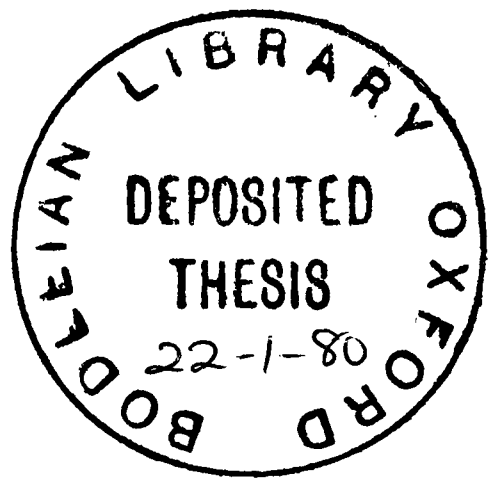
HORMONE SECRETION IN THE CORPUS LUTEUM

by

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Worcester College
OXFORD

TO MY LONG-SUFFERING WIFE, JANE,

WHO KNOWS WHAT IT IS TO BE A

SCIENTIFIC WIDOW

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ABSTRACT

HORMONE SECRETION IN THE CORPUS LUTEUM

D.L. Willcox, Worcester College, Submitted for
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The aim of this thesis was to investigate the mechanism of progesterone secretion from the bovine corpus luteum: in particular, to examine whether steroid hormones are packaged into membrane-bound granules and then transported to the cell membrane where their contents are released by exocytosis.

Morphological examination of bovine mid-cycle corpora lutea showed that luteal cell cytoplasm and extracellular space contained electron-dense granules, 0.2-0.4 μ in diameter. Specific cytochemical staining techniques identified microperoxisomes and lysosomes as being intracellular, and distinguished them from the putative secretory granules.

Morphometric analysis of the bovine corpus luteum was carried out at low and high magnifications on days 6, 13, 15, 17 and 20 of the oestrus cycle. Luteal cells comprised 50% of all cell-types at the beginning and end of the cycle and rose to comprise 74% at mid-cycle. The analysis of luteal cell cytoplasm showed that the relative proportion of cellular organelles involved in the synthesis and secretion of progesterone varied throughout the oestrus cycle. The densely-staining granules occupied 2.5% of the luteal cell volume at day 6, but gradually rose in numbers to reach a maximum of 5.6% around day 17. By day 20 their volume had dropped again to 2%. During mid-cycle, when progesterone secretion was maximal, many of these granules were observed to have undergone exocytosis, whereas

late in the cycle evidence of exocytosis was rare. Thus, the changes in the proportion of aer/ground plasm and densely-staining granules correlated with the changes in the rate of progesterone secretion from the corpus luteum. Extensive protein synthesis was indicated since the proportion of ger and Golgi organelles increased by the time progesterone synthesis began. This was consistent with the need to synthesize a binding protein to which progesterone could be bound within secretory granules.

Biochemical and cytochemical techniques were used to demonstrate the association of progesterone with electron-dense granules in fractions obtained by density gradient centrifugation. Differential centrifugation of luteal tissue obtained at mid-cycle showed that up to one-third of the total progesterone could be sedimented. Most of this particulate hormone banded at a density of less than d=1.118 on sucrose gradients. Marker enzyme analysis of individual gradient fractions showed that mitochondria, lysosomes, microperoxisomes, and most of the membraneous elements banded at densities greater than d=1.118. Electron microscropy and cytochemistry of fixed fractions from three selected regions of the gradient identified microperoxisomes and lysosomes in the two regions with densities $d>1.118$, whilst non-staining electron-dense granules and progesterone were identified in the region with density $d<1.118$.

The corpora lutea of cows and sheep were shown to contain a high-affinity binding protein for progesterone, which was distinct from CBG. This protein was also detected in utero-ovarian and jugular venous plasmas. The bovine corpus luteum contained another progesterone-binding protein which had a greater capacity, but slightly lower affinity, for progesterone.

A role for both these proteins in the biosynthesis of progesterone and its secretion in granules from bovine luteal cells is proposed.

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CHAPTER 1

GENERAL INTRODUCTION

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1.5 MECHANISM OF STEROID SECRETION

1.6 OUTLINE OF THESIS

1.1 STEROID HORMONES

Steroids are small (molecular weight of the order of 300 daltons) rather rigid, lipophilic molecules. They are synthesized and secreted from various endocrine glands and some of them are active as hormones, that is, substances which modulate the physiological processes of cells and tissues in parts of the vertebrate body distinct from their cells of origin. In mammals, the main steroid hormones are the gluco- and the mineralo-corticoids, which are vital for the survival of the individual, and the sex steroids, which are essential to the reproductive processes and thus to maintenance of the species.

Steroid hormones promote a great diversity of effects but their primary interaction with target cells is thought to be similar in all cases (Baulieu, 1975). The changes brought about by steroid hormones may be irreversible ones involving differentiation (e.g., the onset of male puberty stimulated by androgens, King and Mainwaring, 1974) or reversible (e.g., the growth-promoting effect of oestradiol on the immature or castrated adult rat uterus, Jensen and Jacobson, 1962). These major events are brought about by low concentrations of steroid hormones. Furthermore, available evidence suggests that as much as 97-98% of circulating steroid hormones are rendered biologically inactive by being bound to protein (Westphal et al., 1977). Inside cells, steroid action is mediated by binding to specific receptor proteins and binding proteins.

1.2 EVOLUTION OF CORPORA LUTEA

Corpora lutea occur ubiquitously throughout the vertebrates (see Browning, 1973 for references). Two types of corpora

lutea are found: the corpus luteum of atresia (CLA), formed by atresia of a mature or maturing oocyte, and the corpus luteum of ovulation (CLO), which arises after ovulation from the empty follicle (Browning, 1973). CLO predominate in mammals. The granulosa and theca interna cells of the follicle contribute to the formation of both types of corpus luteum, but the latter usually only forms a vascularized capsule of supporting connective tissue. In mammals, the theca interna also becomes luteinized and is an important source of luteal cells. In these animals, the CLO are important in at least the initial stages of gestation (Browning, 1973; Heap et al., 1973). In fact, the life of the CLO generally correlates with the time that the fertilized ovum remains in the duct system of the female (Browning, 1973).

In all vertebrates, only some of the oocytes which commence maturation are actually ovulated, even when large amounts of yolk are not involved, as in Mammalia (Hoar, 1969). The primary function of CLA must be to recover the yolk-rich cytoplasm of such oocytes (Hisaw, 1959), especially in non-mammalian vertebrates (Salisbury and Hart, 1970). In the development of viviparity, the formation of CLA may also promote a more pronounced reduction in the numbers of oocytes achieving ovulation than occurs in oviparity. Viviparous species generally have relatively few young (Amoroso, 1960). In any case, the granulosa cells must have enzymes capable of metabolizing the lipids and cholesterol of vitellogenesis as well as possible steroidogenesis. Thus, synthesis and degradation of yolk constituents may have initially involved the formation of steroids "without their necessarily having any endocrine role"

(Browning, 1973). In view of its stimulation of yolk synthesis by the liver (Hoar, 1969), it seems probable that oestrogen was the first steroid metabolite to evolve as a steroid hormone (Browning, 1973). Progesterone is likely to have always been a major obligatory precursor in the synthesis of oestrogen, so that a hormonal role for it would have readily evolved also. Other specific structures gradually became responsive to circulating concentrations of these steroids and to synchronization of physiological events through gonadotrophin-controlled maturation of oocytes and atresia (Browning, 1973). These would include accessory and secondary sexual characteristics. Both types of corpus luteum could take up the role of a hormonal gland and, as in mammals, become a primary source of oestrogen and progesterone (Ball, 1960). Thus, one or both steroids could come to control the reproductive cycle in oviparous forms as well as gestation in viviparous ones (Browning, 1973).

Mammals lack the facility of yolk storage, so that a higher level of steroid biosynthesis may be required than can be maintained by the granulosa alone. Browning (1973) suggested that this is why the theca interna becomes luteinized and produces steroid in mammals. He also proposed that the independent occurrence of viviparity and ovoviviparity in representatives of various vertebrate taxa implies that there has not been a single evolutionary history of the corpus luteum. Since the ovary produced steroids as part of vitellogenesis, it has remained a convenient source of steroids following the latter's adaptation to function as hormones. Therefore, convergent evolutionary forces have operated so

that now both CLA and CLO fulfill similar roles and support gestation. That is, it is the uses to which the structures are put, and the control which is exerted over them, that has evolved, rather than the structures themselves (Hisaw, 1959).

1.3 THE BOVINE OESTRUS CYCLE

A description of the bovine oestrus cycle is relevant to the subsequent consideration of the secretory function of the corpus luteum. The cow, like other large domestic animals such as the sheep, pig and horse, differs from primates and rodents in that its ovaries lack significant amounts of steroid-secreting interstitial tissue and that normal regression of its corpus luteum requires the presence of the uterus.

1.3.1 Behaviour

Most breeds of dairy cattle have oestrus cycles of 21 to 22 days duration with metoestrus extending from days 1-8, dioestrus from days 9-16, pro-oestrus from days 17-20 and oestrus starting on day 21. According to Trimberger (1948), heifers have slightly shorter oestrus cycles than cows. The mature cow remained in oestrus for a slightly longer period (18 h) than heifers (16 h), and cows and heifers which began oestrus in the afternoon remained in oestrus longer than those which began oestrus in the morning. But neither cows nor heifers exhibited seasonal changes in cycle-length or oestrus behaviour. Nalbandov (1964) reported that older cows exhibited more intensive oestrus activity than heifers. Morrow (1969) confirmed this trend towards more intense libido in older cows, and also observed that the incidence of standing heat increased from the first to the third post-pubertal oestrus cycles. Ovulation occurs 10-12 h after the end of oestrus in mature

animals (Trimberger, 1948; Hansel et al., 1973).

1.3.2 Anatomical Changes

Priedkalns et al. (1968) and Priedkalns and Weber (1968a,b) have carried out the only previous qualitative and quantitative morphological studies of the bovine ovary, so the following description is principally based upon their findings.

1.3.2.1 Development and Structure of the Graafian Follicle

Priedkalns et al. (1968) reported that normal, as well as atretic, Graafian follicles were evident throughout the bovine oestrus cycle, and that during the 2-3 days preceding ovulation there was a terminal enlargement of one or more large follicles. They suggested that it takes approximately 10 days for the Graafian follicle to develop fully from the primordial state and that follicular growth is continuous rather than periodic, so that new follicles arise at any time during the cycle. However, it is the follicles arising from the first wave of growth which generally reach mature size at mid-cycle. This finding conflicts with that of Rajakoski (1960), who concluded that only two waves of follicular growth occurred during the oestrus cycle. He also noted that in the northern hemisphere more small (<5 mm) follicles were present in the ovary during winter and spring. Cole and Snook (1964) reported that the first wave of follicular growth begins about 3-4 days after oestrus, but that these follicles persist until day 11, after which they atrophy. Then a second wave of follicular growth begins during mid-cycle, resulting in a large Graafian follicle which ovulates about 12-18 h after oestrus.

The wall of the Graafian follicle consists of an epithelial layer (granulosa) and a connective tissue layer

(the theca) with the former subdividing into a centrally-projecting cumulus oophorus and a surrounding membrana granulosa. The theca is differentiated into a inner, vascular theca interna and an outer, supportive theca externa, consisting of elongated fibroblasts and abundant collagen fibres. Both thecal layers are highly vascularized (Priedkalns et al., (1968).

During the pre-ovulatory period (days 18-21), the granulosa cells divide less frequently than previously, and increase in size, assuming columnar shapes from the basal layer inward. The majority of the cells of the theca interna remain as fibroblasts during this period but a significant minority differentiate further to resemble large epithelioid cells. Near oestrus, an indentation in the theca interna occurs at the site of ovulation by a process which remains uncertain (Espey, 1978).

Follicles of all sizes undergo atresia. In obliterative atresia of primordial and growing follicles, oocyte degeneration precedes follicle wall degeneration whereas in cystic atresia of Graafian follicles wall degeneration is observed in follicles with apparantly normal ova (Priedkalns et al., 1968).

1.3.2.2 The Corpus luteum

The bovine corpus luteum originated from the membrana granulosa, theca interna and theca externa cell-types of the ruptured follicle wall. During early metoestrus (days 1-4), the membrana granulosa layer resumed mitotic divisions and was invaded by capillaries from the theca interna. By the end of the period of early metoestrus, the granulosa cells had begun to hypertrophy.

Rapid corpus luteum growth occurred throughout late

metoestrus (days 5-8), and was characterized by continued granulosa and theca cell proliferation with an associated vascularization. Two populations of luteal cells evolved at this time: the large luteal cells which developed from granulosa cells, and small luteal cells which developed from the fibroblast-like theca interna cells or, occasionally, paraluteal stromal cells or luteinized theca externa cells (Priedkalns et al., 1968). The large luteal cells attained up to 40 μ in diameter.

During dioestrus (days 9-17), the corpus luteum attained its greatest size (approximately 25 mm in diameter by day 12) and was fully functional according to morphological criteria (Erb et al., 1971). Toward the end of dioestrus, when the progesterone secretion rate was beginning to fall (Hansel and Echternkamp 1972; Heitzman, personal communication) some large luteal cells became vacuolated, the amount of connective tissue increased, and the lumen of blood capillaries became much reduced.

The period of pro-oestrus and oestrus (days 18-21) was characterized by rapid regression of the corpus luteum (Priedkalns and Weber, 1968; Erb et al., 1971) and the cessation of progesterone secretion (Hansel and Echternkamp, 1972; Heitzman, personal communication). Connective tissue proliferation and hypertrophy of blood vessels occurred and the luteal cells showed gross signs of degeneration, such as shrinkage, cytoplasmic darkening, vacuolation and nuclear pyknosis.

