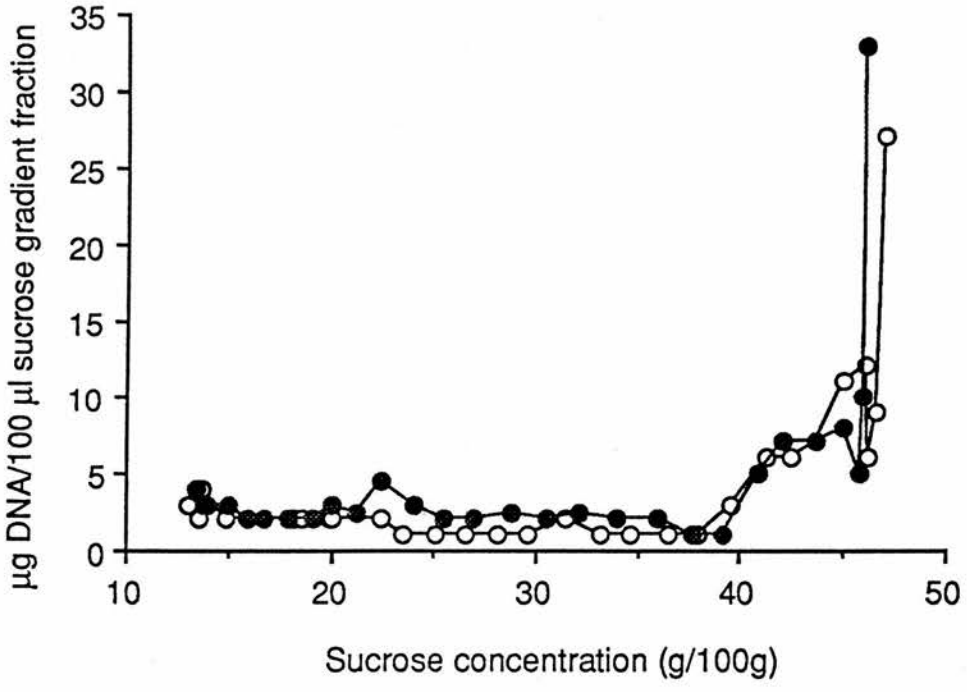


Figure 2.3.18 Distribution of DNA in sucrose gradient fractions of bovine corpus luteum homogenate (mid-luteal phase), with (●) and without (o) digitonin treatment of homogenate prior to fractionation. Figure is representative of one such experiment, assayed in duplicate. No differences in distribution of DNA in sucrose gradients of different mid-luteal homogenates were observed (n=3 homogenates).

2.3.10 Progesterone content of sucrose gradient fractions

Progesterone extraction recoveries

Recovery of progesterone following ether extraction of samples was determined for each sucrose gradient fraction (Figure 2.3.19). Extraction recovery percentages were then used to correct results of progesterone content of extracted samples.

Progesterone content of extracted and non-extracted sucrose gradient fractions

After correction for % recovery of tritiated progesterone, no differences were observed between the progesterone content of extracted and unextracted sucrose gradient fractions (Figure 2.3.20).

Progesterone content of sucrose gradient fractions of bovine corpus luteum homogenate

Levels of endogenous progesterone were highest in a broad peak spanning the buoyant densities 1.10 - 1.14 g/cm^3. High levels of progesterone were observed in cytosolic (top of gradient) fractions and fractions from the bottom of sucrose gradients. However the main peak of endogenous progesterone equilibrated in the aforementioned broad peak, indicating likely association between progesterone and cellular membranes (Figure 2.3.21). Moreover, the buoyant density of the progesterone peak was increased by digitonin, indicating the likely association of progesterone with membranes rich in unesterified cholesterol. In addition, it was observed that preincubation with digitonin reduced the amounts of progesterone recovered in the cytosolic fractions. Levels of progesterone were noted to be highest in mid- and late luteal tissue.

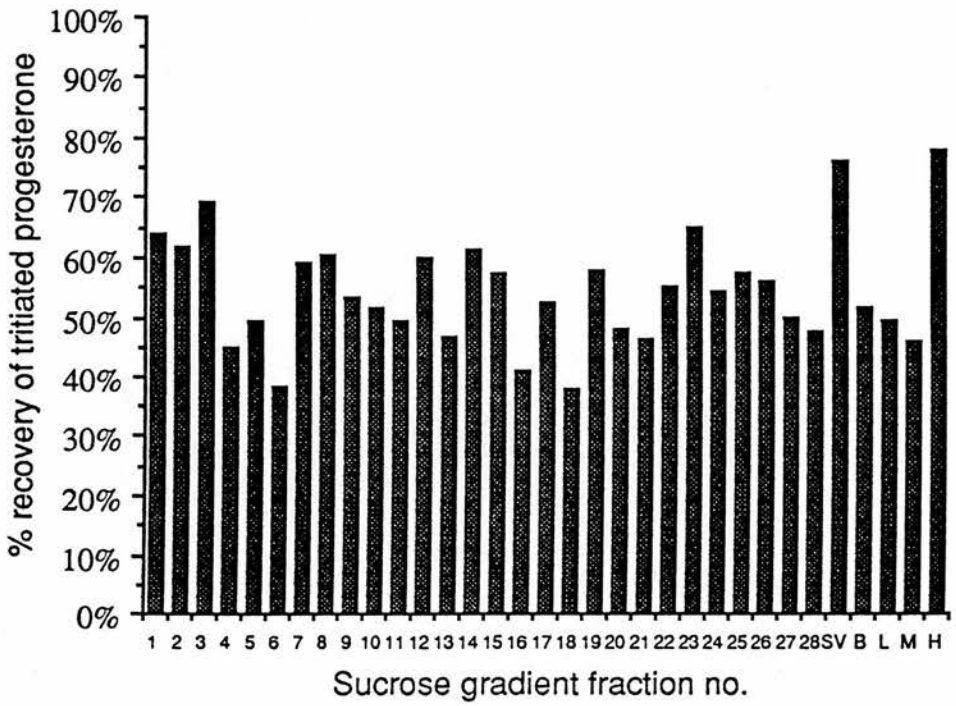


Figure 2.3.19 Recovery efficiencies of solvent extraction of [^{3}H]-progesterone from sucrose gradient fractions of bovine (mid-luteal phase) corpus luteum homogenate (sv=solvent blank, B=buffer blank, L, M & H =low, medium & high progesterone immunoassay quality controls).

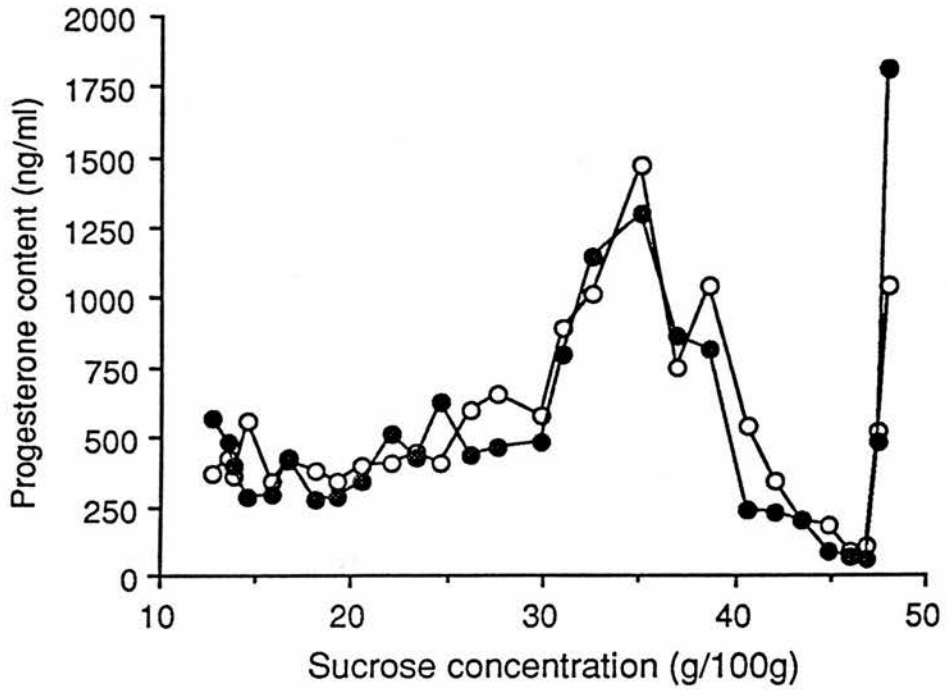
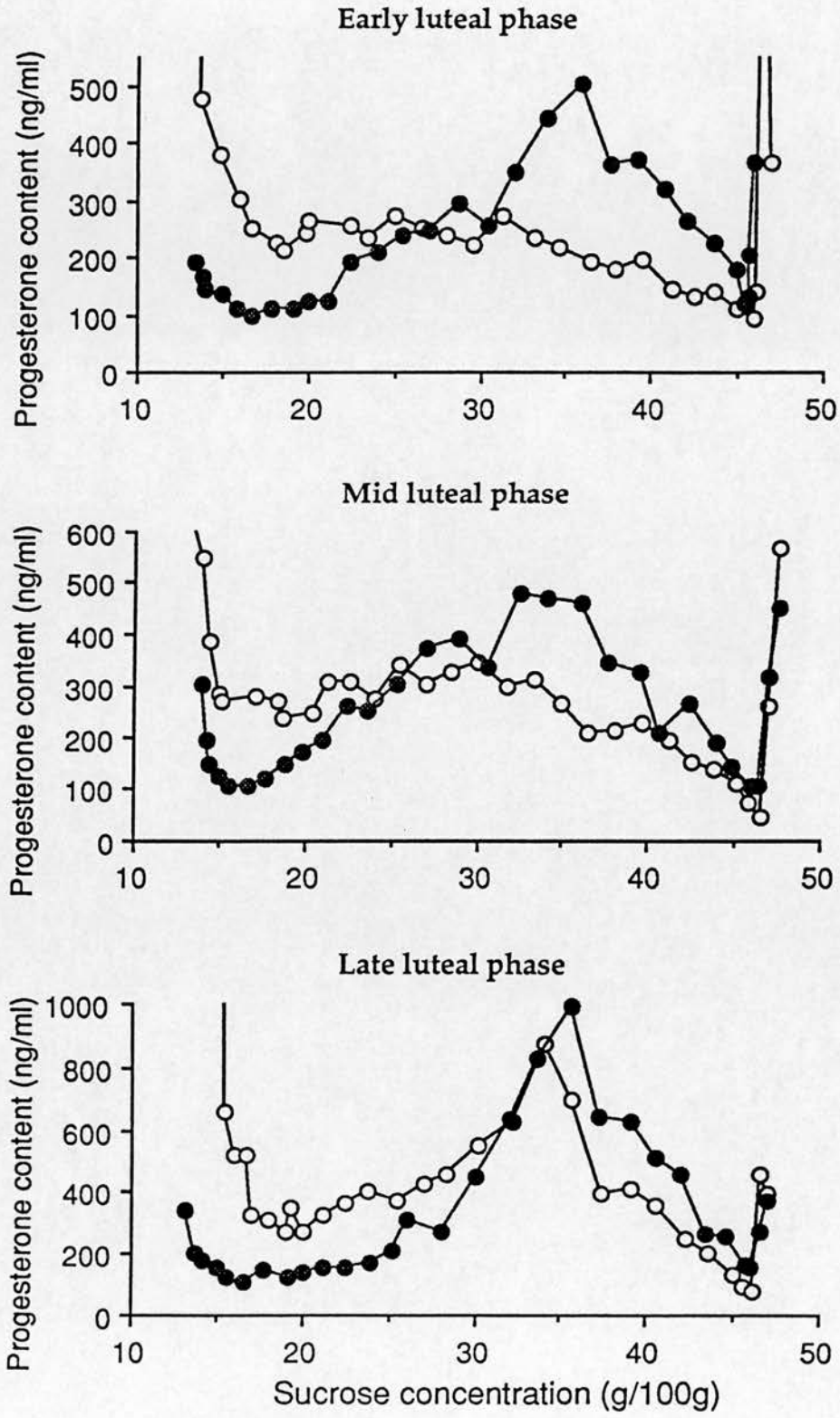


Figure 2.3.20 Comparison of direct progesterone radioimmunoassay of sucrose gradient fractions (o) of (mid-luteal phase) bovine corpus luteum homogenate with progesterone immunoassay of solvent extracted sucrose gradient fractions (●) of (mid-luteal phase) bovine corpus luteum homogenate (after correction of individual extraction recovery efficiencies).

Figure 2.3.21 Progesterone content of sucrose gradient fractions of bovine corpus luteum homogenates from the early, mid- and late luteal phase, with (●) and without (o) digitonin treatment of homogenate prior to fractionation. Data is from one set of luteal homogenates assayed in duplicate. Similar levels and profiles were obtained using a different set of staged luteal homogenates.


Early luteal phase
Progesterone content (ng/ml)
0
100
200
300
400
500
10
20
30
40
50
Mid luteal phase
Progesterone content (ng/ml)
0
100
200
300
400
500
600
10
20
30
40
50
Late luteal phase
Progesterone content (ng/ml)
0
200
400
600
800
1000
10
20
30
40
50
Sucrose concentration (g/100g)

2.3.11 Protein concentrations of sucrose gradient fractions

Figure 2.3.22 shows the distribution of protein over sucrose gradient fractions in control and digitonin pretreated gradients of bovine luteal homogenate from early, mid- and late luteal phase corpora lutea. Levels of total protein and distribution of protein over sucrose gradients were similar at all stages. No digitonin perturbation of protein levels was evident.

2.3.12 Immunoblotting of steroidogenic enzymes

(i) P_{450} side chain cleavage (Plate 2.3.1)

Bands of protein were found to migrate with an apparent M_r of 49 kDa, the molecular weight of P_{450} side chain cleavage. However, additional bands were also detected at approximately 80 kDa, 65 kDa and 46 kDa. These proteins were absent from the cytosolic fractions, though present in all other fractions tested. Heaviest staining occurred in fractions known to contain mitochondrial and microsomal material.

(ii) 3β-hydroxysteroid dehydrogenase (Plate 2.3.2)

Multiple protein bands were detected using this anti-serum, however, the most densely staining bands were found to be approximately 62 and 43 kDa in size. The 62 kDa band was present in all fractions, though staining was found to be present in greater quantity in fractions known to contain microsomal material, which was the predominant location of the 43 kDa band.

(iii) P_{450} 17α hydroxylase/C 17,20 lyase

No detection of this enzyme was found in any of the fractions tested. Positive detection did occur in control lanes (bovine theca cell homogenate) however, indicating that its absence would not appear to be an artifact of the methodology applied (not shown).

Figure 2.3.22 Distribution of total protein in sucrose gradient fractions of bovine corpus luteum homogenates from the early, mid- and late luteal phase, with (●) and without (o) digitonin treatment of homogenate prior to fractionation.

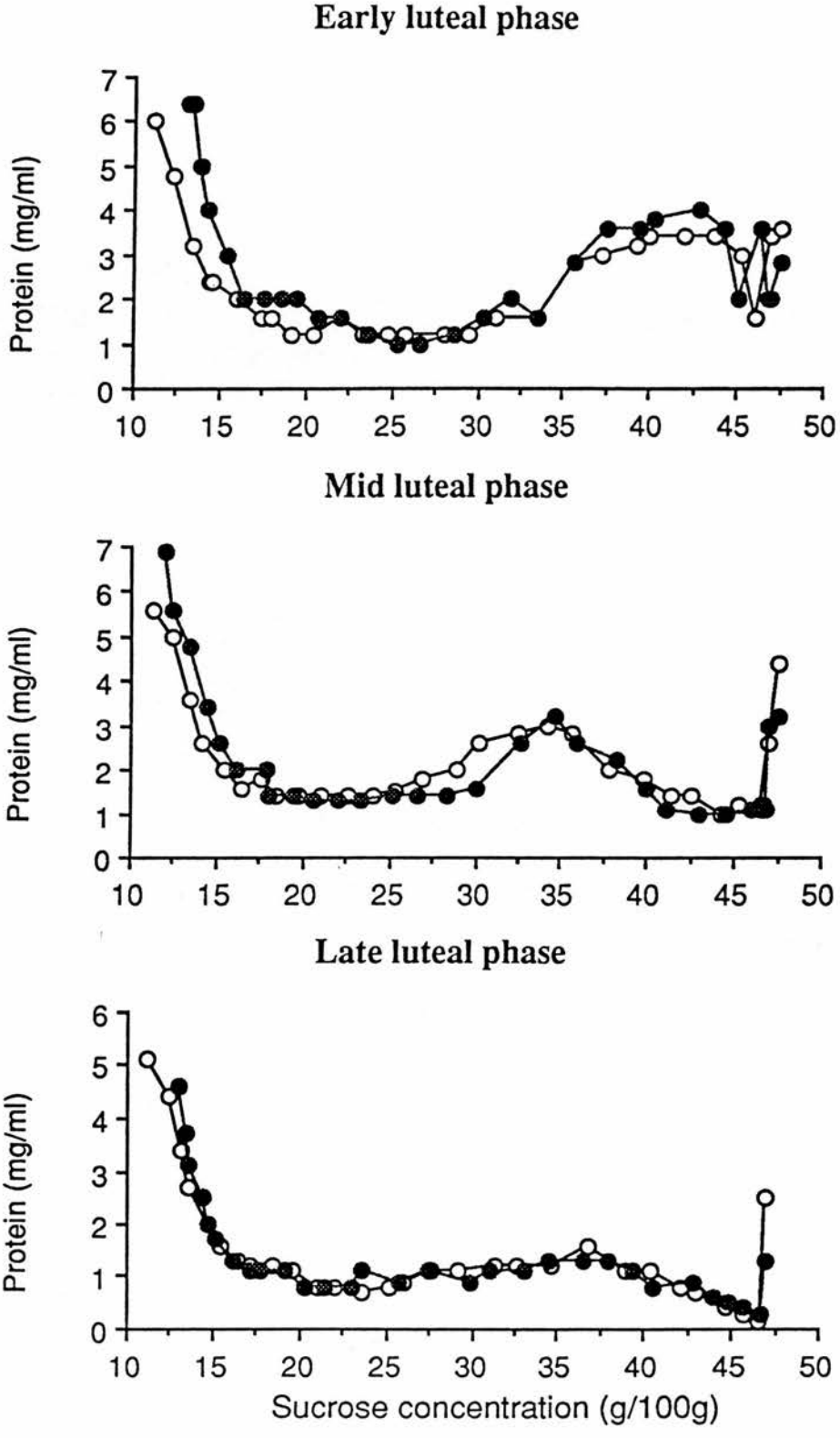
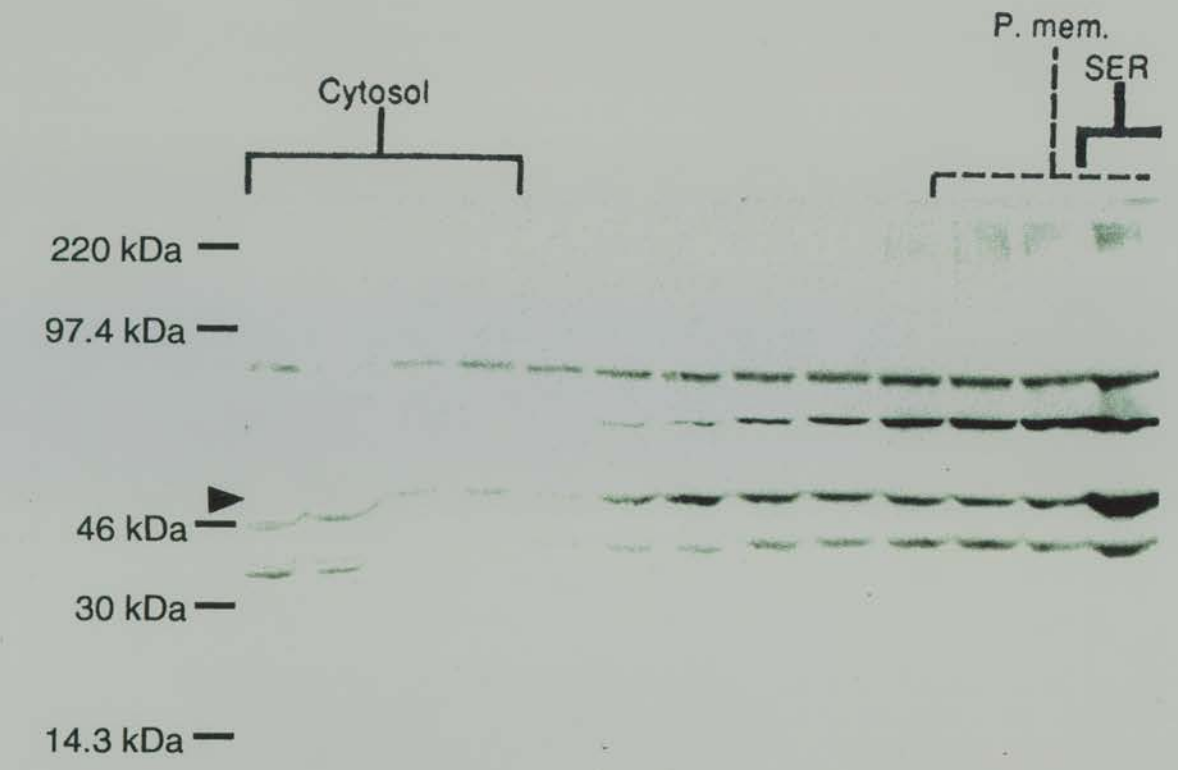

Early luteal phase
Protein (mg/ml)
0
1
2
3
4
5
6
7
10
15
20
25
30
35
40
45
50
Mid luteal phase
Protein (mg/ml)
0
1
2
3
4
5
6
7
10
15
20
25
30
35
40
45
50
Late luteal phase
Protein (mg/ml)
0
1
2
3
4
5
6
10
15
20
25
30
35
40
45
50
Sucrose concentration (g/100g)

Plate 2.3.1 Western blot of P_{450} side chain cleavage distribution in sucrose gradient fractions of bovine corpus luteum homogenate. P_{450} side chain cleavage indicated by arrowhead. Positions of intracellular organelles were determined by organelle marker enzyme assay (P. mem = plasma membrane, SER = smooth endoplasmic reticulum, GERL = Golgi endoplasmic reticulum lysosomal network).


P. mem.
SER
Cytosol
220 kDa
97.4 kDa
46 kDa
30 kDa
14.3 kDa

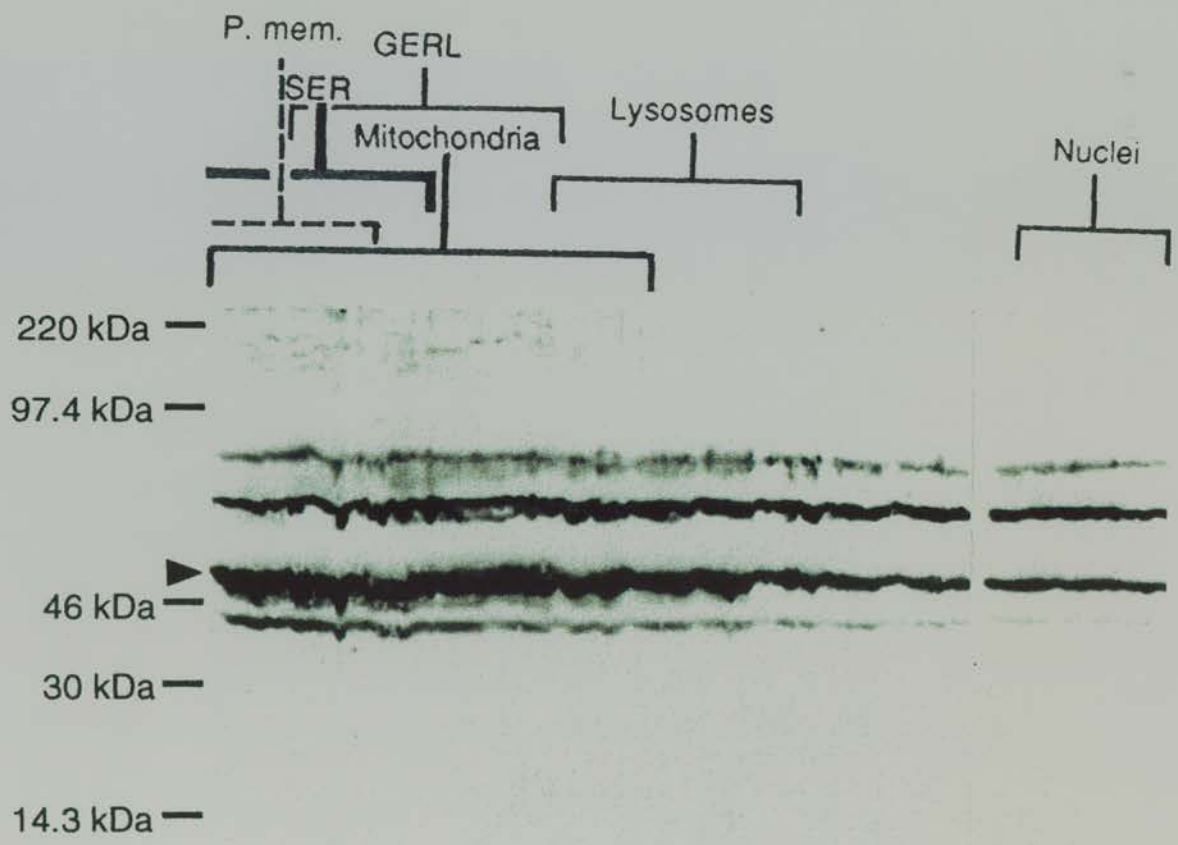
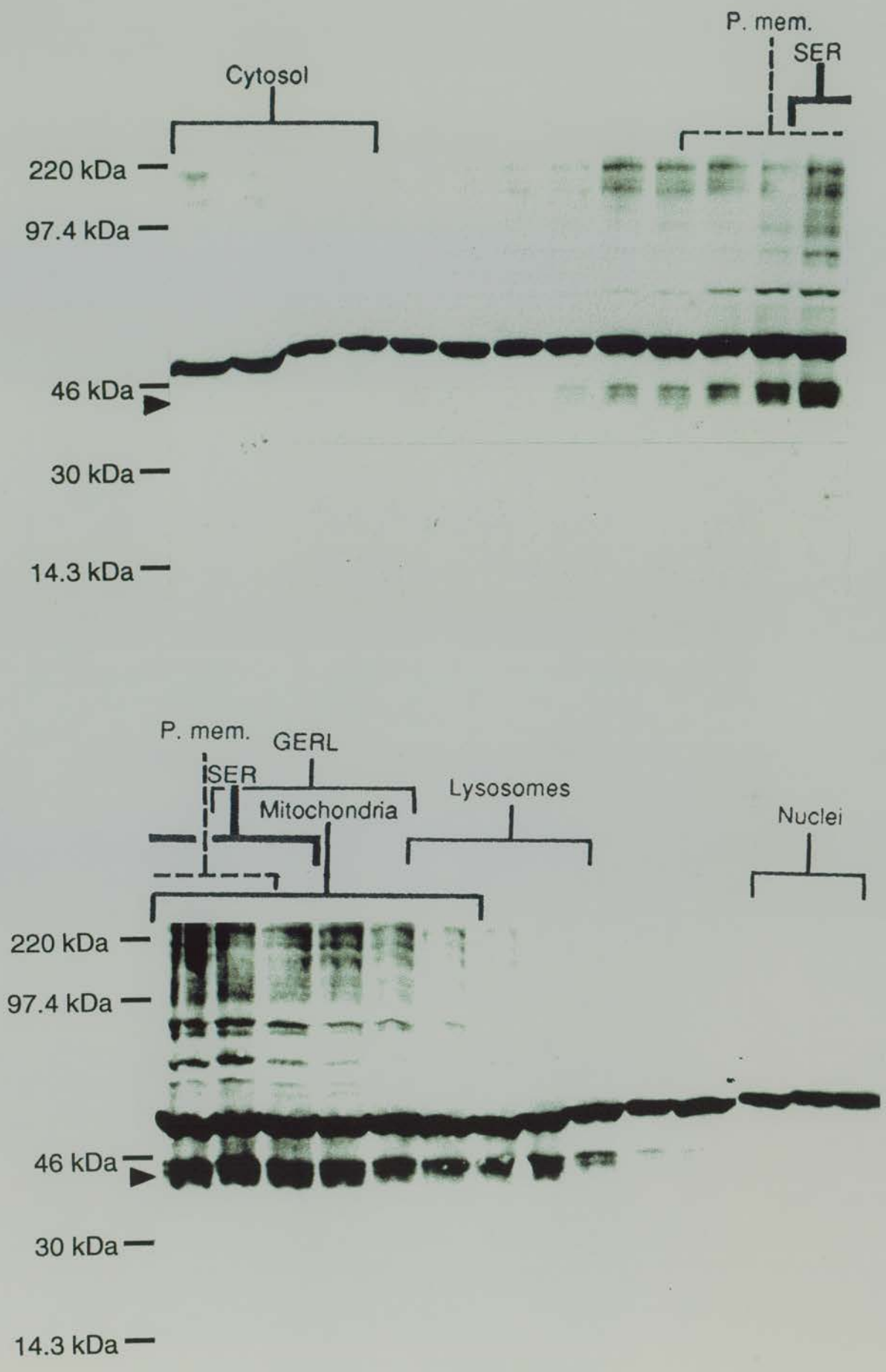

P. mem.
GERL
SER
Mitochondria
Lysosomes
Nuclei
220 kDa
97.4 kDa
46 kDa
30 kDa
14.3 kDa

Plate 2.3.2 Western blot of 3β-hydroxysteroid dehydrogenase distribution in sucrose gradient fractions of bovine corpus luteum homogenate. 3β-hydroxysteroid dehydrogenase indicated by arrowhead. Positions of intracellular organelles were determined by organelle marker enzyme assay (P. mem = plasma membrane, SER = smooth endoplasmic reticulum, GERL = Golgi endoplasmic reticulum lysosomal network).


P. mem.
SER
Cytosol
220 kDa
97.4 kDa
46 kDa
30 kDa
14.3 kDa
P. mem.
GERL
SER
Mitochondria
Lysosomes
Nuclei
220 kDa
97.4 kDa
46 kDa
30 kDa
14.3 kDa

2.4 Discussion

Alkaline phosphatase displayed a pH maximum of 10.8 in bovine luteal homogenates from all stages of the luteal phase. This is similar to the optimal pH of this enzyme in rat ovarian tissue (pH 10.7, Bramley & Ryan, 1978a) and the sheep and porcine corpus luteum (G.S. Menzies, personal communication). Also, the buoyant densities of alkaline phosphatase (when fractionated on sucrose gradients) in sheep and pigs (Bramley & Menzies, 1986; 1988a) were similar to the results presented here for the bovine CL. Alkaline phosphatase assays in rat ovarian tissues have included Mg^{2+} (Bramley & Ryan, 1978a). Studies with different cations demonstrated that magnesium was also highly stimulatory to bovine luteal alkaline phosphatase. Though zinc and calcium were also demonstrated to stimulate activity of alkaline phosphatase in the bovine CL, the concentration ranges at which stimulation occurred were much narrower than the stimulatory concentration range of magnesium. Thus in practice magnesium ions and zinc ions were the two metal salts routinely included in alkaline phosphatase incubations.

From the results of the distribution of alkaline phosphatase, 5'-nucleotidase and LH receptor binding, some disparity in the buoyant densities of the three markers can be observed. In control gradients, alkaline phosphatase peaked at a lower buoyant density (1.12 g/cm^3) in mid-luteal tissue density gradients than in early and late luteal tissue gradients (1.18 and 1.17 g/cm^3 respectively). Digitonin perturbation was similar in early and mid-luteal gradients. However, in late luteal tissue gradients, buoyant density was decreased by digitonin. The reasons for the differences between the buoyant

densities and effects of digitonin at different stages of the luteal phase are unclear. However, a possible suggestion may be that as the luteal phase proceeds, the demand for cholesterol to serve as precursor for steroidogenesis may lead to cholesterol being removed from membranes, as has been suggested for the mechanism of action of SCP2 (sterol carrier protein 2) (Strauss & Miller, 1991). Thus, at times of maximal steroidogenic activity, for example during the mid-luteal phase, membranes may be cholesterol-depleted and would thus equilibrate at a lower buoyant density than cholesterol rich membranes such as those found at the early luteal phase. In late luteal tissue it would appear likely, from the distribution of alkaline phosphatase and the other plasma membrane markers used, that membrane integrity was lost, and degenerative processes associated with luteolysis may have commenced. Indeed, alkaline phosphatase was detected in significant amounts in the upper part of late luteal tissue sucrose density gradients, and was unperturbed by digitonin, indicating that it was no longer associated with a (cholesterol rich) membrane.

The distribution of LH receptor binding follows a very similar pattern to that displayed by alkaline phosphatase in early and mid-luteal tissue gradients. Levels of digitonin perturbation were also similar in both plasma membrane markers. LH receptor numbers were highest during the mid-luteal phase in sheep (Niswender *et al*, 1985a). The results presented here would appear to agree with ovine data, with highest levels of LH binding found in density gradients of mid-luteal tissue. LH binding was very low in density gradients of late luteal tissue, most likely as a consequence of luteolytic changes. The disparity observed between the buoyant densities of alkaline phosphatase/LH receptor/5'-nucleotidase may be due to differences in the

cell types of the corpus luteum in which they are located. It is unclear which cell type (large or small luteal cells) possessed alkaline phosphatase and 5'-nucleotidase activity (or both), and whether the plasma membranes of luteal small and large steroidogenic cells are of similar density. Whether or not large luteal cells contain plasma membrane LH receptors is also unclear, though recent reports appear to indicate the likelihood that both luteal cell types do indeed possess the LH receptor in the bovine corpus luteum (Chegini *et al*, 1991). Thus it may be that the LH receptor is only a marker of theca-lutein cells. Such differences in the buoyant densities of plasma membrane markers have also been suggested to be due to (i) different regions of the plasma membrane having different densities, and (ii) resealing of membrane vesicles produced during homogenization,which may have different buoyant densities (Bramley & Ryan, 1978 b).

The optimum pH determined for total and fluoride-sensitive acid phosphatase was pH 4.7. This was similar to the pH maximum of fluoride-sensitive acid phosphatase in rat ovarian tissue (pH 5.2). In the rat, fluoride-insensitive acid phosphatase activity displayed an optimal activity at pH 6.5 (Bramley & Ryan, 1978a). However, no such difference in optimum pH between the two forms was observed in bovine luteal tissue. Indeed, in the case of the bovine enzyme, activity was maximum from pH 4.4 - 5.0, whilst beyond pH 5.0 activity decreased. No stimulatory activity of metal ions was recorded, though inhibition occurred in the presence of zinc, manganese and calcium ions. Distribution of acid phosphatase was similar at all stages of the luteal phase, as was the bouyant density shift induced by digitonin perturbation. Activity was recorded between 1.15 - 1.17 g/cm^3 in control density gradients, similar to the buoyant densities of this enzyme found in

the sheep and pig corpus luteum, and similar to the peak found in the human corpus luteum (Bramley & Menzies, 1986).

Cytochrome oxidase activity was demonstrated to be minimal below pH 3. Optimum activity was recorded in all bovine luteal homogenates tested at pH 6.8. This was lower than the optimum pH of this enzyme in the rat ovary, which peaked at pH 8.4, though the pH optimum was lowered to pH 6.2 in the presence of phosphate buffers (Bramley & Ryan, 1978a). None of the metal ions tested caused any change in activity. The distributions of cytochrome oxidase in early, mid- and late luteal tissue sucrose density gradient fractions were similar, displaying buoyant densities of 1.16, 1.15 and 1.12 g/cm^3 respectively. Digitonin perturbation was similar in early and mid-luteal fractions, but slightly reduced in fractions of late bovine corpora lutea. Nonetheless, cytochrome oxidase activity increased in buoyant density in response to digitonin in all gradients assayed. In sucrose density gradient fractions of the ovine and porcine corpus luteum, the buoyant density of cytochrome oxidase activity was relatively unaffected by digitonin pretreatment (Bramley & Menzies, 1986; 1988a; c). Thus, bovine mitochondrial membranes may contain higher levels of unesterified cholesterol than the mitochondria of other species.

Activity of N-acetyl-ß-D-glucosaminidase was maximal at pH 5.8, and was considerably enhanced in the presence of magnesium. Lysosomes, represented by the activity of N-acetyl-ß-D-glucosaminidase, equilibrated at a buoyant density of 1.17 g/cm^3. As has been observed in the corpora lutea of sheep and pigs (Bramley & Menzies, 1986; 1988), digitonin pretreatment of bovine luteal homogenate caused the activity of N-acetyl-ß-D-

glucosaminidase to equilibrate at the top of sucrose gradients, ie. in cytosolic fractions, due to detergent (digitonin) permeabilisation of the lysosomal membrane, and release of intralysosomal contents.