Priedkalns et al. (1968) and Priedkalns and Weber (1968a) concluded from morphological criteria that the bovine ovary lacks an interstitial gland and that the epithelioid theca

interna cells of the pre-ovulatory ovary produced oestrogens during pro-oestrus and oestrus, whereas the large luteal cells of the corpus luteum secreted progesterone during dioestrus.

1.3.3 Endocrinology

Until recently, studies of the changes of steroid hormone concentrations during the bovine oestrus cycle were limited to measurements of the concentrations in corpora lutea because the spectrophotometric assays then in use were not sufficiently sensitive to detect circulating steroids (Mars et al., 1962; Gomes et al., 1963; Gomes and Erb, 1965). The advent of radio-immunoassays have allowed measurement of the blood concentrations of steroid. The bovine corpus luteum produces pregnenolone, progesterone and Δ^4-pregnen-20βol-3-one (20β-hydroxy-progesterone) (Mason et al., 1962; Hansel et al., 1973). The peripheral plasma concentration of progesterone is low (less than 1 $ng.ml^{-1}$) for the first two days of the cycle, rises rapidly between days 4 and 12 to a plateau of 7 to 11 $ng.ml^{-1}$, and then declines rapidly on days 17 to 18 of the cycle, although the exact timing of this decline is variable (Stabenfeldt et al., 1969; Swanson et al., 1972; Hansel and Echternkamp, 1972; Heitzman, personal communication). Kazama and Hansel (1970) were unable to demonstrate a pro-oestrus rise in plasma progesterone. Deane et al. (1966) and Erb et al. (1971) found a significant correlation between the concentrations of progesterone in venous plasma and luteal tissue. They suggested that either ovarian venous or peripheral plasma progesterone concentrations provide reliable estimates of luteal function since they correlate with luteal weight, morphology and progesterone content.

A large ovulatory peak of luteinizing hormone (LH) (10-50 $ng.ml^{-1}$) occurs in the plasma near the onset of oestrus and persists for about 6 to 8 h (Swanson and Hafs, 1971; Hansel and Echternkamp, 1972). Smaller peaks in plasma LH also occur around days 8 to 10 of the cycle (Schams and Karg, 1969; Hansel and Snook, 1970). A significant rise in plasma LH does not occur until plasma progesterone levels decline below 1 $ng.ml^{-1}$, suggesting that the cyclic release of pre-ovulatory amounts of LH is inhibited by progesterone (Hobson and Hansel, 1972; Hansel et al., 1973; Karsh et al., 1977). Hansel and Echternkamp (1972) reported three minor peaks of plasma oestradiol and oestrone in addition to the major peak proceding oestrus, corresponding roughly with the periods of accelerated follicle growth described by Rajakoski (1960) and Priedkalns et al. (1968). However, Wetteman et al. (1972) and Henricks et al. (1971) did not find these minor oestrogen peaks during dioestrus in cattle.

The surge of LH at ovulation is triggered by oestradiol since the latter reaches its highest circulating concentration of around 10 $pg.ml^{-1}$ just before the gonadotrophin peak (Henricks et al., 1971; Hansel et al., 1973). Injection of oestradiol-17β into lutectomized heifers at days 13-15 of the cycle (Hobson and Hansel, 1972) or into anoestrus or ovariectomized ewes (Goding et al., 1972) evoked large plasma LH peaks some 24-36 h later. From studies in the sheep, LH may also act by increasing blood flow in the corpus luteum (Thorburn and Hales, 1972; Niswender et al., 1976).

The role (if any) of prolactin in the bovine oestrus cycle remains unclear; plasma levels have been measured throughout the cycle in both the cow and ewe (Walton et al., 1977),

but few significant changes have been found. Sinha and Tucker (1969) reported that prolactin decreased around oestrus in heifers whereas Swanson and Hafs (1971) and Davies et al. (1971) observed slightly increased plasma concentrations at, or just prior to, oestrus. However, the concentration of prolactin in bovine plasma undergoes a definite circadium rhythm according to Malvern and Hansel (1972), being lowest during the day and highest during the night. Prolactin appears to be involved in promoting the transition from anoestrus to breeding activity in sheep (Walton et al., 1977); it may act similarly in cows.

The classical interpretation of the role of gonadotrophins suggests that follicle stimulating hormone (FSH) causes follicular development, whilst LH stimulates steroid (principally oestrogen) secretion by the developing follicles and ultimately brings about ovulation (Short, 1972). However, the role of FSH, in the bovine oestrus cycle at least, may be more complex. Bovine pituitary concentrations of FSH have been reported to decline rapidly either during oestrus (Rakha and Robertson, 1965; L'Hermite et al., 1972) or 24 h before its onset (Hackett and Hafs, 1969), and in the ewe about 12 h before the onset of oestrus (Robertson and Rakha, 1966). The plasma membrane fraction of bovine corpus luteum possesses binding sites for FSH and LH (Haour and Saxena, 1974; Rao and Mitra, 1977). It is possible that FSH acts synergistically with LH to promote ovulation and that its steady secretion in small amounts (together with LH and possibly prolactin) throughout the luteal phase is necessary for follicular growth and corpus luteum function (Short, 1972; Rao et al., 1978).

In summary, circulating concentrations of oestradiol

remain low, except for minor, transient peaks, during the luteal phase of the cycle but increase markedly during the three days preceding oestrus and decrease rapidly after the onset of oestrus. Plasma progesterone is low at oestrus, increases about the second day after oestrus as the corpus luteum becomes functional, and then declines rapidly at the end of the luteal phase, as regression of the corpus luteum becomes evident. The pro-oestrus peak of oestrogen stimulates the ovulatory surge of LH (and possibly a smaller surge of FSH) which lasts for 6-8 h and precedes behavioural oestrus by 29-31 h (Henricks et al., 1970; Swanson et al., 1971). Changes in blood flow generally parallel the increase and decrease in progesterone secretion in the sheep and may also be important in the cow by mediating the supply of gonadotrophic hormones to the luteal cells (Niswender et al., 1976). The corpus luteum of the rabbit has been reported to produce a factor which promotes its own vascularization during early oestrus (Gospodarowicz and Thakral, 1978).

1.3.3.1 Luteotrophic Mechanisms

Corpus luteum weight and progesterone content in the cow increase rapidly between days 3 and 12 of the cycle, and remain relatively constant until day 16 (Erb et al., 1971). LH is luteotrophic in the cow (Simmons and Hansel, 1964; Hansel, 1966; Hansel, 1967). LH, or preparations containing LH, alone among the gonadotrophic preparations tested, were able to overcome the inhibitory effects of concurrently injected oxytocin on corpus luteum growth and progesterone content (Armstrong and Hansel, 1959). Exogenous LH was also capable of prolonging the oestrus cycle of intact animals (Donaldson and Hansel, 1965).

It was also effective in potentiating the secretion of progesterone *in vitro* when added to slices of ovine (Savard et al., 1965; Marsh et al., 1966) and bovine (Armstrong and Black, 1966; Hansel, 1971) luteal tissue possibly by activation of a cyclic AMP-dependent protein kinase (Ling and Marsh, 1977). McCracken et al. (1971) established the luteotrophic effect of LH in the ewe in a series of experiments where purified pituitary hormones were directly infused into the arterial supply of ovaries autotransplanted to the neck; LH, but neither FSH nor prolactin, produced an increase in the secretion of progesterone. Heap et al. (1973) raised the possibility that LH may affect the bovine corpus luteum at two sites of the progesterone biosynthetic pathway, one before cholesterol formation and the other at the cholesterol-pregnenolone conversion.

Robinson et al. (1976) recently suggested that the corpus luteum is subjected to both positive (luteotrophic) and negative (inhibitory) influences, and that the balance between the two determines the level of progesterone secretion. They observed small peaks of PGF in the utero-ovarian venous plasma of sheep, which were accompanied by transient decreases in the concentration of progesterone in the utero-ovarian vein. They suggested that PGF could depress corpus luteum function temporarily without inducing luteolysis. Larger amounts of PGF released at pro-oestrus would induce luteolysis. However, the balance between production of PGE and PGF may also be a factor (Henderson et al., 1977). At different times of the oestrus cycle, exogenous oestrogens are luteolytic in the cow (Hansel et al., 1973) whereas they are luteotrophic in the rabbit, rat and sow (Robson, 1967; Begdanove, 1966, in Hansel et

al., 1973). In the ewe, exogenous oestradiol is luteotrophic early in the cycle, apparently by stimulating LH release, whereas later in the cycle (days 9-12) it is luteolytic (Hawk and Bolt, 1970). Robinson et al. (1976) suggest that these varying responses to oestrogens are partly due to the inability of oestrogen later in the cycle to trigger LH release when the plasma progesterone concentration is high. Subsequently, when the uterus has been primed with progesterone, oestrogen stimulates the release of $PGF_{2\alpha}$, which overcomes the effect of the existing LH levels and provokes luteolysis. In this context, Kimball and Hansel (1974) have demonstrated that the bovine corpus luteum contains a specific oestrogen receptor whose levels fluctuate during the oestrus cycle. Its greatest concentration was reached at pro-oestrus, when sufficient oestrogen may be bound to prevent LH release.

1.3.3.2 Luteolytic Mechanisms

Regression of the bovine corpus luteum commences just after the rapid decline in the rate of secretion of progesterone around days 17 to 18 of the cycle (Priedkalns et al., 1968; Hansel et al., 1973). The morphological features of regressing luteal cells have been described in detail in the sheep (Deane et al., 1966; Bjersing et al., 1970; Gemmell et al., 1976; Stacy et al., 1976), pig (Bjersing, 1970b) human (Adams and Hertig, 1969a) and cow (Priedkalns and Weber, 1968a). They include a general shrinking of the cells, the build-up of lipid bodies, autophagocytic bodies and lysosomes, and a general breakdown of fine structure. Connective tissue elements become more prominent and the lumina of many arterioles become occluded.

Kenny (1964, in Hansel et al., 1973) has hypothesized that the cessation of progesterone synthesis results from an "uncoupling" of the steroid biosynthetic system from the metabolic systems which supply it with energy and precursors. He bases this idea on the fact that isocitrate and glucose-6-phosphate dehydrogenase enzyme activities are sustained beyond day 16, the time at which the decline in progesterone synthesis begins. If this hypothesis is correct, and it has not been substantiated, it would have important implications for the understanding of the basis of the rapid ultrastructural changes that accompany functional involution of the corpus luteum. Supporting evidence comes from experiments where colchicine was administered to ewes at mid-cycle. The concentration of progesterone in the blood was lowered whilst the luteal tissue concentration of hormone was raised correspondingly (Gemmell and Stacy, 1977).

Kenny (1964) also suggested that regression of the corpus luteum as a tissue is caused by a reduction in its blood supply, either before, during, or after the "uncoupling" of progesterone synthesis from the intracellular sources of energy. This idea has recently received support from the work Thorburn and Hales (1972) and Niswender et al. (1976), who investigated the physiological regulation of blood flow in sheep ovaries throughout the oestrus cycle and whose findings are relevant to consideration of the bovine oestrus cycle. Blood flow to the luteal ovary in sheep increased some 3- to 7-fold during the luteal phase of the cycle so that significant (if not increased) quantities of trophic hormones continued to reach the corpus luteum even though their plasma concentrations were low

(Niswender et al., 1976; Brown et al., 1976). In addition, this increase in blood flow to the corpus luteum provided it with increased quantities of oxygen, glucose, acetate, cholesterol, etc. which may stimulate (or at least enable) the luteal cells to secrete progesterone at maximal rates. Niswender et al. (1976) make the point that it is difficult to accept that LH is "the" luteotrophin in man, monkeys, sheep, pigs and cattle, since its circulating levels are minimal when secretion of progesterone is maximal. The situation is further complicated by the findings that LH stimulates the synthesis of progesterone when incubated with slices of corpora lutea from a number of species (see above) and that it promotes luteinization of primate (Channing, 1969; Channing, 1970a) and porcine (Channing, 1970b) granulosa cells in culture.

During luteal cell regression, Niswender et al. (1976) observed that arteriole-venule shunting of blood occurred within the corpus luteum. They suggested that this helped promote luteal cell regression by reducing both trophic support and the supply of nutrients. Similar results have been previously reported in the sheep by Thorburn and Hales (1972) and in the rabbit by Pharriss et al. (1970) and Bruce and Hillier (1974). Administration of $PGF_{2\alpha}$ to sheep caused a drop in flow through the luteal ovary but it was not established if arteriole-venule shunting within the corpus luteum also occurred (Niswender et al., 1976). Similarly, $PGF_{2\alpha}$ caused a redistribution of blood flow in the rabbit ovary, away from the corpus luteum and into the interstitial and follicular tissues (Novy and Cook, 1973).

Bolt et al. (1971) showed that exogenous human chorionic gonadotrophin (HCG), or FSH plus LH, given prior to oestradiol injections on day 10 of the sheep cycle, reduced the luteolytic effect of oestradiol. The luteolytic effects of exogenous oestrogen in the cow and ewe are significant since plasma oestrogens rise in both species before the onset of luteal regression (Hansel et al., 1973). However, it now appears that luteolysis is triggered by local uterine release of large amounts of $PGF_{2\alpha}$ (more than 6 $ng.ml^{-1}$ in sheep) during pro-oestrus (McCracken et al., 1972; Thorburn et al., 1973; Cox et al., 1973; Stacy et al., 1976). Infusion of 50 $\mu g.h^{-1}$ of $PGF_{2\alpha}$ into the ovarian artery of the intact cow for 6 h at mid-cycle caused a rapid decline in the peripheral concentration of progesterone. However, these levels of $PGF_{2\alpha}$ were insufficient to cause luteolysis when infused into the peripheral circulation (Hansel et al., 1973). Later Hansel (1975) showed that $PGF_{2\alpha}$ was luteolytic in the cow as it was in sheep. He suggested that oestrogens are involved in both naturally occurring- and $PGF_{2\alpha}$-induced regression of the bovine corpus luteum, and that in both sheep and cow luteolysis is an active process, involving steroidogenesis and requiring an interaction between oestrogen, $PGF_{2\alpha}$ and a specific oestrogen receptor protein (Kimball and Hansel, 1974; Hansel, 1975).

The luteolytic effect of prostaglandins in cows and sheep explains how the functional lifespan of the corpus luteum is greatly prolonged following hysterectomy. Furthermore, removal of the bovine corpus luteum, either at the time of hysterectomy (Malven and Hansel, 1964) or 75 days after hysterectomy (Anderson and Bowerman, 1963) is followed by ovulation and

formation of a new corpus luteum, which, in turn, is maintained for an extended period of time. However, the role of prostaglandins in luteolysis is not unequivocal, since Speroff and Ramwell (1970) and Hansel et al. (1973) have shown that prostaglandins E_2, E_1, $F_{2\alpha}$ and A_1 are all luteotrophic, rather than luteolytic, when added to slices of bovine luteal tissue *in vitro*.

It has been suggested that $PGF_{2\alpha}$ initiates luteolysis in the luteal cell by directly inhibiting LH-activated adenyl cyclase activity (Henderson and McNatty, 1975; Lahau et al., 1976). Henderson et al. (1977) demonstrated that when prostaglandins E_2 and $F_{2\alpha}$ were infused simultaneously into corpora lutea of ewes bearing ovarian transplants, the luteolytic action of $PGF_{2\alpha}$ was antagonized by PGE_2. They suggested that there are two pools of cyclic AMP available to stimulate luteal synthesis of progesterone: one pool, due to LH-mediated activation of adenyl cyclase, which can be inhibited by the action of $PGF_{2\alpha}$, and another pool brought about separately by stimulation of adenyl cyclase through PGE_2-sensitive receptors, and which bypasses the $PGF_{2\alpha}$-sensitive LH receptor. The primary event in the induction of ovine luteolysis by $PGF_{2\alpha}$ may be either a decline in the concentration of luteal receptors for LH or decreased binding of LH to receptor (Nett et al., 1976; Diekman et al., 1978a). However, $PGF_{2\alpha}$ does not promote luteolysis through decreasing the amount of receptor-bound LH since the $PGF_{2\alpha}$-induced decrease in progesterone secretion by the sheep corpus luteum preceded by 15 h changes in the occupancy of receptors for LH (Diekman et al., 1978b).

The differing effects of prostaglandin under *in vitro* and *in vivo* conditions remains to be reconciled. The report by

Thomas et al. (1978) that the rapid action of prostaglandins E_2 and $F_{2\alpha}$ in isolated luteal cells is brought about by blocking the LH-dependent production of cyclic AMP which, in turn, reduces the secretion rate of progesterone, may point the way to the answer, since in many *in vitro* studies LH is routinely added to the medium to augment the rate of progesterone secretion. From other *in vitro* studies, it seems that cyclic AMP also mediates stimulation of progesterone synthesis via a beta-adrenergic agonist (isoproterenol) in the bovine corpus luteum. However, it is not known if catecholamine receptors are involved in the LH-stimulated production of cyclic AMP *in vivo* (Condon and Black, 1976; Godkin et al., 1977).

1.4 STEROIDOGENESIS

1.4.1 Problems in the Study of Steroidogenesis

The cytological features of active luteal cells have been extensively catalogued and continue to accumulate for different species and different conditions (Christensen and Gillim, 1969; Enders, 1973). (These features are reviewed and discussed in Chapter 2). The steroidogenic pathways of luteal, testicular and adrenal cells of many mammalian species under a variety of *in vivo* and *in vitro* conditions are also well documented (Grant, 1968; Armstrong et al., 1969; Aakvaag and Eik-Nes , 1969; Tamaoki et al., 1969; Savard, 1973; Dorrington, 1977). However, the mechanisms of steroid biosynthesis and secretion at the subcellular level remain obscure. This is in contrast to the situation with regard to protein, polypeptide and catecholamine secretions. The cellular pathway of protein and glycoprotein can be followed from synthesis on polysomes of the granular endoplasmic reticulum (ger), to the tubules of

agranular endoplasmic reticulum (aer), addition of saccharide side chains and terminal sugars in the Golgi, condensation and formation of vesicles in the Golgi, migration of the vesicles to the apical surface, and their release to the exterior by membrane fusion (Fawcett et al., 1969). Secretion of insulin in zymogen granules from the pancreas (Palade, 1975), of catecholamines in chromaffin granules from the adrenal medulla (Douglas, 1966, 1974b) and of gonadotrophins from the anterior pituitary (Smith and Farquhar, 1966; Fawcett et al., 1969) are all considered to occur similarly.

Such information is currently unavailable for steroid secretion, including the secretion of progesterone from the bovine corpus luteum. However, by extrapolating the information obtained from studies of adrenal and testicular secretion, plus that obtained from studies of the corpora lutea of a number of species, it is possible to provide a preliminary description of the steroidogenic process at the subcellular level, and to suggest probable mechanisms of steroid secretion in the bovine corpus luteum and other steroid endocrine tissues.

The main reason for the lack of progress in elucidating subcellular events in steroid endocrine cells lies in a consideration of the differing solubilities of proteins and steroids (Enders, 1973). Unlike proteins, steroids are highly soluble in the organic solvents normally used in making cytological preparations suitable for autoradiography and immunocytochemistry, especially at the ultrastructural level (Enders, 1973). Autoradiography is especially useful in determining the sequence and timing of biosynthetic and secretory events, as in the study of the formation and secretion of zymogen

granules from the pancreatic beta cells (Jamieson and Palade, 1967a,b; Palade, 1975). In addition, steroid cells do not accumulate stores of active steroid, nor are they generally thought to condense a secretion product (Fawcett et al., 1969; Porter and Bonneville, 1973; Enders, 1973), although this must be queried in the light of recent findings (Belt et al., 1965; Gemmell et al., 1974, 1977a,b).

Biochemical information concerning the localization of the enzymes involved in steroid biosynthesis has come mainly from cell fractionation studies using centrifugation techniques (Christensen and Gillim, 1969; Fawcett et al., 1969). When steroid-secreting cells are homogenized, the mitochondria are usually swollen due to the use of hypertonic media but are otherwise fairly intact. However, the endoplasmic reticulum and Golgi elements break up into vesicles of varying size and density to form the principal constituents of the microsomal fraction (Christensen and Gillim, 1969). Enzymatic activities are usually assigned to the mitochondrial or microsomal fractions of the homogenate or to the supernatant which is believed to contain the soluble components of the cytoplasmic matrix (Fawcett et al., 1969). The assignment of an enzyme to one of these subcellular compartments must be considered only tentative, since disruption of the tissue by homogenization and its subsequent centrifugation can lead to serious leakage-adsorption artifacts, whereby true subcellular localizations become obscurred (Sheele et al., 1978). The composition of the separate fractions should be assessed by electron microscopy, although this is not always done (de Duve, 1971). Although the microsomal pellet contains fragmented membranes of the aer,

ger, Golgi and even plasmalemma, it is usually interpreted as being principally composed of aer, the major membraneous component of steroid-secreting cells. This assumption is reasonable, but further fractionation of microsomes into their discrete components would provide greater precision to the localization of the different enzymes (Fawcett et al., 1969). An example of where this has been achieved was in the localization of 11β-hydroxylase in the inner mitochondrial membrane of the adrenal cortex (Dodge et al., 1970).

Another complication in studying steroid synthesis, as compared to protein synthesis, is that only the early stages of cholesterol synthesis are additive. Beyond cholesterol, steroidogenesis involves microsomal, soluble and mitochondrial compartments, so that there is no single compartment comparable to the passage of a peptide along the cisternae of ger. Furthermore, the terminal reactions in the formation of progesterone via pregnenolone from cholesterol involve removal of the side chain of cholesterol and a shift in unsaturation and dehydrogenation, rather than the addition of easily labelled amino acids or sugars to a polypeptide chain (Enders, 1973).

1.4.2 Cholesterol Biosynthesis

The various steroid hormones are synthesized from cholesterol (Tamaoki and Pincus, 1961; Savard et al., 1965; Major et al., 1967), which in turn is derived from acetate. Two molecules of acetate, as acetyl-CoA, are built up into the 6-carbon structure, β-hydroxy-β-methylglutaryl-CoA, which is reduced to mevalonic acid. A sequence of phosphorylation and decarboxylation reactions then convert mevalonic acid to the

fundamental isoprenoid unit, isopentyl pyrophosphate, three molecules of which condense to form the 15-carbon compound farnesyl pyrophosphate. Two of these join to make squalene. Squalene is oxidized to squalene-2,3-oxide, the immediate percursor of lanosterol, which has the characteristic steroid ring structure. Subsequent demethylations and double-bound shifts give rise to cholesterol, containing 27 carbon atoms (Dorfman and Ungar, 1965; Dempsey, 1974; Goad, 1975).

Separate pools of cholesterol exist within the cell and only a portion is readily available for steroidogenesis. The remaining cholesterol cannot be utilized for steroidogenesis, and is not responsive to LH (Armstrong et al., 1964; Flint and Armstrong, 1971a,b). There are three major sources of free cholesterol in the ovary: *de novo* synthesis from acetate and mevalonate, uptake of performed cholesterol from the blood, and hydrolysis of esterified or free cholesterol stored in lipid droplets (Dorrington, 1977). Free cholesterol readily diffuses through cell membranes so that radioactive cholesterol taken up from blood equilibrates rapidly with the existing pools of free cholesterol within the cell (Flint and Armstrong, 1971b). Equilibration between free and esterified cholesterol proceeds much more slowly (Major et al., 1967).

The large lipid droplets characteristic of steroid-secreting cells store cholesterol, principally in the form of esters (Major et al., 1967; Moses et al., 1969). However, there are considerable differences between species both in the total cholesterol content and the proportion which is esterified. The bovine corpus luteum is unusual in that most of its intracellular cholesterol is unesterified (Armstrong

and Black, 1966). Specific mechanisms must exist for the regulation of the supply of cholesterol in lipid droplets to mitochondria, where it is utilized in steroidogenesis. For example, Beckett and Boyd (1977) have found that the cholesterol ester hydrolase of bovine adrenocortical cells is activated by a phosphorylation which is catalysed by a cyclic AMP-dependent protein kinase. Deactivation of the hydrolase is accomplished by dephosphorylation catalysed by a phosphoprotein phosphatase, dependent on magnesium or calcium ions. These authors have suggested that the cyclic AMP-dependent activation of the protein kinase is one way in which ACTH and LH activate the cells of the adrenal cortex and corpus luteum, respectively (Boyd et al., 1975). In rabbit interstitial tissue, progesterone and 20β-hydroxypregn-4-en-3-one are *in vitro* inhibitors of cholesterol ester synthetase (Flint et al., 1973).

1.4.3 Progesterone Biosynthesis

The cholesterol side chain cleavage enzyme complex (CSCC) is common to all steroid-forming tissues (Constantopoulos and Tchen, 1961); it removes the side chain of cholesterol to form pregnenolone and isocaproaldehyde (Sulimovici and Boyd, 1969; Savard, 1973). The complex consists of two hydroxylases and a lyase, and requires cytochrome P450 and a nonheme iron protein for activity (Kimura and Ono, 1968). It is a mixed function oxidase, using NADPH and molecular oxygen (Boyd, 1972; Gower, 1975a). The CSCC and associated electron transport-cytochrome system and NADPH-generating systems in the mitochondria of luteal cells are directly comparable to those of the adrenal cortex and other steroidogenic tissues (Savard, 1973). Therefore data obtained from studies of the adrenal

cortex apply to the corpus luteum as well. On the basis of differential centrifugation studies, the CSCC is usually considered to be located on the inner mitochondrial membrane (Ichii et al., 1963; Hall and Kortiz, 1964; Gower, 1975a; Negrie et al., 1977) but studies by Flint and Armstrong (1971a,b) have suggested that it is also found in the microsomal fraction, at least in the luteal cells of the cow and rat. Since the amount of cytochrome P450 was lower in the microsomal fractions (Flint and Armstrong, 1971a,b) it is possible that the presence of cholesterol side chain cleavage activity in the microsomal fractions of rat and cow corpora lutea was artifactual, brought about by leakage from mitochondria and adsorption onto microsomes (Sheele et al., 1978). However, if a microsomal location for the CSCC is valid, then there exists the possibility of two discrete and totally compartmentalized steroidogenic pathways from cholesterol (Flint and Armstrong, 1971a).

The mechanism of the reaction catalyzed by the CSCC is currently unresolved. Objections have recently been raised to the validity of the "classical" sequential hydroxylation pathway of pregnenolone synthesis (Schimizu et al., 1962), which proposed that cholesterol first undergoes hydroxylation to (20S)-20-hydroxycholesterol or (22R)-22-hydroxycholesterol; that these monohydroxycholesterols are then converted to (20R, 22R)-20,22-dihydroxycholesterol, which is in turn cleaved to pregnenolone and isocaproaldehyde. Thus, it has been suggested that the hydroxylated intermediates are not directly involved in the conversion of cholesterol to pregnenolone but are products of side reactions (Hochberg et al., 1974; Kraaipoel

et al., 1975a,b). There is evidence that the NADPH required for the reactions is supplied via the associated cytochrome P450 and various Krebs cycle intermediates (McIntosh et al., 1971) and that reversed electron transport coupled to an energy-dependent NAD-NADP transhydrogenase is involved (Omura et al., 1965; Uzgiris et al., 1971).