DNA sedimented at the bottom of sucrose density gradients, as has been previously observed (Bramley & Menzies, 1986; 1988). Unlike the sheep and pig corpus luteum, no NADPH cytochrome c reductase activity was observed in the bovine corpus luteum. Whether this enzyme was indeed absent from the bovine CL is unclear. However, under a range of conditions suitable to demonstrate activity of this enzyme in other species, no activity could be recorded. NADH cytochrome c reductase activity on the other hand was readily measurable in the bovine CL, and demonstrated to be optimal at pH 7.0. In the rat ovary, this enzyme was considered to be located in the mitochondrion. However, in the human, ovine and porcine corpus luteum, it was associated with the smooth endoplasmic reticulum, or a subset of smooth endoplasmic reticulum vesicles. It has been demonstrated in the ovine corpus luteum that two forms of this enzyme were present, a lighter form whose buoyant density was slightly decreased by digitonin, and a more dense form that was unaffected by digitonin treatment (Bramley & Menzies, 1988). This dense form of activity was considered to be of mitochondrial origin. In the density gradients assayed in this study, digitonin increased the buoyant density of NADH cytochrome c reductase. Activity peaked at 1.14 - 1.15 g/cm^3 in control gradients, similar to the buoyant density of ovine mitochondrial NADH cytochrome c reductase (Bramley & Menzies, 1988). In digitonin pretreated gradients increases in the buoyant density of NADH cytochrome C reductase were somewhat inconsistent; in some gradients the shift was greater than others. However,

in those gradients which displayed a large shift in the buoyant density of NADH cytochrome C reductase no increased shift in the buoyant density of progesterone was observed, suggesting that perhaps the two were not associated directly. However, it was clear that on the basis of digitonin induced increases in buoyant density, the locations of cytochrome oxidase and NADH cytochrome c reductase were distinct. Also, cytochrome oxidase activity displayed a much sharper peak of activity than did NADH cytochrome c reductase. More work is required to elucidate whether or not NADH cytochrome c reductase is of use as a marker of the endoplasmic reticulum in the bovine corpus luteum, but since cytochrome oxidase has been localised to the mitochondrion, it would appear unlikely that the activity of NADH cytochrome c reductase is mitochondrially derived, from the two enzymes' distinct behaviour in sucrose gradient fractions.

Of great interest was the location of endogenous progesterone in sucrose density gradients of the bovine corpus luteum. Though a significant amount of progesterone was found in the cytosolic fractions, the main peak of progesterone equilibrated in a broad peak, spanning the buoyant density range 1.10 - 1.16 g/cm^3. Its distribution showed a similar pattern to that of the distributions of plasma membrane markers and NADH cytochrome c reductase. However, in all cases, progesterone equilibrated at slightly lower buoyant densities than NADH cytochrome c reductase. Moreover, digitonin induced an increase in the buoyant density of progesterone, demonstrating progesterone to be associated with particulate material containing unesterified cholesterol. Associations between cellular membranes and progesterone secretion have been previously suggested. Rice *et al* (1986) reported that associations between progesterone and the plasma membrane

of sheep luteal cells represented non-specific intercalation of progesterone into the membrane. Indeed, the accepted theory of progesterone secretion is that due to its hydrophobic nature, it simply diffuses across the luteal cell plasma membrane (Enders, 1973). Thus, the location of progesterone in sucrose density gradients may be simply non-specific intercalation into the luteal plasma membrane as it diffuses across. However, it would be expected that under the conditions of this study that intercalation could occur into all other cellular membranes also. Since this was clearly not the case, and work in other species has demonstrated specific, membrane association of progesterone (Bramley & Menzies, 1986; 1988 a; b; 1994), the subcellular location of progesterone may be of importance. Others have demonstrated the existence of specific binding proteins for progesterone in the bovine corpus luteum (Willcox & Thorburn, 1981; Willcox & Alison, 1982; Willcox, 1983). However, since these proteins were located in the cytosol of luteal cells, binding to these proteins cannot account for association of endogenous progesterone with a particulate membrane fraction. Since the theory of progesterone secretion has not gone unchallenged, it was decided to investigate further the nature of the interaction between progesterone and cellular membranes (see Chapter 3).

The intracellular locations of the different steroidogenic enzymes has been well documented. The accepted subcellular locations of the main steroidogenic enzymes have been determined by differential centrifugation, followed by identification of enzymatic activity for each particular steroidogenic enzyme, often in conjunction with electron microscopy analysis of the fractions obtained (see Lieberman & Prasad, 1990, for review). The accepted location of the cholesterol side chain cleavage system, which

catalyses the formation of pregnenolone from cholesterol, is the inner mitochondrial membrane (Strauss & Miller, 1991). However, early studies demonstrated the absence of this enzyme in mitochondrial fractions (Lynn *et al*, 1954; 1955). From the results presented here, it is clear that P_{450} scc is found in very small quantities in the cytosol, and in largest quantities in the fractions thought to contain the endoplasmic reticulum and mitochondrial material. It is unclear from these experiments whether or not this is simply a case of contamination of microsomal fractions with mitochondria (since microsomal preparations may also contain inner and outer mitochondrial membranes), or whether in other organelles, besides mitochondria, side chain cleavage enzyme may be located. Incubation of 20-methyl-5-pregnen-3ß-ol with adrenal microsomes has been shown to result in the production of corticosterone (Tait & Hodge, 1985). This conversion requires a number of enzymatic activities, one essential process being the cleavage of the side chain from the substrate. Thus, given current understanding of the enzymatic production of steroids from sterol precursors, there is evidence to suggest that P_{450} scc may be located in either the endoplasmic reticulum and/or the mitochondrion.

Bovine cholesterol side chain cleavage has been identified as a 49,000 Da protein (Rodgers, 1986a, Young *et al*, 1995). In studies where antibodies have been used to detect the presence of P_{450} scc by immunoblotting, positive results in the corpus luteum and follicles of cows have confirmed this size (Rodgers *et al*, 1986a; b; c). In this study, a band of approximately 49,000 Da was detected. However, other bands of different sizes were also evident. Recently, it has been demonstrated in the bovine corpus luteum that cross-linking between P450scc and its electron donor adrenodoxin occurs (Young

et al, 1995). The formation of the 1:1 covalent, non-disulphide-linked cross-linked protein is thought to be counteracted by the presence of anti-oxidants such as ß-carotene present in the bovine corpus luteum (Young *et al*, 1995). The molecular weight of the cross-linked product is 63 kDa (Young *et al*, 1995). In this study, bands of approximately 65 kDa were evident. It would appear that this band is likely to be P_{450} scc cross-linked to adrenodoxin. The bands of 46 kDa and 80 kDa that were detected may be artifactual in nature, due to either the use of an antibody raised against ovine P_{450} scc, or due to incomplete reduction/dissociation prior to electrophoresis. Thus, it is not possible to say with certainty whether bands other than that at 49,000 Da are the result of isoforms of P_{450} scc modified by post-transcriptional modifications, or due simply to non-specific antibody binding. From experiments performed with antibodies raised against purified bovine P_{450} scc, it appears that the cow corpus luteum contains only one form of P_{450} scc (Rodgers *et al*, 1986). It has been demonstrated that humans possess only one gene coding for P_{450} scc, but that the protein is made in an immature form which is larger than the active enzyme, and then transported to the mitochondria where it is activated by a specific endoprotease (Matocha & Waterman, 1985). The higher molecular weight bands observed here may therefore represent immature, inactive bovine P_{450} scc.

No P_{450} 17αhydroxylase/C17,20 lyase was observed in immunoblots of sucrose density gradient fractions of bovine corpora lutea. The antibody used was raised against the bovine form of this enzyme, and indeed was shown to blot positively in control tissues (bovine theca cell homogenate). This is in agreement with results obtained by others using different antibodies. Rodgers *et al*, (1986c), observed that the P_{450}

17αhydroxylase/C17,20 lyase content of bovine follicles increased with follicular development then was dramatically reduced to below detection levels in corpora lutea. Levels of steroidogenic enzymes tend to reflect the steroidogenic activity of the tissue examined (Rodgers *et al*, 1986c). Since it is this enzyme that provides the substrate for the aromatase enzyme in the preovulatory follicle, it would appear that the loss of P_{450} 17αhydroxylase/C17,20 lyase from the bovine corpus luteum is the critical factor in the inability of the cow corpus luteum to form oestradiol. Indeed, mRNA levels for both P_{450} 17α hydroxylase and P_{450} aromatase have been observed to fall dramatically after the LH surge in cattle (Voss & Fortune, 1993).

The accepted intracellular location of 3ß-hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase (3ß-HSD) is the endoplasmic reticulum. In the human corpus luteum it has been localized to the perimitochondrial endoplasmic reticulum (Strauss & Miller, 1991). The reported size of bovine 3ß-HSD is 42 kDa (Couet *et al*, 1990). In the results presented here, a band of approximately 43 kDA was evident in fractions enriched in mitochondrial and microsomal enzyme activities. It would appear likely from the molecular size that this band was 3ß-HSD. Another band of approximately 65 kDa was evident in all fractions. It is possible that SER vesicles could have formed during homogenization of the luteal tissue, thus giving rise to the almost uniform distribution of this protein in the fractions immunoblotted in this study, however it is not known whether or not this protein is related to 3ß-HSD. Hawatashi *et al*, (1985) have suggested the existence of a number of isoforms of 3ß-HSD, and previous studies in the bovine ovary have indicated the presence of up to 4 bands of different sizes in the bovine corpus luteum (Couet *et al*, 1990). However, care must be exercised in the interpretation of

the results of this latter study, since, as in the experiments reported here, bovine tissue was blotted using antisera raised against the human form of the protein and the homology between the two forms is only 79% (Couet *et al*, 1990). In these studies, the heaviest protein bands equilibrated at approximately 43 kDa and 65 kDa. Previous studies have also noted the existence of a larger protein, of approximately 68 kDa (Couet *et al*, 1990) in the bovine corpus luteum. It is likely that the 65 kDa band detected in these experiments is the same protein that others have identified as the 68 kDa band. It is not known whether or not this larger band could represent cross-linking of the 3β-HSD to another steroidogenic component such as an electron transport protein. However, Young *et al* (1995) suggest that this may possibly occur by oxygen radical damage with steroidogenic enzymes other than P450 scc. Though the existence of multiple forms of this enzyme has been suggested (Gibb & Hagerman, 1976), it is not known whether these are substrate or tissue specific (Strauss & Miller, 1991).

It was clear, however, that progesterone was not associated with the steroidogenic enzymes immunoblotted in this study. Moreover, since these enzymes represent mitochondrial and microsomal proteins, the exclusive association of these organelles with endogenous progesterone does not appear likely, in agreement with observations from experiments involving organelle marker assays. The mechanism of progesterone release from the luteal cell has not yet been demonstrated convincingly to be either active, or passive diffusion across luteal membranes; together with the possibility of intracrine effects of progesterone on luteal cells, the phenomenon of the association of progesterone with material of membrane origin was investigated further in following chapters.

Chapter 3

Investigation of association between progesterone and luteal membranes

3.1 Introduction

In the previous chapter it was demonstrated that progesterone equilibrated in a broad peak spanning the buoyant density 1.10 - 1.14g/cm^3 in a 10 - 50% (v/v) sucrose gradient. Whilst progesterone could not be unequivocally localized to one of the major subcellular organelles, its buoyant density was increased by the saponin, digitonin. Since digitonin does not interact directly with progesterone, this suggested that the progesterone was tightly associated with a membrane-bound component that was affected by digitonin.

Previous studies on steroid binding in the porcine (Bramley & Menzies, 1988a; 1993; Menzies & Bramley, 1994), equine (Bramley *et al*, 1995) and ovine (Bramley & Menzies, 1988c; 1994) corpus luteum have demonstrated specific binding sites for [^{3}H]-progesterone. After incubation of luteal homogenates with labelled progesterone, and fractionation on continuous sucrose gradients, fractions which accumulated radiolabelled progesterone also contained the majority of endogenous progesterone present. This fraction appeared to be associated in those species with NADH-cytochrome C reductase activity, but not with other endoplasmic reticulum markers (Bramley & Menzies, 1988a; 1993; 1994). Although other workers have described progesterone binding

activity in the bovine corpus luteum, binding was found in the luteal cytosol preparations (Willcox & Thorburn, 1981; Willcox & Alison, 1982; Willcox, 1983). Binding of progesterone to a protein was thought to be a mechanism whereby progesterone could be sequestered in a secretory granule (Willcox & Thorburn, 1981; Willcox & Alison, 1982; Willcox, 1983). Others have attempted to demonstrate the existence of progesterone in a secretory granular bound form (Gemmell *et al*, 1974; Gemmell & Stacy, 1977; 1979; Quirk *et al*, 1979; Sawyer *et al*, 1979; Gemmell *et al*, 1983). These studies were fuelled by the demonstration of a luteal population of electron dense single-membrane-bounded granules of about 250 nm in diameter which were morphologically and cytochemically distinct from other cell organelles (Quirk *et al*, 1979; Parry *et al*, 1980; Paavola & Christensen, 1981). However, subsequent studies demonstrated these granules contained only oxytocin, not progesterone (Rice & Thorburn, 1985; Rice *et al*, 1986).

The mechanism of secretion of progesterone remains enigmatic. A few isolated studies based on ultrastructural observations have suggested (a) that steroid may be transported in lipid droplets encased in smooth endoplasmic reticulum (Rhodin, 1971) or, (b) transported within canilicular endoplasmic reticulum (Yoshimura *et al*, 1968). However, these have gained little acceptance and the mechanism of progesterone secretion reported in most textbooks which address this topic ascribe secretion to simple passive diffusion down a concentration gradient from the cell in which it was synthesised, according to the general steroid secretion mechanism proposed some years ago (Enders, 1973). Indeed, the

ability of progesterone to intercalate into cholesterol-containing phospholipid bilayers has been demonstrated (Carlson *et al*, 1983).

The scope of the work in this chapter was therefore to ascertain (i) whether or not the subcellular fractions of the bovine corpus luteum which sequestered endogenous progesterone could also bind exogenous progesterone and, if so (ii) to examine the characteristics of progesterone binding further to elucidate the physiological role of these binding sites.

3.2 Materials & Methods

3.2.1 General materials

All substrates, fine chemicals, inhibitors, reagents and unlabelled steroids were purchased from Sigma Chemical Co., Poole, Dorset, U.K., from BDH, Poole, Dorset, U.K. or from Amersham International plc, Amersham, Bucks, U.K. Epostane (Stirling-Winthrop), the 3ß-hydroxysteroid dehydrogenase inhibitor was a gift from Dr. R. Webb, Institute of Animal Physiology & Genetic Research, Roslin, Midlothian, and the progesterone receptor antagonist (RU 38486) was the gift of Dr. K. J. Thong, Department of Obstetrics & Gynaecology, The University of Edinburgh.

3.2.2 Tracers

Radiolabelled steroids used were: [1,2,6,7-^{3}H]-labelled progesterone (100 Ci/mmol), [1,2,6,7-^{3}H] testosterone (80 Ci/mmol) from Amersham International plc, Bucks., U.K. and [7-^{3}H] pregnenolone (10 Ci/mmol) from New England Nuclear Research Products, Du Pont (U.K.) Ltd., Stevenage, Herts, U.K.

3.2.3 Ovarian Tissue

Ovaries were recovered from presumed non-pregnant cows slaughtered at a local abattoir, and transported to the laboratory in ice-cold phosphate buffered saline (PBS; sodium chloride (0.15 mol/l) in sodium phosphate (10mmol/l; pH 7.4) within 1 hour of death. The stage of the luteal phase was assessed by gross morphology (Figure 2.1), and corpora lutea were dissected free from connective tissue and stroma, pooled according to their assigned stage (early, mid-and late luteal phase), minced with

scissors and homogenized in ice-cold sucrose (0.3mol/l)/ EDTA (1mmol/l)/Tris-HCl (10 mmol/l), pH 7.4 (SET medium; 200 mg tissue/ml) using a Polytron homogenizer (Kinematica, Lucerne, Switzerland) with two 10-s bursts at full speed, interspersed with a 1-min cooling period on ice. Homogenates were then filtered through two layers of cheesecloth.

3.2.4 Sucrose density gradient fractionation

Continuous sucrose density gradients (30 ml; 10-50 % w/w) were prepared by the method of Stone (1974) (see Chapter 2). Aliquots of homogenate (2.5 ml) were mixed with either 0.5 ml SET medium (controls) or 0.5 ml SET medium containing digitonin (10 mg/ml) and to both controls and digitonin-treated gradients, 100,000 cpm of radiolabelled steroid, [^{3}H]-pregnenolone, [^{3}H]-progesterone, or [^{3}H]-testosterone was added. To establish the *in vitro* progesterone binding assay to gradients of early, mid- and late luteal tissue, no radiolabelled steroid was added. After a 30 min incubation on ice, 2.5 ml aliquots were gently layered over the sucrose density gradients and centrifuged at 30,000 g_{av} for 2h in a Sorvall VTR 50 vertical tube rotor (4^{o}C). Each gradient was then fractionated using a Buchler-Searle Autodensiflo gradient fractionator equipped with a meniscus-sensitive probe, and fractions (1ml) frozen at -20^{o}C until assay. In one representative experiment, fractions were assayed for progesterone binding before and after freezing : no significant differences were observed (data not shown). Where radiolabelled steroid was added, 250µl aliquots of 1 ml fractions collected from these gradients were immediately mixed with 4 ml of scintillant fluid (Ecoscint A, National

Diagnostics, Atlanta, Georgia) in scintillation vials, then counted for tritium using an LKB Rackbeta II scintillation counter.

Aliquots of sucrose gradient fractions were assayed for progesterone binding activity, endogenous progesterone content (by radioimmunoassay), protein content and organelle marker enzyme activities. Pools of fractions enriched in progesterone binding activity were stored at -20^{o}C for further analyses.

3.2.5 Assays

Measurement of the sucrose content of density gradient fractions was performed using an Abbe refractometer (Atago, Japan). Protein was measured by the method of Lowry *et al* (1951) using bovine serum albumin (BSA) as a standard.

Marker enzyme activities specific for the major subcellular organelles and luteal plasma membranes in the bovine corpus luteum were measured using previously described optimised assays (Chapter 2).

Progesterone content of sucrose density gradient fractions was determined as described in Chapter 2.

3.2.6 Steroid binding to bovine luteal fractions *in vitro*

Aliquots (5-50µl) of bovine luteal fractions from sucrose gradients were incubated at 4°C for two hours (except where indicated) in a system containing 40 mmol/l Tris-HCl (pH 7.4), 0.1% (w/w) BSA, [^{3}H]-progesterone (or other steroid where indicated) (50,000 cpm) and digitonin (250 µg/tube, except where indicated otherwise), with a final incubation volume of 0.5 ml. Bound tracer was separated from free by adding 1.0 ml of dextran-coated charcoal (DCC) (2.5g activated charcoal, (250-350 mesh) (Sigma) and 0.25g Dextran-T (Sigma) in Tris-BSA assay buffer). Tubes were centrifuged at 3000g for 10 min (4°C), then supernatants decanted and mixed with 4ml scintillant, shaken and counted for tritium by liquid scintillation spectrometry. Tubes with no membranes but with digitonin, and tubes with membranes but no digitonin were routinely included as controls. In binding specificity experiments, steroids were dissolved in ethanol, added to the assay tubes, then dried down at room temperature under a stream of nitrogen. Assay buffer was then added and the tubes vortexed vigorously (30 sec) before proceeding with the addition of the remainder of the assay cocktail and the normal incubation. Where other substances (such as cofactors, other saponins, etc.) were added to the assay, these were dissolved in assay buffer (Tris-BSA), and the incubation volume maintained at 0.5 ml. Where low solubility of particular test substances necessitated dissolving in ethanol prior to addition to the assay, control tubes containing an equal volume of ethanol alone were included.

3.2.7 HPLC analysis

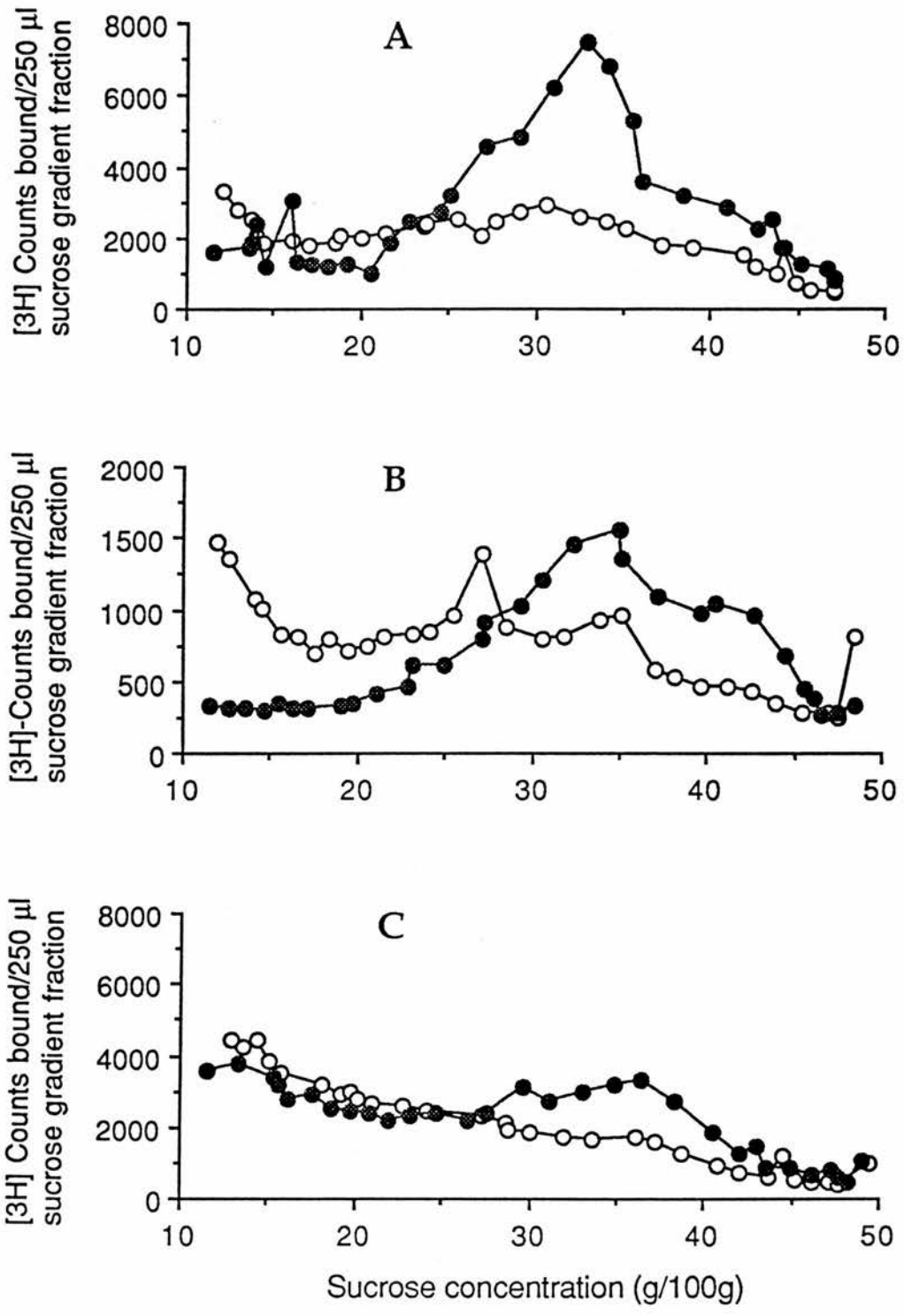
Bound radiolabelled progesterone was extracted from the binding site by mixing 1ml bound fraction (DCC supernatant, taken after binding equilibrium was reached) with 2.5 ml petroleum ether and vortexing vigorously for 10 minutes. The resulting emulsion was then centrifuged at 2000 rpm, (4°C) for 10 minutes. The supernatant was decanted and placed in a dry-ice/alcohol freezing solution. The solvent was decanted from the frozen aqueous phase and dried down under a stream of nitrogen before reconstitution in 100 µl of absolute ethanol. The column used was a reverse-phase Bio-Rad Hi-pore column (250 x 4.6 mm). Solvent (acetonitrile/H_20) was run isocratically (1 ml/min) until all substances has been cleared from the column (ca. 30 min). As standards, [^{3}H]-progesterone and unlabelled progesterone were also run.

3.3 Results

3.3.1 Fractionation of bovine luteal homogenates preincubated with steroid tracer (Figure 3.3.1)

Fractionation of bovine luteal homogenates on control gradients in the absence of digitonin indicated little binding of [^{3}H]-progesterone, [^{3}H]-pregnenolone and [^{3}H]-testosterone (Fig. 3.3.1, A, B & C). However, preincubation of tracer with homogenates in the presence of digitonin increased the amount of progesterone (Figure 3.3.1 A) and pregnenolone recovered in the gradient (Figure 3.3.1 B), with both steroids displaying a broad peak of binding, equilibrating at a buoyant density of 1.10 - 1.14 g/cm^3. In the absence of digitonin, binding of [^{3}H]-testosterone was low, and did not display the peak observed for progesterone and pregnenolone (Figure 3.3.1 C), even in the presence of digitonin. The effect of digitonin on steroid binding was more pronounced with progesterone than with pregnenolone. In the absence of digitonin, little steroid tracer entered the gradient; however, with digitonin present, increased amounts of all three steroid tracers entered the gradients (though no specific binding was apparent in the case of testosterone). A secondary effect of digitonin was that it appeared to sharpen the peak of [^{3}H]-progesterone binding (Figure 3.3.1 A). Binding of [^{3}H]-progesterone was consistently greater than [^{3}H]-pregnenolone binding for a number of different homogenates treated in this manner (n=4).

Figure 3.3.1 Fractionation of bovine luteal homogenate incubated with radiolabelled steroids in the presence (•) and absence (o) of digitonin. A = luteal homogenate incubated with [^{3}H]-progesterone, B = luteal homogenate incubated with [^{3}H]-pregnenolone, C = luteal homogenate incubated with [^{3}H]-testosterone.


A
B
C
[3H] Counts bound/250 µl sucrose gradient fraction
[3H]-Counts bound/250 µl sucrose gradient fraction
[3H] Counts bound/250 µl sucrose gradient fraction
8000
6000
4000
2000
0
2000
1500
1000
500
0
10
20
30
40
50
Sucrose concentration (g/100g)

3.3.2 Steroid binding to bovine luteal fractions *in vitro*

3.3.2 (i) Protein concentration

In the absence of digitonin, [^{3}H]-progesterone binding remained at non-specific levels, even when high concentrations of luteal membranes were added to the assay. However, in the presence of digitonin binding of [^{3}H]-progesterone increased linearly, reaching a plateau at high membrane concentrations (Figure 3.3.2).

3.3.2 (ii) Effects of digitonin

In the absence of digitonin, binding of both progesterone and pregnenolone tracers remained low. Binding of progesterone increased markedly with increasing digitonin content, but only in the presence of luteal membranes (Figure. 3.3.3 A), reaching a plateau at a digitionin concentration of 0.25 mg/tube. [^{3}H]-pregnenolone binding also increased with increasing digitonin concentrations in the absence of membranes (Figure 3.3.3 B). However, binding was always much greater in the presence of membranes, reaching a plateau at lower digitonin concentrations (30 - 40 μg/tube) than was required in the case of progesterone (0.25 mg/tube). Thus the remainder of these studies were conducted using [^{3}H]-progesterone.

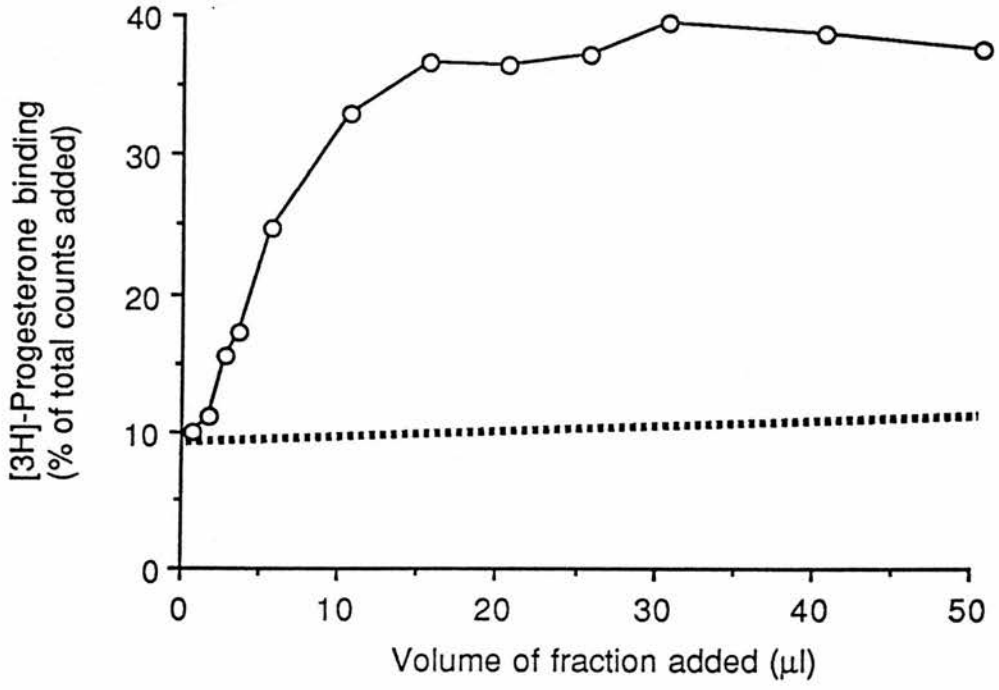
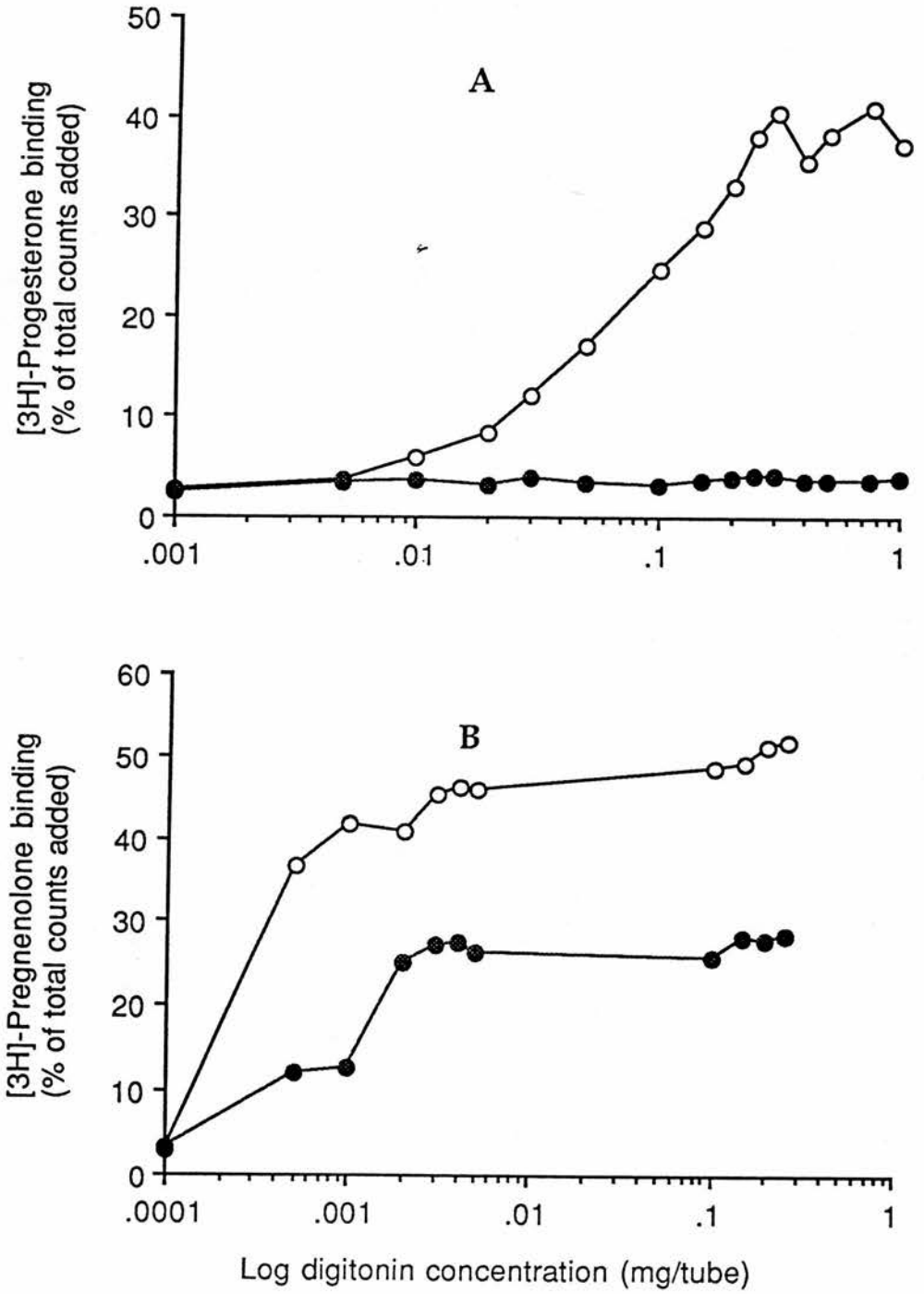


Figure 3.3.2 [^{3}H]-progesterone binding to increasing concentrations of bovine luteal homogenate sucrose gradient fraction pooled from fractions enriched in progesterone binding activity. Binding of progesterone was detectable only in the presence of digitonin. In the absence of digitonin, binding did not increase beyond non-specific levels (heavy dotted line).

Figure 3.3.3 Effects of increasing digitonin concentration on [^{3}H]-progesterone and [^{3}H]-pregnenolone binding to pooled sucrose gradient fractions enriched in progesterone binding activity. A = progesterone binding. Binding of [^{3}H]-progesterone was low (ca. 4%) in the absence of digitonin. B = pregnenolone binding, in the presence of sucrose gradient fraction (o) and in the absence of sucrose gradient fraction (●). The above figure displays data representative of two such sets of experiments. Similar results were obtained using two different bovine corpus luteum homogenates.


A
[3H]-Progesterone binding
(% of total counts added)
50
40
30
20
10
0
.001
.01
.1
1
B
[3H]-Pregnenolone binding
(% of total counts added)
60
50
40
30
20
10
0
.0001
.001
.01
.1
1
Log digitonin concentration (mg/tube)

3.3.2 (iii) pH of incubation

Progesterone binding to bovine luteal membranes was found to be dependent on the pH of the incubation cocktail. Binding of progesterone to membranes was absent below pH 4.5, but increased sharply with increasing pH, reaching a plateau above pH 6.5 (Figure 3.3.4). Again, only non-specific levels of binding (4-6%) were found in the absence of digitonin over the pH range tested.

3.3.2 (iv) Duration and temperature of incubation

Radiolabelled progesterone binding to bovine luteal membranes increased rapidly at all temperatures tested (4°C, 20°C, 37°C) reaching a maximum at 4°C after 90 minutes (Figure 3.3.5). These levels were reached in the first 10 minutes of incubation at 20°C and 37°C. However, binding began to fall after this time point at 37°C, indicating a possible loss of binding sites at this temperature. Once again, in the absence of either digitonin or bovine luteal membranes, [^{3}H]-progesterone binding did not exceed non-specific levels (data not shown).

3.3.3 Specificity of bovine luteal progesterone binding sites

Unlabelled progesterone displaced the binding of tritiated progesterone at low levels (IC_{50}, 63 nM) (Figure 3.3.6). Testosterone was 100-fold less active. Pregnenolone displaced [^{3}H]-progesterone binding only at micromolar concentrations, reaching half-maximal displacement only at the maximum concentration tested (200 µg/ml final assay concentration). Other steroids such as oestradiol did not displace binding significantly. Steroid precursors such as cholesterol, inhibitors of steroidogenic enzymes (epostane and ketoconazole - inhibitors of the 3ß hydroxysteroid

dehydrogenase and C17,20-lyase enzymes respectively) and the potent progesterone receptor antagonist RU 486 did not compete for binding sites with the progesterone tracer. (Figure 3.3.6 and Table 3.1)

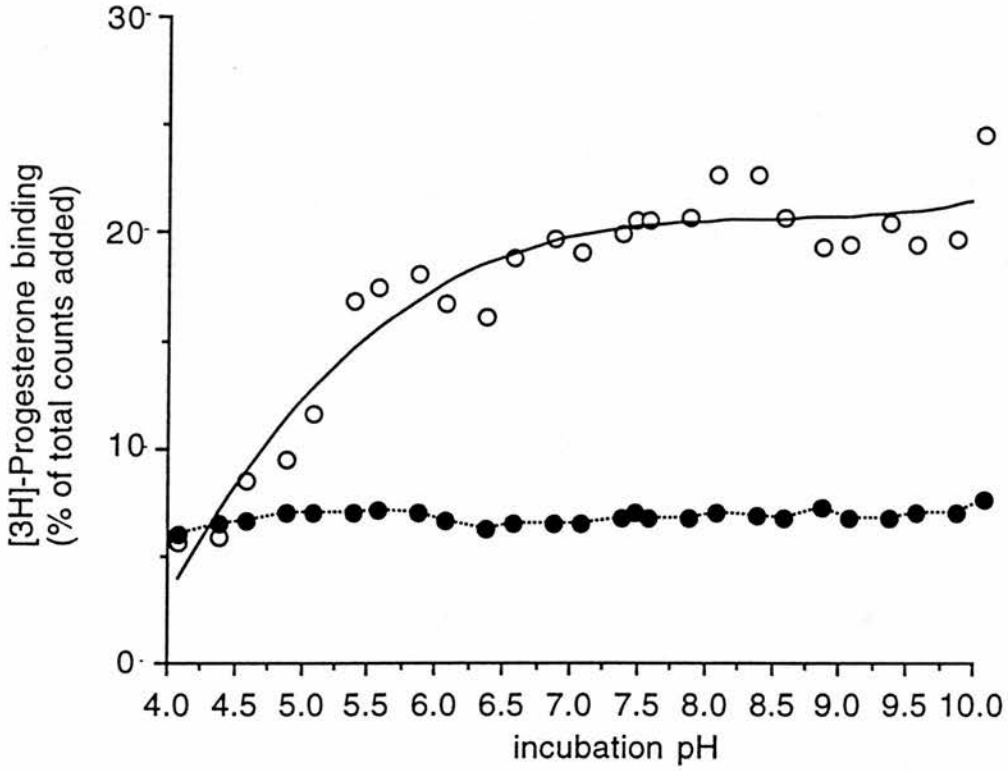


Figure 3.3.4 Determination of optimal pH for progesterone binding activity. (o) = in the presence of sucrose gradient fraction (10µl), (●) = no sucrose gradient fraction added. Binding was measured in the presence of digitonin (0.25 mg/tube). In the absence of digitonin, binding remained at non-specific levels (5 - 6 %) at all pH intervals tested (data not shown).