The factors requlating the flux of cholesterol through mitochondria are: (i) the transport of cholesterol into the organelle, (ii) the availability of molecular oxygen, (iii) the transformation of oxygen for hydroxylation to an activated state (by an electron transport system), (iv) the supply of reducing equivalents by suitable enzymatic generating cycles, and (v) the transport of pregnenolone out of the mitochondrion (Savard, 1973).

The conversion of cholesterol to pregnenolone is the rate-limiting step of steroidogenesis (Stone and Hechter, 1954) and the site of the pathway at which LH (and ACTH) influences the rate of steroid production (Dorrington, 1977). Recent studies using adrenocortical and luteal cells have suggested that transportation of cholesterol within the mitochondrion is the rate-limiting event in the overall CSCC reaction and that labile protein(s) may be involved in this and other aspects of the reaction (Boyd et al., 1975). In the adrenal cortex, calcium ions do not affect the CSCC directly, although they are involved in the stimulation of steroidogenesis by ACTH at the cell membrane and secretion of steroids (Laychock and Rubin, 1977). Steroidogenesis in isolated adrenal cells is also stimulated by excess pregnenolone which bypasses the CSCC.

Pregnenolone is converted to progesterone in a two-step

process by 3β-hydroxysteroid dehydrogenase (3β-HSD) and an isomerase. The enzymes have been found in microsomal fractions prepared from testes (Tamaoki et al., 1969) and in several ovarian preparations (Ryan and Smith, 1965). However, in the corpus luteum and adrenal cortex of the cow, these enzymes are both microsomal and mitochondrial (Savard, 1973; Simpson and Boyd, 1967).

Several NADPH-generating systems have been demonstrated in bovine and human corpora lutea (Nielson and Warren, 1965; Savard et al., 1973). The following enzyme activities were recovered in supernatant fractions: glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase, and malate dehydrogenase. Histochemical studies show some of these enzymes to be associated with the aer (Lobel and Levy, 1968).

Progesterone is produced by the corpora lutea of all mammals, but there is considerable diversity among species with respect to the overall range of steroids produced (Savard, 1973). The bovine corpus luteum produces the simplest steroidal product, namely progesterone and 20β-hydroxyprogesterone (Mason et al., 1962). This tissue does not synthesize C_{19} steroids because it lacks the necessary 17-hydroxylase and C-17,20-lyase enzymes essential for androgen production (Savard and Telegdy, 1965). It also does not form oestrogens because of the lack of the 19-hydroxylase-aromatase system necessary for the conversion of androgens to oestrogens (Savard and Telegdy, 1965). The microsomes of the luteal cells of the cow have been reported to lack cytochrome P450, needed for 17- and 19-hydroxylase activities (McIntosh et al., 1971), but Flint and

Armstrong (1971a,b) would question this.

At the other extreme is the human corpus luteum, which contains the enzymes necessary to synthesize progesterone and additional progestogens, androgens and oestrogens (Huang and Pearlman, 1963; Ryan and Smith, 1965). Between these two extremes in steroidogenic capabilities lie all of the other species studied so far - for example, the rabbit (Telegdy and Savard, 1966; Dorrington and Kilpatrick, 1969), the dog (Aakvaag and Eik-Nes, 1969), the pig (Cook et al., 1967) and the sheep (Kattenbach et al., 1967). As Savard (1973) has pointed out, although the different tissues comprising the rabbit and human ovaries all contain the complete array of steroidogenic enzymes, each tissue elaborates a distinctive range of steroids under normal physiological conditions. To account for this, it has been proposed that there is a sub-cellular compartmentalization of these microsomal enzymes, and that the steroids undergo modification bound to protein (Savard, 1973; Gower, 1975b). Only when the terminal end is reached is the steroid released as an androgen or oestrogen (Savard, 1973). This may explain why incubations after disruption of the sub-cellular organization of steroidogenic tissues by freezing and thawing and/or homogenization provide only a qualitative estimate of the total enzymatic array of the tissues. If perfusion of the organ is not possible a physiologically-meaningful determination of the range of steroids produced by the tissue may be obtained by incubations of slices or intact cells (Channing, 1969b).

1.4.4 Role of Intracellular Cholesterol and Steroid Binding Proteins

From the foregoing, it is suggested that steroid endocrine cells show much interspecies variation and do not always produce the range of steroids of which they are enzymatically-capable. It is also apparent that in these cells the enzymes utilized sequentially in the production of some steroids (e.g., glucocorticoids) are variously located within the membranes of mitochondria and endoplasmic recticulum, or are soluble (but not necessarily freely-diffusible) within the cytoplasmic matrix. This seems an unnecessarily complicated arrangement, unless mitochondria are the only sites in the cell where the oxidative environment is sufficiently favourable for the cholesterol chain cleavage reaction and 11β-hydroxylation of deoxycorticosterone to occur (Enders, 1973). Thus, compartmentalization of the steroidogenic enzymes may have been used to advantage with respect to control of the range and rate of steroids produced. Whatever the reason, it is possible that specific intracellular carrier or binding proteins for cholesterol and steroids form part of the intracellular transport system necessary to steroid biosynthesis.

Three cholesterol binding proteins, one of them analogous to the sterol carrier protein described by Ritter and Dempsey (1971, 1973) have been identified in adrenocortical, luteal and testicular cells (Scallen et al., 1975). Separate binding proteins for pregnenolone and its sulphate have been identified in the adrenal cortex of guinea pigs and rats (Strott, 1977; Kream and Sauer, 1977; Strott and Lyons, 1978). The bovine corpus luteum contains at least one specific progesterone-

binding protein (Leymarie and Gueriguian 1969, 1970) and a glucocorticoid-binding protein has been found in the bovine adrenal cortex (Cochet et al., 1977). A more extensive discussion of the properties of these and other intracellular binding proteins for cholesterol and steroids is given in Chapter 4.

1.5 MECHANISM OF STEROID SECRETION

It has generally been assumed that steroids diffuse from endocrine cells, even though little direct evidence for such a means of release is available. For example, early studies on the production of glucocorticoids from the adrenal assumed that steroid reached the bloodstream by diffusion (Vogt, 1943, 1944, 1951) and this view has largely prevailed, not only for adrenocortical cells (Pollard et al., 1977) but for all steroid-secreting cells (Fawcett et al., 1969; Christensen and Gillim 1969; Porter and Bonneville, 1973; Enders, 1973; Heap et al., 1973). Gillim et al. (1969) suggested from morphological studies of the human corpus luteum that steroids might pass through cell membranes into extracellular space by either diffusion or active transport.

The reason for acceptance of such a secretory mode for steroids principally resides in the reputed lack of evidence to the contrary: since membranes are readily permeable to steroids and steroid-secreting cells contain only a few minutes supply of hormone (Short, 1965; Kuhn and Briley,1970; Illingworth, 1970), it was considered that there was no reason for, nor evidence of, secretion by vesicles or droplet (Fawcett et al., 1969; Enders, 1973). The low aqueous solubility of progesterone and other steroids implied that their continuing

release in small quantities would be more suitable than their ejection as large droplets (Enders, 1973). However, the wide variation in the concentration of progesterone in the luteal tissue of different animals has led Heap et al. (1973) to propose that intracellular binding to tissue proteins may regulate the rate at which steroids are released from endocrine tissues.

The identification of various unusual ultrastructural features in steroidogenic tissues such as the adrenal cortex and corpus luteum have given rise to other suggested secretory modes. Yamada and Ishikawa (1960) noted that the luteal cells of the mouse were highly convoluted at the surface adjacent to the extracellular space and that some of these luteal cell protrusions penetrated into the capillary lumen. This led them to propose that progesterone could be secreted by an apocrine mechanism. Green and Macqueo (1965) observed microvilli in human luteal cells and concluded that the tips of the villi were pinched off and disintegrated in the extracellular space, similar to the proposal of Yamada and Ishikawa (1960).

According to Green et al. (1968), human luteal cells possess an extensive system of canaliculi. They combined this finding with previous results to propose that steroid hormones were transported intracellularly via the canaliculi and were secreted by a microapocrine mechanism. However, Enders (1962) found no evidence for apocrine secretion by the luteal cells of the armadillo, mink or rat and Gillim et al. (1969) specifically excluded the existence of both canaliculi and such a secretory mechanism in their study of human luteal cells. However, the presence of large intracellular channels and an aprocrine secretion of progesterone from the human corpus

luteum is unresolved since Crisp et al. (1973), although not observing canaliculi, did not exclude the possibility that there existed channels for the movement of protein-bound steroid or relaxin.

The presence of coated vesicles in the Golgi regions of opossum adrenocortical cells and their similarity to coated invaginations at the cell surfaces led Long and Jones (1974) to suggest that steroids were secreted in these specialized membraneous elements.

Mechanisms which entail the coalescence of progesterone into discrete droplets prior to its secretion have also been proposed. Tokida (1962) suggested that the coated vesicles they observed in human luteal cells contained progesterone, and were secreted by reverse pinocytosis, although such a mechanism was discounted by Gillim et al. (1969). However, the interrenal cells of the brown pelican (avian homologues of mammalian adrenocortical cells) contained small dense bodies (Sheridan et al., 1963). The latter were also observed at the lateral margins of the interrenal cells, between adjacent plasmalemmas, especially near the apical surfaces of the cells (Belt et al., 1965). These authors suggested that these dense bodies contained steroid hormones which were expelled from the cell by reverse pinocytosis. These studies were among the first to suggest that steroid hormones may be packaged into, and secreted via, membrane-bound granules, although endocrine cells producing hormones of such widely differing secretions as glucagon, oxytocin, vasopression, calcitonin, ACTH, serotonin and catecholamines all store their product in granules (Fawcett et al., 1969). Previous ultrastructural studies have

shown that small densely-staining granules, about 0.2-0.4 μ in diameter, occur widely in steroid-secreting cells (Christensen and Gillim, 1969 and see Chapter 2).

In a combined ultrastructural and subcellular fractionation study of the ovine corpus luteum, Gemmell et al. (1974) demonstrated a correlation between the formation and exocytosis of these granules with the known changes in the rate of progesterone secretion throughout the oestrus cycle. The granules were first observed within the luteal cells at day 3 of the cycle; they increased in number as the cycle progressed and at day 6 the first signs of secretion were noticed. Maximum secretion of granules occurred at days 10 and 11, when the rate of progesterone production was maximal (Thorburn and Mattner, 1971), and this was followed by a progressive decline in secretory activity from days 12 to 15, when regression of the luteal cells commenced (Gemmell et al., 1974, 1976; Stacy et al., 1976). The secretory granules appeared to be formed in the Golgi membranes and then transported to the cell membrane and released in the manner described for the protein hormones (Fawcett et al., 1969). Differential contrifugation of homogenates of corpora lutea were not sufficiently refined to yield a fraction consisting purely of densely-staining granules. Nonetheless, densely-staining granules were observed in the particulate fraction obtained by centrifugation at 10,700 g, together with about 25% of the total progesterone (Gemmell et al., 1974).

Densely-staining granules, analogous to those observed in the sheep, and evidence for their secretion into the extracellular spaces, was obtained in the luteal cells of the goat, cow and pig. The luteal cells of the rabbit, rat and guinea

pig contained granules, although they were not observed in the extracellular spaces (Gemmell and Stacy, unpublished observations).

Granular secretion of steroid hormones may not be restricted to the corpus luteum but may be a general phenomenon, since it has also been reported in the cat adrenal gland. The ACTH-induced increase in intracellular and secreted steroid was accompanied by a 4-fold increase in the number of densely-staining granules in the cortical cells. These granules appeared to originate from the Golgi region and were not lysosomes since marker enzymes for this organelle did not show increased activity (Gemmell et al., 1977a).

The sequestration of steroids in densely-staining granules implies that they must also contain protein, since steroids are not osmiophilic in glutaraldehyde-fixed tissues (Enders, 1962). Such protein(s) may be required for binding of the steroids in order to allow high intragranular concentrations. In studies of the perfused adrenal gland, ACTH-induced secretion of glucocorticoids was temporally related to protein release (Laychock and Rubin, 1974; Rubin et al., 1974). This secretion of protein was dependent on calcium ions, as was steroid secretion (Laychock and Rubin, 1977). Synthesis and release of protein together with steroid hormone has also been reported from *in vitro* studies of corpora lutea of ewes and bitches (Abel et al., 1977; Sawyer et al., 1977) and in isolated Leydig cells from rat testis (Janszen et al., 1977). These findings are consistent with the view that the granules containing protein-bound steroid are expelled from the cell by exocytosis, or by a closely-similar process. Pollard et al.

(1977) have suggested that membrane-permeable anions in the external medium, such as chloride and hydroxyl ions, may play an important role in regulating exocytosis whereas calcium ions are intimately involved in the stimulus-secretion process, (Douglas, 1966, 1974a; Dreifuss, 1977). Many neurotransmitters, hormones and enzymes are stored in intracellular secretory vesicles and, in response to appropriate stimuli, are released into the extracellular compartment by exocytosis (Allison and Davies, 1974). The process seems well defined ultrastructually but the chemical and energetic basis for exocytosis remains obscure. In secretory systems such as the exocrine pancreas (Jamieson and Palade, 1974; Palade, 1975), adrenal medulla (Douglas, 1966, 1974b), neurosecretory cells (Morris, 1976; Dreifuss, 1977), and mast cells (Lawson et al., 1977), exocytosis has been visualized by electron microscopy. The process appears to consist of a number of discrete steps. Individual secretory vesicles contact the plasmalemma forming a pentalaminar or "fusion" complex by an as yet poorly defined process which requires calcium (Douglas, 1966, 1974a). The fusion becomes more intimate, and in some cases it has been shown that the submembrane particles in the membrane portion of the fusion complex move aside. "Fission" of the bilayer finally occurs, resulting in exposure of the vesicle or granule contents to the exterior of the cell, and secretion results. The mechanism for the latter step is also not known and may vary according to the substance being secreted (Douglas, 1974b; Satir, 1974). Extensive exocytosis is usually accompanied by extensive endocytosis (Douglas, 1966, 1974b; Allison and Davies, 1974). Endocytosis of microvesicles and other membraneous structures

is generally thought to provide a means of conserving the membrane of secretory vesicles and granules, and to prevent the endocrine cell from increasing its surface area (Allison and Davies, 1974). Microvesicles are not especially abundant in luteal and adrenocortical cells, although there is evidence of extensive exocytosis in luteal cells (Gemmell et al., 1974; Gemmell et al., 1977a,b). This may imply that the membrane of the secretory organelles is conserved by the cells in some other way, such as secretory bursts being accompanied by alternate swelling and shrinking of the cells (Allison and Davies, 1974).