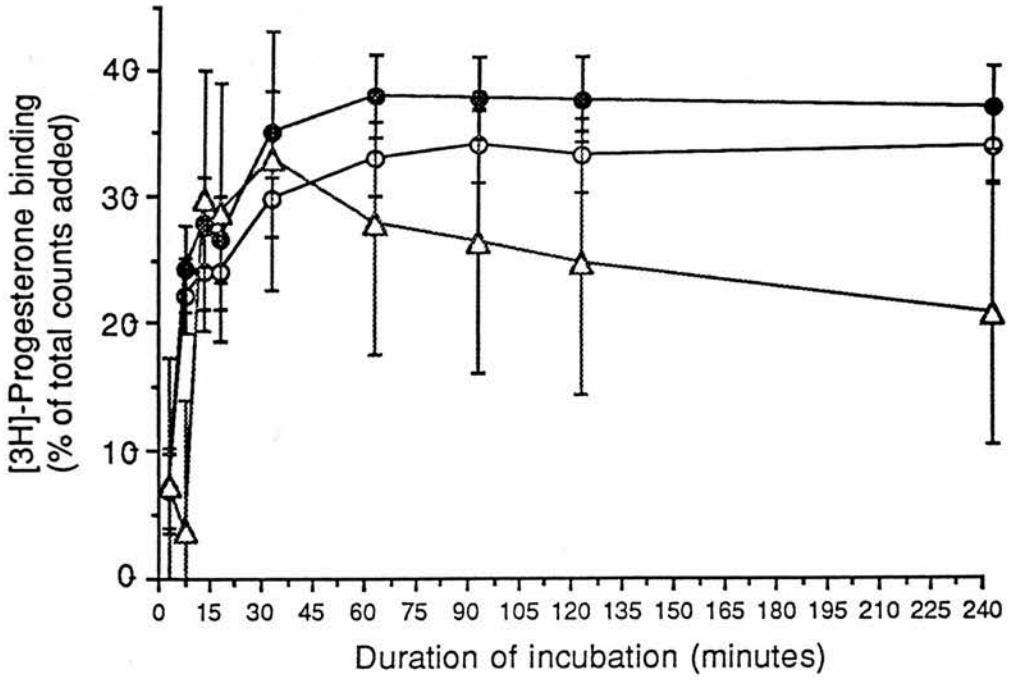


Figure 3.3.5 Effects of duration and temperature of incubation on progesterone binding to pooled sucrose gradient fractions (10 µl) enriched in progesterone binding activity. (•) = 4°C, (o) = 20°C, (Δ) = 37°C. Graph shows mean binding for each time and temperature of incubation tested, ± S.E.M. (n=3).

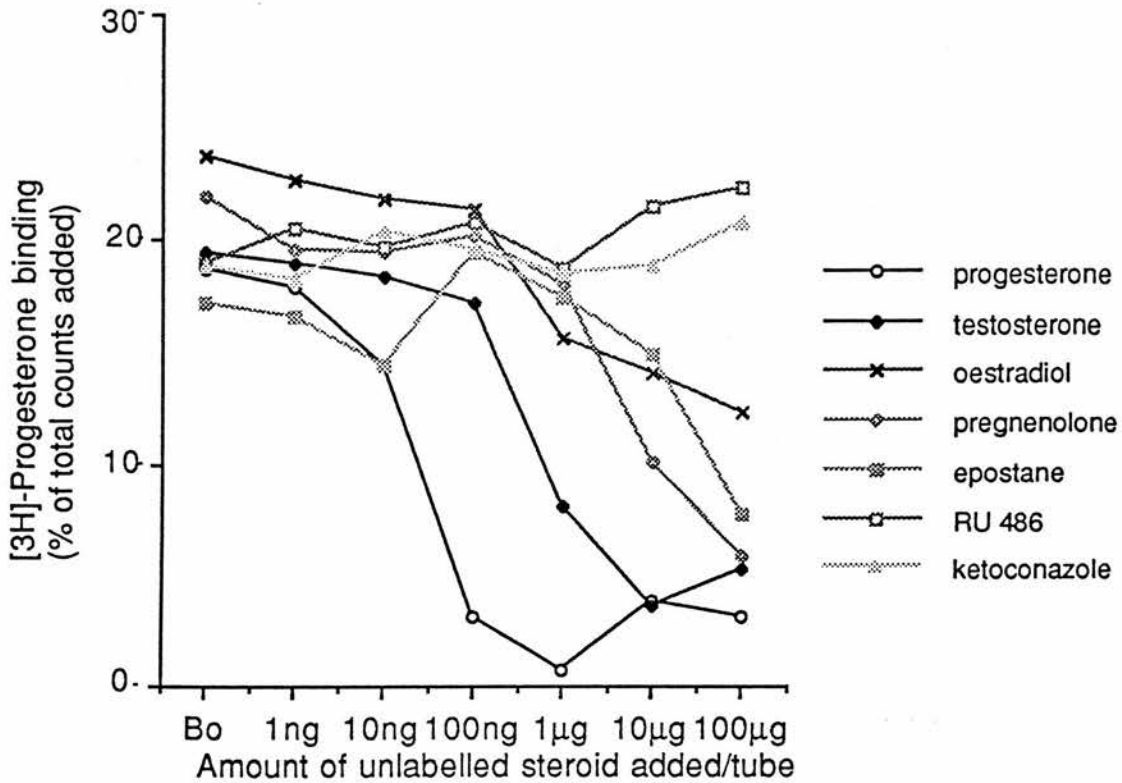
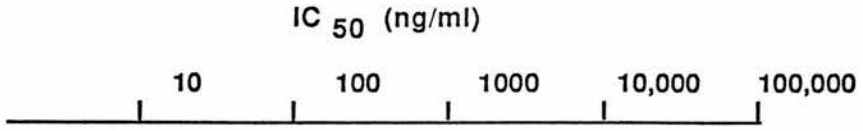


Figure 3.3.6 Displacement of [^{3}H]-progesterone binding by progesterone and other steroids, receptor antagonists and steroid metabolizing enzymes. Bo = no added unlabelled steroid. Each unlabelled steroid was assayed in triplicate, and each displacement experiment repeated using three different homogenates. For clarity, only a selection of data is displayed in the above figure, without error bars, since each membrane preparation used in these experiments gave similar results with respect to displacement of bound [^{3}H]-progesterone. Differing Bo values are due to different membrane preparations used for the above data sets. For full description of displacement potencies see Table 3.1.



Steroid:

Progesterone

20 α OH progesterone
20 β OH progesterone

DHA
17 OH progesterone
Δ4 Androstene 3-17 dione
Testosterone

DHT
Pregnenolone

Oestrogens
Oestrone-3-sulphate
Oestradiol

Progestagens
Pregnanediol
RU 486
17 α OH preg-
-nenolone
R5020

Adrenal Steroids
Corticosterone
Deoxycortico-
-sterone

Substrates
Cholesterol

Inhibitory agents
Ketoconazole
Epostane
ORG 2058
ouabain
ANS

Table 3.3.1 Potencies of steroids, precursors and inhibitory agents for [^{3}H]-progesterone binding sites in bovine corpus luteum sucrose gradient fractions.

A number of progesterone analogues were tested for their abilities to displace the binding of radiolabelled progesterone. The only progesterone analogues tested that showed displacement similar to that of progesterone were 20α-hydroxyprogesterone and 20ß-hydroxyprogesterone. Alterations on the C3 - C11 side of the molecule resulted in a reduction in potency, suggesting that these positions may be important in the specificity of the binding site for progesterone. Indeed, there was no observable binding of iodinated progesterone (the iodinated form of progesterone tested had the ^{125}I attachment at C11) (Figure 3.3.7).

3.3.4 Subcellular localization of bovine luteal progesterone binding sites

The marker enzyme assays (as described in Chapter 2) used were cytochrome oxidase (mitochondrial marker), acid phosphatase (GERL marker), alkaline phosphatase, LH receptor and 5' nucleotidase (plasma membrane markers), N-acetyl glucosaminidase (lysosomal marker), DNA (nuclear marker) and NADH-cytochrome C reductase (smooth endoplasmic reticulum marker). From Figure 3.3.8 it can be seen that progesterone binding equilibrated in fractions enriched in activities of plasma membrane markers and to a lesser extent smooth endoplasmic reticulum markers. However, it can be observed that in digitonin pretreated subcellular fractions the perturbation of the buoyant density of the progesterone binding site (30% sucrose to 32.7% sucrose) was more similar to that of the plasma membrane markers (33% sucrose to 34.6% sucrose) than the endoplasmic reticulum marker (30.2% sucrose to 35% sucrose).

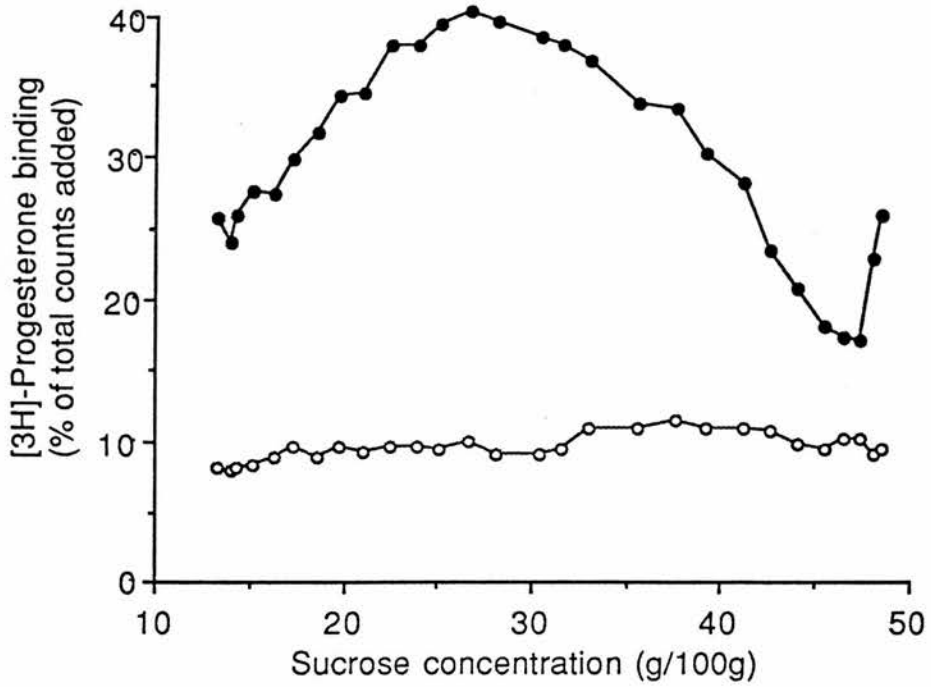


Figure 3.3.7 Distribution of binding sites for tritiated progesterone (•) and iodinated progesterone (o). No specific binding of either tracer was observed in the absence of digitonin. However, in the presence of digitonin, only tritiated progesterone was bound.

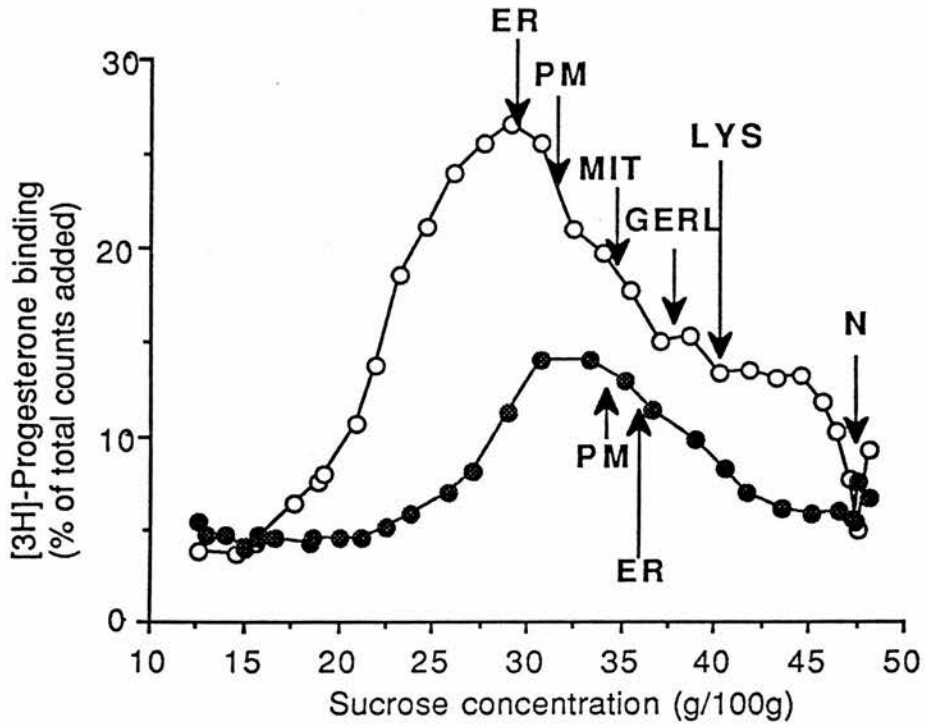


Figure 3.3.8 Comparison of the distribution of major intracellular organelles with the pattern of progesterone binding sites observed in fractions of bovine luteal homogenates subjected to sucrose density gradient fractionation, in the presence (•) or absence (o) of digitonin. (PM = plasma membrane, ER = endoplasmic reticulum, MIT = mitochondia, GERL = Golgi-endoplasmic reticulum-lysosomal complex, LYS = lysosomes, N = nuclei).

3.3.5 Subcellular localization of endogenous progesterone in sucrose gradient fractions

Endogenous progesterone content was present in high levels in cytosolic and nuclear fractions (Figure 3.3.9), which would appear to be free steroid since it was readily removable by dextran-coated charcoal treatment (data not shown). However, progesterone content equilibrated at the same buoyant density (1.10 - 1.14 g/cm^3) as fractions enriched in [^{3}H]-progesterone binding activity. Moreover, the buoyant density of progesterone content was perturbed by digitonin pretreatment to the same extent as the buoyant density of [^{3}H]-progesterone binding (Fig 3.3.9), suggesting that in the luteal cell endogenous progesterone is associated with this particulate fraction.

3.3.6 Levels of progesterone binding throughout the bovine luteal phase

In density gradients of early luteal tissue, progesterone binding was present at levels comparable to those observed in the mid-luteal phase, (Figure 3.3.10). In the late luteal phase, [^{3}H]-progesterone binding levels were somewhat lowered.

Figure 3.3.9 Progesterone content (o) and progesterone binding (•) activity of sucrose density gradient fractions of bovine luteal homogenate. A = control gradient, B = digitonin pretreated gradient. Progesterone binding activity was measured in the presence of digitonin (250 µg/tube).

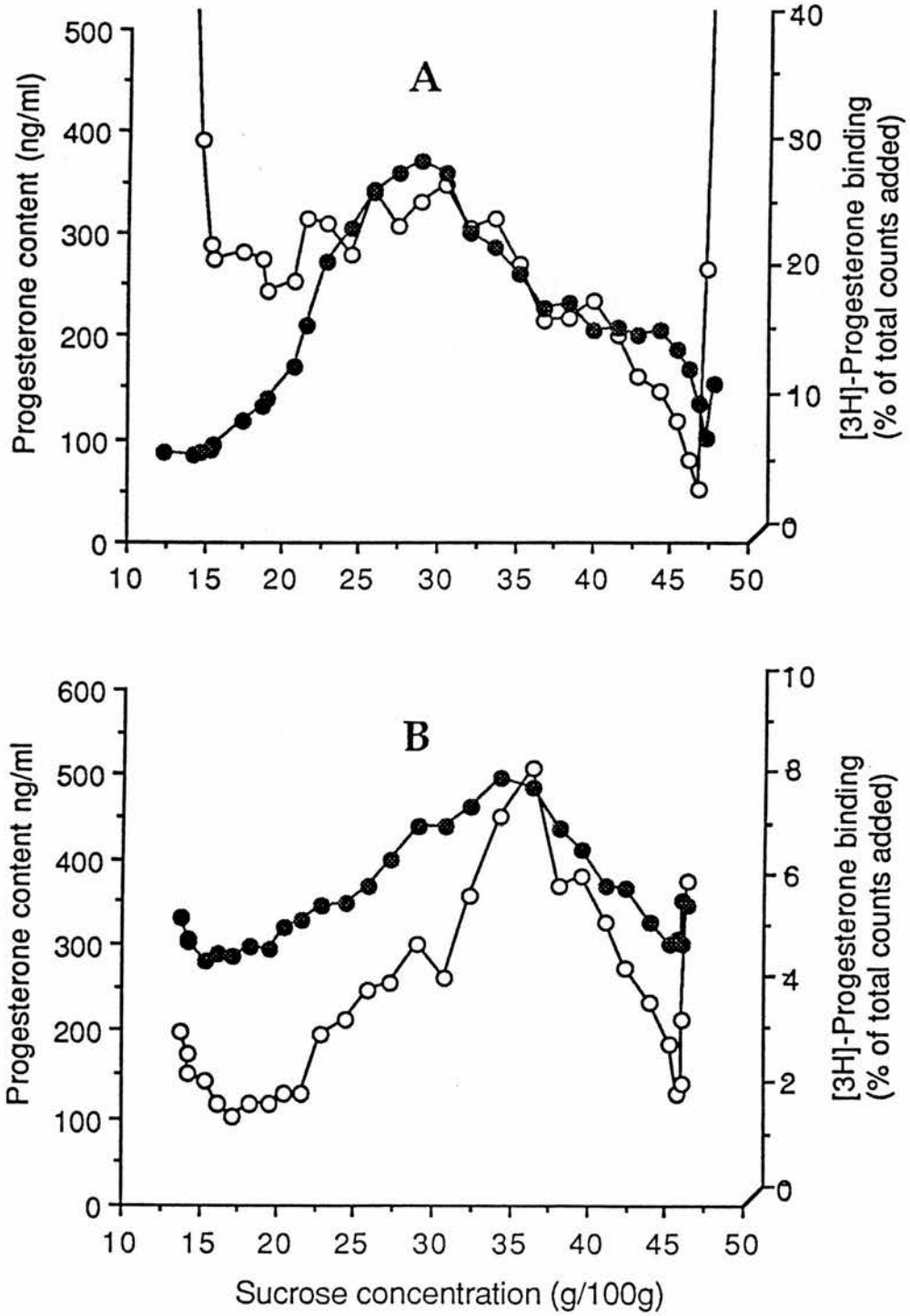
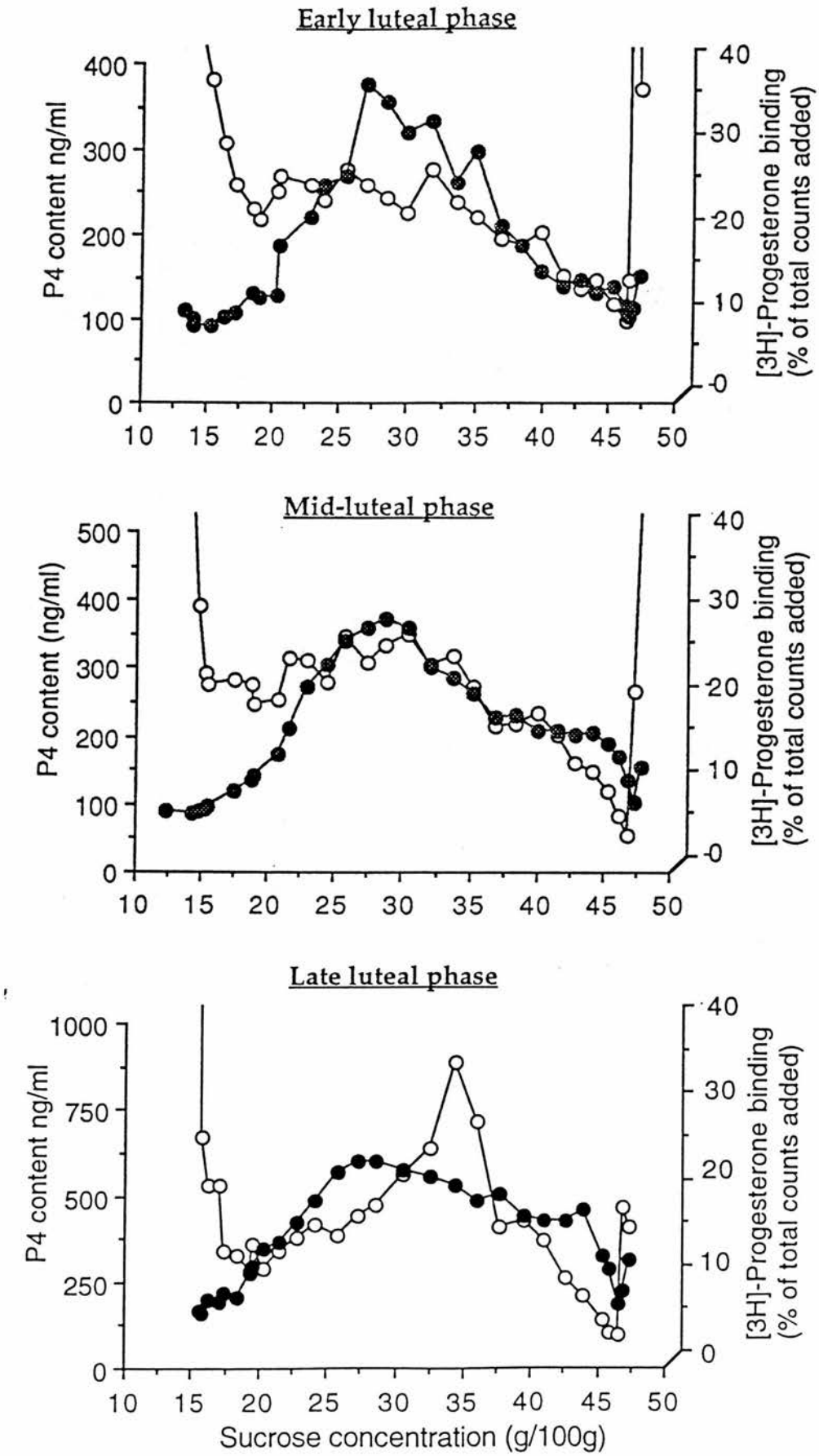

A
Progesterone content (ng/ml)
[3H]-Progesterone binding
(% of total counts added)
500
400
300
200
100
0
40
30
20
10
0
10 15 20 25 30 35 40 45 50
B
Progesterone content ng/ml
[3H]-Progesterone binding
(% of total counts added)
600
500
400
300
200
100
0
10
8
6
4
2
0
10 15 20 25 30 35 40 45 50
Sucrose concentration (g/100g)

Figure 3.3.10 Levels of progesterone content (o) and progesterone binding (•) during the early, mid and late luteal phase. Progesterone binding activity was measured in the presence of digitonin (250 µg/tube). Figure represents mean values of two experiments using two sets of morphologically staged CL.


Early luteal phase
P4 content ng/ml
[3H]-Progesterone binding (% of total counts added)
Mid-luteal phase
P4 content (ng/ml)
[3H]-Progesterone binding (% of total counts added)
Late luteal phase
P4 content ng/ml
[3H]-Progesterone binding (% of total counts added)
Sucrose concentration (g/100g)

3.3.7 HPLC analysis of steroid recovered from binding site

[^{3}H]-progesterone tracer extracted from the progesterone binding site after binding to equilibrium had the same retention time on reverse phase HPLC as progesterone and [^{3}H]-progesterone tracer, demonstrating that no metabolism of progesterone had occurred (Figure. 3.3.11).

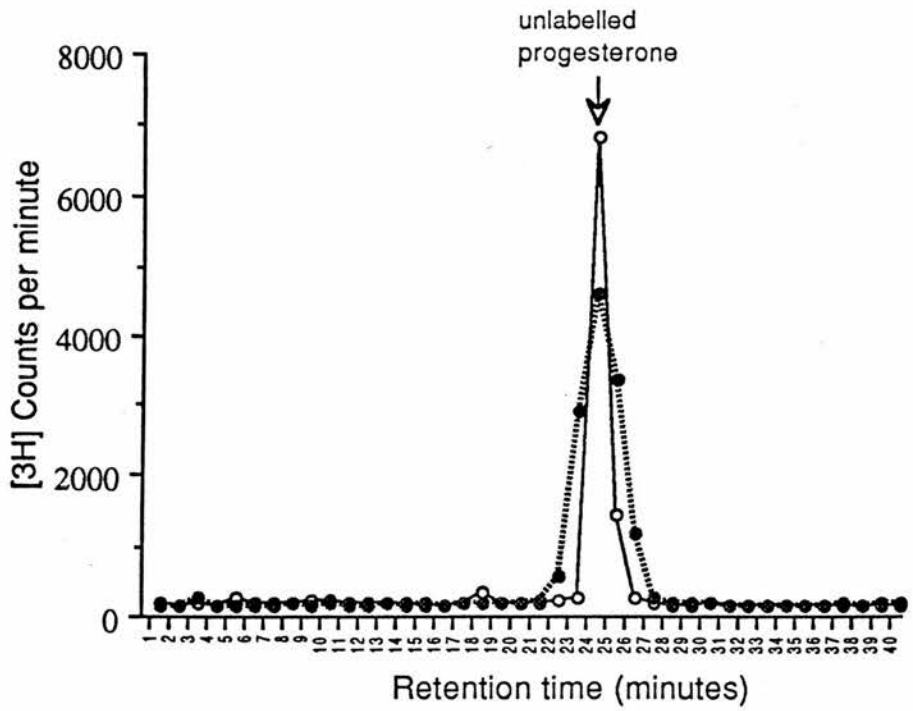


Figure 3.3.11 Reverse phase HPLC analysis of bound progesterone extracted back from the luteal binding site (--•--) and fresh [^{3}H]-progesterone (o). Also indicated is the retention time of unlabelled progesterone.

3.4 Discussion

It has been demonstrated previously that both exogenous and endogenous progesterone were sequestered by a unique particulate fraction of the porcine, ovine and equine corpus luteum (Bramley & Menzies, 1988a; 1993; 1994; Bramley *et al*, 1995). In the porcine and ovine corpus luteum, the only intracellular organelle marker enzyme activity consistently associated with progesterone binding activity was NADH-cytochrome C reductase, a marker of the smooth endoplasmic reticulum (Bramley & Menzies, 1988a; 1994). However, in the equine corpus luteum, progesterone binding activity was more closely associated with luteal cell surface membranes than any other organelle (Bramley *et al*, 1995)

In the present study of the bovine corpus luteum, NADH-cytochrome C reductase equilibrated at a slightly higher buoyant density (1.14 g/cm^3) than [^{3}H]-progesterone binding activity (1.10 - 1.14 g/cm^3) (Figure 3.3.8). However, bovine luteal plasma membrane markers followed an almost identical pattern to that of progesterone binding, suggesting an association between the plasma membrane and [^{3}H]-progesterone binding. Association of progesterone with luteal plasma membranes has been suggested by other workers in the past, but was reported to be due to non-specific intercalation of progesterone into luteal membranes (Rice *et al*; 1986). The results presented here demonstrated clearly that luteal steroid binding sites were highly specific for progesterone. Furthermore, if the binding observed in this study was simply due to the partitioning of hydrophobic steroids into hydrophobic lipid bilayer membranes, then one

would expect that binding would occur to other luteal membranes. This was clearly not the case, as only plasma membranes, and to a lesser extent the membranes of the smooth endoplasmic reticulum, were associated with progesterone binding. Other membranes (eg. mitochondrial, nuclear and lysosomal membranes) did not bind [^{3}H]-progesterone appreciably. Furthermore, inclusion of the detergent digitonin in the binding assay was essential in order to demonstrate binding (Figure 3.3.3). If steroid binding were simply due to steroid dissolution into luteal membranes then it would be expected to occur in the absence of digitonin also. It could be argued that digitonin treatment resulted in the formation of lipid micelles into which progesterone could partition more readily. However, the solubilization of the progesterone binding site from bovine luteal membranes with the detergent octyl glucoside could be demonstrated, with minimal losses in activity (see Chapter 5). In digitonin-pretreated subcellular fractions, perturbation of the endoplasmic reticulum was slightly greater than that of the progesterone binding site and that displayed by the plasma membrane markers (Figure 3.3.8). However, when compared to results obtained in Chapter 2, it can be noted that the perturbation of NADH cytochrome c reductase was not consistent - in some gradients very little increase in bouyant density with digitonin treatment was observed, however in other gradients a much greater shift was evidient. The reasons for this inconsistency are unclear. Nontheless, in control gradients and digitonin pretreated gradients, both progesterone content and binding were consistently found to equilibrate in similar positions to the plasma membrane markers regardless of the position of NADH cytochrome c reductase, and thus would appear to be more closely associated with the plasma membrane than the endoplasmic

reticulum, similar to experiments performed on the horse corpus luteum (Bramley *et al*, 1995). However, due to the nature of enzymatic identification of positions of organelles in density gradients and contamination of eg. fractions enriched in plasma membranes with membranes of the endoplasmic reticulum, it is difficult to ascertain the exact location of the progesterone binding site, though from the data presented here it would appear to be more likely to be associated with luteal cell surface membranes rather than intracellular membranes. To this end, differences in some samples between the buoyant densities of the plasma membrane markers alkaline phosphatase and LH receptor were observed, suggesting that these two activities may be located either in different areas of the plasma membrane, or in areas differently enriched in unesterified cholesterol. To resolve this question, more detailed subcellular fractionation studies of bovine plasma membranes would be required.

In the previous studies mentioned (Bramley & Menzies 1988a; 1993; 1994), preincubation of progesterone tracer with luteal homogenates prior to fractionation led to recovery of most of the radiolabelled steroid in cytosolic fractions which failed to enter the sucrose gradients. However, preincubation with digitonin caused the majority of steroid to enter the gradient, and to equilibrate at the same buoyant density as progesterone binding activity (Bramley & Menzies, 1988a; 1983; 1994). The experiments presented here showed the same behaviour in bovine luteal subcellular fractions. Moreover, in agreement with previous studies, binding of steroids other than progesterone and pregnenolone was poor or absent. In all species studied, including these experiments in the cow corpus

luteum, binding of radiolabelled progesterone was significantly greater than binding of pregnenolone. Preincubation of luteal homogenates with digitonin prior to fractionation increased the buoyant density of the progesterone binding site, but decreased the recovery of progesterone binding activity, as compared to levels found in control gradients. If digitonin unmasked progesterone binding sites by inducing membrane disruption (Bramley and Menzies, 1994), then the decrease in binding activity following digitonin pretreatment may reflect inactivation of binding sites during fractionation. Alternatively, preincubation of luteal homogenate with digitonin may activate progesterone binding sites which are not already active, allowing binding of endogenous progesterone which is normally unbound (free cytosolic progesterone) to be bound by the particulate binding site prior to fractionation, thus effectively reducing the numbers of binding sites available to be measured.

Digitonin forms non-covalent unimolecular complexes with steroids possessing a 3ß-hydroxy-Δ^5 configuration and a C17 side chain (Miller, 1984; Severs & Robeneck, 1983; Murphy & Martin, 1985). This interaction is responsible for the perturbation of the buoyant density of cell surface membranes which contain high levels of unesterified cholesterol (Bramley & Menzies, 1988). This interaction also occurs with pregnenolone, resulting in higher levels of non-specifically bound pregnenolone tracer in the absence of luteal membranes. In contrast, progesterone, which does not interact with digitonin, was not bound in the absence of membranes. Dextran-coated charcoal did not appear to absorb [^{3}H]-pregnenolone complexed to digitonin, as expected at the

levels of digitonin used in the binding assay incubation cocktail (data not shown). It is possible therefore that the binding potency of pregnenolone relative to [^{3}H]-progesterone binding may be grossly underestimated, since there was no way of ascertaining how much of the pregnenolone added to the assay cocktail was actually available to compete for binding sites, and how much had been complexed by the digitonin present in the assay incubation cocktail. The observation that only digitonin was able to stimulate progesterone binding, and that all other membrane perturbants tested failed to do so, demonstrated clearly that disruption of membrane structure was not the sole mechanism by which digitonin acted to stimulate steroid binding (see Chapter 6). Indeed, no other membrane perturbants tested mimicked the effects of digitonin, indicating that this effect was not mediated by membrane permeabilization alone (see Chapter 6). However, digitonin may displace an endogenous steroid from its binding site, allowing the progesterone tracer to bind. Perhaps luteal binding sites are normally occupied by high levels of endogenous steroid in the cell. Indeed, dextran-coated charcoal treatment of membranes can increase the amount of radiolabelled progesterone bound (see Chapter 5), suggesting occupation of a large proportion of binding sites by endogenous steroid. Indeed, since the buoyant densities of endogenous progesterone and progesterone binding were identical, then it would appear that a significant proportion of the endogenous progesterone found in the bovine corpus luteum was bound to this membrane binding site.

Binding of progesterone *in vitro* was shown to be dependent on the temperature, duration and pH of incubation, and binding increased

linearly with increasing concentrations of bovine luteal membranes added. Binding was specific for [^{3}H]-progesterone. Radiolabelled pregnenolone was bound to a lesser degree (though this may be at least partly due to interactions between the radiolabelled pregnenolone and digitonin in the absence of membranes). Binding of other steroid tracers was poor or absent. Unlabelled progesterone competed for binding of tritiated progesterone at very low concentrations (IC_{50} = 63 nM). From Table 3.3.1 it is clear that androgenic steroids and progesterone analogues have lower potency for [^{3}H]-progesterone binding sites than does progesterone itself. Furthermore, substances which inhibit steroidogenic enzymes such as ketoconazole (inhibitor of 17 α hydroxylase/C17,20 lyase) and epostane (inhibitor of 3ß-hydroxysteroid dehydrogenase) had no effect on progesterone binding, demonstrating that binding of progesterone was not due to binding of steroid to the active site of these steroid metabolizing enzymes. Other steroids, steroid precursors, and progesterone receptor antagonists showed little or no activity (Table 3.3.1), confirming observations in other species (Bramley & Menzies, 1992; 1994) and emphasising that luteal progesterone binding sites herein were distinct from progesterone receptors.

It has been demonstrated that 3ß-hydroxysteroid dehydrogenase is extremely sensitive to end-point inhibition, and has a six-fold greater affinity for its product (progesterone) than it has for its substrate, (pregnenolone) (Caffrey *et al*, 1979a). However, testosterone and oestradiol are also potent inhibitors of this enzyme through the same end-point inhibition that is exerted by progesterone (Caffrey *et al*, 1979a). Hence, if binding of progesterone was due to binding to the active site of

this enzyme, then androgens and oestrogens would be expected to show potent progesterone binding competition. From the results presented in Table 3.3.1 this was clearly not the case. Moreover ANS, which blocks steroid binding to cortisol binding globulins, had no effect on luteal progesterone binding. Finally, solvent extraction and reverse phase HPLC of progesterone tracer bound to bovine luteal binding sites demonstrated that >95% of radiolabel recovered had an identical retention time to progesterone and [^{3}H]-progesterone, indicating that metabolism of progesterone had not occurred during binding.

The significance of the luteal progesterone binding site examined in these experiments remains unclear. The data presented here agree with previous data from the porcine corpus luteum in that binding sites were of high affinity and were specific for progesterone, and that endogenous progesterone was associated with these binding sites (Bramley & Menzies, 1994), and may indicate that these binding sites are involved in sequestration of steroid, perhaps for secretion from the luteal cell. The mechanism of secretion of progesterone has remained unresolved, despite investigations by various workers over the past twenty-five years. Progesterone binding to a specific binding protein enclosed by electron dense granules has been proposed (Gemmell & Stacy, 1979; Quirk *et al*, 1979; Sawyer *et al*, 1979), and Quirk *et al* (1979) demonstrated fractions of the bovine corpus luteum rich in secretory granules and progesterone, though these granules were subsequently shown to contain oxytocin, and further studies demonstrated that oxytocin and progesterone were secreted independently of each other (Sernia *et al*, 1982; Rice *et al*, 1986; Luck, 1988). Other workers described progesterone binding proteins

isolated from the cytosol of the bovine corpus luteum, suggesting a possible role in luteal steroidogenesis and/or secretion (Willcox & Thorburn, 1981; Willcox, 1983). However, these proteins also bound R5020 (a synthetic progestagen with high affinity for mammalian progesterone receptors); unlabelled R5020 was unable to compete for binding sites with [^{3}H]-progesterone bound to particulate luteal binding sites described here.

It has been suggested that progesterone may play an important role in the regulation of the corpus luteum (Rothchild, 1981). It is possible, therefore, that the binding described here may represent a binding to a non-classical receptor for progesterone which is involved in the autocrine regulation of luteal function. Studies which have addressed the role of progesterone in the control of luteal function have thus far concentrated on the genomic progesterone receptor, as have the majority of studies of steroid function in general. However, non-genomic effects of steroids have been known for a number of years, and thus the binding site studied herein may play a role in the transduction of as yet uncharacterised non-genomic effects of progesterone. The autocrine role of luteal progesterone and possible involvement of the particulate binding site studied herein is dealt with in more detail in following chapters.

Due to the sensitivity of steroidogenic enzymes to end-point inhibition, it has been suggested that there must be some modification of this feedback, or progesterone synthesis would quickly halt. Suggestions have included steroid-binding proteins which sequester progesterone and thereby

effectively remove its inhibitory effects on the 3β-hydroxysteroid dehydrogenase enzyme (Niswender & Nett, 1994). Since luteal concentrations of pregnenolone are low as compared to luteal concentrations of progesterone, it would appear likely that some mechanism of removing progesterone to relieve its negative feedback on the enzyme 3β-hydroxysteroid dehydrogenase (which converts pregnenolone to progesterone) is required (Caffrey *et al*, 1979a). Thus it is possible that the progesterone binding sites studied here may function as (i) a steroid binding protein whose function is to bind progesterone to remove it from actively inhibiting its own formation, (ii) a binding protein to shuttle progesterone in an aqueous soluble form to other parts of the cell as part of an autocrine system, or (iii) to participate in the secretion of progesterone from the luteal cell.

In conclusion, the bovine corpus luteum contains particulate binding sites specific for progesterone. In later chapters of this thesis this binding site is examined more fully, with attempts to purify it, studies of its distribution in the preovulatory follicle and the specific cell types of the bovine corpus luteum, and further elucidation of the mechanism of action of digitonin.

Chapter 4

Cellular distribution of progesterone binding sites in the bovine corpus luteum and bovine preovulatory follicle

4.1 Introduction

Early studies in the porcine and guinea pig CL indicated that the cell types of the corpus luteum were derived from both the thecal and granulosa cells of the preovulatory follicle (Loeb, 1906; Corner, 1919). This theory of the origins of luteal cells is still accepted today, and has been described in a number of species including the cow (Donaldson & Hansel, 1965; Priedkalns *et al*, 1968), sheep (McClellan *et al*, 1975; O'Shea *et al*, 1980), pig (Corner, 1919) and human (Guraya, 1971).

The experiments detailed in this chapter were designed to investigate the cellular distribution of the progesterone binding site studied in previous chapters in both the bovine corpus luteum and the bovine preovulatory follicle, if indeed this binding site is present prior to luteinization. This knowledge, coupled with that from experimental work detailed in earlier chapters, should provide deeper understanding of the physiological role of this progesterone binding site in the bovine ovary.

4.2 Methods and Materials

4.2.1 Ovarian tissue

Ovaries were recovered from presumed non-pregnant cows slaughtered at a local abattoir and transported to the laboratory in ice-cold phosphate buffered saline (PBS; sodium chloride (0.15 mol/l) in sodium phosphate (10mmol/l; pH 7.4) within 1 hour of death. The stage of the luteal phase was assessed by gross morphology (Figure 2.2.1), and mid-luteal phase corpora lutea were dissected free from connective tissue and stroma.

3.2.2 Recovery of granulosa and thecal cells

Granulosa cells were aspirated from non-atretic (clear, blood-free) small (<5 mm diameter), medium (5-10 mm diameter) and large (>10 mm diameter) follicles of normal appearance using a fine-gauge needle and syringe. The cells were recovered from the follicular fluid by centrifugation at 800 g_{av} for 5 min, washed twice in 10 volumes of isotonic PBS and homogenized in ice cold SET medium (2ml per ml packed cells) with 15 strokes of an all-glass tight fit Dounce homogenizer. Theca cells were obtained by peeling the thecal layer away from surrounding tissue (after careful scraping and rinsing in ice-cold PBS to remove adherent clumps of granulosa cells) then homogenized in SET (200 mg homogenate/ml) as described for granulosa cells.

4.2.3 Sucrose density gradient fractionation

Continuous sucrose density gradients (30 ml; 10-50% w/w) were prepared by the method of Stone (1974) (see Chapter 2). Aliquots of granulosa and thecal homogenate (2.5 ml) were mixed with either 0.5 ml SET medium (controls) or 0.5 ml SET medium containing digitonin (10 mg/ml). After a 30 min incubation on ice, 2.5 ml aliquots were gently layered over the sucrose density gradients and centrifuged at 30,000 g_{av} for 2h in a Sorvall VTR 50 vertical tube rotor (4^{o}C). Each gradient was then fractionated using a Buchler-Searle Autodensiflo gradient fractionator equipped with a meniscus-sensitive probe, and fractions (1ml) frozen at -20^{o}C until assay.

4.2.4 Luteal cell dispersion

Corpora lutea were minced finely with scissors, then placed in dispersion buffer (25 mls buffer/CL) (Dulbeccos phosphate-buffered saline, pH 7.3 (Sigma), 0.1 mM EDTA, 0.5% w/v BSA, DNAase I (8units/ml), heparin (25 units/ml), Pen-Strep (Sigma; 100 units penicillin/ml, 100 μg streptomycin/ml) and agitated gently for 1 minute at room temperature. The resulting suspension was then filtered through two layers of cheesecloth to remove debris. This filtered suspension was then loaded on to an elutriator for cell separation.

4.2.5 Large and small luteal cell elutriation

All parts of the elutriation system were cleaned and sterilised by flushing with 2% H_2O_2 solution for 30 minutes. Elutriation buffer (Dulbeccos phosphate-buffered saline, pH 7.3 (Sigma), 0.1% w/v BSA, DNAase I (1000 units/ml), heparin (25 units/ml), Pen-Strep (Sigma; 100 units penicillin/ml, 100 μg streptomycin/ml) was flushed through the system to remove any

traces of peroxide. Cell suspensions (20 ml) were loaded into the elutriation chamber (Beckman) by direct injection into the system, by-passing the loading chamber. The initial pump (Masterflex) speed was 1, and the elutriator rotor (Beckman) was adjusted to 750 gav. Cells were observed to enter the elutriation chamber (by means of a strobe light, adjusted to the speed of the centrifuge). Concomitant with injection of cell into the elutriation system, 50 ml fractions were collected. After 2 fractions had been collected (small luteal cells and erythrocytes), the pump speed was increased to 2, and a further 2 fractions collected. Rotor speed was now reduced (500 gav), and the chamber inspected visually for disruption of the cell pellet remaining. Rotor speed was reduced until the cell pellet in the elutriation chamber was observed to disperse and cells to move towards the outlet. At this point 4 x 50 ml fractions were collected, to flush the small cells trapped in the cell pellet. Finally, the most dense cells (large luteal cells) were elutriated from the chamber by increasing the pump speed to >3, and decreasing the rotor speed to 250 gav.

50 ml fractions were then spun (250 g.av) for 10 minutes to pellet cells. Supernatant was removed, and cells resuspended in 5 ml elutriation buffer. Cell suspensions were then examined microscopically, and enriched populations of small and large luteal cells were checked for viability by trypan -blue exclusion (Sigma). Viability, as assesed by this method, was found to be typically between 50 - 65 %.

4.2.6 Progesterone binding ability of isolated luteal cell sub-populations

Enriched cell populations of large and small luteal cells were pelleted by centrifugation at 250 gav (10 minutes) then resuspended in sterile PBS, pH

7.3. This wash step was repeated, to ensure removal of BSA present in the elutriation buffer, which would otherwise interfere in measurements of protein content. Cells were then homogenized (Dounce tight fit, 10 strokes) and resuspended in PBS. Homogenates were then frozen at -20°C until assay. Progesterone binding was assayed at suitable dilutions as described in Chapter 2.

4.2.7 Protein determination

Protein levels of homogenates were determined by the method of Lowry *et al* (1951) using bovine serum albumin (BSA) (Sigma) as a standard.

4.2.8 Progesterone binding to theca and granulosa cell homogenates

4 follicles from each group (small, medium and large) were processed as detailed above. Progesterone binding was assayed *in vitro* as described in Chapter 3, at a range of homogenate concentrations (10 µl - 50 µl). Protein content of each homogenate was determined and results expressed as [^{3}H]-progesterone counts bound/ mg protein. Mean binding was calculated for each follicular group and results presented $\pm$ S.E.M. Statistical significance of differences in progesterone binding activity between theca and granulosa cells from each follicular group was assessed by students t-test, and differences between cell populations from different groups by ANOVA.

4.2.9 LH binding to large and small luteal cell homogenates

LH binding to large and small bovine luteal homogenates was performed as described in Chapter 1.

4.2.10 SDS PAGE separations and Western blotting of bovine small and large luteal cell homogenates

SDS PAGE gels were prepared as described in Chapter 2 (2.2.12). 30 μg (total protein) of large and small cell homogenates were loaded onto each gel. Western blotting was carried out as described in Chapter 2 (2.3.13). The antibodies used were rabbit-anti-ovine P_{450} side chain cleavage, rabbit anti-human 3ß - hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase and rabbit-anti-bovine P_{450} 17 α hydroxylase/C17,20 lyase (kindly donated by Professor I. Mason, Dept. of Clinical Chemistry, The University of Edinburgh). As positive controls, gels to be blotted for P_{450} side chain cleavage and P_{450} 17 α hydroxylase/C17,20 lyase were run with a lane containing 30 μg bovine theca cell homogenate, and gels to be blotted for 3ß - hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase run with a lane containing 30 μg human placental microsomes.

4.3 Results

4.3.1 Progesterone binding to theca and granulosa cell homogenates from small, medium and large sized follicles

Both granulosa and theca cell homogenates bound [^{3}H]-progesterone, though only in the presence of digitonin. Binding was found to be almost ten-fold greater in theca cell homogenates than in granulosa cell homogenates. Little difference was found between the binding capabilities of the different follicle sizes (Figure 4.3.1), though a decrease in binding activity of theca cells with increasing follicular size was apparent, though not statistically significant.

4.3.2 Progesterone binding to theca and granulosa cell homogenate sucrose gradient fractions

Both theca cell and granulosa cell homogenates subjected to sucrose density gradient fractionation displayed progesterone binding activity when assayed using the *in vitro* assay developed in the cow corpus luteum (Chapter 3). Binding was observed to be greater in theca homogenate gradients than in granulosa homogenate gradients. The buoyant density of the peak of progesterone binding activity was higher in granulosa homogenate gradients (1.12 - 1.15 g/cm^3) (Figure 4.3.2) than in theca gradients (1.08 - 1.13 g/cm^3) (Figure 4.3.3) or luteal homogenate gradients (1.1 - 1.14 g/cm^3). In addition, theca homogenate gradients displayed a peak of binding activity at a much lighter buoyant density (1.05 - 1.07 g/cm^3), as well as a peak in the same position as was found in luteal homogenate gradients. In experiments where theca and granulosa cell homogenates were incubated with digitonin prior to fractionation, binding was markedly reduced. No binding was observable in

digitonin pretreated granulosa cell homogenate gradients, however, in theca cell homogenate sucrose gradients pretreated with digitonin a peak of binding was observed at a lower buoyant density (1.14 - 1.16 g/cm^3) than that found in control homogenate gradients. No peak of binding at the top of the gradient was observable in digitonin pretreated theca homogenate gradients.

4.3.3 Progesterone binding characteristics of theca and granulosa sucrose gradient fractions

Binding of [^{3}H]-progesterone to granulosa and theca cell homogenate sucrose gradient fractions was found to increase linearly with increasing concentration of (sucrose density gradient) fraction added, reaching a plateau at high membrane concentrations (Figures 4.3.4 and 4.3.5) Binding was measurable only in the presence of digitonin. This plateau was reached at lower membrane concentrations in theca cell homogenate (Figure 4.3.5) sucrose gradient fractions (20 µl/0.5 ml assay volume) than in granulosa cell homogenate (Figure 4.3.4) fractions (30 µl/0.5 ml assay volume). In the absence of digitonin, no binding was observable. Binding was found to be higher in fractions of theca cell homogenate sucrose density gradients than the fractions of granulosa cell homogenate sucrose density gradients.

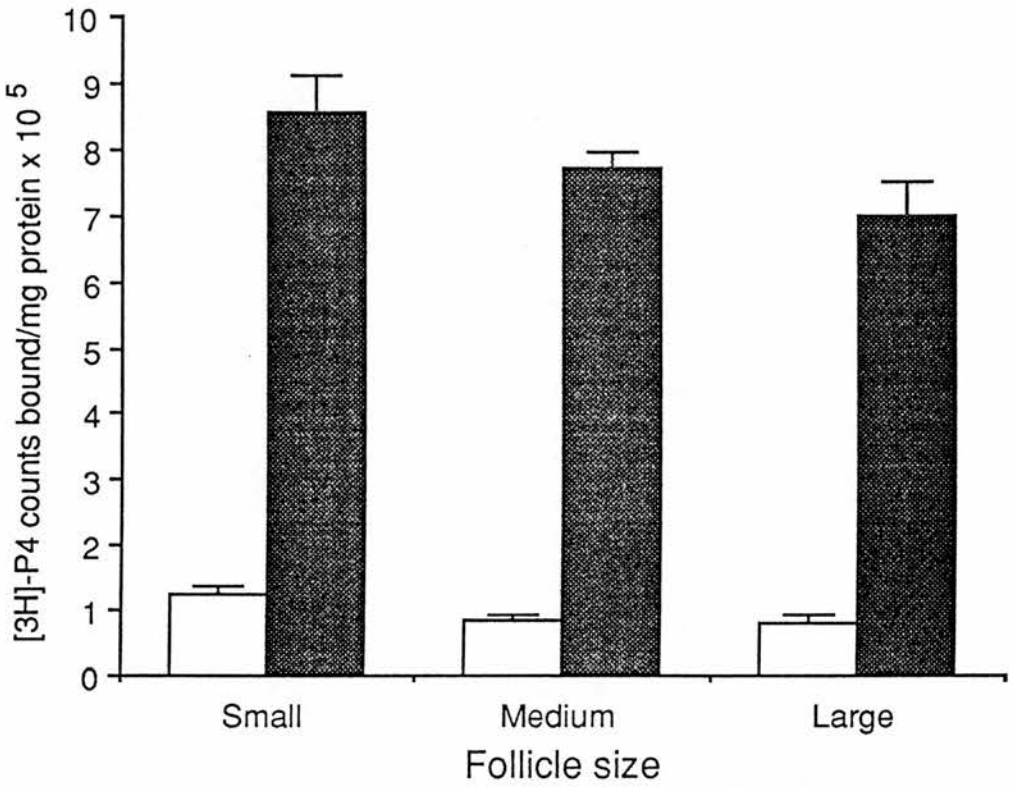


Figure 4.3.1 [^{3}H]-progesterone binding to theca (shaded bars) and granulosa cell (open bars) homogenates of small (< 5 mm diameter), medium (5 - 10 mm diameter) and large (> 10 mm diameter) bovine preovulatory follicles. Figure shows mean progesterone binding for each follicle group ± S.E.M. Differences in progesterone binding activity between theca and granulosa cells from each group were highly significant ($P<0.01$) (t- test). No significant difference was found between granulosa cells from each group, nor was any significant difference found between theca cells from each group (ANOVA).

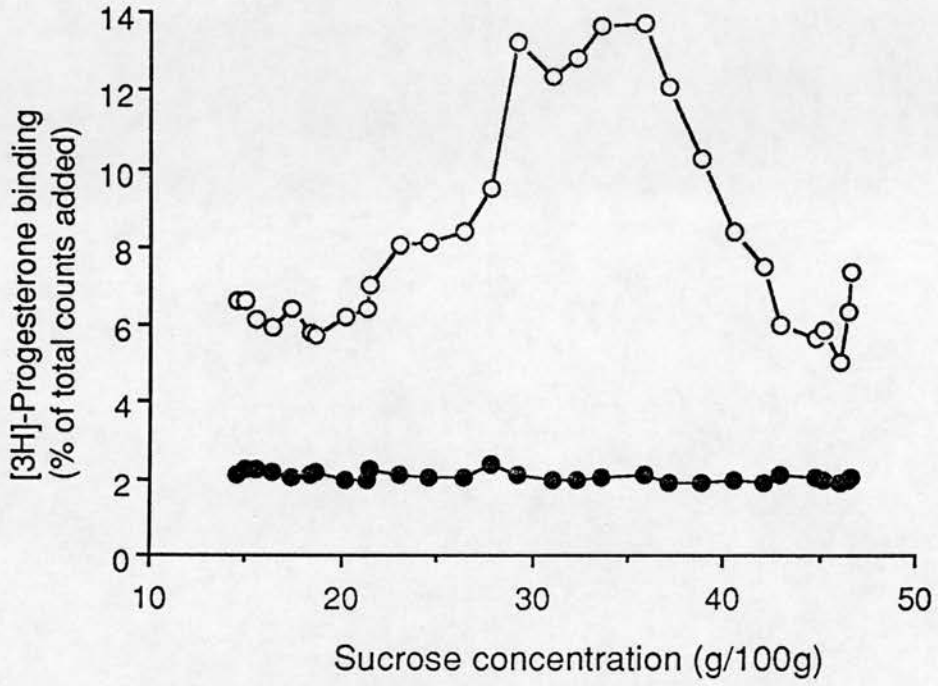


Figure 4.3.2 [^{3}H]-progesterone binding to sucrose gradient fractions of bovine granulosa cell homogenate, with (●) and without (o) digitonin treatment of homogenate prior to fractionation.

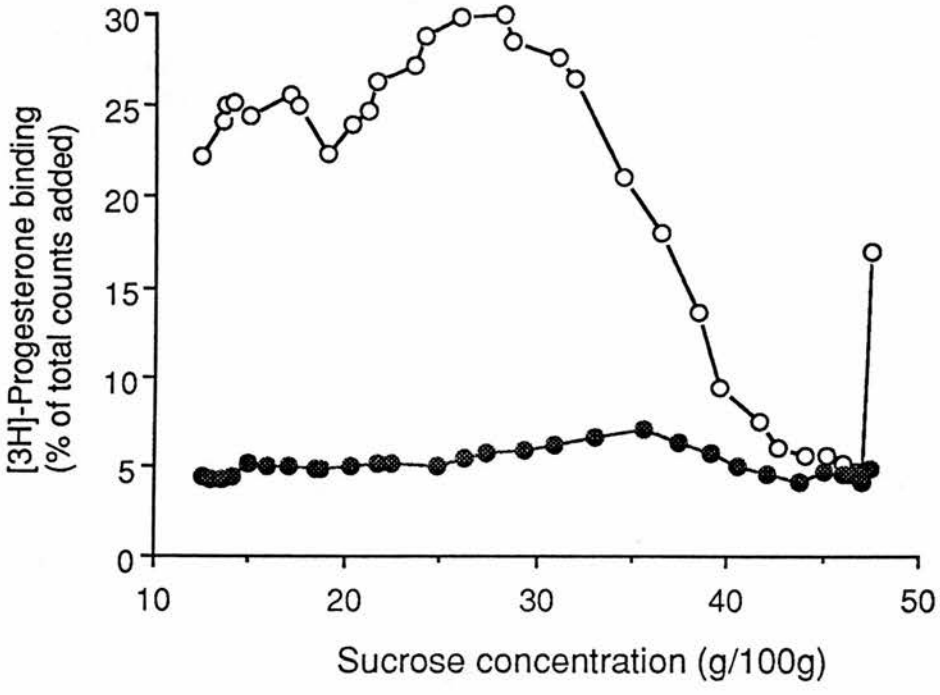


Figure 4.3.3 [^{3}H]-progesterone binding to sucrose gradient fractions of bovine theca cell homogenate, with (●) and without (o) digitonin treatment of homogenate prior to fractionation.

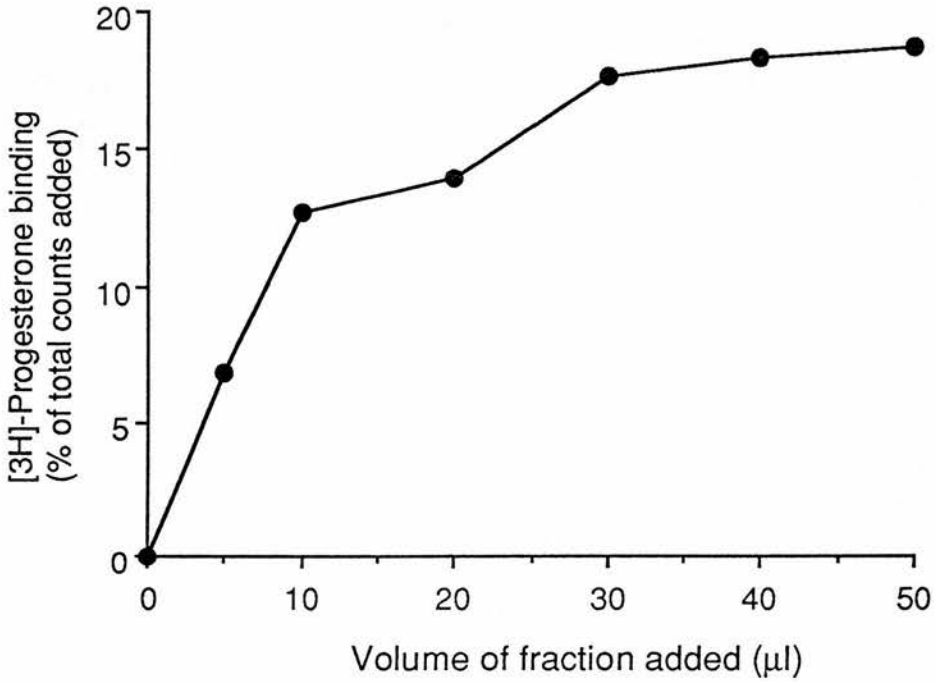


Figure 4.3.4 [^{3}H]-progesterone binding to increasing amounts of membrane fraction pooled from sucrose gradient fractions of bovine granulosa cell homogenate enriched in progesterone binding activity.

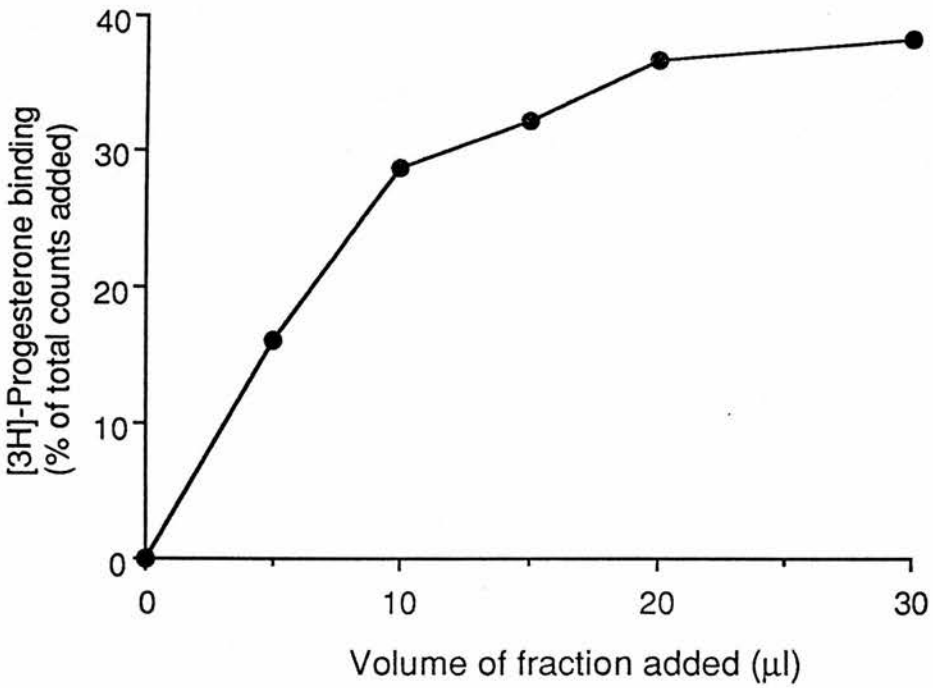


Figure 4.3.5 [^{3}H]-progesterone binding to increasing amounts of membrane fraction pooled from sucrose gradient fractions of bovine theca cell homogenate enriched in progesterone binding activity.

4.3.4 Specificity of preovulatory binding sites for progesterone

Figure 4.3.6 shows the results of steroid displacement of progesterone binding to granulosa cell homogenate sucrose density gradient fractions enriched in progesterone binding. Progesterone displayed half-maximal displacement of binding between 10 - 100 ng of cold progesterone added, whereas other steroids (testosterone, pregnelolone) reach this level of displacement only at concentrations 10 - 100 fold greater than that of progesterone, or, in the case of oestradiol-17ß, showed little or no displacement potency.

Displacement experiments were performed on theca cell homogenate sucrose density gradient fractions from both peaks of progesterone binding activity. Similar results were obtained with both the lower and higher buoyant density progesterone binding peaks found over sucrose gradient fractions (Figure 4.3.7). Unlabelled progesterone competed for binding sites with [^{3}H]-progesterone at low levels (10 - 100 ng added cold progesterone) whereas testosterone displayed this level of displacement of progesterone binding only at levels 100-fold greater than this. Pregnenolone displayed less potent displacing ability over progesterone binding in fractions pooled from the more dense peak of binding, than fractions pooled from the less dense peak of progesterone binding activity in theca cell homogenate sucrose density gradient fractions.

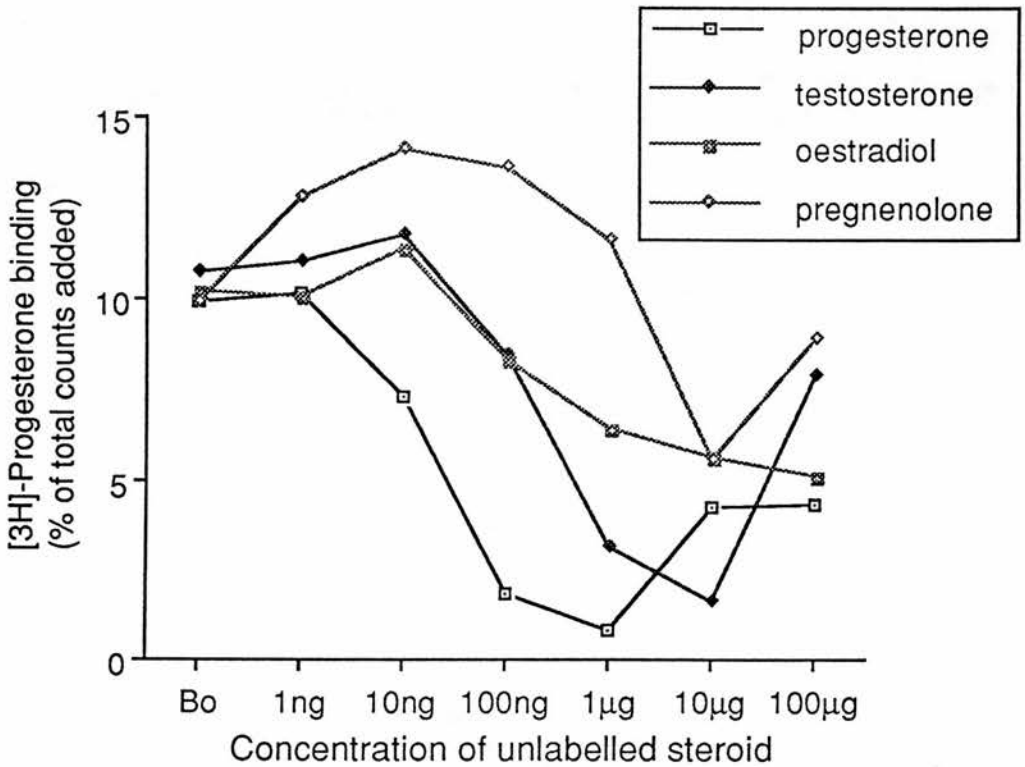


Figure 4.3.6 Displacement of [^{3}H]-progesterone binding by unlabelled steroids to pooled sucrose gradient fractions of bovine granulosa cell homogenate enriched in progesterone binding.

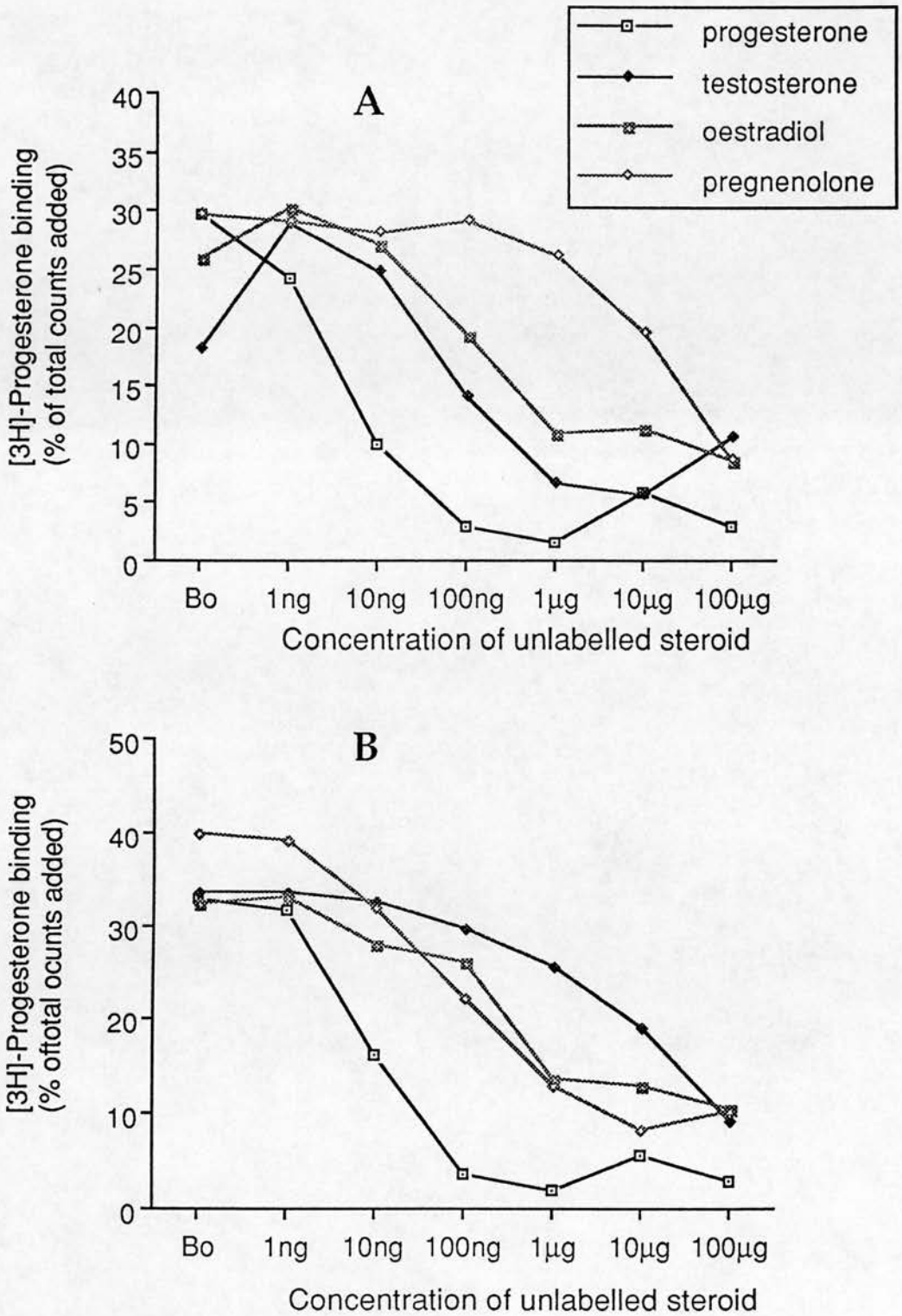


Figure 4.3.7 Displacement of [^{3}H]-progesterone binding by unlabelled steroids to pooled sucrose gradient fractions of bovine theca cell homogenate enriched in progesterone binding. A = fractions pooled from low density binding peak, B = fractions pooled from high density peak.

4.3.5 Luteal cell separations and progesterone binding to separated small and large luteal cells

Separation of luteal cells yielded enriched small cell populations with some red blood cell contamination (typically 70% small luteal cells, 30% red blood cells). Enriched large cell populations were approximately 80% large luteal cells, the remainder being made up of clumps of small luteal cells and clumps of cell debris.

Low levels of [^{3}H]-progesterone binding were observed in enriched small luteal cell populations. However, binding was observed to be almost 3-fold greater in large luteal cell populations than in fractions enriched in small luteal cells. In all cases binding was only observable in the presence of digitonin (250 μg/tube) (Figure 4.3.8).

4.3.6 LH binding to isolated large and small luteal cells

Using the LH receptor binding assay detailed in Chapter 2, high levels of binding were observed in both small and large luteal cells. However, no quantification of the numbers of binding sites present could be determined since binding could not be displaced by unlabelled hCG, and thus no indication of specific binding vs. non-specific binding of LH tracer could be calculated.

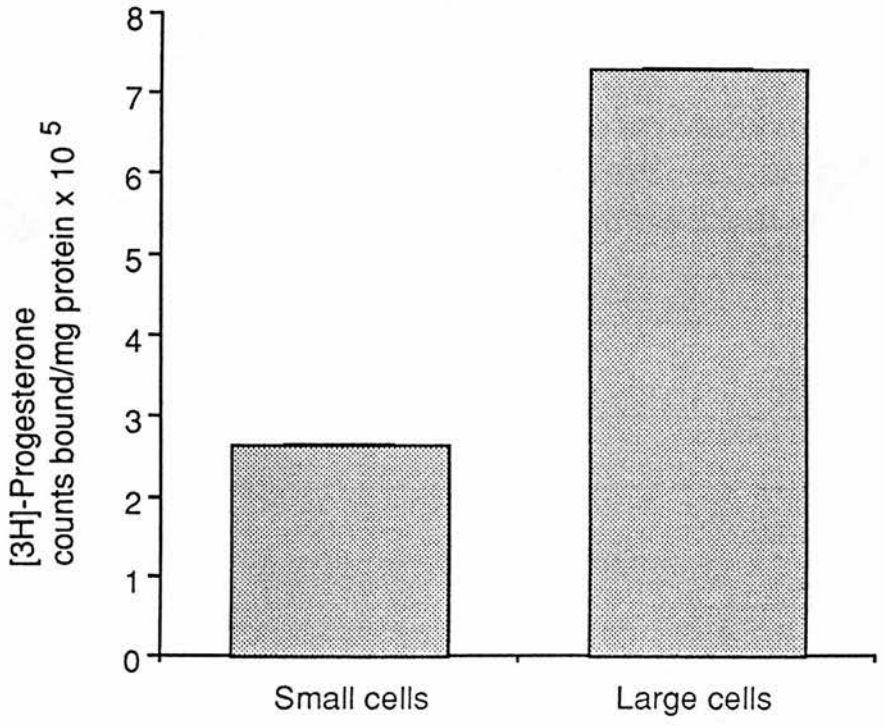


Figure 4.3.8 [^{3}H]-progesterone binding to separated small and large luteal cells. Progesterone binding assay was performed in triplicate on each cell type. Graph represents the mean of two such experiments, using dispersed cells from two sets of mid-luteal phase CL.

4.3.7 Western blots of steroidogenic enzyme content of isolated luteal cell sub-populations (Plate 4.3.1)

(i) 3ß - hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase: Both large and small cells displayed strong detection of 3ß-HSD. The molecular weight of the bands detected in these experiments was 44 kDa. The reported molecular weight of bovine 3ß-HSD is 42 kDa (Couet *et al,* 1990). It was also observed that stronger detection was present in large luteal cells than in small luteal cells fractionated on these gels.

(ii) The reported size of P_{450} side chain cleavage is 49 kDa (Rodgers *et al,* 1986a). In these experiments faint bands of 49 kDa are evident in both luteal cell types. However, much denser bands are evident, equilibrating at a molecular weight of approximately 68 kDa. Of these 68 kDa proteins, stronger detection was found in fractionated large luteal cells than small luteal cells.

(iii) P_{450} 17 α hydroxylase/C 17,20 lyase: Positive detection, though very slight, was evident in both large and small luteal cells when immunoblotted with anti-sera raised against P_{450} 17 α hydroxylase/C 17,20 lyase .