Microtubules may be involved obligatorily in the intracellular transport of secretory vesicles to the periphery of cells and at least two theories have been proposed to account for this transport. Lacy and colleagues proposed a microtubule-microfilament system linking the insulin secretory vesicles with the plasma membrane. Stimulation of secretion involves "contraction" of this system with resultant transport to the cell surface (Lacy et al., 1968). Forbes and Dent (1974) found 100 Å filaments parallel to the microtubules in the gonadotrophic cells of a lizard. They suggested that these filaments were involved in the movement of secretory vesicles, possibly through a direct attachment between the filaments and the secretory granules, with the filaments providing motive force in some undefined way. Microtubules have also been implicated in the secretion of glucagon (Leclercq-Meyer et al., 1974), catecholamines (Poisner and Berstein, 1971) and albumin (Le Marchand and Malaisse, 1974). However, in all these secretory systems close contact between granules and micro-

tubules has only been infrequently observed, although microtubules assembled *in vitro* bound isolated porcine pituitary granules containing either growth hormone or prolactin in the presence of 4 M glycerol (Sherline et al., 1977). Antimitotic drugs such as colchicine, vincristine and vinblastine disrupt the microtubular system (and other components) of cells (Stephens and Edds, 1976), and have also been shown to interfere with the process of secretion (Forbes and Dent, 1974; Reaven and Reaven, 1975).

Microtubules are present in steroidogenic cells (Enders, 1973), and colchicine treatment was found to inhibit the secretion of progesterone by the ovine corpus luteum, producing specific derangements of the fine structure of the luteal cells (Gemmell et al., 1977c). The suppression of progesterone secretion could not be attributed to decreased synthesis since the hormonal content of the luteal tissue rose after colchicine treatment (Gemmell et al., 1977c). However, Stephens and Edds (1976) have cautioned against the interpretation that the microtubular system is directly involved in the movement of all secretory granules, since some drugs previously thought to interact only with tubulin are now known to also disrupt other cellular structures and functions which are part of normal secretory activity. Furthermore, antimitotic drugs do not depress neurohypophyseal secretion significantly (Russell and Thorn, 1973; Dreifuss, 1977), and some secretory cells are stimulated by colchicine or vinblastine (Edwards and Howell, 1973; Ray and Strott, 1978).

1.6 OUTLINE OF THESIS

The objective of the thesis was to investigate the

mechanism of progesterone secretion from the bovine corpus luteum. In Chapter 2, the morphology of mid-luteal cells is described, with particular reference to the presence of electron-dense granules in the cytoplasm. Cytochemical techniques are used to distinguish lysosomes and microperoxisomes from all other electron-dense granules. The variation in the relative proportions of the subcellular organelles of luteal cells throughout the oestrus cycle are quantitatively assessed. Chapter 3 describes the investigation of the subcellular location of progesterone in the bovine corpus luteum using biochemical fractionation techniques. Specific proteins are postulated to participate in the biosynthesis and secretion of steroids (see above). The properties of soluble progesterone binding proteins in the corpus luteum are described in Chapter 4, and Chapter 5 attempts to interprete the results of the previous chapters and to assimilate them into current ideas of the mechanism of steroid secretion.

CHAPTER 2

ULTRASTRUCTURE AND CYTOCHEMISTRY OF THE BOVINE CORPUS LUTEUM

2.1 INTRODUCTION

2.1.1 Relationship Between Structure and Function

2.1.2 Steroidogenic Cells

2.1.3 The Corpus Luteum

2.1.3.1 Ultrastructure of the Luteal Cells of the Functional Bovine Corpus Luteum

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2.2 METHODS

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2.3.1 Morphology of the Large Luteal Cell

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2.3.2.1 Microperoxisome Cytochemistry

2.3.2.2 Lysosome Cytochemistry

2.3.3 Morphometric Analysis of the Bovine Corpus Luteum Throughout the Oestrus Cycle

2.3.3.1 The Corpus Luteum

2.3.3.2 Cytoplasm of the Large Luteal Cell

2.4 DISCUSSION

2.4.1 Morphological and Cytochemical Considerations

2.4.2 Ultrastructural Changes Throughout the Bovine Oestrus Cycle: Correlation of Luteal Structure and Function

2.1 INTRODUCTION

2.1.1 Relationship Between Structure and Function

The study of the structure and function of steroid-secreting cells has largely consisted either of the histochemical and morphological description of the various steroidogenic cell types or of the characterization of the nature and range of steroids produced by these different cell types. With the near completion of this early phase it has recently become pertinent to direct questions toward the interrelation between structure and function at the subcellular level. Such a progression in experimental approach is necessary in order to define more fully the subcellular sites and mechanism of steroid biosynthesis, and to elucidate both the mode of secretion of steroid hormones and their mechanisms of action upon target cells.

For example, biochemical and ultrastructural techniques have now been combined in the study of the mechanism of progesterone secretion from the corpus luteum (Gemmell et al., 1974) and in the study of the mode of steroid release induced by ACTH in the perfused cat adrenal (Gemmell et al., 1977a). Also, cytochemical identification of lysosomes at the subcellular level has been employed in investigation of the role of lysosomes in corpus luteum regression (McClellan 1977) and ovarian steroidogenesis (Elfont et al., 1977). O'Malley's group have used a wide range of biochemical techniques to elucidate much of the mechanism of progesterone and oestrogen receptor action at the nuclear level in target cells of the chick oviduct (Schwartz et al., 1976; O'Malley et al., 1976).

A general discussion of the ultrastructural features

common to all steroidogenic cells will be followed by consideration of those ultrastructural features specific to the corpora lutea of a number of different species, including the cow. This will be related to the overall aim of the work reported in this chapter, which was to correlate the presence of secretory granules in the large luteinized granulosa cells of the bovine corpus luteum with their principal function, namely secretion of progesterone.

2.1.2 Steroidogenic Cells

A number of cell types are specialized for steroid hormone secretion in higher mammals. The adrenal cortex is divided into two distinct zones: the zona glomerulosa, which secretes aldosterone, and the inner zones, which mainly secrete cortisol or corticosterone. Androgens are principally derived from the Leydig or interstitial cells of the testis. The ovary contains a range of steroid-producing cells whose composition varies throughout the course of the oestrus cycle. Thus, the theca interna and in some cases the interstitial cells of the follicle secrete oestrogens, whereas the luteinized granulosa cells of the corpus luteum secrete progesterone. In pregnancy, the placenta can produce progesterone, oestrogens and other steroids (Christensen and Gillim, 1969; Turner and Bagnara, 1971).

Steroid-producing cells are usually polyhedral in shape and vary from 10 to 20 μ in diameter, although luteal cells can reach a diameter of 40 μ in some species (Enders, 1973; Christensen and Gillim, 1969; Fawcett et al., 1969). Steroidogenic cells are readily distinguisable under the electron microscope from the endocrine cells which secrete protein and

polypeptide hormones. They characteristically contain a very extensive network of agranular endoplasmic reticulum (aer), often arranged in large, concentric whorls, and sparsely distributed granular endoplasmic reticulum (ger). Mitochrondria invariably have tubular cristae rather than the lamelliform cristae common to other cell types. Golgi complexes are prominent, and lipid droplets may be abundant (Christensen and Gillim 1969; Enders, 1973).

2.1.3 The Corpus Luteum

The corpus luteum develops primarily from the granulosa cells of the post-ovulatory follicles, although a varying component is derived from the theca interna. It remains functional for specific periods of time during both the oestrus cycle and pregnancy, depending on the species. During pregnancy, its progesterone secretory function may be supplemented (rat, mouse, guinea pig, sheep, dog and cat) or supplanted (human, monkey and goat) by the placenta (Short, 1959; Heap et al., 1973; Baird, 1977). In all cases the corpus luteum eventually degenerates (or regresses) into a mass of hyaline connective tissue, the corpus albicans, which does not secrete steroids. The transition of granulosa cell to luteinized granulosa (or luteal) cell in the formation of a functional corpus luteum is characterized at the ultrastructural level by the replacement of ger with aer and characterized at the physiological level by the onset of progesterone secretion (Blanchette, 1966, Christensen and Gillim, 1969).

Numerous ultrastructural studies have been conducted on the cells of the follicle and corpus luteum, throughout the oestrus and menstrual cycles and during pregnancy. These

studies have shown that the corpora lutea of the pig (Bjersing, 1967a,b), rat (Long, 1973), mouse (Yamada and Ishikawa, 1960), sheep (Deane et al., 1966), monkey (Kirton and Koering, 1973) and human (Crisp et al., 1970; Crisp et al., 1973; Green and Maqueo,1965; Gillim et al., 1969; Adams and Hertig, 1969a,b) all typically possess those ultrastructural features considered to be characteristic of steroid-producing cells: abundant tubular aer, lipid droplets, dispersed Golgi elements, and mitochondria with tubular cristae.

The corpus luteum is composed of two major cell types, the granulosa lutein and the theca lutein cells. In the follicle, the granulosa cells do not produce steroids but instead nourish the developing ovum. It is only after luteinization and incorporation into the corpus luteum that they produce progesterone. On the other hand, the follicular theca interna cells and, in some cases, the interstitial cells, synthesize most of the oestrogens in the pre-ovulatory ovary (Ryan and Smith, 1965; Baird, 1977). After luteinization, the theca interna cells of the corpus luteum continue to secrete oestrogens in a number of species (Huang and Pearlman, 1963; Baillie et al., 1966; Rodway et al., 1976) possibly including the cow (Priedkalns and Weber, 1968a).

The corpora lutea of species such as the human (Crisp et al., 1970; Gillim et al., 1969), pig (Bjersing, 1967b) and cow (Priedkalns and Weber, 1968a) consist mainly of large luteal cells interspersed with a number of small luteal cells. The former are the luteinized granulosa cells which secrete progesterone (and related progestogens depending on the particular species),whereas the latter are the luteinized

theca interna cells, which secrete oestrogens (see above). This difference in cell size and function is overlooked in many ultrastructural studies, which have usually confined their observations to the large luteal cells (for example, Yamado and Ishikawa, 1960; Green and Maqueo, 1965).

Christensen and Gillim (1969) discuss the often-reported appearance of light and dark cells in electron micrographs of corpus luteum, and conclude with Enders (1962) that this variation in electron-density between cells has no physiological basis but, rather, is an artifact of preservation. They also point out that the vesiculated appearance of aer in steroidogenic cells is an artifact of immersion fixation with glutaraldehyde which is avoided by perfusion fixation, and that in the living cell aer probably consists of an interconnected system of tubules rather than as closely-packed vesicles. This view was received support from a recent scanning electron microscope study of the rat corpus luteum (Van Blerkom and Motta, 1978).

2.1.3.1 Ultrastructure of the Luteal Cells of the Functional Bovine Corpus Luteum

Priedkalns and Weber (1968a) observed "large" and "small" luteal cells in the late metoestrus-dioestrus corpus luteum. At the ultrastructural level, the large cells were characterized by the presence of numerous, large mitochondria whereas the small luteal cells were characterized by numerous lipid bodies. This led the authors to suggest that the latter acted as sites of lipid storage whereas the large luteal cells synthesized and secreted progesterone.

Their description of the large luteal cell indicated that

its structural features were typical of those of steroid-producing cells generally. The mitochondria contained tubular cristae, agranular endoplasmic reticulum predominated, Golgi elements were abundant and lipid droplets were present. "Lysosomes, membraneous bodies, multivesicular bodies, and dense bodies were also observed in some midcycle luteal cells" (Priedkalns and Weber, 1968a). Many of the dense bodies or transitional bodies seen by these authors were undoubtedly analogous to the secretory granules described in the luteal cells of the sheep corpus luteum (Gemmell et al., 1974; Gemmell et al., 1976).

The small luteal cells (15-20 μ diameter) varied in electron density and their mitochondria (also with tubular cristae) were less abundant than in the large luteal cells. The endoplasmic reticulum was mostly agranular, although granular membranes were also present, and the concentration of both endoplasmic reticulum and Golgi apparatus did not differ from that of the large cells. Thus, steroidogenesis by the small luteal cells was not necessarily precluded, as suggested by Priedkalns and Weber (1968a), especially as the highest concentrations of lipid bodies were observed in early metoestrus (immediately after ovulation) and during corpus luteum regression. At this time progesterone secretion is minimal so that lipid precursors would be expected to accumulate (Deane, 1962).

2.1.3.2 Correlation of Luteal Cell Structure and Function

Correlation of cell ultrastructure with steroid biosynthesis in the corpus luteum has been attempted in a number of species (Cavazos, 1972), including the pig (Bjersing, 1967b; Cavazos et al., 1969; Belt et al., 1970), the rat (Long,

1973) and the sheep (Gemmell et al., 1974, 1976).