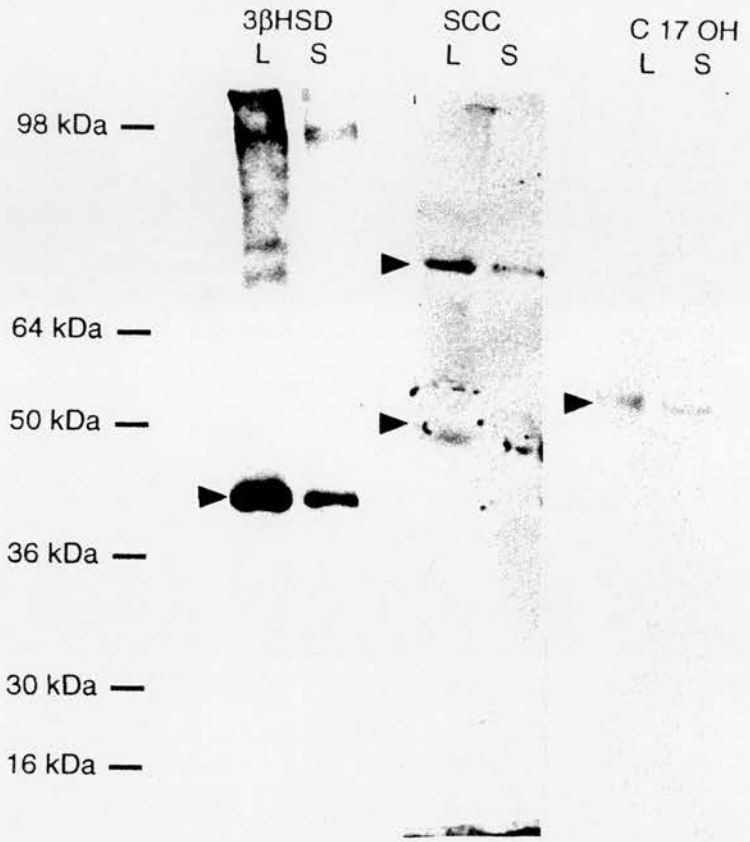


Plate 4.3.1 Western blots of separated large and small luteal cell homogenates for steroidogenic enzymes. 3βHSD = 3ß - hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase, SCC = P_{450} side chain cleavage, and C17OH = P_{450} 17 α hydroxylase/C 17,20 lyase. Arrowheads indicate protein of interest (see text for details, sections 4.3.7 and 4.4)

4.4 Discussion

The experiments presented in this study were designed to investigate whether or not the novel progesterone binding site described in Chapter 3 was present in the preovulatory follicle and to attempt to elucidate a possible function for this protein with respect to its cellular distribution in the follicle. In addition, differences between small and large luteal cells with respect to their LH receptor populations and steroidogenic enzyme content were also investigated, as was the cellular distribution of the novel bovine progesterone binding site in the corpus luteum.

Progesterone binding activity was found to be present in both cell types of the bovine preovulatory follicle. Interestingly, much higher levels of progesterone binding were found in the theca cells than in the granulosa, in small, medium and large sized follicles. Prior to ovulation, the vast majority of bovine follicular steroidogenesis is directed to the production of oestradiol (Dieleman *et al*, 1983b). However, from 20 hours post LH surge, up until ovulation, progesterone concentrations in follicular fluid rise (Dieleman *et al*, 1983b). In sheep, this rise is thought to be simply an accumulation of progesterone in the follicular fluid, and not a reflection of the follicles progesterone synthesizing ability (Murdoch & Dunn, 1982), however, it has been demonstrated in cattle that this increase in follicular progesterone is indeed a reflection of the steroidogenic output of the follicle, that is, increased rate of synthesis of progesterone (Dieleman & Blankenstein, 1985). This increase in progesterone synthesis occurs at the same time as morphological luteinization of the granulosa cells occurs (Dieleman *et al*, 1983b). Thus it has been suggested that, in cows, the granulosa cells are the main source of follicular progesterone at this stage of the oestrous cycle

(Dieleman & Blankenstein, 1985). Since it was the theca cells which contained the highest levels of progesterone binding activity, it would appear unlikely that this progesterone binding site is involved in the packaging of progesterone for secretion in the bovine preovulatory follicle. However, it was unknown at what stage the follicles used in this study were, and from which follicular wave they were taken. Hence it remains a possibility that even the largest of the follicles assayed here were pre-LH surge, and were thus not producing progesterone at increased rates. Indeed, before the LH surge, and up to 20 hours post surge, thecal progesterone production has been demonstrated to increase (Fortune & Hansel, 1979).

Fractionation of theca and granulosa cells recovered from bovine follicles gave similar profiles of progesterone binding activity to that seen in the case of fractionated bovine corpora lutea (Chapter 3). In theca cell fractionations two peaks of binding were observed in control gradients, whereas only one peak was evident in digitonin-pretreated gradients, equilibrating in a similar position to that found in sucrose gradients of corpora lutea. This digitonin-pretreated peak displayed similar levels of digitonin perturbation as compared to perturbations seen in corpora lutea sucrose gradients, and was also much reduced as compared to its control gradient equivalent, in the same manner as was observed in sucrose gradient fractions of corpora lutea. The secondary peak of progesterone binding activity found at the top of the gradients of theca cell fractionations is likely to be due to membrane vesicles created during homogenization procedures. Homogenization of theca cells was performed using a hand-operated Dounce homogenizer; the tough nature of this tissue meant repeated strokes to attain a smooth homogenate, - conditions that are likely to produce membrane vesicles. Thus, in control

gradients, these membrane vesicles may float to the top of the gradient along with their associated progesterone-binding protein, but, as is reflected by the results of these experiments, in homogenates pretreated with digitonin, vesicles would be perturbed and would not float, but would instead become sedimented at the correct buoyant density for such membranes.

In sucrose gradient fractions of granulosa cell homogenates, progesterone binding was found to form only one peak, at approximately the same position as the peak of binding found in gradients of bovine corpora lutea. The lack of binding activity in digitonin-pretreated gradients may be due to degradation of binding sites as appears to be the case in corpora lutea sucrose gradients. Since binding was very low in granulosa cells, then any degradation of binding sites would leave any progesterone binding activity so low as to be undetectable. Alternatively, since only low levels of binding were found in granulosa cells, activation of the progesterone binding site prior to fractionation may have allowed the saturation of binding sites with endogenous progesterone, making the measurement of progesterone binding difficult.

The differences between the buoyant densities of the progesterone binding peaks between theca and granulosa cells may be accounted for by differences in the membrane composition of the two cell types. If indeed this is the case, then if these differences are carried over after luteinization, then this may possibly explain why such a broad peak of progesterone binding activity is observed in luteal gradients, that is, it may be speculated that in luteal gradients the single peak of progesterone binding activity may be two

overlying peaks of binding activity, whose origins are cell specific and whose associated membranes display slightly different buoyant densities.

Separation of luteal cells has, over the past fifteen years, become a common practice in studies of the corpus luteum of many species. In all studies performed in sheep and cows, enzymes such as collagenase have been used to facilitate the dispersion of luteal tissue into single cells, which can then be separated according to size and/or density. Recently however, a study of the equine corpus luteum revealed that luteal cell types show different sensitivities to the rigours of dispersion, most notably, to the use of dispersion enzymes (Broadley *et al*, 1994). Indeed, it was demonstrated that the LH receptor population of large luteal cells was more sensitive to damage than that of small luteal cells (Broadley *et al*, 1994). Thus in this study, mechanical dispersion of luteal cells was used to avoid any differential damage to the two cell types' LH receptor populations. This mechanical dispersion yielded large numbers of separated small and large luteal cells, with reasonable cell viability, typically in excess of 50%. In previous studies it has been demonstrated that numbers of LH receptors found on small cells are much higher than numbers localized to large cells, highlighting an important functional difference (Fitz *et al*, 1982). The control of luteal steroidogenesis is exerted by both intra-ovarian paracrine factors and endocrine factors produced elsewhere in the body. Pituitary-derived LH is considered to be the single most important factor involved in the support and function of the corpus luteum in cattle. Large cells cultured *in vitro* however, show little or no response to LH concentrations that maximally stimulate progesterone production in small luteal cells (5-10 ng/ml), and require concentrations of up to 100 ng/ml LH to elicit a

response in increased progesterone production (Alila & Dowd, 1991). Thus it appears likely that *in vivo*, large cells do not respond to LH stimulation, since the levels of LH found in the circulation of ruminants is only of the order of 5-10 ng/ml (Alila & Dowd, 1991). In sheep large luteal cells, which possess LH receptors, little or no response to LH is observed (Harrison *et al*, 1987). Since other trophic substances such as PGE_2 which mediate their effects (increase in progesterone secretion) through the cAMP second messenger system do have effects on these cells, as indeed do exogenous cAMP analogues (Fitz *et al*, 1984; Silvia *et al*, 1984; Alila *et al*, 1988 a; b), it appears that large luteal cells LH receptors are uncoupled from the cAMP second messenger system (Hoyer *et al*, 1984; Hoyer & Niswender, 1986; Harrison *et al*, 1987; Davis *et al*, 1989). The mechanism of this apparent uncoupling is as yet unknown (Alila & Dowd, 1991). It is also possible that this apparent uncoupling may be simply an artifact produced by the process of tissue disruption. Unfortunately, no information on the stability or existence of LH receptors of large and small luteal cells could be obtained from the experiments detailed in this chapter. This was due to the inability of the unlabelled hCG used to measure non-specific binding to displace specifically bound LH tracer in LH binding assays of isolated luteal cell sub-populations. This result was surprising since the same assay was used with no such problem on sucrose gradient fractions of bovine corpora lutea. It is not clear why difficulties in measuring specific binding of LH to isolated luteal cell homogenates arose. The possibility that the LH receptors have been damaged during mechanical disruption of the corpora lutea and the subsequent elutriation of cells may be one explanation. However, no such problems were encountered in measuring LH binding to homogenized bovine corpora lutea (Chapter 1). Hence if receptor damage has occurred

then it would appear likely that this damage occurred due to agents found in the dispersal or elutriation buffers. However, this problem was not observed in similar studies in the equine corpus luteum (Broadley *et al*, 1994). It would be of interest to obtain anti-sera specifically raised against the bovine forms of the LH receptor and PGF 2α receptor to use as indicators of purity of luteal cell sub-populations (by Western blot of SDS-PAGE fractionated large and small luteal cell homogenates), and to provide information on LH receptor protein levels that did not rely on maintaining intact, functional LH receptors. Also, it would be of value to characterise the LH receptor assay for use on such samples. For example, it may be that unlabelled LH is more suitable for displacement of bound LH tracer than is unlabelled hCG in such experiments. Unfortunately these experiments were outwith the timescale of this project.

In large luteal cells from the bovine corpus luteum, basal progesterone secretion is 10-20 fold greater than the basal secretion of progesterone from small luteal cells (Koos & Hansel, 1981; Alila *et al*, 1988). Indeed, in the sheep corpus luteum 80% of progesterone output is large luteal cell derived. Results presented in this study demonstrate that in the bovine corpus luteum large luteal cells posses almost three times the amount of progesterone binding activity as do small bovine luteal cells. Thus, if the progesterone binding protein studied here is a component of the mechanism by which progesterone is secreted, then the distribution of binding correlates with the progesterone output of these cells in an unstimulated state. To further this theory, it would be of interest to measure progesterone binding and secretion from LH-stimulated and basally secreting small luteal cells, to ascertain if indeed progesterone binding activity can be correlated with

progesterone secretion. Pilot experiments have been performed to address this question, but due to difficulties of tissue availability and time constraints no firm conclusions have yet been drawn.

Also possible is that the progesterone binding protein studied here may be involved in the relief of end-point inhibition of steroidogenic enzymes. End product inhibition occurs with all of the enzymes involved in progesterone biosynthesis (Niswender & Nett, 1994). For example, the affinity of 3B-HSD for progesterone, its product, is approximately six times greater than that of its substrate, pregnenolone (Caffrey *et al*, 1979a). It is suggested that binding proteins sequester progesterone and thus remove its negative feedback effects on its own production (Caffrey *et al*, 1979a). However, since oestradiol and testosterone have similar high affinities for this enzyme (3β–HSD) (Caffrey *et al*, 1979a), then the protein studied here, since it does not bind either of these steroids with a high affinity, may be specific for the relief of end point-inhibition exerted by progesterone only, and thus would be an integral part of a system of autocrine regulation of progesterone synthesis by progesterone. There have been suggestions of this type of self-regulatory system previously. Basal bovine luteal progesterone secretion has been shown to be pulsatile (Rossmanith *et al*, 1991). Pulse frequency was unaffected by hCG stimulation to tissue slices of bovine corpora lutea perfused *in vitro*. However the amplitude and release rates of progesterone were increased (Rossmanith *et al*, 1991) as a result of this treatment. This pulsatile progesterone release was suggested to be due to large luteal cells since it appeared to be independent of LH stimulation. Furthermore, the antiprogesterone ZK 96.734 increased the amplitude and frequency of the progesterone pulses, suggesting that progesterone exerted inhibitory effects

on its own release by short, local inhibitory progesterone feedback in the bovine corpus luteum (Rossmanith, 1991). As a secondary system the high blood flow to the corpus luteum may also help to keep levels of steroid down (Caffrey *et al*, 1979a) by efficient removal into the blood. Conversely, progesterone has been suggested to up-regulate its own synthesis and secretion (Rothchild, 1981). Hence, if the progesterone binding site was a non-genomic receptor for progesterone involved in this type of autocrine regulation, then it would be expected to be present in the cell type which secreted the most progesterone. Since large luteal cells contained more progesterone binding activity than small cells, and these are the cells responsible for a large amount of the progesterone secreted by the corpus luteum, it could be that this binding site operates through binding of progesterone to exert stimulation of progesterone synthesis and/or secretion. Increased progesterone production occurs in bovine follicles post LH surge, and continues until ovulation (Dieleman & Blankenstein, 1985). This increase is suggested to reflect morphological luteinization of granulosa cells, however, prior to this time, follicular progesterone is thought to be mainly theca derived (Dieleman *et al*, 1983b). If indeed the follicles examined in these experiments were removed prior to the preovulatory LH surge, then the cellular distribution of progesterone binding sites would also fit in with this hypothesis. However, from the actual levels of binding activity found theca and granulosa cells, and the observation that although progesterone secretion from theca cells increases after the LH surge, it is the granulosa cells which contribute the majority of follicular progesterone (Fortune & Hansel, 1979), then it would appear that in the follicle the progesterone binding site would be more likely to be involved in another role. It would be of interest to repeat these experiments on follicular cells

and measure the actual amount of progesterone in follicular fluid and the theca and granulosa cells ability to synthesise progesterone in culture, to see if any correlation between progesterone binding and progesterone synthesising ability was evident. Thus it is unclear at present why there is an apparent switch in the cell type which contains the most progesterone binding from the theca in the follicle to the granulosa derived large luteal cells in the corpus luteum.

Results of Western blots of steroidogenic enzymes in isolated bovine large and small luteal cells showed that large luteal cells contain more 3ß - hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase than do small luteal cells, reflecting that in large luteal cells from the bovine corpus luteum, basal progesterone secretion is 10-20 fold greater than the basal progesterone secretion of small luteal cells (Koos & Hansel, 1981; Alila *et al*, 1988). Both cell types appeared to contain surprisingly little P_{450} side chain cleavage (49 kDa). Interestingly, however, immunodetection of P_{450} side chain cleavage revealed bands of approximately 68 kDa in both cell types. This band was found in greater quantities in large luteal cells than in small luteal cells. It has been reported that 1:1, covalent, non-disulphide linked cross-linkage of bovine P_{450} side chain cleavage and its electron donor adrenodoxin occurs (Young *et al*, 1995). The cross-linked product has a reported molecular weight of 63 kDa (Young *et al*, 1995). It is likely that the approximately 68 kDa band evident in these experiments is due to this cross-linkng between P_{450} side chain cleavage and adrenodoxin. Thus, though approximately equal amounts of P_{450} side chain cleavage were observed in large and small luteal cells, if no cross-linking of this type had occurred, there may have been increased amounts of P_{450} side chain cleavage detected in large luteal cells as compared to small luteal cells. Indeed, there is evidence to suggest that greater quantities of P_{450} side chain cleavage are found in large luteal cells than in small luteal cells (Rodgers *et al*, 1986b). This cross linkage may occur in the tissue before experimental procedures commence. It is suggested that low levels of anti-oxidants lead to this cross-linkage (Young *et al*, 1995), and thus it would be of interest to determine the effects of inclusion of anti-oxidants in the separation procedures employed here. In Chapter 2

when bovine luteal sucrose gradient fractions were immunoblotted for the detection of P_{450} 17 α hydroxylase/C 17,20 lyase no detection was evident. It was of interest to note that there was a small amount of this protein detected in homogenates of large and small luteal cells. Detection sensitivity in these experiments was increased because of the use of enriched populations of luteal cells rather than luteal homogenate which contains a number of non-steroidogenic cell types, thus per mg of total protein the large and small cell homogenates would have contained higher levels of steroidogenic enzymes. It does not appear that this enzyme is active in the bovine corpus luteum (Savard, 1973), however, these experiments show that it is present. Tranfection studies using P_{450} 17 α hydroxylase/C 17,20 lyase -specific DNA constructs have revealed that bovine luteal cells do not express P_{450} 17 α hydroxylase/C17,20 lyase (Lauber *et al*, 1991). It is possible that the P_{450} 17 α hydroxylase/C 17,20 lyase detected may have been synthesised before luteinization and is inactive in the corpus luteum, however, the fact that it is present, even in small quantities may have some importance since androgens are now implicated in the regulation of granulosa cell function in developing follicles, and androgen receptor has been detected in small amounts in the corpus luteum of rats (Tetsuka et al, 1995).

In summary, results presented in this chapter indicated that the progesterone binding protein studied in Chapter 3 is found in different amounts in the cell types of the bovine corpus luteum and the bovine preovulatory follicle.

Chapter 5

Purification of the luteal progesterone binding protein

5.1 Introduction

Previous chapters have demonstrated the presence of a unique particulate progesterone binding site in both the pre-ovulatory follicle and the corpus luteum of cows. The significance of these binding sites to the regulation of progesterone synthesis, or the secretion of this steroid, remains unclear. To further examine the cellular distribution of this binding protein by, for example, immunocytochemistry, necessitates the raising of a specific anti-serum against the binding site. An antibody raised against the progesterone binding protein would also facilitate the study of the regulation of progesterone binding protein at the molecular level, and could be used to probe a bovine luteal cDNA library to facilitate isolation of the gene encoding this progesterone binding protein.

To generate polyclonal antisera specific to the bovine progesterone binding site, a purified sample of progesterone binding protein must first be obtained. This chapter deals with the attempted purification of the bovine luteal progesterone binding site.

Since it has been demonstrated that the binding protein in question is membrane associated, the first stage of any purification requires the solubilization of the protein. To this end, a number of detergents commonly

used for protein extraction and purification were screened for suitability, the criteria being optimal solubilization with minimal losses of activity.

Once solubilized, there are a number of protein purification techniques that are commonly used sequentially to purify proteins of interest. A number of these techniques have been exploited, each being subject to screening for its purification potency and its effects on the activity of the progesterone binding site being purified. The aim of this chapter was to devise a purification strategy which would yield reasonable amounts of purified progesterone binding protein without excessive losses in activity (since at this stage progesterone binding activity is the only measure of the protein of interest). The purified protein could then be micro-sequenced to provide identification of the protein, and to indicate whether it is a novel protein which has not yet been characterised.

5.2 Materials and Methods

5.2.1 Inhibition of endogenous proteases

To minimise degradation of binding sites during solubilization procedures and subsequent purification protocols, a cocktail of protease inhibitors was added to solubilisation and extraction media. This cocktail contained 1mM EDTA (inhibitor of metalloproteinases), 2 μg pepstatin A (inhibitor of carboxy peptidases) and 1 mM PMSF (inhibitor of serine proteases). Progesterone binding was assayed in the presence of this cocktail to ascertain any adverse effects that these protease inhibitors may have on progesterone binding. As a control, progesterone binding was assayed in the absence of protease inhibitor cocktail (buffer control) and in the presence of ethanol at similar levels to that found in the protease inhibitor cocktail (EtOH control) due to PMSF being dissolved in ethanol (10 μl ethanol/PMSF per ml incubation mix). Cocktail inhibitors/ethanol/buffer controls were incubated for 30 min at 4°Cwith pooled sucrose density gradient fractions enriched in progesterone binding, before removal of an aliquot of this mix to be assayed in the *in vitro* progesterone binding assay.

5.2.2 Detergent solubilization of progesterone binding protein

Detergents (Boehringer Mannheim detergent kit, specially purified for membrane research) were screened for their ability to solubilise the progesterone binding site from its associated membranes, without causing loss of binding activity. This screening was achieved by incubating pooled sucrose density gradient fractions enriched in progesterone binding with each detergent at a number of concentrations relative to the critical miscellar concentration (CMC) of each detergent (10xCMC, 1xCMC, 0.5xCMC,

0.3xCMC, 0.1xCMC, 0.05xCMC). Each detergent was dissolved in 10 mM Tris HCl, pH 7.4, 10% glycerol. Solubilization cocktails (detergent, buffer, fractions enriched in progesterone binding and protease inhibitors) were mixed on ice for 1 hour to ensure maximal solubilization, then centrifuged at 30,000 r.p.m for 1 hour at 4°C. Controls consisted of the solubilization mix with no detergent added. Supernatants and pellets were then separated and stored at -20°C until assayed for progesterone binding.

5.2.3 Removal of endogenous steroids

1 ml aliquots of dextran-coated charcoal were centrifuged in assay tubes (1000 g.av 10 min, 4°C). Supernatants were aspirated and discarded, and 0.5 ml aliquots of detergent-solubilised sucrose gradient fraction enriched in progesterone binding (solubilised progesterone b.p.) added to the charcoal pellets. Tubes were then vortexed and incubated on ice for 10 min, before centrifuging (1000 g.av, 10 min, 4°C). Supernatants were then removed and assayed for their progesterone binding ability. To give an indication of the effects of removal of endogenous steroid, samples of unstripped solubilised progesterone b.p. were included in all assays.

5.2.4 Ion exchange chromatography

Anion and cation exchange resins were tested for their ability to bind the progesterone binding protein in a batch test. 1ml aliquots of cation exchange resin (CM-Sepharose, Pharmacia-Biotech) or anion exchange resin (DEAE-Sepharose, Pharmacia-Biotech) were equilibrated with an equal volume of 10 mM Tris HCl buffer, pH 7.4, containing 2 mM n-octyl glucoside. 200 µl of each resin was then added to assay tubes and centrifuged (250 g.av, 4°C, 10 min) to compact the resin. Supernatants were aspirated, and 100 µl of

solubilized progesterone b.p. added to resin pellets. 400 µl of each salt elution buffer (Tris HCl, pH 7.4, 0 - 1M NaCl) was then added and each tube vortexed to resuspend the resin. Each salt elution concentration was set up in duplicate. After a 30 minute incubation (4°C, gentle agitation) tubes were centrifuged (250 g.av, 4°C, 10 min), and supernatants assayed for progesterone binding activity. Knowledge of the salt concentration which caused elution of bound progesterone binding protein from the exchange resin was then used to construct a narrow range of salt elution buffers to ensure good selective elution of the binding protein. Columns (5 ml) of ion exchange resins were prepared, by first equilibrating the resins as described above, then incubating each resin with solubilised progesterone b.p. (30 min, 4°C, stirred), before pooling and collecting fractions (500 µl) from increasing stepwise salt elutions. Fractions were then assayed for progesterone binding activity. Fractions enriched in progesterone binding activity were then pooled. Protein estimations were routinely carried out to provide an indication of the degree of purification achieved.

5.2.5 HPLC separations

Two types of HPLC were attempted, anion exchange and hydroxylapatite chromatography. Using a commercially available auto-injection system (Bio-Rad), 1 ml of solubilised progesterone b.p. was loaded on to an HPHT hydroxylapatite column (Bio-Rad). A micro-computer-controlled gradient module was used to set up a linear gradient from 10 - 300 mM sodium phosphate eluent solution buffer (10 - 300 mM NaH_2PO_4/Na_2HPO_4 buffer, 0.01 mM $CaCl_2$, pH 6.8). Elution of protein was monitored by U.V. detection (250 nm) and fluorimetry for 30 minutes, the time taken for the NaCl

gradient to run from 0 - 100%, during which eighty 0.5 ml fractions were collected.

Anion exchange chromatography was carried out on the above apparatus using a Bio-Rad anion exchange column (50 X 7.8 mm MA7Q) . In a trial experiment it was observed that the progesterone binding was eluted early in the run, though other material remained bound to the column with much longer retention times. Thus sufficient separation was achieved with a 15 minute run, running isocratically for 5 minutes (20 mM Tris HCl, pH 8.5), then forming a linear gradient (20 mM Tris HCl, pH 8.5 to 20 mM Tris HCl, 500 mM NaCl, pH 8.5).

5.2.6 Affinity Chromatography

A number of progesterone analogues were tested for their ability to displace bound [^{3}H]-progesterone from solubilised progesterone binding protein (as detailed in Chapter 3 for displacement experiments using sucrose gradient fractions). 20 ß hydroxy progesterone (20ß-OH-P_4) was found to be the most effective of these compounds. The affinity matrix chosen, Epoxy-activated Sepharose CL-4B (Pharmacia), was deemed suitable because of its long spacer arm, which theoretically should give the affinity ligand maximum spatial freedom, allowing it to interact as fully as possible with the progesterone binding protein.

A 1 mg/ml solution of 20ß-OH-P_4 was prepared in coupling solution (1:1 dimethylformamide : distilled H_2O) and the pH adjusted to pH 11 with 1N NaOH. This was mixed with 2 g of Epoxy-activated Sepharose CL-4B, which was previously swollen in H_2O, and washed under flowing distilled H_2O on

a sintered glass plate for 1 hour. Coupling was then allowed to proceed for 16 hours in a shaking water bath at 30°C.

After coupling, the gel was washed in fresh coupling solution, followed by distilled H_2O, on a sintered glass plate. Next, the gel was washed alternately in 0.1M sodium acetate buffer, 0.5 M NaCl, pH 4 and 0.1 M Tris HCl, 0.5M NaCl, pH 8 (3 cycles of each buffer). Finally, any uncoupled reactive oxirane groups were blocked by incubation of the gel in 1M ethanolamine for 16 hours at 45°C. Gels were then washed thoroughly in sample application buffer (10 mM Tris HCl, pH 7.4). As a control to determine the specificity of interactions between the affinity construct and the progesterone b.p., a sample of gel was processed in the above manner with no ligand linking, in which all reactive groups were blocked with ethanolamine.

200 µl aliquots of DCC-stripped solubilized progesterone b.p. were applied to 0.5 ml pellets of each gel (control gel and 20ß-OH-P_4---affinity construct), in the presence and absence of 160 µl 10 mg/ml digitonin (amount of digitonin required for maximum stimulation of 200 µl of solubilised progesterone b.p.), and incubated for 2 hours at 4°C. Tubes were then centrifuged (1000 g.av, 10 min, 4°C). Supernatants were removed and transferred to fresh tubes. Pellets were then eluted by two methods, (i) increasing ionic strength (addition of 0.5 ml 1 M NaCl) or (ii) by lowering pH (addition of 0.1 M sodium acetate buffer, pH 4). From ion-exchange experiments it was noted that 1M NaCl had little effect on the activity of the progesterone binding site. Thus no further processing of these elutions was required. However, elutions of pH 4 buffer were spun to equilibrium in 30,000 Da nominal molecular weight limit (nmwl) cut-off filters (Millipore),

and resuspended in 10 mM Tris HCl, pH 7.4. Supernatants and eluted fractions from affinity and control gels were assayed for progesterone binding ability.

5.2.7 Chromatofocusing

20 mls PBE 94 (Pharmacia) were equilibrated by rinsing with 100 mls of 0.025 M ethanolamine acetate buffer, pH 9.4. Gel was then poured into a 15 x 0.5 cm column. To ensure correct column packing, all columns were tested with 100 µl of 10 mg/ml horse heart cytochrome c (Sigma). After connecting the column outflow to a U.V monitor and plotter (Bio-Rad) set to detect at 280 nm, 5 ml of eluent buffer (PBE 96, pH 6) (Pharmacia) were applied, followed by application of 1 ml of solubilised progesterone b.p., which had been pre-equilibrated in eluent buffer. Eluent was then applied at a flow rate of 1 ml/min, and 0.5 ml fractions collected until the pH of the collected fractions stabilized at pH 6, as measured by pH paper (Whatman, pH 4 - 10). Fractions were then assayed for the ability to bind [^{3}H]-progesterone, as described previously.

5.2.8 Gel filtration

5 cm x 0.5 cm columns of Sephadex G-100 (Pharmacia) were prepared by swelling the dried gel in 0.1 M ammonium bicarbonate overnight. Columns were then poured and equilibrated with eluent buffer (10 mM Tris HCl, pH 7.4, containing 2 mM n-octylglucoside). Aliquots (100 - 300 µl) of DCC-stripped solubilised progesterone b.p. were then applied, and elution buffer applied at a flow rate of 1 ml/min. 0.5 ml fractions were collected until the column had been eluted with five times the column bed volume of eluent buffer. Fractions were then assayed for [^{3}H]-progesterone binding ability.

5.2.9 Centrifugal separators

DCC-stripped solubilised progesterone b.p. (100 µl) was applied to 5000 Da, 10,000 Da, 30,000 Da (Millipore) and 100,000 Da (Centricon) nominal molecular weight limit (nmwl) cut-off filters. These were then centrifuged (500 g.av., 4°C) until all of the applied sample had been filtered. Retentates were resuspended in 10 mM Tris HCl, pH 7.4, containing 2 mM n-octylglucoside, and the centrifugation/filtration step repeated. Retentates were then resuspended in the same buffer and retentates and filtrates were assayed for the ability to bind [^{3}H]-progesterone. Also, the effects of repeated re-suspension and filtration in 100 kDa nmwl cut-off filters with respect to progesterone binding activity were investigated.

5.2.10 Freeze drying

Samples to be freeze dried were centrfuged to equilibrium in 30,000 Da nmwl cut-off filters (Millipore). Retentates were then resuspended in 0.1 M ammonium bicarbonate, transferred to fresh tubes, and frozen on solid CO_2 for 1 hour. Samples were transferred to freeze-drying apparatus and freeze dried overnight.

5.2.11 Gel electrophoresis

Denaturing SDS-PAGE (7.5%) gels were prepared as described in Chapter 2. Non-denaturing PAGE gels were prepared in the same manner, omitting SDS and 2-mercaptoethanol from gels, and both running and sample buffers. Samples were applied to non-denaturing gels without boiling.

After each gel run was complete, gels were removed from the running apparatus (Bio-Rad Protean xi) and fixed overnight in 4:1:5 methanol : glacial

acetic acid : distilled H_2O. After removal from fixative solution, gels were stained using a commercially available silver nitrate kit (Sigma). Once background bleaching was complete, gels were examined and re-stained if necessary. Once satisfactory staining was achieved, gels were then photographed, labelled, then re-photographed.

5.3 Results

5.3.1 Effects of protease inhibitor cocktail

As displayed in Figure 5.3.1, the cocktail of protease inhibitors had no deleterious effect on progesterone binding.

5.3.2 Detergent solubilization of progesterone binding protein

One of the detergents tested, (decanoyl-N-methylglucamide), caused a loss of activity at high concentrations and was thus unsuitable for solubilization of the progesterone b.p. Of the other detergents tried, n-dodecylglucoside failed to solubilize the binding site. However, both n-dodecyl-ß-D-maltoside and n-octylglucoside effectively solubilized the binding site from its associated membrane material. n-Octylglucoside was chosen as the detergent for solubilization procedures because it was most effective at its CMC and because of its biochemical characteristics (see Discussion 5.4) (Figure 5.3.2).

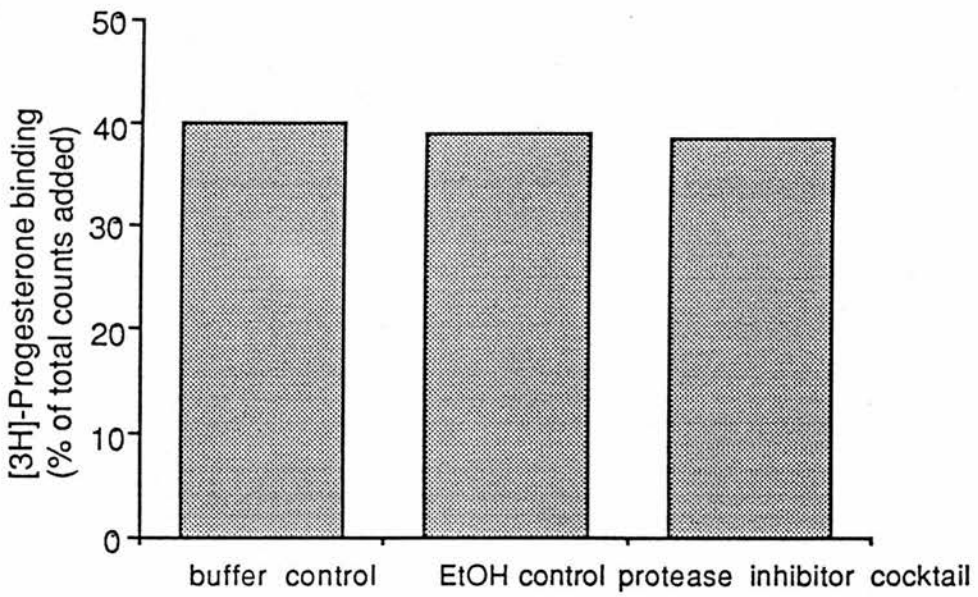
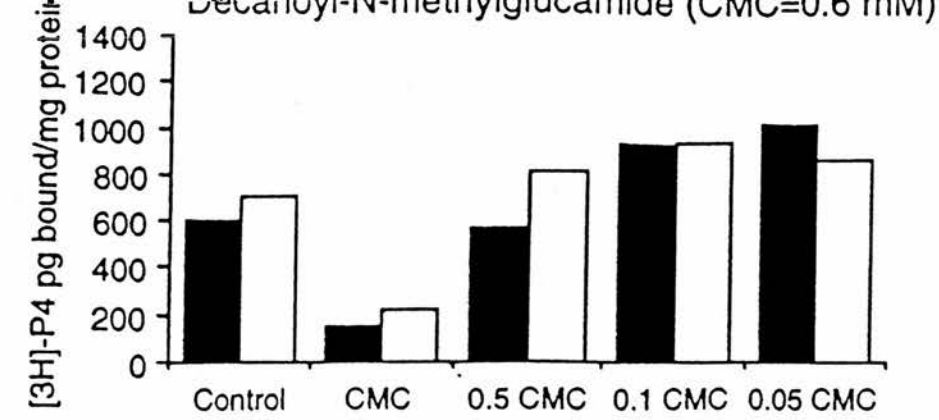
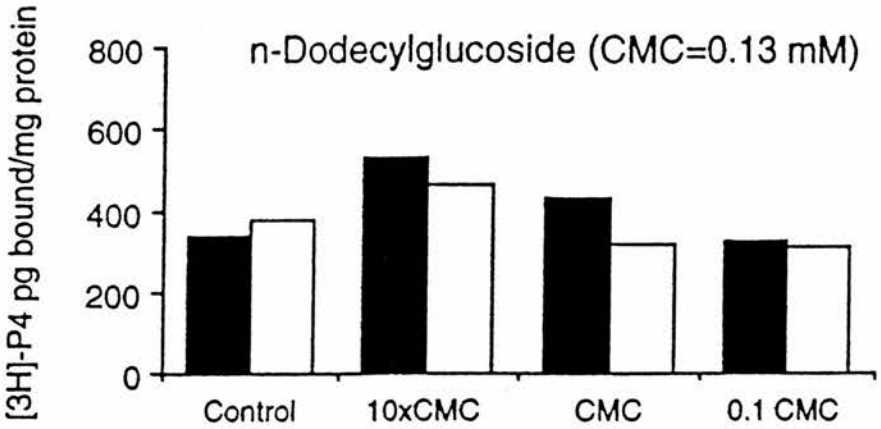
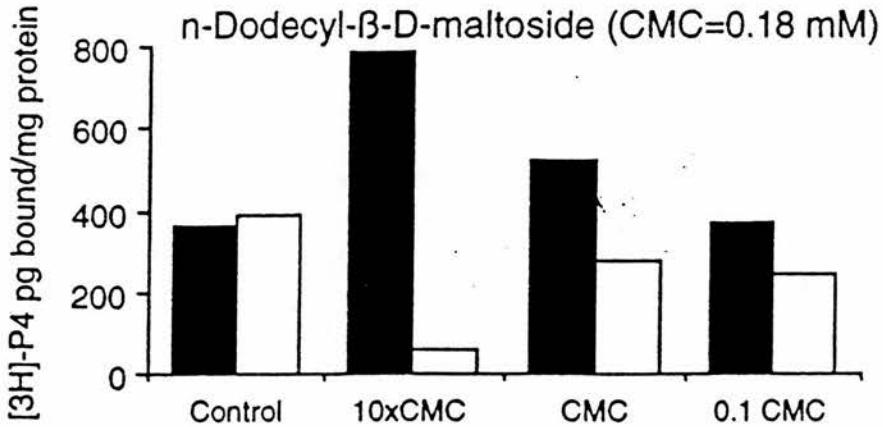


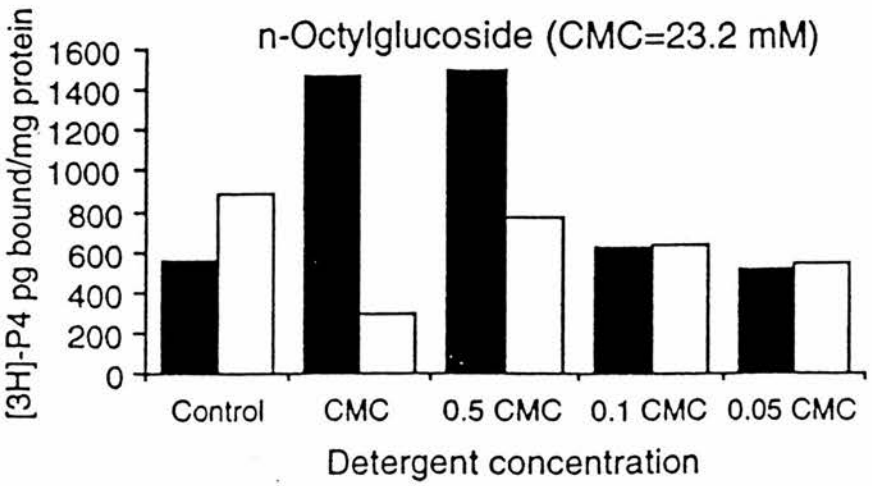
Figure 5.3.1 Lack of effect of protease inhibitor cocktail on progesterone binding activity of sucrose gradient fraction enriched in progesterone binding activity.

Figure 5.3.2 Trial of detergents to solubilize the progesterone binding protein away from associated membranes. Open bars represent [^{3}H]-progesterone binding to 30,000 rpm pellet, shaded bars represent [^{3}H]-progesterone binding to 30,000 rpm supernatant. Results for each detergent were calculated from the mean of two separate such experiments. All assays were performed in triplicate, and results expressed as pg of [^{3}H]-progesterone bound/per mg of protein. See Results (5.3) and Discussion (5.4) for further details.


Decanoyl-N-methylglucamide (CMC=0.6 mM)
[3H]-P4 pg bound/mg protein
1400
1200
1000
800
600
400
200
0
Control
CMC
0.5 CMC
0.1 CMC
0.05 CMC


n-Dodecylglucoside (CMC=0.13 mM)
[3H]-P4 pg bound/mg protein
800
600
400
200
0
Control
10xCMC
CMC
0.1 CMC


n-Dodecyl-ß-D-maltoside (CMC=0.18 mM)
[3H]-P4 pg bound/mg protein
800
600
400
200
0
Control
10xCMC
CMC
0.1 CMC


n-Octylglucoside (CMC=23.2 mM)
[3H]-P4 pg bound/mg protein
1600
1400
1200
1000
800
600
400
200
0
Control
CMC
0.5 CMC
0.1 CMC
0.05 CMC
Detergent concentration

5.3.3 Stripping of endogenous steroid

Dextran stripping of solubilised progesterone b.p. increased the amount of binding present by up to 60% (Figure 5.3.3)

5.3.4 Ion exchange chromatography

A typical elution profile of progesterone b.p from a CM-sepharose column is shown by Figure 5.3.4. From batch tests, no binding of progesterone b.p. to the cation exchange resin was evident. However, it was clear that though the progesterone b.p. does not bind to CM-Sepharose, a significant amount of inactive material does bind and was thus removed by this resin.

Figure 5.3.4 (lower graph) shows a typical elution profile of progesterone b.p. from a column of DEAE-Sepharose. Results from batch tests demonstrated that the progesterone b.p. was bound by anion exchange resin, indicating that the binding protein has a net negative charge at neutral pH. The salt concentration at which the progesterone b.p became dissociated from the anion exchange resin was found to be 400 mM NaCl. During both types of ion exchange chromatography, losses in activity were low (5 - 10 %).

HPLC anion exchange chromatography resulted in minimal losses of progesterone binding activity. The progesterone b.p. was eluted early in each HPLC run, before commencement of the elution gradient, indicating a difference in the binding properties of anion exchange gels and anion exchange HPLC matrices used (Figure 5.3.5). No progesterone binding activity could be recovered from the hydroxylapatite HPLC column; thus this step was omitted from purification strategies (data not shown).

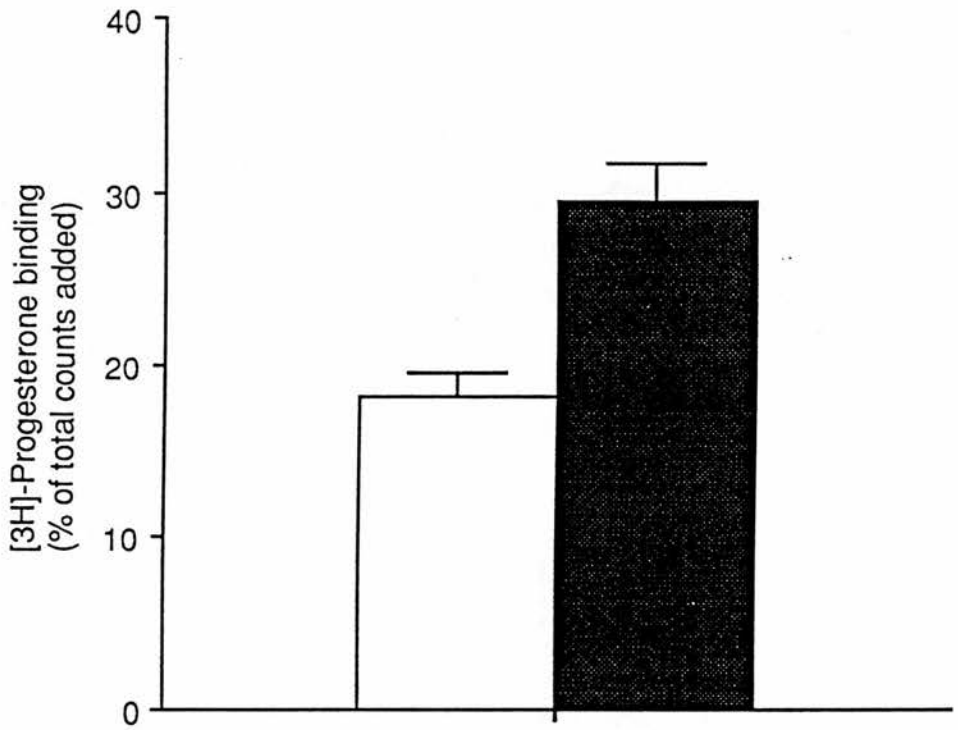


Figure 5.3.3 Effect of removal of endogenous steroid by dextran coated charcoal stripping on progesterone binding activity of solubilised progesterone b.p. Open bar represents untreated solubilised progesterone b.p., shaded bar represents dextran coated charcoal stripped solubilised progesterone b.p. $\pm$ S.E.M, (n=4 experiments).

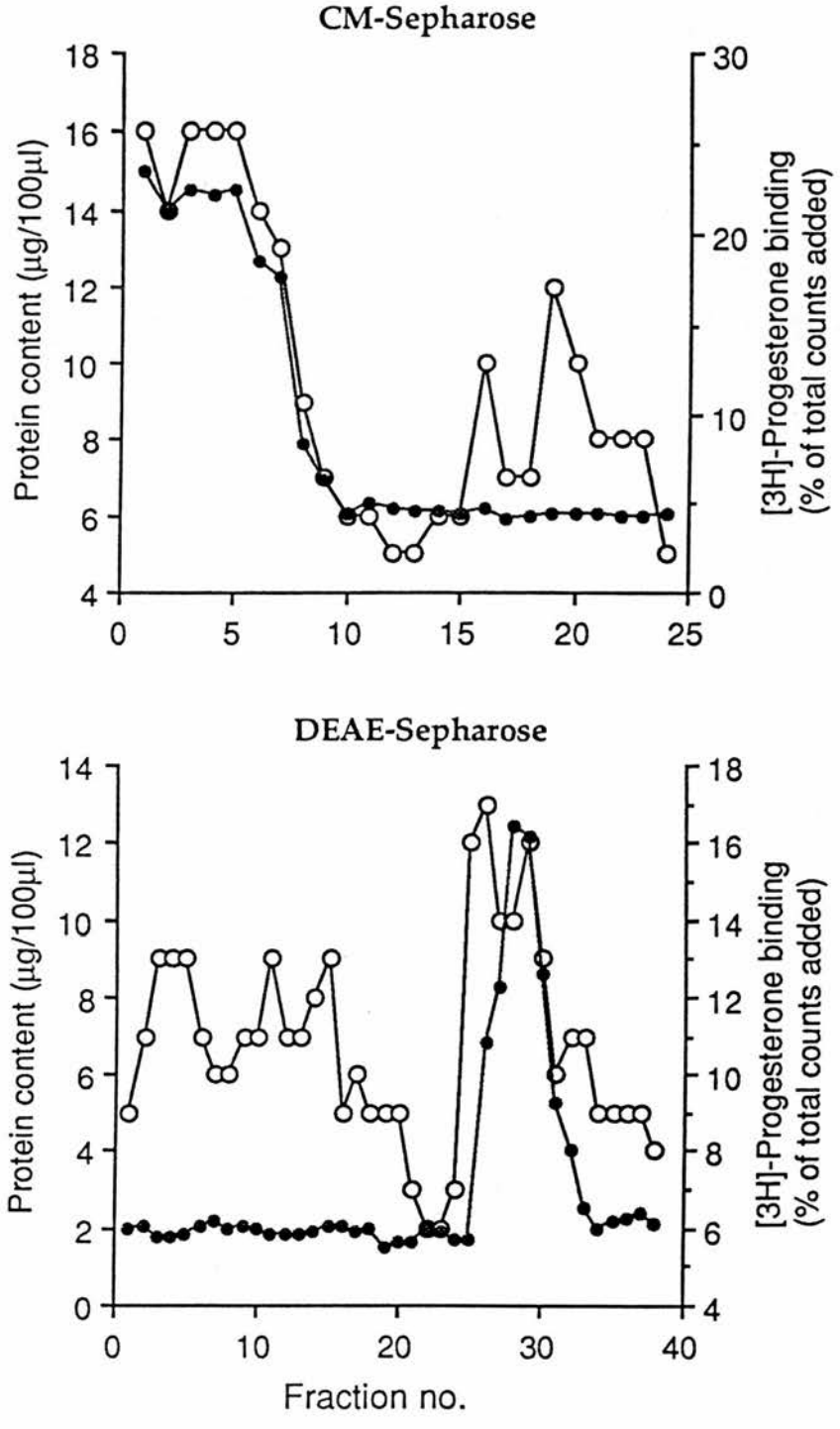


Figure 5.3.4 Elution profiles of progesterone b.p from CM-Sepharose and DEAE-sepharose ion exchange gels. Elution was achieved with stepwise NaCl gradients. (o) = Protein content, (•) = [^{3}H]-Progesterone binding.

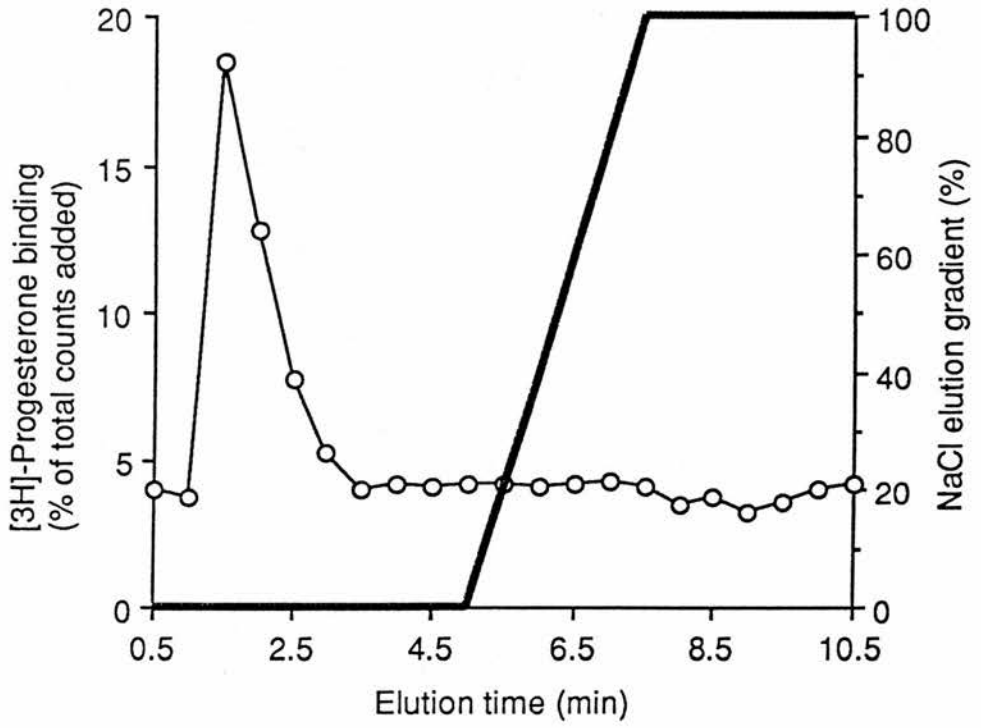


Figure 5.3.5 Elution profile of progesterone b.p from an HPLC anion exchange matrix. (o) = progesterone binding activity; heavy black line = NaCl gradient (0% = 0 M, 100 % = 0.5 M)

5.3.5 Gel filtration

In experiments where solubilised progesterone b.p was applied to a G-100 column and eluted with buffer containing no detergent, binding activity passed quickly through the column, with a short retention time, and appeared to run as an aggregate. Also, large losses in activity were observed. Where elution buffer contained detergent (2 mM n-octylglucoside) the binding activity was observed to pass through the column more slowly than in the absence of detergent, being eluted much later (Figure 5.3.6). However, 150 - 200 µl of fraction was necessary to show maximal binding, as compared to 30 µl of solubilized progesterone b.p before G-100 fractionation, with the result that activity losses of between 40 - 70% commonly occurred. These losses were investigated by mixing fractions obtained from gel filtration experiments together to see if binding activity could be reconstituted. However, these were unsuccessful, and binding activity could not be restored. Also, the possibility of the binding site adsorbing to G-100 was examined by testing the effects of inclusion of gel on progesterone binding activity in a batchwise test as detailed for the ion exchange matrices. However, no direct interaction between G-100 and the progesterone binding site was evident. In practice, shorter G-100 columns were used (5 cm) in an effort to keep losses to a minimum. However, activity losses were still substantial. Thus, gel filtration was suitable only for initial purification stages, prior to ion exchange chromatography.

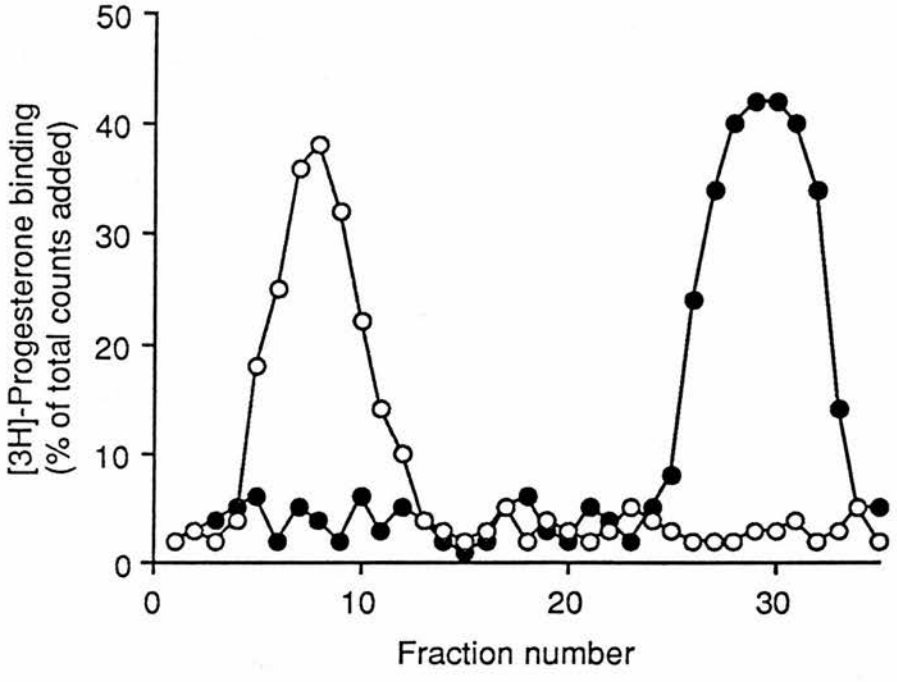


Figure 5.3.6 Elution of progesterone b.p from Sephadex-G100 gel filtration column, with (•) and without (o) 2 mM n-octylglucoside in eluent buffer.

5.3.6 Affinity chromatography

It could not be ascertained whether or not the progesterone b.p. bound to the immobilised 20β OH-progesterone because no activity could be recovered in these experiments. However, this was also the case for unlinked, fully blocked affinity matrix. In batchwise tests it was ascertained that the affinity matrix, whether bound to 20β OH-progesterone or not, had the ability to completely inactivate progesterone binding activity present.

5.3.7 Chromatofocusing

As was the case with the affinity matrix, no activity could be detected after passing solubilised progesterone b.p. through a chromatofocusing column. In batchwise tests, it was demonstrated that contact with the chromatofocusing matrix destroyed all binding activity present. This effect was exerted by the gel, since the buffers used in this process were demonstrated to have no effect on progesterone binding activity.

5.3.8 Freeze drying

It was demonstrated that freeze drying of semi-purified extracts rich in progesterone binding activity followed by reconstitution of the freeze dried residue in 10 mM Tris HCl, pH 7.4 caused some loss of activity, but the progesterone binding activity could be reconstituted (ca. 15% losses).

5.3.9 Molecular weight cut-off filters

Binding was recovered in the retained fractions of all filters (Figure 5.3.7 A). This indicated that the progesterone binding site was larger than 100 kDa (highest nmwl filter) in its active form. Repeated resuspension and recentrifugation caused an approximately 10% loss of activity with each resuspension and refiltration cycle with 100 kDa cut-off filters (Figure 5.3.7 B).

5.3.10 Electrophoresis of progesterone binding site

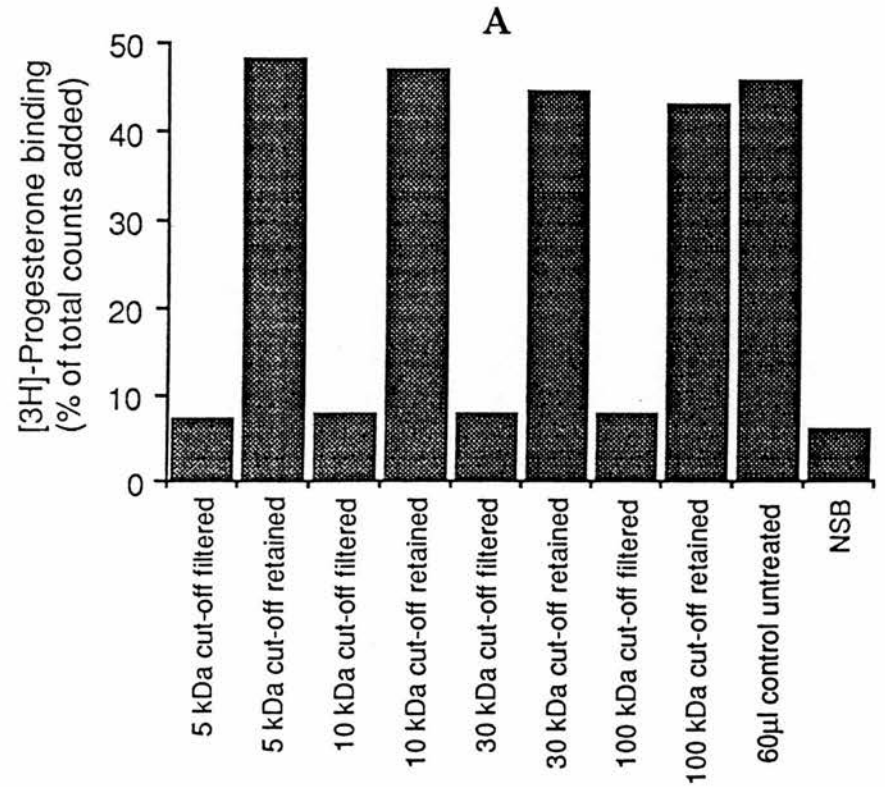
Plate 5.3.1 shows the proteins present in bovine corpus luteum whole homogenate (lane 1), proteins present in a sample of solubilised progesterone b.p. (lane 2) and those proteins present after cation and anion exchange chromatography of this sample (lane 3). From this gel it can be observed that these initial purification stages greatly reduced the total number of proteins present in samples of progesterone b.p. as compared to the initial protein content of a luteal homogenate.

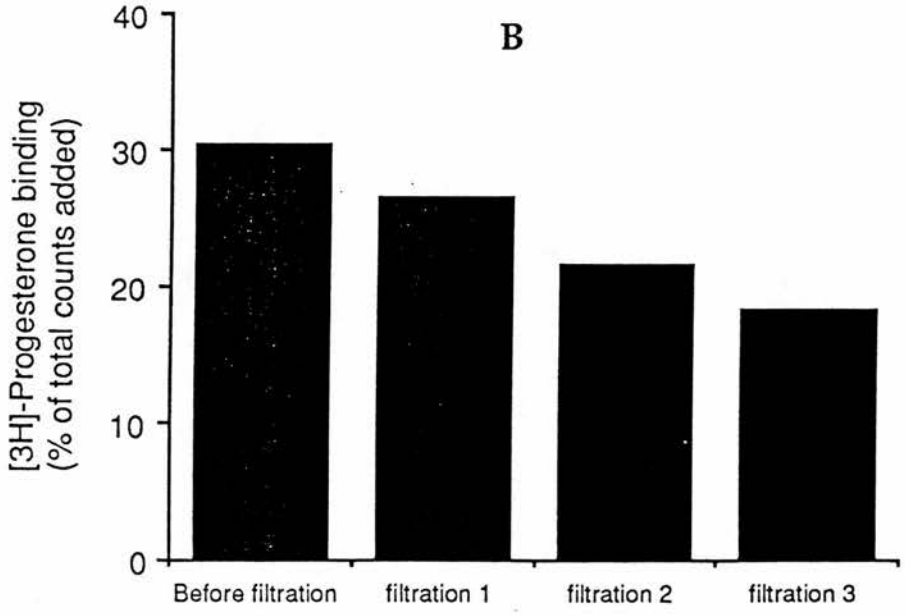
Plate 5.3.2 shows the total proteins present in a sample of solubilised progesterone b.p. after gel filtration following ion-exchange chromatography. This gel was a non-denaturing gel, and it can be seen that much of the protein appears to have aggregated, since very few protein bands are apparent and those which are present detect very strongly.

Plate 5.3.3 shows the results of electrophoresis of a sample of semi-pure solubilised progesterone b.p. after ion-exchange and gel-filtration chromatography and ion-exchange HPLC. Lane 1 contains protein from filtrates of 100 kDa nmwl cut-off filtration, which contained no progesterone binding activity. Lane 2 contains the semi-pure solubilised progesterone b.p. Lane 3 contains the retentate from 100 kDa nmwl cut-off filters, which

contains progesterone binding activity. It can be observed that there are two bands of protein (indicated by arrowheads) present in both the 100 kDa nmwl retentate and unfiltered semi-pure progesterone b.p. samples (both contain progesterone binding activity) (lanes 2 and 3) which are both absent in the sample of filtrate from 100 kDa nmwl filtration of semi-pure progesterone b.p. (which contained no progesterone binding activity).

Figure 5.3.7 A = Test of molecular weight cut-off filters. In all cases progesterone binding activity was found exclusively in retentates recovered from filter cups. B = Repeat resuspension and filtration of progesterone binding site in 100 kDa cut-off filters caused losses of 5 - 10% of [^{3}H]-progesterone binding activity with each filtration. No activity was found in filtrates from these experiments.


A
[3H]-Progesterone binding
(% of total counts added)
50
40
30
20
10
0
5 kDa cut-off filtered
5 kDa cut-off retained
10 kDa cut-off filtered
10 kDa cut-off retained
30 kDa cut-off filtered
30 kDa cut-off retained
100 kDa cut-off filtered
100 kDa cut-off retained
60µl control untreated
NSB


B
[3H]-Progesterone binding
(% of total counts added)
40
30
20
10
0
Before filtration
filtration 1
filtration 2
filtration 3

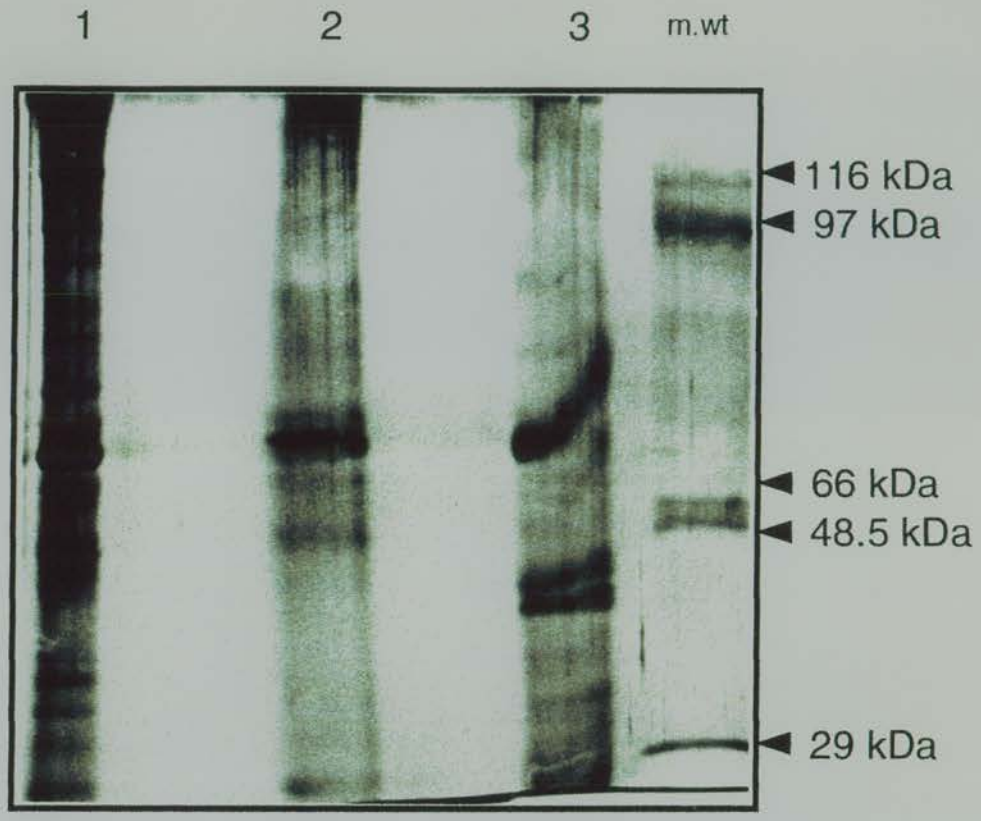


Plate 5.3.1 SDS-PAGE electophoresis gel (sliver nitrate stained) of whole bovine luteal homogenate (lane 1), n-octylglucoside solubilised progesterone b.p. (lane 2) and n-octylglucoside solubilised progesterone b.p. after anion and cation exchange chromatography (lane 3).

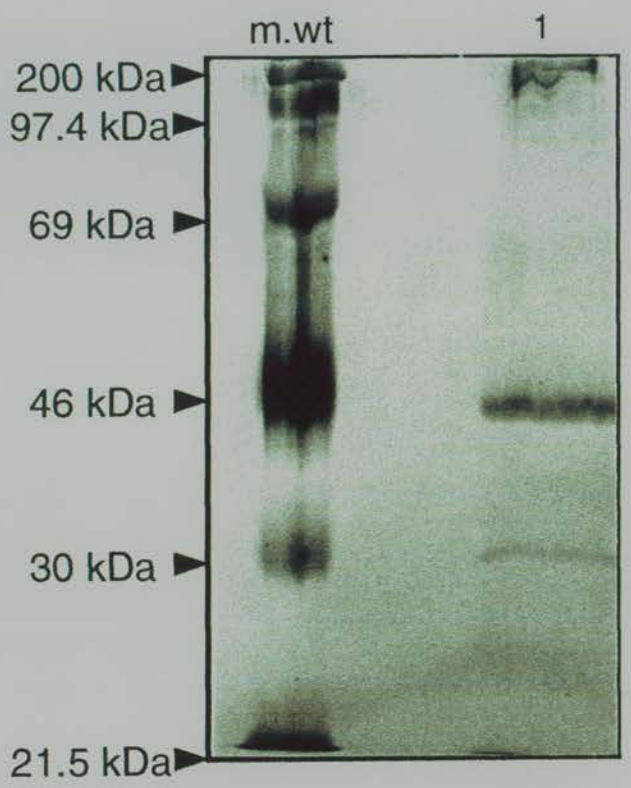


Plate 5.3.2 Non-denaturing PAGE gel of n-octylglucoside solubilised progesterone b.p. which had been purified by anion and cation ion exchange chromatography and G-100 gel filtration (lane 1).

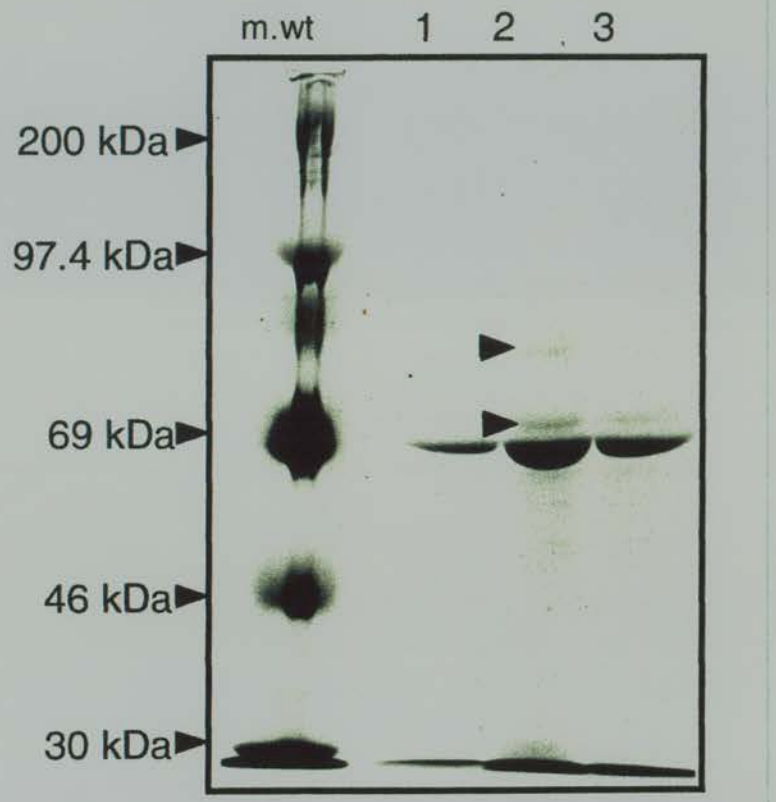


Plate 5.3.3 SDS-PAGE gel of purified progesterone b.p. Lane 1 contained a sample of the filtrate obtained by centrifuging n-octylglucoside solubilised progesterone b.p. (which had undergone ion-exchange chromatography (anion, cation and HPLC anion) and gel filtration) in a 100 kDa nmwl cut-off filter. Lane 2 contained a sample of the same semi-pure preparation without 100 kDa nmwl filtration, and lane 3 contained the retentate from such filtration experiments. Arrowheads indicate the presence of protein bands found in lanes 2 and three, but absent from lane 1 (see text for details).

5.4 Discussion

Protein purification systems employ a number of techniques to achieve the goal of obtaining a pure sample of the protein of interest which can then be used for further characterization and identification. The experiments detailed in this chapter were designed to allow purification of the progesterone binding protein studied in previous chapters.

From results of sucrose gradient fractionation of luteal homogenates it was observed that the progesterone binding protein under study was membrane associated. However, it was not known whether or not this membrane association was intrinsic or extrinsic. Since detergent solubilization can be used to separate either type of protein from the membrane, this was the method chosen to remove the progesterone binding protein from its membrane location.

From the results of detergent solubilization experiments, it can be seen that control tubes (no added detergent) appeared to exhibit a degree of solubilization (Figure 5.3.2). It should be noted, however, that although the fraction containing some of the progesterone binding activity of controls does not sediment at centrifugal forces powerful enough to sediment membranes, this did not indicate that solubilization has occurred. Under a number of conditions, formation of microvesicles can occur. These require centrifugal speeds in excess of 100,000 g to sediment, and even then the supernatant may still contain a wide range of molecular species (Findlay, 1990), some of which may not be fully solubilized. Also, the more effective a detergent is at solubilizing protein from membranes, the greater the

likelihood of activity losses occurring as phospholipid is stripped away from the protein (Findlay, 1990). It is common therefore to include 10 - 50% glycerol in the solubilization buffer to avoid complete removal of membrane material and ensure lipid replacement with only detergent. A low concentration of glycerol (10%) was used to avoid the necessity of using centrifugal speeds of 200,000 g to pellet microvesicles through the denser medium; inclusion of glycerol may be partially responsible for the seemingly spontaneous "solubilization" that appears to occur. Indeed, when greater centrifugal speeds were used the amount of binding activity found in the supernatants of controls was reduced (data not shown). However, for practical reasons detergents were tested using lower speeds so that a microcentrifuge could be used to facilitate a higher throughput of samples. Inclusion of protease inhibitors was designed to complement the inclusion of glycerol by giving some degree of protection against unwanted proteolytic degradation of the progesterone binding protein.

From the results of detergent screening, it can be seen that both n-dodecylglucoside and decanoyl-N-methylglucamide had adverse effects on progesterone binding activity. However, both n-octylglucoside and n-dodecyl-ß-D-maltoside caused solubilization of the progesterone binding site from the membrane without unacceptably high activity losses. The detergent chosen for further use was n-octylglucoside. This decision was based on two factors; firstly n-octylglucoside displayed a wider solubilization concentration range, and secondly had a higher critical micellar concentrations (CMC). Detergents with low CMC values, such as dodecyl-ß-D-maltoside (CMC=0.18 mM), are more difficult to remove than detergents with high CMC values, such as n-octylglucoside (CMC=23.2

mM), which can be removed by simple dialysis (Findlay, 1990). Also, n-octylglucoside has the advantage over a wide range of ionic detergents available that it is uncharged. The use of an uncharged detergent avoids complications of alteration of the charge of the protein to be purified which may arise if, as in this case, the charge properties of the protein are to be exploited in its subsequent purification.

Dextran coated-charcoal stripping of solubilized progesterone b.p. was observed to increase binding activity by up to 60%. This was most probably due to the removal of endogenous progesterone that competes with the [^{3}H]-progesterone added to measure binding in the *in vitro* assay (Chapter 3). Thus, to obtain maximum binding from the smallest possible sample, all solubilized extracts were treated in this way before subsequent purification steps were employed.

From the results of ion-exchange chromatography, it can be seen that the progesterone b.p. adsorbs only to anion exchange gel, displaying a net negative charge. Inclusion of cation exchange chromatography in the purification strategy was deemed worthwhile however, since this method removed other contaminants with a net positive charge. Non-ionic detergent (n-octylglucoside) was included in all elution buffers to prevent protein aggregation and to maintain the progesterone b.p.'s native charge. Interestingly, the progesterone b.p. did not appear to adsorb to the HPLC anion exchange column. This may be explained by the higher elution pressures utilised in HPLC, or the differences in adsorption properties between HPLC matrices and ion-exchange gels. Hydroxylapatite HPLC caused complete loss of activity, possibly due to separation of one or more

sub-units of the progesterone b.p., because of differing elution profiles of these subunits.

Gel filtration experiments were limited in their successful applicability in this study. Large losses of activity (40 - 70%) accompanied all procedures giving size separations. From experiments designed to examine the nature of this activity loss, it did not appear that the actual matrix (Sephadex G-100, Pharmacia) had any direct interaction with the progesterone b.p. which could account for the losses of activity. It appears from these experiments that the progesterone b.p may dissociate into two or more components required to confer progesterone binding activity. The separated components may be protein subunits, cofactors or other prosthetic groups. Re-mixing of fractions obtained from gel filtration experiments did not restore activity. Thus once separated, the components of the progesterone binding site could not be reconstituted. This was also apparent with non-denaturing PAGE gels; no activity could be measured in fractions eluted from these gels.

Results obtained from centrifugation with molecular weight cut-off filters appeared to indicate that the progesterone b.p. was larger than 100 kDa, since all activity was retained by filters with a nmwl > 100 kDa. Interestingly, when the retained fraction was resuspended and refiltered, similar losses in activity were noted each time. This may also be indicative of subunit separation. At the centrifugal speeds employed, a sub-unit < 100kDa may become separated and filtered through the cut-off membrane. Indeed, electrophoresis of retentates of these filters, demonstrated that bands of protein less than 100 kDa were routinely observed, even after repeated washing and refiltering, suggesting that the active progesterone b.p. can be

resolved into two or more sub-units by SDS/2-mercaptoethanol reduction. Unfortunately, because of the lack of a pure sample of progesterone b.p. to raise antibodies, it was not possible to identify which protein bands were involved in progesterone binding activity, and were part of the progesterone binding complex.