In the latter studies, Thorburn and coworkers showed that changes in the levels of secretory granules during the course of the oestrus cycle of the sheep correlated with the known pattern of progesterone secretion throughout the oestrus cycle of this animal (Thorburn and Mattner, 1971). Thus, small densely-staining granules (0.2 μ diameter) were observed in the large luteal cells by the second day of the oestrus cycle. The granules increased in number as the cycle progressed so that at day 6, when secretion of progesterone was high, some were observed to have undergone expulsion from the cell, probably by exocytosis. At days 10-11 of the cycle, when progesterone secretion was maximal, secretory granules appeared to be most numerous and their exocytosis was most frequently observed (Gemmell et al., 1974). With the decline in hormone secretion and the start of luteal cell regression at day 15, few granules were present and their exocytosis had ceased. In morphological studies of regressing corpora lutea of the ewe, it was observed that the first signs of luteolysis at day 12 of the oestrus cycle coincided with a decrease in the secretion of these electron-dense granules and with the emergence within the large luteal cells of organelles (autophagocytic bodies and lysosomes) associated with intracellular degradation (Corteel 1975; Gemmell et al., 1976; Stacy et al., 1976).

Densely-staining granules (0.2-0.4 μ in diameter) have frequently been observed in micrographs of steroid-secreting cells published by other workers. Their presence has either been overlooked, or they have been attributed to various

classes of organelles such that their relation to the secretory process has not been elucidated. They are observed in the interstitial cells of the mouse testis (Yamada and Ishikawa, 1960; Enders, 1962; Christensen and Gillim, 1969) and human testis (Fawcett and Burgos, 1960), and in the luteal cells of the following species:

(i) rat (Enders, 1962; Long, 1973)

(ii) mink, hamster, and four-eyed opossum (Enders, 1973)

(iii) pig (Bjersing, 1967b; Cavazos et al., 1969)

(iv) sheep (Gemmell et al., 1974; Corteel, 1975; Gemmell et al., 1976; McClellan et al., 1977)

(v) dog (Crisp et al., 1972; Abel et al., 1975a,b)

(vi) cow (Priedkalns and Weber, 1968a)

(vii) deer (Sinha et al., 1971)

(viii) goat (Gemmell et al., 1977b)

(ix) monkey (Kirton and Koering, 1973; Gulyas, 1974; Gulyas and Yuan, 1975)

(x) human (Green and Maqueo, 1965; Adams and Hertiga, 1969; Christensen and Gillim, 1969; Crisp et al., 1973; Enders, 1973)

In fact, Christensen and Gillim (1969) pointed out that steroid-secreting cells contain various cytoplasmic granules which may vary in size and appearance depending on the organ, species and method of fixation. They state that it is unclear whether these granules, variously described as "dense bodies", "lysosomes", "small bodies", "cytosomes", "microbodies", "lipofuscin or lipochrome pigment granules", etc. represent several different structures or are diverse forms of a single organelle.

Deane et al. (1966) reported the presence of "numerous small dense bodies, each invested by a single membrane" in the ovine corpus luteum and Bjersing et al. (1970) called them "cytosomes", and suggested that they were primordial autophagic vacuoles and autolysosomes, even though they noted that their numbers decreased, rather than increased, in degenerating luteal cells. Christensen and Gillim (1969) argued against electron-dense granules in the luteal cells of a number of species being lysosomes on the basis they did not have acid phosphatase activity (Frank and Christensen, 1968; Gemmell et al., 1976), that they occurred in opossum testicular interstitial cells, where pigment granules and other residual bodies are rare (Christensen and Fawcett, 1961), and that they were very numerous in frog testicular interstitial cells at the time of the year when these cells were most steroidogenically active (Brokelman, 1964; in Christensen and Gillim, 1969). The observations made on the sheep corpus luteum by Gemmell et al., 1974,1976) substantiate the latter point (see above).

Small granular structures called microperoxisomes are reported to occur in all animal tissues and are thought to be ontogenetically related to the larger peroxisomes of mammalian liver and kidney and to the glyoxysomes of plants (Holmes and Masters, 1978). They range from 0.15-0.25 μ in diameter and contain catalase and variable oxidase activities (Novikoff and Novikoff, 1973; Herzog and Fahimi, 1974; Goldenberg et al., 1975a,b; Gulyas and Yuan, 1975). They possess numerous slender connections to the aer, and may also have specific, spatial associations with other cellular inclusions such as lipid droplets and zymogen granules (Novikoff, 1976). Gulyas and

Yuan (1977) have confirmed the intimate association of micro-peroxisomes with aer in the granulosa lutein cells of the rhesus monkey. They are probably derived from the endoplasmic reticulum (Novikoff and Novikoff, 1973; Gulyas and Yuan, 1977).

Peroxisomes in liver have been implicated in lipid metabolism (Goldfischer, Roheim and Edelstain, 1971), which has led Gulyas and Yuan (1975) to hypothesize that the enzymes in the microperoxisomes of luteal cells may have a role in the intracellular transport and/or utilization of cholesterol or other intermediates in steroidogenesis.

Cytochemically, microperoxisomes can be identified in aldehyde-fixed tissue incubated in alkaline 3,3'-diamino-benzidine (DAB, Novikoff and Goldfischer, 1969). In this medium they are identified (stained) because, in the presence of H_2O_2, catalase enzyme acts peroxidatically to oxidize DAB. This rapid polymerization of DAB results in amorphous osmiophilic polymers which are insoluble in water, lipid or plastic. The osmiophilic product is highly electron-opague and enables the visualization of microperoxisomes under the transmission electron microscope in non-counterstained sections.

Cavazos et al. (1969) stated that the electron-dense granules of the pig corpus luteum were lysosomes, whilst Long (1973) observed two classes of granules in the corpus luteum of the rat, one of which stained for acid phosphatase and another, non-lysosomal type of granule, which he hypothesized contained relaxin. Belt et al. (1970) refer to the presence of two types of granules in the pig corpus luteum during pregnancy: "small granules, commonly clustered and larger more hetero-geneous granules". However, as Gemmell et al. (1974) point out,

this distinction does not seem to have been maintained in subsequent publications in which it was suggested that the granules (type unspecified) may store relaxin (Belt et al., 1971). Recently, a granular fraction has been prepared from the corpora lutea of pregnant rats which showed enrichment of relaxin activity, as determined by bioassay (Anderson and Long, 1978). Electron micrographs of this fraction revealed the presence of mitochondria and a heterogeneous population of granules, which were similar in appearance to the relaxin granules and lysosomes of the intact luteal cell (Long, 1973).

Both the porcine and human corpora lutea are rich sources of relaxin during pregnancy (Steinetz et al., 1960; Weiss et al., 1977) and Weiss and coworkers report that relaxin secretion correlates well with luteal progesterone secretion at mid-pregnancy and term in the human. The localization of relaxin in granules in the corpora lutea of one pregnant sow has recently been reported using the immunoperoxidase technique (Kendall et al., 1978). Unfortunately the antigen used in this (and the above) studies to obtain the antiserum to relaxin was "microheterogeneous" so that the presence and location of relaxin in these tissues remains uncertain (Sherwood and O'Byrne, 1974; Larkin et al., 1977). It is reported that there are various high molecular weight precursors to relaxin (Kwok et al., 1978). In the corpora lutea of non-pregnant women, however, O'Byrne et al. (1978) found that relaxin was not detectable and Chamley et al. (1975) reported that during the oestrus cycle of sheep peak circulating concentrations of progesterone and relaxin did not coincide. Therefore, the possible intragranular sequestration of relaxin may be a

phenomenon restricted to pregnancy. It is significant that Cavazos et al. (1969) and Belt et al. (1969, 1971) did not obtain evidence for the secretion of granules from the luteal cells of non-pregnant sows. Thus, it seems that the corpora lutea of pregnancy in women, sows, ewes, cows and other species contain two populations of membrane-bound granules, one of which stores relaxin and is not secreted, and another which contains progesterone and is secreted. In this context, it would be of interest to look for the presence both of granules and of relaxin in the placentae of these species.

Priedkalns and Weber (1968b) carried out a quantitative ultrastructural anaylsis of the cells of the bovine ovary with a view to defining the roles of the different cell-types in ovarian synthetic processes. They used a linear scanning method (Carpenter and Lazarow, 1962) and applied Delesse's (1847) and Rosiwal's (1898) theories to determine the relative volumes of various cytoplasmic components from their linear measurements. They concluded from morphological criteria that during late metoestrus and early dioestrus (days 9-18 of the bovine oestrus cycle), the large luteal cell synthesized progesterone and that its cytoplasm contained maximal percentage volumes of mitochondria and aer, and minimal percentage volumes of lipid bodies and lysosomes. Conversely, during pro-oestrus and oestrus (days 18-21), the theca interna cell synthesized oestrogens and its cytoplasm contained maximal percentage volumes of mitochondria and aer, and minimal percentage volumes of lipid bodies and lysosomes. These workers did not specifically measure changes in the cytoplasmic content of electron-dense granules in luteal cells. In fact, they called

them small lipid bodies and grouped them with microbodies and large lipid droplets. However, it was significant that "the smaller, round lipid bodies were encountered in the 'active' stages of presumed steroid synthesis, while the larger, irregular lipid bodies were present most commonly during cell regression" (Priedkalns and Weber, 1968b).

In a study of the changes in ovarian blood flow in the sheep during the oestrus cycle, Niswender et al. (1976) found that the capillaries of the corpus luteum were most abundant during the mid-luteal phase. They suggested that this reflected the need of the luteal cells at this time for an increased supply of nutrients and support by gonodotrophic hormones However, the removal of progesterone from its site of synthesis would also be facilitated by the increase in blood supply through the corpus luteum.

2.1.4 Experimental Approaches

The objectives of the work reported in this chapter were to confirm the presence of electron-dense granules in the large, progesterone-producing luteal cells of the bovine corpus luteum, analogous to those described in the ovine corpus luteum (Gemmell et al., 1974). Thus, the ultrastructural features of the large cell of the mid-luteal corpus luteum of the cow were investigated, with emphasis being placed on the presence and nature of membrane-bound, electron-dense granules. In particular, evidence was sought for their secretion from the cell, possibly by exocytosis.

Subsequently, cytochemical techniques were used to identify microperoxisomes and lysosomes in tissue sections of bovine corpus luteum, so that these organelles might be

distinguished from the putative secretory organelles. These studies were intended to provide a basis for the subsequent biochemical fractionation experiments of the mid-luteal corpus luteum described in Chapter 3.

Morphometric analysis of the bovine corpus luteum at different stages of the oestrus cycle was also undertaken in order to correlate the varying proportions of subcellular organelles with the functional state of the cell. The variation in the proportion of large luteal cells comprising the corpus luteum at different stages are determined by analysis of low-magnification micrographs. This was followed by analysis of high-magnification micrographs of the cytoplasm of the large luteal cell in order to determine variations in the numbers of electron-dense granules throughout the oestrus cycle. It was hoped that the morphometric studies would allow the correlation of luteal cell structure with function so that any change in the numbers of secretory granules could be related to known endocrine events.

2.2 METHODS

2.2.1 Morphology

Collection of Tissues: Bovine corpora lutea were obtained within 5 minutes of death from Friesen animals on known days of the oestrus cycle from the Agricultural Research Council Laboratories at Compton, Berkshire.

Fixation and Embedding: Representative tissue pieces of maximum dimension 1 mm^3 were treated as follows:

1) fixed at 0-4^{o}C for 2 h in 3% v/v glutaraldehyde, buffered by 0.1 M sodium cacodylate, pH 7.4.
2) washed two to three times in 0.1 M sodium cacodylate, pH 7.4 and left in buffer overnight at 4^{o}C.
3) post-fixed in cacodylate-buffered 1% v/v osmium tetroxide for 30 min at room temperature (22^{o}C).
4) dehydrated through an alcohol series (70% v/v ethanol and 90% v/v ethanol for 5 min, and ethanol for 2 h).
5) immersed in propylene oxide for 1 h, followed by a 1:1 v/v mixture of propylene oxide: epoxy resin (Araldite) overnight.
6) embedded in Araldite in plastic capsules (Beem capsules) and the Araldite polymerized at 60^{o}C for a minimum of 36 h.

Sectioning: The Araldite-embedded tissue blocks were trimmed by hand and approximately 100 nm sections cut using either an LKB Ultratome III or a Cambridge-Huxley microtome. The sections were flattened by exposure to chloroform vapour, collected on uncoated copper grids and then post-stained with saturated methanolic uranyl acetate and Reynold's lead citrate (Reynold, 1963).

The specimens were viewed and photographed using a Philips

301 electron microscope, operating at 80 kV.

2.2.2 Morphometric Analysis

Low power micrographs (final magnification 4,130 X) were taken for the analysis of the proportion of luteal cell types in corpora lutea from different stages of the oestrus cycle. The micrographs were obtained using a systematic random sampling procedure whereby the two bottom corners of every grid square were photographed.

For the analysis of the distribution of subcellular organelles, high power micrographs (final magnification 14,030 X or 17,500 X) of large luteal cell cytoplasm were obtained randomly as above.

The area fractions of selected cells, or subcellular organelles, were estimated using random point-counting analysis according to Glagoleffaa (1933). The method is also described in Williams (1977), and involved laying over the micrographs a transparant plastic sheet upon which was drawn a grid of lines some 16.5 mm apart. The cell types, or subcellular organelles, lying beneath the points of intersection of the lines were identified and tabulated.

The measured area fractions (or percentage areas) have been shown to be directly proportional to the actual volume fractions (or percentage volumes) by various people, including Delesse (1847) and Sorby (1856).

2.2.3 Microperoxisome Cytochemistry

Fresh bovine corpora lutea from the same source (see 2.2.1) were sliced with a razor blade into approximately 20 μ sections, and fixed for 3 h in 3% v/v glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 containing 7% v/v sucrose.