The complete loss of all activity in experiments using chromatofocusing and affinity media was somewhat surprising. Given that each of these matrices has a reasonably high porosity the likelihood of the protein-detergent complex, (which may be large) being completely sterically hindered is unlikely. Hydrophobic interaction chromatography resin was also tested (results not shown) in a batch test without any sucessful recovery of activity, however, this was not considered to be an ideal tool for the purification of this particular protein, since the presence of any detergent will effectively attenuate hydrophobic interactions between protein and gel. In the case of the affinity gel, it was demonstrated that the gel itself was responsible for the loss of activity. The reason for this was unclear, since losses occurred in the absence of digitonin, and thus could not be accounted for by instability of the activated binding protein compared to its inactive, un-(digitonin)-stimulated state. Inactivation occurring with hydrophobic interaction chromatography may possibly be accounted for by separation of subunits, with the differential elution of each subunit of the progesterone b.p. No logical explanation can be given for the inactivation that occurred when the progesterone b.p was in contact with the chromatofocusing gel. This technique is a relatively recent innovation compared to the other purification strategies employed. Its advantages include its compatability with detergents. The buffers used in all of these procedures were tested

separately, but had little or no deleterious effect on progesterone binding activity. Also, these losses cannot be accounted for by some spurious size separation caused by the matrix, since batch tests revealed that activity was lost to the same degree as was observed in column studies. Had this been the case, then it would have been expected that losses of the same order would also be encountered when using ion-exchange gels. Clearly this was not the case. Proteolysis is also unlikely to be the cause, since increasing protease inhibitor concentrations had no effect on conservation of progesterone binding activity after contact with these gels. It is thus difficult to speculate on the causes of these losses of activity. A number of other gels are commercially available for these methods of protein purification, but due to constraints of time, the testing of other available products was not possible in this study. A number of new protein purification strategies have recently been developed which may provide future progress with this work, such as the 'Rotofor' system developed by Bio-Rad. This system allows the electrophoresis (from preparative stages up to final purification stages) of proteins in free solution, similar to PAGE/isoelectric focusing using polyacrylamide gels. Powerful techniques such as this were outwith the timescale imposed by completion of this thesis, however.

Given the relative instability of the progesterone b.p. as indicated by the large losses of activity encountered during attempts to purify it, it was somewhat surprising that activity could be reconstituted from freeze-dried extracts. The fact that this was possible, will enable long term storage of semi-pure progesterone b.p. without any inherent problems of proteolytic degradation upon thawing of frozen aqueous extracts.

From the results of the SDS/PAGE purity checks, it is clear that the goal of purifying the progesterone b.p. was not achieved. Nonetheless, it can be seen from Plate 5.3.3 that a reasonably pure sample of progesterone b.p. was obtained. This gel showed that the filtrate from 100 kDa nmwl cut-off filters (where there is no progesterone binding activity) lacked 2 protein bands which were present in the retentate and semi-pure progesterone b.p. preparations. It would appear likely from the heavy amounts of protein present in both filtrate and retentate that the 69 kDa band may be a serum protein. This protein cannot be the progesterone b.p. however, because of its presence in the filtrate. Thus it is possible that the progesterone b.p. is either one of the bands larger than 69 kDa, or indeed, a complex of these bands. A progesterone binding complex would appear to be the most likely, since binding activity was retained by 100 kDa filters, yet (when run out under reducing conditions) the retentate appeared to contain 2 bands (in addition to the heavy 69 kDa band), both of which had molecular weights less than 100 kDa. A combination of these bands yields a molecular weight greater than 100 kDa, and would thus be retained by these filters. This possibility of a multimeric progesterone binding complex of two or more subunits may also explain the activity losses encountered in gel filtration experiments. Further experiments would be required to test whether the progesterone binding site consists of smaller protein subunits which can be separated by size, and which do not appear to re-associate when re-mixed together.

In summary, attempts were made to purify the progesterone binding protein from the bovine corpus luteum. Though some degree of success was achieved, results from techniques commonly employed in protein purification were disappointing. Losses of activity were high, and more

importantly, could not be fully explained even after experimental investigation. It would appear likely, however, that the progesterone b.p. is not a single moiety, but more probably is composed of two or more subunits.

Chapter 6

Investigation of the mechanism of action of digitonin in activation of bovine luteal progesterone binding sites

6.1 Introduction

In previous chapters it was demonstrated that both the bovine corpus luteum and follicle possessed progesterone binding sites which were distinct from the genomic progesterone receptor. These binding sites appeared to be localised to the plasma membrane.

To demonstrate progesterone binding to these binding sites, the presence of the saponin digitonin was required in all binding incubations. The work detailed in this chapter was designed to attempt to determine the action of digitonin on these novel progesterone binding sites, and through this to gain a deeper understanding of the possible physiological action of these binding sites. Given the difficulties encountered during the purification of the progesterone binding protein, further elucidation of the way in which digitonin interacts with the binding protein may provide another avenue through which new purification rationales based on digitonin : binding site interactions may be utilised.

Digitonin and other digitalis analogues such as digoxin, digitoxin etc. are derived from the foxglove *Digitalis purporea*. The main medicinal use of digitalis-derived saponins has been in the treatment of congestive heart failure and some cardiac arrhythmias, usually in the form of digoxin. It is for this reason that the parent digitalis compounds and their analogues are often classified as cardiotonic steroids

Interactions between digitonin and cholesterol have been exploited in a number of biochemical techniques, most notably freeze-fracture membrane studies. Studies demonstrated that by complexing digitonin to membrane-localised cholesterol, losses of cholesterol during further experimental procedures leading to electron microscopy could be minimised (Darrah *et al*, 1971). The experiments which lead to this discovery utilised digitonin as a marker of 3-ß-hydroxysterols, to which it binds in a unimolecular manner (Severs & Robeneck, 1983).

More recently, it has been suggested that an endogenous digitalis-like factor may be present in certain tissues, notably the human ovary (Jakobi *et al*, 1991). It has been suggested that there may be some correlation between increased levels of this endogenous digitalis-like factor and hypertension during pregnancy (Gusden *et al*, 1984; Graves & Williams, 1984; Poston *et al*, 1989), though evidence for this link, and indeed evidence for the existence of the endogenous digitalis-like factor remains incomplete (see Chapter 7, General Discussion for further details).

Digitonin forms unimolecular non-covalent complexes not only with cholesterol, but also with other steroids which possess a 3-ß-hydroxy-Δ5 configuration and a C17 side chain (Miller, 1984; Severs & Robeneck, 1983; Murphy & Martin, 1985). Thus, whereas digitonin will complex with cholesterol and pregnenolone, no interaction would be expected with progesterone. From the results presented in Chapter 3, this would appear to be the case, since progesterone binding does not occur in tubes containing only digitonin and [^{3}H]-progesterone without the inclusion of luteal

membranes. Moreover, HPLC analysis of progesterone recovered from the binding site (thus, after exposure to digitonin) indicated that the only steroid recovered was free progesterone. It is the interaction between digitonin and cholesterol which causes the increase in buoyant densities of cellular membranes containing unesterified cholesterol when homogenates of corpora lutea are incubated in the presence of digitonin prior to fractionation.

The experiments in this study were designed to examine the role of digitonin in stimulating progesterone binding in the bovine corpus luteum, by first identifying whether or not the interaction between the progesterone binding site and digitonin is direct, and focussing on the mechanism of this interaction. Secondly, the specificity of this interaction with regard to whether or not other saponins and compounds with similar actions could mimic the effects of digitonin on progesterone binding was investigated.

6.2 Methods and materials

6.2.1 Trials of various commercial preparations of digitonin.

Preparations of digitonin were purchased from a number of suppliers in various grades, and tested for their ability to stimulate progesterone binding. The preparations used were :-

a/ BDH standard grade (prepared pre-1990)

b/ BDH standard grade (prepared post-1990)

c/Sigma standard grade

d/Aldrich standard grade

e/Calbiochem standard grade

f/ Calbiochem 'super-pure' (>99% pure digitonin)

Each preparation was dissolved in Tris HCl, pH 7.4, 0.1% BSA (progesterone binding assay buffer) with gentle warming, to a concentration of 10 mg/ml. Where the solubility of a preparation was too low to reach this concentration in aqueous media, a lower concentration was prepared and the amount added to the assay tubes was adjusted accordingly to enable direct comparisons of the different preparations abilities to stimulate progesterone binding at all concentrations tested. Progesterone binding assays were then performed on pooled sucrose gradient fractions enriched in progesterone binding as described in Chapter 3, using each of the digitonin preparations.

6.2.2 TLC separation of digitonin preparations

Solutions of Sigma digitonin and BDH digitonin were prepared in absolute ethanol (1 mg/ml). 200 µl of each solution was spotted on to silica gel TLC plates (Sigma) which had been equilibrated in running solvent, and

separated using a solvent system of 9:1 chloroform: methanol (running solvent). This solvent system was tested with pure digitonin (Calbiochem) and was found to cause little or no movement of digitonin from the origin. However, all other steroids tested with this system showed good migration. Hence, any steroidal impurity in Sigma digitonin should be clearly resolved from digitonin. As control/steroid identification lanes, 200 μg (1 mg/ml solutions in absolute ethanol) of progesterone, testosterone, pregnenolone, oestradiol, 17-α-hydroxyprogesterone and DHA were spotted on to the TLC plate also. When the solvent front reached 4 cm from the top of the plate, TLC plates were removed, air dried, then evenly sprayed with an aerosol of 20% (v/v) H_2SO_4 (in absolute ethanol). After air drying, plates were then incubated at 100°C until charring of separated compounds was evident. Once cooled, plates were photographed.

6.2.3 Use of digitonin analogues and other membrane perturbants to mimic effects of digitonin

In the same way that different digitonin preparations were tested for their ability to stimulate progesterone binding in Section 6.2.1, the following substances were tested for their ability to stimulate progesterone binding. Due to the relatively low solubilities of these substances in aqueous buffers, all (apart from those marked by *) were dissolved in ethanol (10 mg/ml), added to assay tubes and dried down under a stream of nitrogen. As controls, tubes containing ethanol alone were also dried down. As a positive control, an identical set of tubes was prepared and digitonin dissolved in ethanol (10 mg/ml) added, then treated in the same way as tubes containing the other compound tested. In the case of the two aqueous soluble saponins, controls consisted of buffer alone, with no dissolved saponin, and a set of

positive controls consisting of digitonin dissolved in assay buffer (Tris HCl, pH 7.4), spanning the same concentration range as the compounds being tested. In each case the progesterone binding assay was carried out as described in Chapter 3. In experiments where the test compound had been dissolved in ethanol and dried down, careful vortexing ensured maximum resuspension of the dried residue in the incubation cocktail. The compounds tested for their ability to mimic the stimulatory effects of digitonin were :-

1/ ouabain
2/ tomatin
3/ fillipin
4/ gitoxigenin
5/ digoxigenin
6/ digoxigenin 3, 12 diacetate
7/ digoxin
8/ digitoxin
9/ digitoxigenin
10/D-(+)-digitoxose
11/ gitoxin
12/ saponin *†
13/ saponin*‡
14/ smilagenin
15/ tigogenin
16/ diosgenin
17/hecagenin
18/ streptolysin A
19/ streptolysin O
20/ triton-X-100
21/triton-X-114
22/ nystatin

†from Quillaga bark
‡ Saponaria spp.

In addition, each of these substances was also tested for their ability to inhibit binding of [^{3}H]-progesterone, as described for steroid displacement assays in Chapter 3. With the exception of 18/ and 19/, each of the above substances was assayed at least twice for both their ability to stimulate or to displace [^{3}H]-progesterone binding to two different pooled membrane fractions enriched in progesterone binding. These two substances, the streptolysins A and O, were each assayed only once due to the extremely hazardous nature of these substances and subsequent disposal procedures.

6.2.4 Determination of the importance of the sterol complexing activity of digitonin during progesterone binding

Cholesterol digitonides and pregnenolone digitonides were prepared by dissolving 1 mg of digitonin in ethanol (770 µmoles digitonin) and adding either cholesterol or pregnenolone dissolved in ethanol at an excess of 100 µmoles. Thus, at these concentrations, all digitonin should be saturated, ie. no free digitonin should be present. These mixtures were allowed to complex overnight, at 4°C, then dried down under a stream of nitrogen. The resultant crystals were re-dissolved in absolute ethanol, and spotted on to a silica gel TLC plate. As a control, 1 mg/ml digitonin was also dried down, re-dissolved in absolute ethanol, then spotted on to a TLC plate. A solvent mix of 9:1 chloroform : methanol was used to separate digitonides from free steroid. The area in which the digitonides and digitonin equilibrated was ascertained by running identical plates and charring as described above. Since the digitonides showed little or no migration, whereas free steroid did, ascertaining the area of silica to be removed was straightforward. Digitonides (and control digitonin) were scraped off the TLC plate, and eluted into absolute ethanol by incubating the silica scrapings in 500 µl ethanol overnight at 4°C. To ensure elution of digitonides and control digitonin from the silica, a sample of the eluted samples was run on a TLC plate. From the molar ratios of digitonin and steroid present, the weight of digitonide (and control digitonin) which corresponded to the amount of digitonin required to demonstrate stimulation of progesterone binding was calculated, and added to assay tubes at concentrations below and above the threshold of digitonin stimulation of progesterone binding. Tubes were then

dried down as described above, then assay cocktail added (with no additional digitonin present) and progesterone binding determined.

6.2.5 Interaction of progesterone binding sites with digitonin

This experiment was designed to ascertain whether having activated progesterone binding, digitonin remains bound, or, if this is not the case, whether a steroid complexed to the digitonin can be demonstrated after stimulation of binding. This required the purchase of [^{3}H]-labelled digitonin. Since radiolabelled digitonin was not commercially available, a contract was taken out with DuPont (New England Nuclear Research products) to prepare tritiated digitonin. The purest grade of digitonin available (Calbiochem) was exposed to a stream of tritium gas for two weeks. However, TLC of the radiolabelled digitonin supplied revealed that massive degradation of digitonin had occurred; so that at best, only 0.5% - 1% of the total product was represented by intact digitonin. Even if purified to homogeneity, this would not have been sufficient to use experimentally. At the time of writing this problem has not been resolved.

6.2.6 Investigation of the interaction between digitonin, progesterone binding sites, and progesterone

To ensure that digitonin was not affecting binding by prior formation of a steroid:digitonin complex, the progesterone binding assay was set up as described previously but with delayed addition of membranes at various times thereafter. The assay cocktail, including 25 µl 10 mg/ml digitonin was added to tubes. Membranes were added to control tubes immediately, whilst other tubes had membranes added at 15 minute intervals, up to 4

hours. The experiment was performed in triplicate. Incubation was for two hours (4°C) before separation of bound and free [^{3}H]-progesterone.

6.2.7 Is digitonin required throughout progesterone binding ?

The aim of this study was to determine whether, once membranes had been exposed to digitonin, removal of digitonin returned the progesterone binding site to its inactive state, or if, once stimulated by digitonin, the progesterone binding site remained in an active, stimulated state in the absence of digitonin. 500 µl of pooled sucrose gradient fraction enriched in progesterone binding was incubated with 1.5 ml assay buffer (Tris HCl, pH 7.4) containing 12.5 mg digitonin (equivalent amount of digitonin to normal progesterone binding assay i.e. 10 µl of membranes incubated with 250 µg digitonin) for 1 hour, at 4°C. As a control, 500 µl of the same pooled fractions were incubated with buffer alone. The mixtures were loaded into separate 30,000 nmwl centrifugal separators (Millipore) and centrifuged at 750 gav until all liquid had been removed. Membranes were then resuspended in Tris HCl, 0.1 % BSA, pH 7.4 (containing no digitonin) (assay buffer) and re-centrifuged. This wash step was repeated twice. Membranes were finally resuspended in assay buffer to the original volume (500 µl). Progesterone binding was assayed on 15 µl aliquots of each membrane preparation (digitonin-stimulated and control membranes) in triplicate, with or without digitonin added to the assay (250 µg). Progesterone binding was then assayed as described previously.

6.3 Results

6.3.1 The potency of different digitonin preparations stimulation of progesterone binding

All of the digitonin preparations tested were capable of stimulating [^{3}H]-progesterone binding to pooled bovine luteal sucrose gradient fractions enriched in progesterone binding activity (Figure 6.3.1). However, the different preparations displayed different potencies. Calbiochem (pure digitonin) was the most potent of the preparations tested. However, at concentrations above 500 μg/tube, decreased binding was observed. The other preparations of digitonin did not decrease binding until concentrations in excess of 1mg/tube were reached. Digitonin supplied by Sigma was the least potent stimulator of progesterone binding activity. TLC of Sigma and BDH digitonin preparations revealed no differences in migration or the presence of migrating impurities using the solvent system described (Plate 6.3.1).

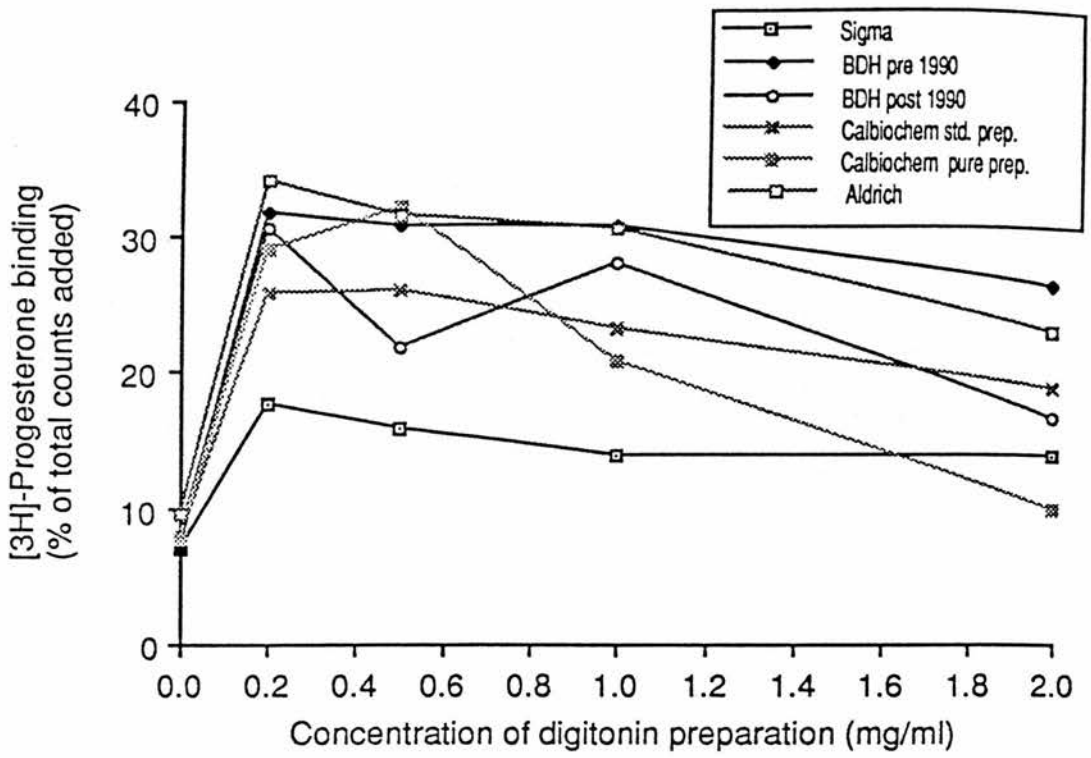


Figure 6.3.1 Trial of abilities of different commercial preparations of digitonin to stimulate [^{3}H]-progesterone binding to bovine luteal membranes enriched in progesterone binding activity.

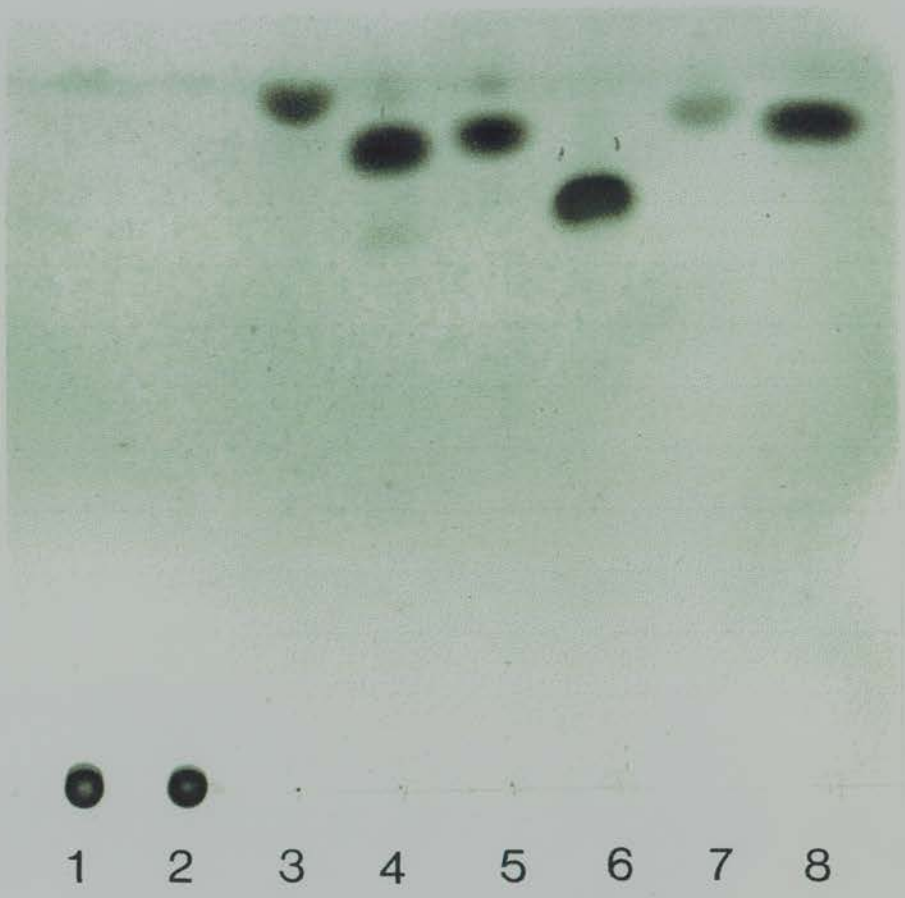


Plate 6.3.1 Thin layer chromatography of BDH digitonin and Sigma digitonin, using a 9 : 1 chloroform : methanol running solvent. 1=200µg BDH digitonin, 2=200µg Sigma digitonin, 3=200µg progesterone, 4=200µg testosterone, 5=200µg pregnenolone, 6=200µg oestradiol-17b, 7=200µg 17-hydroxyprogesterone, 8=200µg DHA.

6.3.2 Ability of digitonin analogues, cardiotonic steroids and other membrane perturbants and detergents to stimulate or displace progesterone binding.

Apart from digitonin, none of the substances tested displayed any stimulation of progesterone binding activity, nor did any of these substances inhibit/compete for progesterone binding sites. Digitonin stimulated [^{3}H]-progesterone binding to the same extent after being dissolved in ethanol, dried down and reconstituted in assay buffer as was observed using digitonin dissolved in the usual manner.

6.3.3 TLC of pregnenolone and cholesterol digitonides to remove free steroid

Plate 6.3.2 shows the mobility of the digitonides and free steroids present in digitonide preparations. As can be seen from this plate, digitonides do not migrate using a solvent system of 9 : 1 chloroform : methanol, however, both pregnenolone and cholesterol showed considerable migration, allowing separation of digitonides from free steroids.

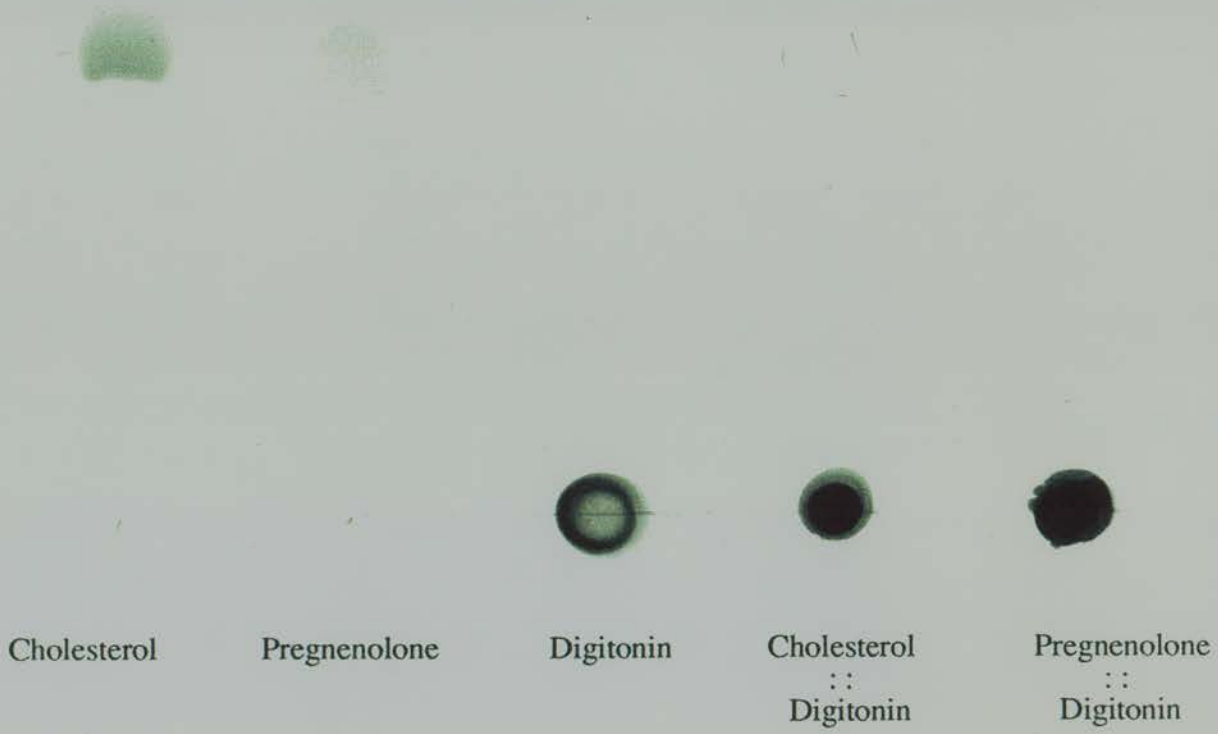


Plate 6.3.2 TLC of pregnenolone and cholesterol digitonides using a 9 : 1 chloroform : methanol solvent system.

6.3.4 Effects of pregnenolone and cholesterol digitonides on progesterone binding

Pregnenolone : digitonide was unable to stimulate progesterone binding above non-specific levels. Though apparent slight stimulation of progesterone binding occurred at low concentrations of cholesterol digitonide, increasing the cholesterol : digitonide concentration lowered progesterone binding. Digitonin which had been processed in the same manner but not complexed to steroid retained its ability to stimulate progesterone binding (figure 6.3.2).

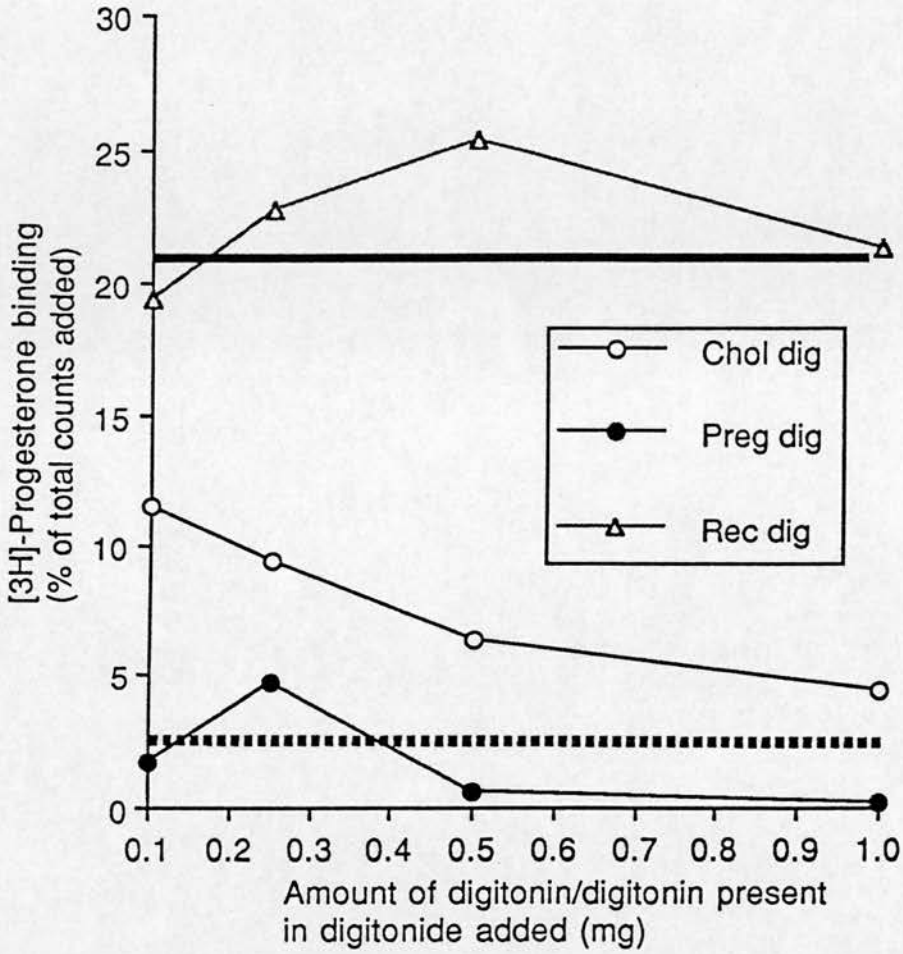


Figure 6.3.2 Ability of cholesterol and pregnenolone digitonides to stimulate progesterone binding. The digitonin tested in these experiments had been processed (see Section 6.2.4 Methods and Materials) in the same manner as the digitonides. The amount of digitonin present in each digitonide preparation was calculated and the amount of digitonide added to the assay adjusted accordingly so that each point contained the same amount of digitonin (as digitonide) as the assay containing uncomplexed digitonin. Heavy black line indicates levels of stimulation achieved using fresh digitonin that was dissolved in assay buffer. Heavy black broken line indicates levels of non-specific binding. Preg dig = pregnenolone digitonide, chol dig = cholesterol digitonide, Rec dig = digitonin recovered from TLC plate (uncomplexed - see methods and materials).

6.3.5 Stimulation of membranes with digitonin followed by subsequent removal of digitonin

Membranes which had been stimulated with digitonin remained in an active stimulated state after removal of digitonin. No further digitonin stimulation was required for these membranes to bind [^{3}H]-progesterone. When digitonin-prestimulated membranes were assayed in the presence of digitonin, no additional stimulation was observed. Control membranes which had not been previously stimulated by digitonin showed binding only in the presence of digitonin added during the assay; no binding was observed in the absence of digitonin in the assay cocktail (Figure 6.3.3).

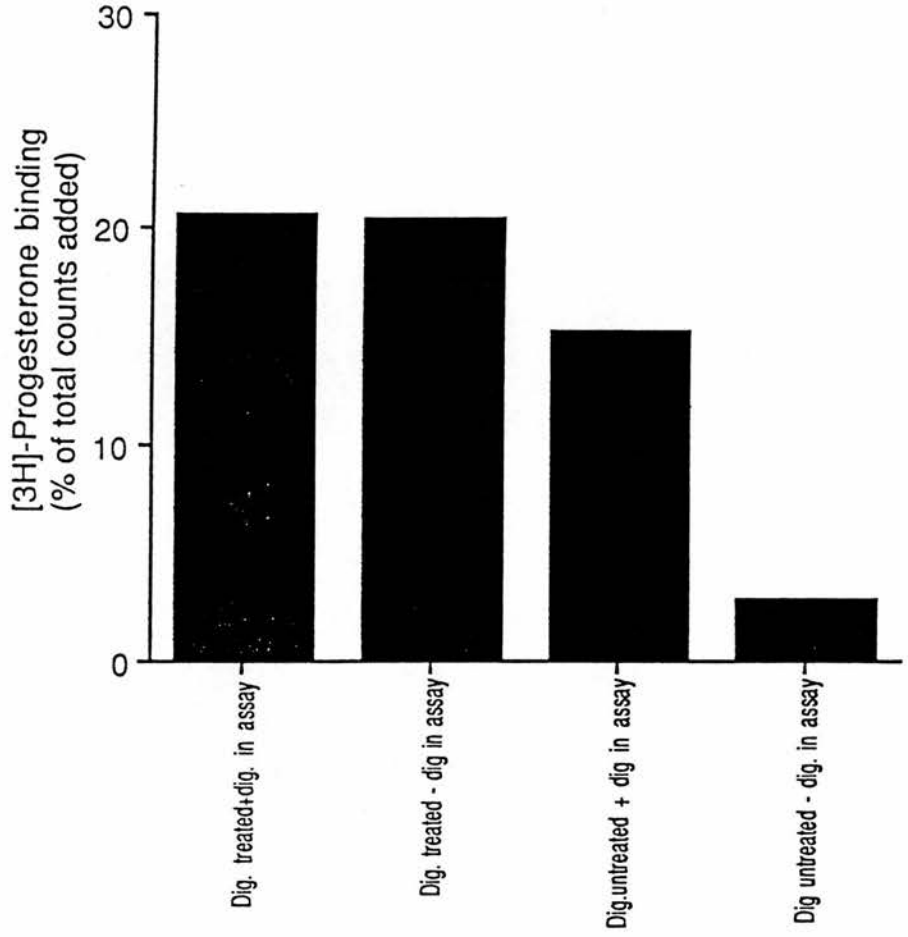


Figure 6.3.3 Effects of stimulation of luteal membranes with digitonin and subsequent removal of digitonin prior to assay for [^{3}H]-progesterone binding $\pm$ digitonin added to the assay.

6.3.6 Preincubation of membranes and [^{3}H]-progesterone with digitonin prior to assay (Figure 6.3.4)

Preincubation of [^{3}H]-progesterone with digitonin prior to addition of luteal membranes had no effect on binding of that [^{3}H]-progesterone to pooled luteal membranes (enriched in progesterone binding activity), relative to levels of binding measured when [^{3}H]-progesterone was preincubated with buffer alone, then added to the rest of the assay. Preincubation of luteal membranes with digitonin prior to assay had little effect on binding, as compared to levels observed when [^{3}H]-progesterone was incubated with digitonin or buffer. However, preincubation of membranes with buffer prior to assay caused a reduction in [^{3}H]-progesterone binding (15% bound as compared to 25% bound in samples where membranes had been incubated with digitonin prior to assay) which was time dependent. Decreased binding was not observed until membranes and buffer had been incubated for over 1 minute. After this time no further reductions in binding were observed.

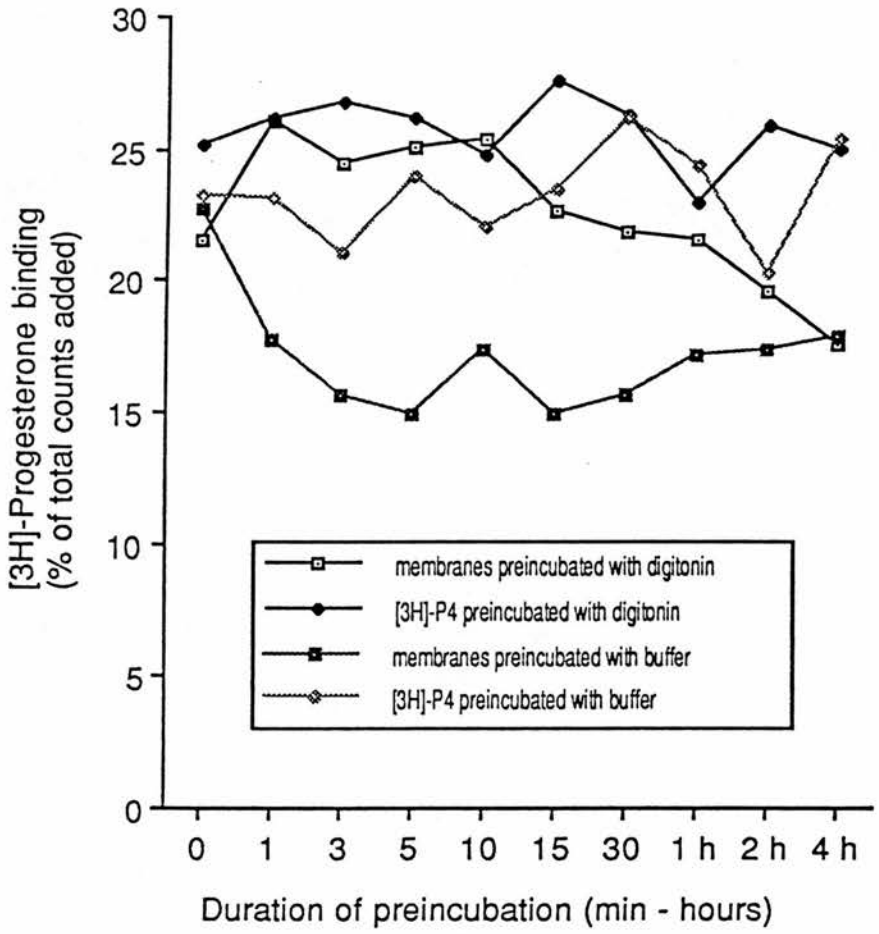


Figure 6.3.4 Effects of preincubation of membranes and of [^{3}H]-progesterone with digitonin prior to addition of rest of assay cocktail. Figure is representative of two such experiments.

6.4 Discussion

Digitonin was found to be the major saponin component present in preparations of digitalis derived from the foxglove *Digitalis purpurea*. Since then, the presence of another saponin, gitonin, in preparations of digitonin has been recognised (Fieser & Fieser, 1959). Whereas the glycosidic sidechain of digitonin is composed of two glucose, two galactose and one xylose units, that of gitonin consists of only 3 galactose and one xylose. In commercial preparations of digitonin there may be up to 10 - 20% contamination by gitonin (Fieser & Fieser, 1959), unless the preparation states otherwise (eg. Calbiochem "pure" digitonin).

The major use of digitonin has been in freeze fracture membrane studies. These techniques have exploited the 1:1 non-covalent interaction between digitonin and unesterified cholesterol and other 3ß-hydroxysterols (Severs & Robeneck, 1983). When digitonin interacts with cholesterol in membranes, characteristic lesions are produced which are indicative of the presence of sterols when the membrane is viewed by freeze fracture (Severs & Robeneck, 1983). Two other chemicals are commonly used for such techniques, the saponin tomatin and the polyene antibiotic fillipin, both of which appear to have similar actions to digitonin (Severs & Robeneck, 1983). Another major use of digitonin has been in the processing of tissue for electron microscopy, since incubation of tissue with digitonin alleviates the problem of loss of cholesterol during tissue preparation (Napolitano & Scallen, 1969; Sterzing & Napolitano, 1972; Napolitano *et al*, 1972). Since then, the uses of digitonin have multiplied, but in essence all of its uses pertain to its interaction with cholesterol. Originally, in this study, digitonin was used to perturb the

buoyant densities of membranes containing unesterified cholesterol as a tool to examine associations between luteal progesterone and luteal intracellular organelles (Chapter 2). However, the experiments described in this chapter were designed to elucidate the mechanism by which digitonin stimulated progesterone binding to isolated bovine luteal membranes.

It was of interest to note the different potencies of different preparations of digitonin with respect to the stimulation of [^{3}H]-progesterone binding to bovine luteal sucrose gradient fractions. Since the pure digitonin preparation from Calbiochem stimulated binding, it would appear that it is indeed digitonin that causes stimulation of progesterone binding, not an impurity present in digitonin from other commercial suppliers. This preparation decreased binding at concentrations above 500 µg/tube. The reasons for this decrease are unclear, though digitonin at such high concentrations may interfere with the binding site. The solubility of BDH preparations of digitonin in aqueous buffers appeared to have lessened. Before 1990, digitonin was supplied to BDH by a Swiss company, but after 1990 it was supplied by a German company. How this resulted in a change in the solubilities of the two preparations is unclear. However, BDH themselves were unaware of this change, so no further information was forthcoming. It was of interest to note that the digitonin preparation supplied by Sigma was the least potent stimulator of [^{3}H]-progesterone binding of all the preparations tested. Though the supplier was consulted on the purity of their product and the purity checks which were employed, no information was forthcoming on why this digitonin preparation appeared to be different to other digitonin preparations tested. Certainly chromatography on TLC plates revealed no differences between Sigma and

BDH preparations. However, these experiments used only one solvent system, and it is possible that chromatography under different conditions may have revealed an impurity. The presence of an impurity in Sigma digitonin preparations may inhibit binding, or, if the level of impurity is high enough less Sigma digitonin would be added to binding assays relative to other preparations tested. The impurity present runs similarly to digitonin on TLC, and it may be gitonin (Fieser & Fieser, 1959). Since gitonin is not commercially available it was not possible to test it for any stimulatory or inhibitory effects on steroid binding.

None of the digitonin analogues tested stimulated [^{3}H]-progesterone binding to membranes, nor competed for binding when [^{3}H]-progesterone binding was measured in the presence of digitonin. Also, none of the other detergents or cholesterol perturbants tested either stimulated or inhibited binding in the presence of digitonin. Of particular interest was the lack of effect of tomatin and filipin. Both of these compounds have been used extensively for electron microscopy and freeze fracture techniques (Severs & Robeneck, 1983; Elias *et al*, 1979; Steer *et al*, 1984). Both tomatin and filipin have been observed to cause similar lesions in membranes to those caused by digitonin (Elias *et al*, 1979). However, filipin cannot interact with membrane cholesterol in regions of membrane coated with clathrin. When clathrin is removed, filipin : cholesterol interactions occur as normal (Steer *et al*, 1984). Thus the progesterone binding site may have a similar domain which is not activated by these cholesterol binding agents, but is activated by digitonin. This would imply that digitonin alone can penetrate to interact with membrane steroids. The action of digitonin on the progesterone binding site is not due simply to a detergent effect, or to a cholesterol

perturbation effect. From the results of preincubation experiments, it is clear that digitonin did not directly interact with [^{3}H]-progesterone. This is backed by a wealth of literature on this subject which rules out digitonin - progesterone interaction at physiological pH. However, the action of digitonin on membranes must be extremely rapid, since no differences in binding were observable between any of the time points in this study. A rapid reduction of binding occurred when membranes were incubated with buffer prior to assay (ca. 1 min), but binding did not decrease any further. However, when membranes were incubated with buffer and digitonin prior to assay, a decrease in binding occurred only after 15 minutes of incubation time. It is likely that these decreases were due to endogenous proteolysis, since no protease inhibitors were included in the preincubation cocktails. The delay in the decrease of binding in the presence of digitonin may indicate an inhibitory effect of digitonin on proteolytic enzymes.

From the results of the experiments in which digitonin was complexed to cholesterol or pregnenolone, it is clear that the sterol binding domain of digitonin is critical to the stimulation of progesterone binding activity. However, since the mode of binding between digitonin and sterols is unknown, it is not certain whether or not the digitonin-sterol binding site is directly involved with stimulation of progesterone binding to luteal membranes, or whether the lack of stimulation by digitonide is due to formation of a larger, differently shaped complex which is unable to interact with the progesterone binding site. If the sterol binding domain is indeed directly involved in the stimulation of progesterone binding, then it may be speculated that digitonin may complex with a (endogenous) sterol which occupies this binding site, resulting in the release of a sterol : digitonide from

the binding site, thus allowing progesterone tracer to bind. However, since (as demonstrated in Chapter 2) a large proportion of endogenous luteal progesterone is associated with this progesterone binding site, these progesterone binding sites are probably normally occupied by progesterone. Attempts have been made to resolve this point by extracting digitonin from progesterone binding cocktails after digitonin treatment of luteal membranes, and chromatographing (TLC) the extracted digitonin to see if any other steroid is associated with it. However these experiments have met with little success because of the extremely high detection sensitivities required and the high levels of steroid co-extracted along with the digitonin from assay incubations. It would appear likely that the mechanism of action of digitonin may be complex, since other agents which will mimick at least some of the known actions of digitonin (detergents, cardiotonic steroids, sterol complexing and cholesterol perturbing agents) had no stimulatory effect on [^{3}H]-progesterone binding.

I have demonstrated that membranes remain in an activated state after digitonin removal; that is, once the progesterone binding site has been activated by digitonin, it no longer requires digitonin to be present in the assay cocktail to bind progesterone. This provides further evidence that digitonin interacts with the binding site directly rather than with progesterone. Digitonin may bring about a conformational change in the progesterone binding protein/complex which enables access of [^{3}H]-progesterone to the binding site. However, since digitonin is still required for binding site activation even when the binding site has been solubilised from its membrane by octylglucoside (Chapter 5), digitonin stimulation of

progesterone binding is not dependent on enabling access of progesterone across the luteal membrane on which the binding site is located.

The problems encountered when attempting to incorporate tritium (^3H) on to molecules of digitonin were unexpected. Degradation of digitonin to the extent which occurred is difficult to explain, since digitonin is a relatively stable compound, requiring no special conditions (ie. temperature, humidity control) for long term storage. During consultation with the laboratory which attempted this radiolabelling proceedure, it became evident that this problem had occurred in the past with attempts to radiolabel digitonin, though no explanation for this failure had been proposed. At the time of writing the matter remains unresolved. Preparation of radiolabelled digitonin will enable completion of this study.

Recently, a number of studies have been published in which progesterone binding to sperm surface membranes has been reported. A recent article has indicated the involvement of digitonin in this progesterone binding (Ambhaikar & Puri, 1995). Interestingly, the studies reported in this article indicated that progesterone binding was not observed in the absence of detergents. However, in the presence of detergents, binding of progesterone was evident. The most potent of the detergents tested was digitonin (Ambhaikar & Puri, 1995). Similar concentrations of digitonin stimulated [^3H]-progesterone binding to the rat sperm cell surface membranes as were required to promote [^3H]-progesterone binding to bovine luteal cell surface membranes. In sperm, RU 486 was unable to compete with [^3H]-progesterone for binding to spermatozoa, and 17 α-hydroxyprogesterone and R5020 showed only partial displacement of [^3H]-progesterone bound

(Ambhaikar & Puri, 1995). Unlabelled progesterone displaced bound [^{3}H]-progesterone at nanomolar concentrations (Ambhaikar & Puri, 1995). Thus a similar progesterone binding site to that studied here is present in spermatozoa. Progesterone exposure to sperm surface causes an influx of calcium. This may initiate the acrosome reaction (Blackmore *et al*, 1990). It has been suggested that digitonin may unmask progesterone binding sites on the spermatozoa cell surface (Ambhaikar & Puri, 1995). However, this work is at present in its early stages. It will be of interest to follow the progress of investigators studying the role and characteristics of this binding site in sperm cells.

In summary, the action of digitonin which brings about stimulation of progesterone binding is unlikely to be one of the 'classical' actions of digitonin, since other membrane perturbants, cholesterol complexing agents, saponins and detergents all fail to mimick the action of digitonin. Further work is required to solve this enigma. The use of radiolabelled digitonin should answer the question of how digitonin stimulates progesterone binding, whilst purification of the protein complex which forms the progesterone binding site may provide useful insights. It will be of particular interest to follow the progress of workers who study progesterone binding to sperm surface membranes, since the question of how digitonin acts to elicit the activation of progesterone binding must also be fundamental to their studies.

Chapter 7

General Discussion

In this section the results presented in the preceding chapters will be discussed further, evidence will be presented to clarify some of the experimental results obtained, and literature on the possible relevance of the progesterone binding site will be discussed.

7.1 The progesterone binding site is not a steroidogenic enzyme

In Chapter 3, it was observed that inhibitors of 3ß - hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase, P_{450} 17 α hydroxylase/C 17,20 lyase did not compete with [^{3}H]-progesterone for luteal progesterone binding sites. Moreover, steroids which have equal or greater affinities than progesterone for the steroidogenic enzymes did not displace [^{3}H]-progesterone binding to the extent that would be expected if steroid binding were due to binding to one of these enzymes. It has been demonstrated that 3ß-hydroxysteroid dehydrogenase is extremely sensitive to end-point inhibition, and has a greater affinity for its product (progesterone) than it has for its substrate, (pregnenolone) (Caffrey *et al*, 1979a). However, testosterone and oestradiol are also potent inhibitors of this enzyme through the same end-point inhibition that is exerted by progesterone (Caffrey *et al*, 1979a). Hence, if binding of progesterone was due to binding to the active site of this enzyme, then androgens and oestrogens would be expected to show potent progesterone binding competition. Results of Western blots of fractions of cow corpus luteum did not appear to correlate with the distribution of progesterone binding sites in sucrose gradient fractions, although some

overlap between the locations of the steroidogenic enzymes and progesterone binding site was evident. To ascertain that progesterone binding was completely distinct from steroidogenic enzymes the following experiment was performed. Bovine luteal microsomes were prepared from freshly prepared bovine luteal homogenate, resuspended in SET buffer, 10 mM n-octylglucoside, and mixed for 2 hours at 4°C. This mix was then recentrifuged (100,000 gav 2 hours, 4°C), and the supernatant (solubilised bovine luteal microsomes) removed. The solubilised microsomes were then extracted batchwise by DEAE-Sepharose (Pharmacia). Extracted samples were stored, and the binding site eluted from the gel with salt (see Chapter 5, **5.3.4**). To SDS PAGE gels, 30 μg of solubilised luteal microsomes, 30 μg (total protein, measured by Lowry method) of DEAE extracted solubilised microsomes, and 30 μg of (400 mM) NaCl elution from DEAE elution was added. Finally, each gel was run including molecular weight markers and suitable positive control tissues (human placental microsomes in blots for immunodetection of 3ß - hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase, P_{450} SCC and adrenodoxin, and bovine theca homogenate for P_{450} 17 α hydroxylase/C 17,20 lyase). Western blotting was carried out as described in Chapter 2 (2.3.13), using the antibodies described therein. Fractions were also assayed for progesterone binding activity. It was found that solubilised luteal microsomes and the fraction eluted from DEAE-Sepharose with (400 mM) NaCl contained comparable amounts of progesterone binding activity. The fraction that had been DEAE extracted was found to be depleted of progesterone binding activity, as was expected from experiments detailed in Chapter 5. Results of the Western blots (Figure 7.1) showed that (i) when immunoblotted for P_{450} side chain cleavage, no detection of this enzyme is evident in either the DEAE extracted fraction (progesterone binding

depleted) or the DEAE eluted fraction (progesterone binding enriched) indicating that P_{450} SCC is extracted by DEAE but not eluted by 400 mM NaCl and thus cannot be the progesterone binding site, (ii) when immunoblotted for P_{450} 17 α hydroxylase/C 17,20 lyase, both the DEAE eluted and extracted fractions showed no detection of this enzyme, and, since the DEAE eluted fraction contained progesterone binding activity, progesterone binding cannot be due to this enzyme, and finally (iii) when immunoblotted using an antibody directed against 3ß - hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase, no detection was evident in DEAE extracted fractions (progesterone binding depleted). Detection of this enzyme was observable in DEAE eluted fractions (progesterone binding enriched), however, much lower detection of 3ß - hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase as compared to levels of detection in solubilised bovine luteal microsomes was evident. Given that solubilised bovine luteal microsomes and the fraction eluted from DEAE-Sepharose with 400 mM NaCl contained comparable amounts of progesterone binding activity, progesterone binding cannot be due to binding to this enzyme. Thus, the possibility of the progesterone binding studied here being due to steroid binding to one of the steroidogenic enzymes can be ruled out.

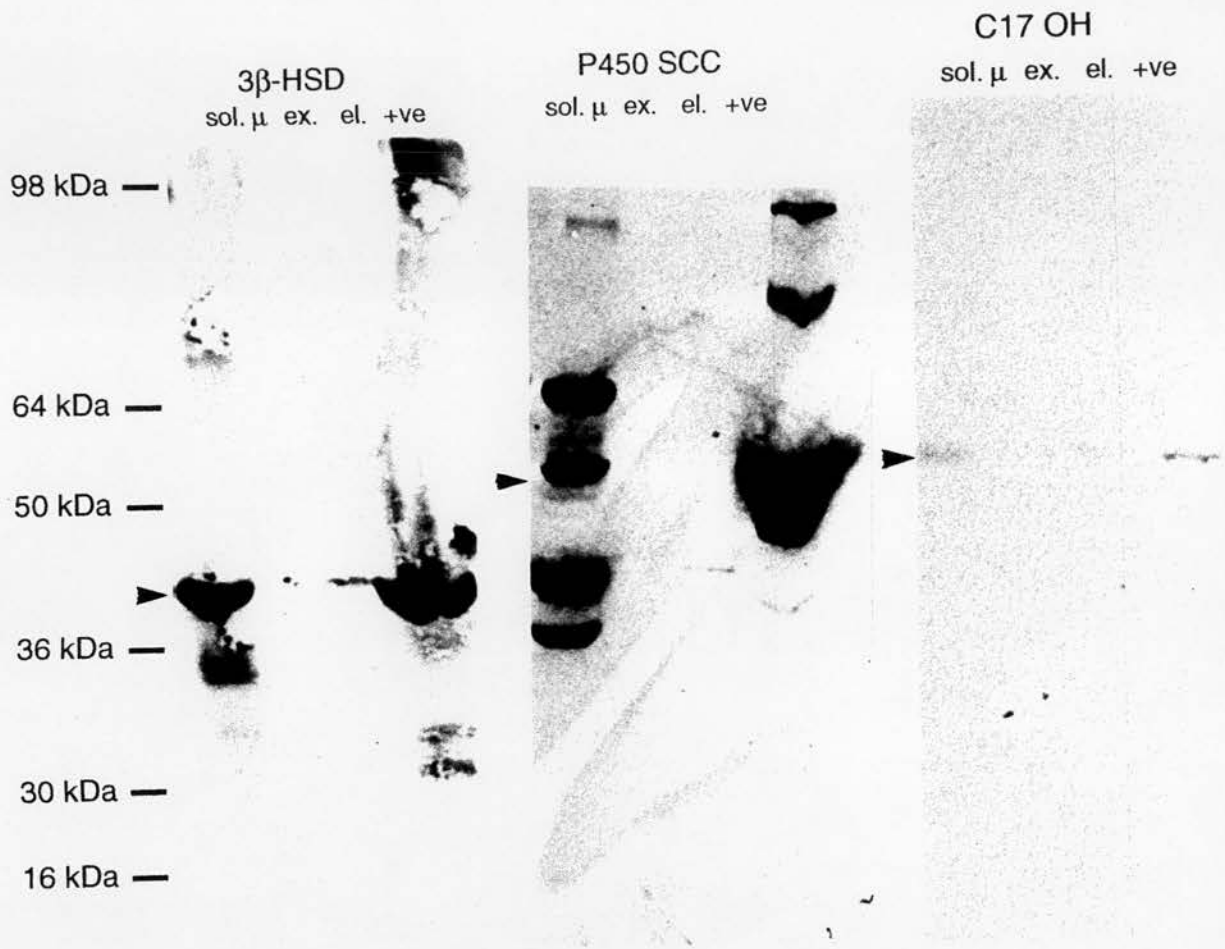


Figure 7.1 Western blotting of bovine luteal microsomal extracts for P450 side chain cleavage, 3β-hydroxysteroid dehydrogenase/Δ^4-Δ^5 isomerase and P_{450} 17 α hydroxylase/C 17,20 lyase; sol. μ = n-octylglucoside solubilised luteal microsomes, ex. = DEAE extracted solubilised luteal microsomes, el. = 400 mM NaCl elution from DEAE, and +ve = positive control tissue for protein of interest. See text (**7.1**) for further details.

7.2 Is there an endogenous digitonin-like activator in luteal tissue ?

The existence of an endogenous digitalis-like factor / naturetic hormone (DLIF) which has the ability to inhibit Na^+, K^+ ATPase has been suggested by a number of studies. The existence of this substance(s) is often associated with conditions involving fluid volume expansion (Gruber *et al*, 1980; Graves & Williams, 1984; Jakobi *et al*, 1987; 1989). The presence of DLIF in follicular fluid of gonadotrophin treated women has been demonstrated (Jakobi *et al*, 1989). The ovarian follicles of such patients have undergone a marked volume expansion. The source of DLIF has been suggested to be the ovary (Jakobi *et al*, 1991). However, other investigations have indicated the source of DLIF to be the adrenal glands, (Tamura *et al*, 1986), cardiac tissue, (Fagoo *et al*, 1986) and hypothalamic tissue (Anner *et al*, 1990). Much attention has focused on the adrenal as a source of DLIF. Using stimulators and inhibitors of cortisol secretion from the adrenal cortex, it has been demonstrated that increased DLIFlevels in plasma occur in conjunction with stimulation of cortisol secretion (Vinge *et al*, 1993).

False-positive results have been reported when serum from third-trimester pregnant women was subjected to radioimmunoassay for digoxin (Kerkez *et al*, 1990). A link between elevated DLIF levels and pregnancy-induced hypertension has been suggested (Gusden *et al*, 1984; Graves & Williams, 1984; Poston *et al*, 1989). However, other workers have refuted this suggestion (Gonzalez *et al*, 1987) and studies designed to investigate DLIF levels as a clinical indicator of hypertension have concluded that the correlation between DLIF levels and hypertension was too low to be of use clinically (Kerkez *et al*, 1990).

Digoxin is derived from the foxglove *Digitalis purpoea*, and shares a similar structure to digitonin, which is also derived from this source. This raises the possibility of a substance of ovarian origin (DLIF) which may be structurally similar to digitonin. Such a substance may act as an (endogenous) activator of progesterone binding protein in the luteal cell. Experiments to determine DLIF levels in homogenates of bovine corpora lute and bovine luteal sucrose gradient fractions were undertaken. However, the commercial assay kit to detect digoxin that most workers have used to determine DLIF levels (RIANEN, New England Nuclear) was no longer available. An anti-digoxin antibody purchased (Sigma) had only very low cross-reactivity with digoxin. Though a number of batches of this antibody were tested, the antibody titre that gave reasonable detection of digoxin was so low that assay sensitivity was lost. Information from the supplier revealed that the antibody was raised against a digoxin-conjugate, which because of its large size and shape produced an antibody which recognised free digoxin poorly. At the time of writing no other anti-digoxin antibodies are available to resolve this problem.

Other experiments involved TLC of solvent extracts of bovine corpora lutea to ascertain if compounds which migrated similarly to digoxin, digitonin and a number of other digitonin analogues were present. No such endogenous substances were detected in these experiments (data not shown). However, this does not discount the possibility that such a substance may exist, since the efficiency of extraction of such compounds from luteal tissue was not determined, and the sensitivity of detection (H_2SO_4 charring) is known to be low.

If indeed the bovine corpus luteum did contain an endogenous activator of progesterone binding that operated in a similar way to digitonin in *in vitro* studies, (whether or not this substance is related to digitonin) it may be present in extremely small quantities. Given the high steroid concentration in this tissue, detection would be difficult. Conversely, the source of progesterone binding activator may not be the ovary, and activation of luteal progesterone binding effects may be regulated in an endocrine fashion by another organ. Progesterone binding protein /non-classical progesterone receptors in the corpus luteum would remain inactive until secretion of endogenous activator stimulated them. Clearly, this is an area that requires further investigation. However, activation of the progesterone binding site occurs *in vivo*, since fractionation of bovine corpora lutea without exposure to digitonin indicates association of endogenous progesterone with this binding site.

7.3 Are progesterone binding sites present only in steroidogenic tissues ?

To ascertain whether or not the progesterone binding site examined here was present in any other organ apart from the ovary, homogenates of other bovine tissues were preparedand assayed for progesterone binding activity. The tissues treated in this way were bovine lung, kidney, heart, liver, skeletal muscle and adrenal gland. Binding of [^{3}H]-progesterone to these tissues could only be detected in the presence of digitonin. Levels of binding were varied in these tissues, though the specificity of binding sites for progesterone was similar to that determined in the corpus luteum (Table 7.1).

Tissue	Progesterone binding activity
Lung	++
Liver	+++
Adrenal	++
Corpus luteum	++
Muscle	+
Heart	++
Kidney	+

(numbers of + denotes levels of binding activity found)

Table 7.1 Levels of progesterone binding activity found in various tissues.

Thus, it is clear that the progesterone binding site is not exclusive to the bovine ovary. Thus, it appears unlikely that the progesterone binding sites are involved in the secretion of progesterone, as originally postulated. Progesterone and progesterone metabolites are known to have anaesthetic effects in a number of tissues (Backstrom *et al*, 1990). Thus, the binding site studied herein may represent the membrane protein through which these anaesthetic effects are mediated. Furthermore, a large body of evidence exists which shows that steroids, in addition to their classical, genomic actions, also have non-genomic receptor mediated effects on a number of cell types. It is not possible that the progesterone binding studied here is a general, 'fluke' effect that occurs with tissues in the presence of digitonin. Evidence to refute this possibility came from experiments in which bovine corpora lutea were fractionated without any exposure to digitonin. Endogenous progesterone was found to be localised in the same fractions (by radioimmunoassay) as the progesterone binding site, thus the progesterone binding site was occupied by progesterone before any

artifactual effects of digitonin could have occurred. Levels of binding differed between tissues, and, between cell types of the same tissue. Thus, different levels of progesterone binding activity were observed in theca and granulosa cell membranes from the same bovine pre-ovulatory follicles, and between small and large luteal cells isolated from the same corpus luteum.

7.4 The progesterone binding site may be a non-genomic steroid receptor

The significance of progesterone binding to membranes from other tissues remains unclear. However, these progesterone binding sites do not appear to be involved in the mechanism of progesterone secretion (though, in the absence of studies of progesterone synthesis in other tissues, this possibility cannot be completely discounted). Indeed, progesterone may have a range of effects which at present are unknown. The nature of progesterone binding to these other tissues and effects of progesterone on these tissues clearly requires more work, however, this was outwith the scope of this thesis which is concerned only with the ovarian progesterone binding site. Indeed, a recently published report has demonstrated the presence of genomic progesterone receptor mRNA in male peripheral veins and blood vessel walls (Bergqvist *et al*, 1993), though as yet there is no physiological role of progesterone on such tissues. The presence of progesterone receptor mRNA has also been demonstrated prostate stromal cells (Mobbs *et al*, 1990). Furthermore, the presence of 3β–HSD and the synthesis of progesterone in Schwaan cells has also been reported, where it may play a role in promotion of myelin repair (Koenig *et al*, 1995). Hence, the actions of progesterone may be much wider and more diffuse than its functions as a sex steroid. Indeed, the progesterone binding site may actually be a form of non-genomic, non-classical, plasma membrane located progesterone receptor. Putative plasma

membrane receptors for steroids have been previously suggested. Specific progesterone binding to synaptosomal membrane preparations of the mediobasal-hypothalamic-anterior hypothalamic-preoptic area of rat brains has been demonstrated (Tischkau & Ramirez, 1993). In common with the binding site investigated herein, alterations to the C11 position of progesterone decreased binding potency, however C11 iodinated progesterone was bound to some degree. Alterations to the C3 position did not appear to affect binding. Although digitonin was not required to stimulate binding in these preparations, digitonin was the detergent used to solubilise membrane fractions. Thus, progesterone binding to these fractions may have been stimulated by digitonin. These experiments concluded that the hypothalamic membrane progesterone binding site were sex-specific. Moreover, the levels of progesterone binding were oestrogen-dependent (Tischkau & Ramirez, 1993). Recently, progesterone has been shown to increase oxytocin receptor mRNA in the hypothalamus of lactating rats (Thomas *et al*, 1995). Moreover, this effect was observed in cells in which few, if any, genomic progesterone receptors can be demonstrated, and was suggested to perhaps be mediated through a non-genomic steroid receptor (Thomas *et al*, 1995).

Progesterone has been observed to affect brain cell excitability. These effects were observed to be extremely rapid, and, furthermore, were demonstrated in the cerebellum and where few intracellular steroid receptors could be observed (Smith *et al*, 1987). Indeed, conclusions reached from such studies point to a mechanism of progesterone action other than that mediated via classical intracellular receptors, with suggestions including progesterone interactions with membrane binding sites (Smith *et al*, 1987). Furthermore,

progesterone acts to expand the area occupied by oxytocin receptors in the ventromedial hypothalamus of oestradiol-primed female rats (Schumacher *et al*, 1990). This effect was observable within 30 minutes of progesterone exposure and lasted up to 8 hours (Schumacher *et al*, 1990). It was concluded that the effects of progesterone on these tissues were due to either a direct effect of progesterone binding to the cell membrane or to an effect on the oxytocin receptor, perhaps by the induction of a conformational change in the oxytocin receptors from a low affinity state to an active high affinity state (Schumacher *et al*, 1990), or the modification of the oxytocin receptors lipid environment by intercalation of progesterone into the lipid bilayer. This intercalation has been demonstrated in the corpus luteum (Carlson *et al*, 1983a). Nonetheless, these effects all appeared to be specific for progesterone. Other steroids (5α-dihydroprogesterone, 3α-hydroxy-5α-pregnane-20-one, cholesterol and oestradiol) all failed to affect the oxytocin receptors (Schumacher *et al*, 1990). Thus, a recent review of steroid hormone action concluded that steroids may act in two ways, (i) through the classical genomic steroid receptors and (ii) by binding to membrane receptors to produce very rapid effects (Wehling, 1994).

It was of great interest to note that other investigators have found a digitonin activatable progesterone binding site with similar characteristics to the luteal NCP4-BP studied herein. Studies have demonstrated that progesterone initiates the acrosome reaction (AR) in sperm, via a rapid influx of calcium (Blackmore *et al*, 1990). Follicular fluid triggers this effect (Thomas & Mizel, 1988), but once stripped with dextran-coated charcoal to remove steroids, this effect was lost. Interestingly, it was noted that progesterone was less potent than follicular fluid at triggering AR; in studies, it was observed that

25 % fewer sperm underwent AR with progesterone than with follicular fluid (Osman *et al*, 1989). It has been suggested that some other factor present in follicular fluid may potentiate the progesterone response (Ambhaikar & Puri, 1995). This raises the possibility of a novel class of progesterone cell surface receptors in a variety of tissues and cell types, whose presence has been previously unacknowledged due to their requirement of digitonin for *in vitro* activation.

7.5 Progesterone binding is not due to either non-specific intercalation of steroid into luteal membranes, or binding to steroid binding globulin

Progesterone binding did not occur to other luteal membranes, only to the plasma membrane. Intercalation of progesterone into luteal plasma membranes has been reported as non-specific interactions (Rice *et al*, 1986). Clearly, the binding studied here was distinct from these interactions, since binding was highly specific for progesterone. Moreover, if steroid intercalation into membranes were responsible for progesterone binding, other steroids, by their hydrophobic nature, would also be bound by the binding site, and progestins such as RU 486 would be expected to show some competition for binding. Moreover ANS, which blocks steroid binding to cortisol binding globulins, had no effect on luteal progesterone binding.

7.6 Possible physiological functions on non-classical progesterone binding protein

In sheep, the onset of luteolysis is triggered by the action of $PGF_2\alpha$ on large luteal cells, via a specific $PGF_2\alpha$ receptor which consists of 362 amino acids (Graves *et al*, 1995). Interestingly, levels of $PGF_2\alpha$ receptor appear to be directly correlated with circulating progesterone levels (Graves *et al*, 1995).

Large luteal cells respond to $PGF_2\alpha$ stimulation with activation of protein kinase C (Wiltbank *et al*, 1989) and calcium release from intracellular pools (Wegner et al, 1990; 1994) . In sheep, progesterone production is regulated by both protein kinase A (PKA) and PKC. Small luteal cells do not possess $PGF_2\alpha$ receptors and thus cannot respond to PGF 2α. However, PKC is inhibitory to progesterone production by both cell types (Wiltbank *et al*, 1993). Associated with $PGF_2\alpha$ binding to large luteal cells, there is a rapid and precipitious drop in 3β HSD mRNA levels in large luteal cells (ca. 80 % decrease after 1 hour) (Hawkins *et al*, 1993). Since the synthesis of this enzyme is constitutive, only a 28% fall in progesterone output from large luteal cells (which account for ca. 80 % of total luteal progesterone) at this time (Hawkins *et al*, 1993). It has been suggested that some form of intercellular communication exists between large and small luteal cells and that this communication transmits the luteolytic message from large cells to small cells (Niswender & Nett, 1994). Thus, from the chronological sequence of events in luteolysis, falling concentrations of progesterone may facilitate the transmission of luteolytic signals from large luteal cells to small luteal cells. These speculations are based on observations made in sheep; however, a similar system would appear likely to function in the bovine corpus luteum, though the acute role of PKC is less well defined. Nonetheless, if progesterone does indeed have autocrine/paracrine actions on its own synthesis, the non-classical progesterone receptors studied here may mediate actions of progesterone in the corpus luteum. The suggestion that progesterone may exert some control over its own synthesis is not new. In 1981, Rothchild postulated that progesterone had the ability to increase its own synthesis and secretion. Hence, higher levels of non-classical progesterone binding protein (NCP_4-BP) found in large luteal cells as

compared to small luteal cells would appear to fit with this hypothesis. If progesterone secretion is increased by progesterone actions mediated via NCP4-BP, then cells with a greater NCP4-BP level would secrete greater amounts of progesterone. Indeed, the large luteal cells of the bovine corpus luteum were demonstrated to contain the highest levels of NCP4-BP. Also, it is not known why basal secretion of progesterone from small luteal cells is so low as compared to basal progesterone secretion by large luteal cells, though it does not appear to be due to cholesterol availability (Wiltbank *et al*, 1993). Thus it may be speculated that the low basal secretion of progesterone from small luteal cells may be due to a lack of sufficient numbers of NCP4-BP to mediate stimulatory effects opf progesterone. Experiments to test this hypothesis would involve culturing separated luteal cells with inhibitors of 3β HSD (eg. epostane, trilostane) to lower endogenous progesterone levels, and then adding exogenous progesterone to the cultures. Initially, we would measure effects on the levels of steroidogenic enzymes and LDL receptor expression, and on IP_3 turnover, calcium influx and pregnenolone formation. The use of inhibitors of classical progesterone receptors (RU 486) would determine whether effects of progesterone observed were due to genomic progesterone receptor binding, or to NCP4-BP. Since the NCP4-BP does not bind RU 486, any effects of progesterone that were not abolished by RU 486 treatment could possibly be mediated by the NCP4-BP.

Studies which have addressed the suggestion that progesterone may have actions on the corpus luteum have thus far concentrated on actions of progesterone mediated via classic, genomic progesterone receptors. From immunohistochemical studies, genomic progesterone receptors have been observed in the theca and stromal cells of monkey (Hild-Petito *et al*, 1988)

and human ovaries (Press & Green, 1988). Post-luteinization, human granulosa-lutein cells stain positively for nuclear progesterone receptor (Horie *et al*, 1992). A relationship between progesterone levels and progesterone receptors in primate corpora lutea from the early and mid-luteal phase has been demonstrated. However, there appeared to be a lack of correlation between serum progesterone, progesterone receptor mRNA and progesterone receptor during the late luteal phase (Duffy & Stouffer, 1995a). However, other recent studies failed to detect any change in levels of progesterone receptor in human corpora lutea over the course of the luteal phase (Duncan *et al*, 1995). LH has also been implicated in the control of expression of progesterone receptor in granulosa cells from monkeys (Stouffer & Duffy, 1995b).

Progesterone may act as a locally produced luteotrophin in the corpus luteum (Rothchild, 1981) which stimulates its own synthesis and secretion. Alternatively, it may play a role in the development and regulation of the corpus luteum, or in the inhibition of antral follicle growth during the luteal phase (Rothchild, 1981). Recent radioligand binding studies have demonstrated progesterone receptor binding in the primate corpus luteum when endogenous progesterone levels are decreased by inhibition of steroidogenesis (Slayden *et al*, 1994), and progestins upregulate the activity of the steroidogenic enzymes responsible for luteal progesterone synthesis in rat granulosa cells (Ruiz de Galarreta *et al*, 1985) and pig theca cells (Tonetta, 1987). Moreover, progestins stimulate LH receptor expression in cultured bovine luteal cells (Jones *et al*, 1992). Studies in chicken granulosa cells have indicated that progesterone may play a role in the stimulation of fibronectin production (Conkwright & Asem, 1995). However, no studies have yet

addressed the question of whether progesterone may also have actions that are not mediated via classical genomic progesterone receptors. From the data presented here, there appears to be a correlation between progesterone content and NCP4-BP levels in the bovine corpus luteum. NCP4-BP levels are highest during the luteal phase when progesterone secretory activity is maximal, however, due to the nature of tissue collection in these experiments no information on circulating progesterone levels was available. Thus it will be of value to examine both NCP4-BP levels in bovine luteal tissue and circulating progesterone levels, as well as classical progesterone receptors and progesterone receptor mRNA in the same animals.

7.7 Non-classical progesterone binding sites may be involved in the relief of end-point inhibition of steroidogenic enzymes

As mentioned previously, progesterone, through end-point inhibition of 3β HSD, has the ability to inhibit its own synthesis. Ovine luteal 3ß-hydroxysteroid dehydrogenase is extremely sensitive to end-point inhibition, and has a six-fold greater affinity for its product (progesterone) than it has for its substrate (pregnenolone) (Caffrey *et al*, 1979a). Thus, there must be a mechanism which removes the inhibitory effects of progesterone on its own synthesis. The most common suggestion is binding proteins which sequester progesterone and thus prevent it competing with pregnenolone for binding to 3β HSD (Caffrey *et al*, 1979a). Hence, higher levels of binding protein would be required to facilitate higher levels of progesterone synthesis. Thus, from the distribution of progesterone binding protein in the cells of the ovary, it may be that in this tissue, non-classical progesterone binding sites may function to alleviate end-point inhibition of steroidogenesis. However, since the binding site is found in non-

progesterone synthesising tissues, a function other than relief of end-point inhibition would appear more likely.

7.8 Conclusion

In conclusion, the experiments presented in this thesis have demonstrated for the first time the presence of non-classical progesterone binding sites associated with the plasma membranes of bovine luteal cells. These non-genomic progesterone binding sites do not appear to be a part of the progesterone secretory mechanism. Present in both the bovine corpus luteum and the bovine preovulatory follicle, these binding sites may be involved either directly (as in the case of a non-genomic steroid receptor) or indirectly (in the case of relief of end-point inhibition of steroid synthesising enzymes by a progesterone binding protein) in the regulation of progesterone synthesis in the bovine corpus luteum.

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