For the determination of peroxidase activity, the tissue sections were incubated in an alkaline DAB medium for 2 h at 37°C according to Novikoff and Goldfischer (1968, 1969) and Novikoff et al. (1973).

Three media were freshly prepared as follows:

Complete medium: 80 mg diaminobenzidine-tetrahydrochloride (DAB)
39.2 ml 0.2 M 2-amino-2-methyl-1,3-propandiol (AMPD) buffer, pH 10
0.8 ml 1% v/v hydrogen peroxide

Incomplete medium: as above but lacking hydrogen peroxide

Control medium (containing a peroxidase inhibitor): complete medium plus 69.6 mg (12 mM) dichlorophenol indophenol (DCPIP).

After incubation the tissue sections were post-fixed dehydrated, embedded, and sectioned as above for Morphology, except that counter-staining with uranyl acetate and lead citrate was omitted.

2.2.4 Acid Phosphatase Cytochemistry

Approximately 20 µ sections (usually prepared together with the microperoxisome sections) were fixed for 30 min in 1% v/v glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 7% w/v sucrose.

Acid phosphatase activity was detected by the enzymatic deposition of lead phosphate, which was osmiophilic. The Barka and Anderson (1962) medium was used, as described in Essner (1973).

Three media were freshly prepared as follows:

Complete medium: 60 mg sodium β-glycerophosphate

4.0 ml double-distilled water

8.0 ml 0.2 M Tris-maleate buffer pH 5

8.0 ml 0.2% w/v lead nitrate

Incomplete medium: as above, but lacking primary substrate, glycerophosphate

Control medium (containing an acid phosphatase inhibitor): complete medium plus 8.4 mg (10 mM) sodium fluoride.

After incubation for 1 h at 37°C, the tissue sections were dehydrated, embedded and sectioned as described for Morphology.

2.3 RESULTS

2.3.1 Morphology of the Large Luteal Cell

A description of the large luteal cells of the bovine corpus luteum was undertaken. These cells secrete progesterone and were found to contain electron-dense, secretory granules. This served as a basis for subsequent investigation of the ultrastructural changes that these cells undergo throughout the course of the oestrus cycle.

The large granulosa lutein cells were the most prominent feature of the mid-luteal corpus luteum, which also contained some small luteal cells interspersed with a few fibroblasts and loose connective tissue. The tissue was highly vascularized so that the large luteal cells were always in close proximity to capillaries (Fig. 2-1).

The large luteal cells ranged between 25 and 40 μ in diameter. Their outer membranes were highly convoluted providing the cell with a large surface area. Junctional complexes between cells were not a significant feature of the bovine luteal cells, unlike those of the human (Adams and Hertig, 1969a) and mouse (Crisp and Browning, 1968).

The nuclei were similar in appearance to those of the luteal cells of other species. They were large, round and approximately 10 μ in diameter with dispersed chromatin, little heterochromatin, and prominent nucleoli. Perichromatin granules, about 600 Å in diameter, were sometines observed near the heterochromatin. The nuclear envelope was a double unit membrane with few pores opening to the surrounding cytoplasm (Fig. 2-2).

Mitochondria were abundant and dispersed throughout the

Fig. 2-1: Bovine corpus luteum from a cow at day 13 of the oestrus cycle

Three luteal cells (LL) are shown adjacent to a capilliary (Cp). The nuclei (N) of the large luteal cells contain prominent nucleoli (n); their cytoplasm contains numerous mitochondria (M) and abundant agranular endoplasmic reticulum (AER). Electron-dense granules (→) are scattered throughout their cytoplasm but are most abundant near the convoluted cell margins. X 4,875.

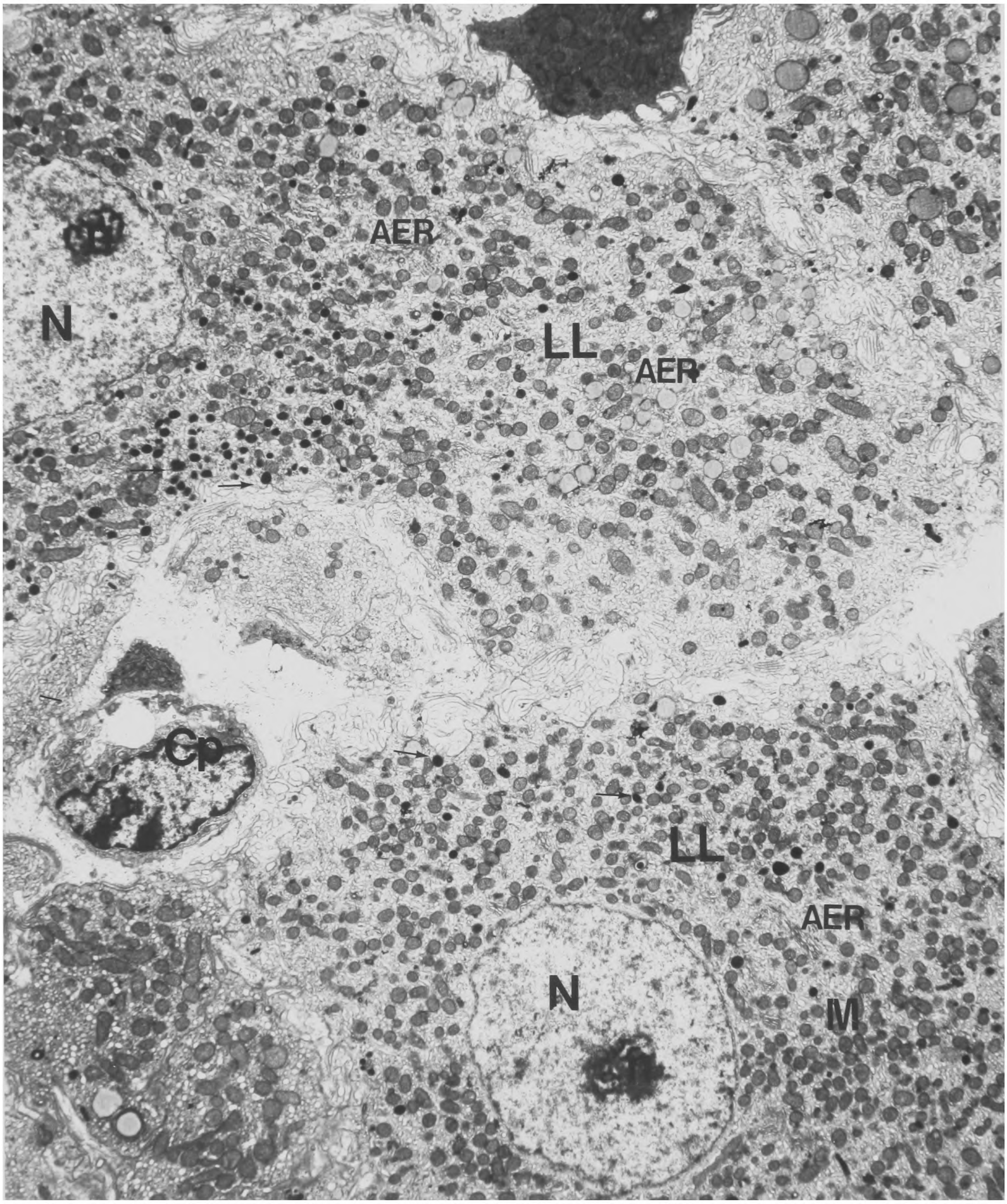
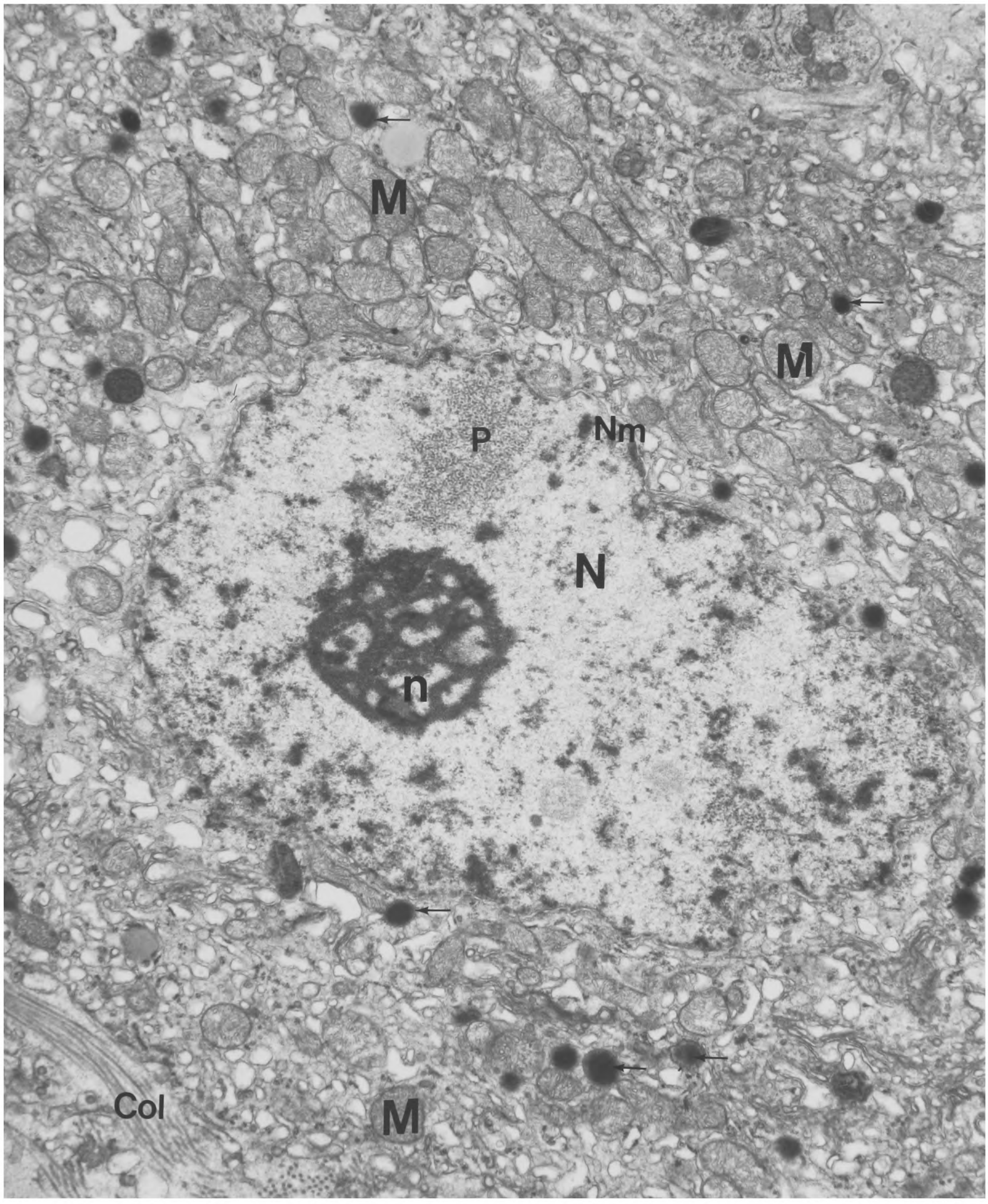

AER
N
LL
AER
Cp
LL
AER
N
M

Fig. 2-2: The nucleus and surrounding cytoplasm of a large luteal cell from a cow at day 13 of the oestrus cycle

The large nucleus (N) contains well-dispersed chromatin, perichromatin granules (P) and a prominent nucleolus (n). The nuclear membrane (Nm) has a double-unit structure. Mitochondria (M) and electron-dense granules (→) are scattered throughout the cytoplasm. Collagen fibres (Col) are seen in the extracellular space. X 13,000.


M
M
P
Nm
N
n
Col
M

cytoplasm. They were generally large and pleomorphic with respect to size and shape (Fig. 2-3). Their cristae were mostly tubular, which is considered to be a characteristic feature of all steroid-secreting cells (Christensen and Gillim, 1969), but varied to lamellar. Mitochondrial inclusions are commonly observed in luteal cells of other species, for example, human (Adams and Hertig, 1969), but were rarely observed in the mitochondria of bovine luteal cells.

Agranular endoplasmic reticulum was the most prominent feature of the cytoplasm. It was distributed extensively and was only occasionally arranged in whorls. In this study, fixation of tissue pieces by immersion in glutaraldehyde yielded micrographs where the aer appeared as a collection of swollen membrane vesicles (Figs. 2-3 and 2-4), whereas in the living cell it is thought to consist of a system of branching and interconnected tubules (Enders, 1962; Christensen and Gillim, 1969).

Granular endoplasmic reticulum occurred only in small amounts as discrete patches scattered throughout the cytoplasm, and was probably contiguous with aer (Figs. 2-3 and 2-4). Its membranes appeared as flattened sacs or cristernae. In addition, numerous free ribosomes and/or polysomes were scattered throughout the cytoplasm (Fig. 2-3).

Golgi complexes were extensive and numerous in the cytoplasm of the large luteal cells, and consisted of stacks of closely-packed cisternae and collections of vesicles (Fig. 2-4). Small, membrane-bound vesicles, which were often electron-dense, were commonly observed in the region of the Golgi complex suggesting that they had been "budded off" from this

Fig. 2-3: Portion of a large luteal cell from a cow at day 13 of the oestrus cycle

The large, pleomorphic mitochondria (M) contain tubular cristae. Membrane-bound lipid droplets (L) and granular endoplasmic reticulum (GER) are seen. Polysomes (Py) are scattered among the vesiculated membranes of the agranular endoplasmic reticulum (AER). An electron-dense granule is present (→). X 33,750.

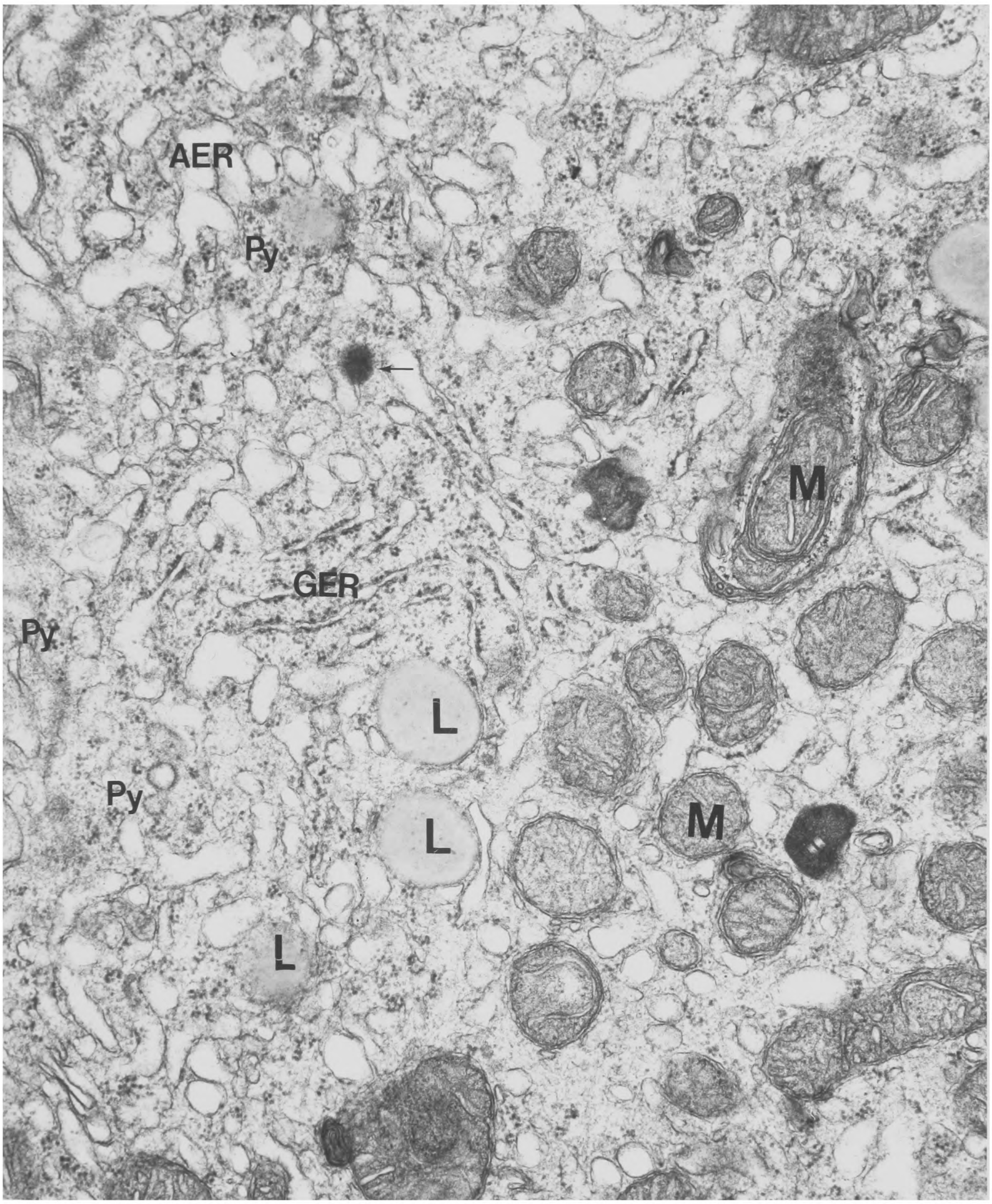
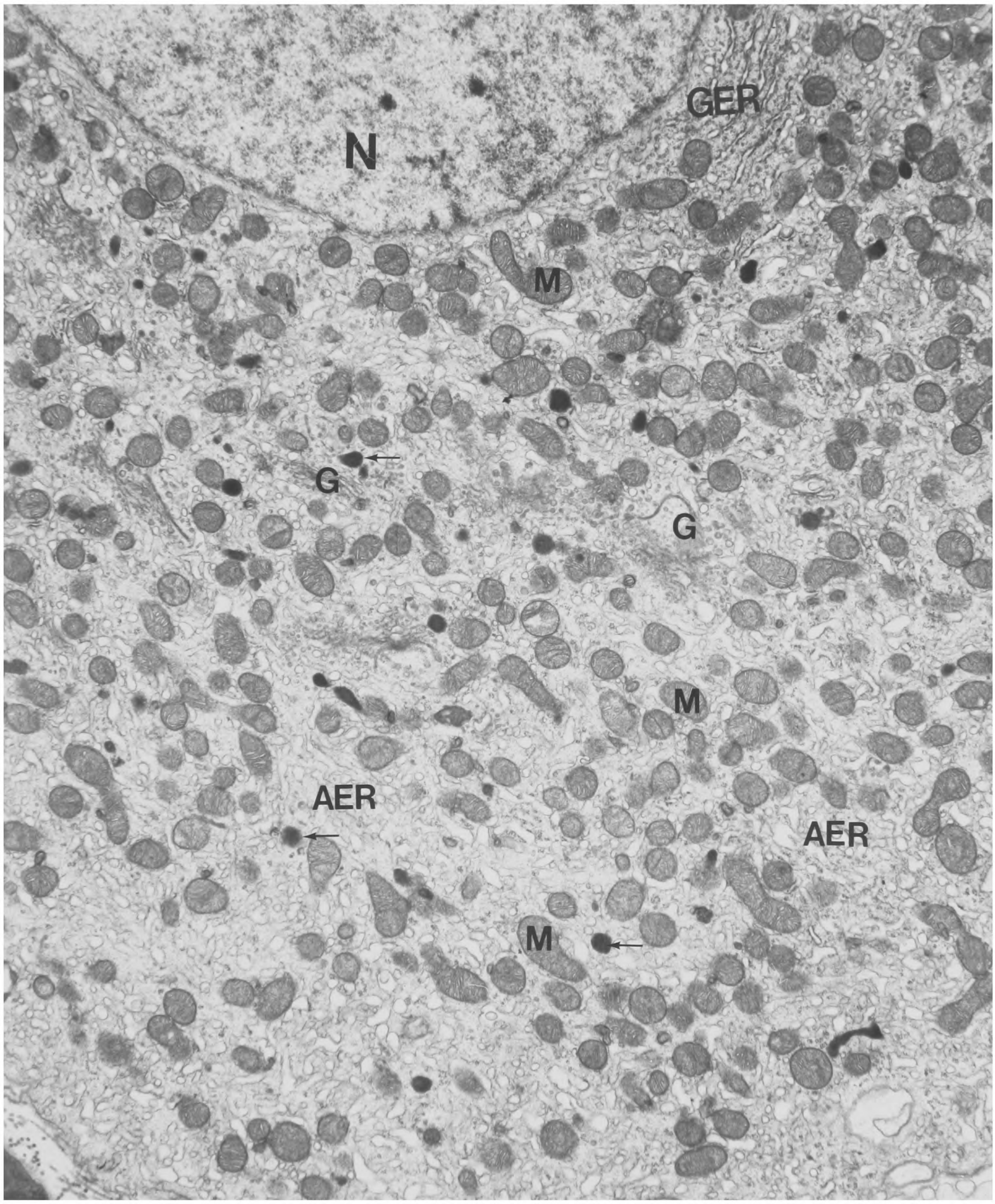

AER
Py
M
GER
Py
L
Py
L
M
L

Fig. 2-4: Portion of a large luteal cell from a cow at day 13 of the oestrus cycle

Mitochondria (M), containing tubular cristae, are dispersed throughout the cytoplasm. Golgi complexes (G) and granular endoplasmic reticulum (GER) occur discretely amongst the extensive (and vesiculated) agranular endoplasmic reticulum (AER) Electron-dense granules (→) occur widely throughout the cytoplasm, and are found close to Golgi membranes. N = nucleus. X 13,500.


N
GER
M
G
G
M
AER
AER
M

organelle (Fig. 2-4).

Lipid droplets were present in some of the cells; their contents were uniformly opaque and were surrounded by a single membrane (Fig. 2-3). In general their concentration varied inversely to the numbers of electron-dense granules present. In those cells with small numbers of electron-dense granules, lipid droplets of varying size were scattered throughout the cytoplasm and were usually found in those areas containing microperoxisomes but where electron-dense granules were either sparsely distributed or absent. Furthermore, in those cells containing significant numbers of lipid droplets, evidence for exocytosis of electron-dense granules was less common.

Microtubules (structural elements considered to be involved in steroid and protein secretion) and microfilaments were occasionally observed, but were not prominent features of the large bovine luteal cell, in contrast to the same cells of the human (Crisp et al., 1970) and dog (Crisp et al., 1972).[1]

Electron-dense secretory granules were very noticeable in the cytoplasm of the large luteal cells of the mid-cycle bovine corpus luteum. They ranged between 0.2 and 0.4 μ in diameter, compared to 0.2 μ in diameter in sheep (Gemmell et al., 1974). A single membrane enclosed the electron-dense contents, which was uniformly opague at high magnification (Fig. 2-5). The granules were usually spherical, although some pleomorphism was observed, such as a "dumb-bell" configuration (Fig. 2-6). They were distributed in clusters throughout the cytoplasm. especially in the vicinity of the Golgi complexes (Fig. 2-8),

---

1: After *in vitro* incubation of slices of bovine or ovine corpus luteum, the microtubules became much more prominent (Quirk, Parry and Willcox, unpublished observations).

Fig. 2-5: The apical region of a large luteal cell from a cow at day 13 of the oestrus cycle

The electron-dense granule in the cytoplasm near the margin of the cell (→) is bounded by a membrane, whilst another electron-dense intracellular structure appears to consist of the partial fusing of a secretory granule (→) with a crystalline body (CB). Two electron-dense granules (↕) have lost their membranes and are located in the extracellular space (Ex), where they appear to have partially disintegrated. X 60,000.

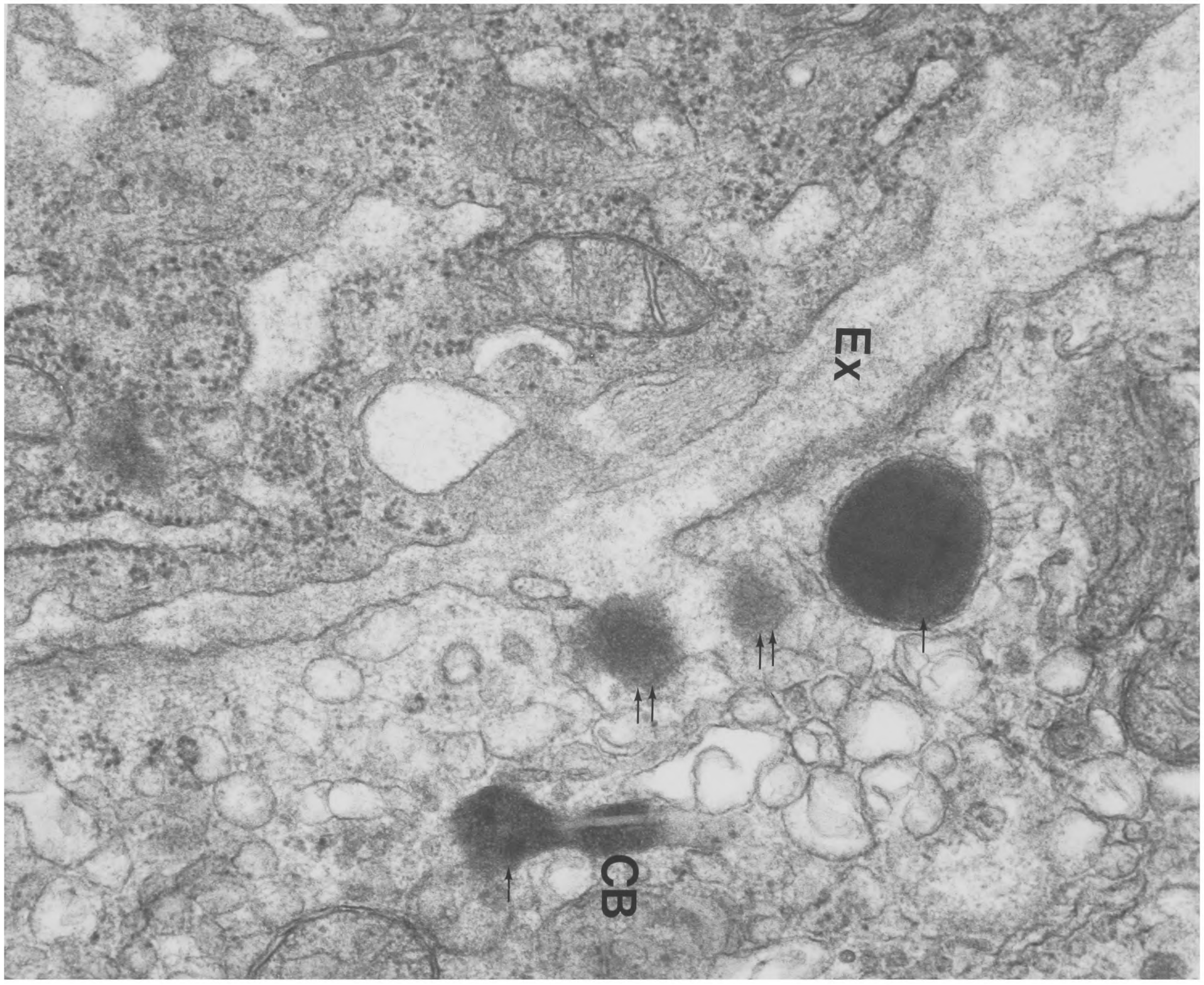
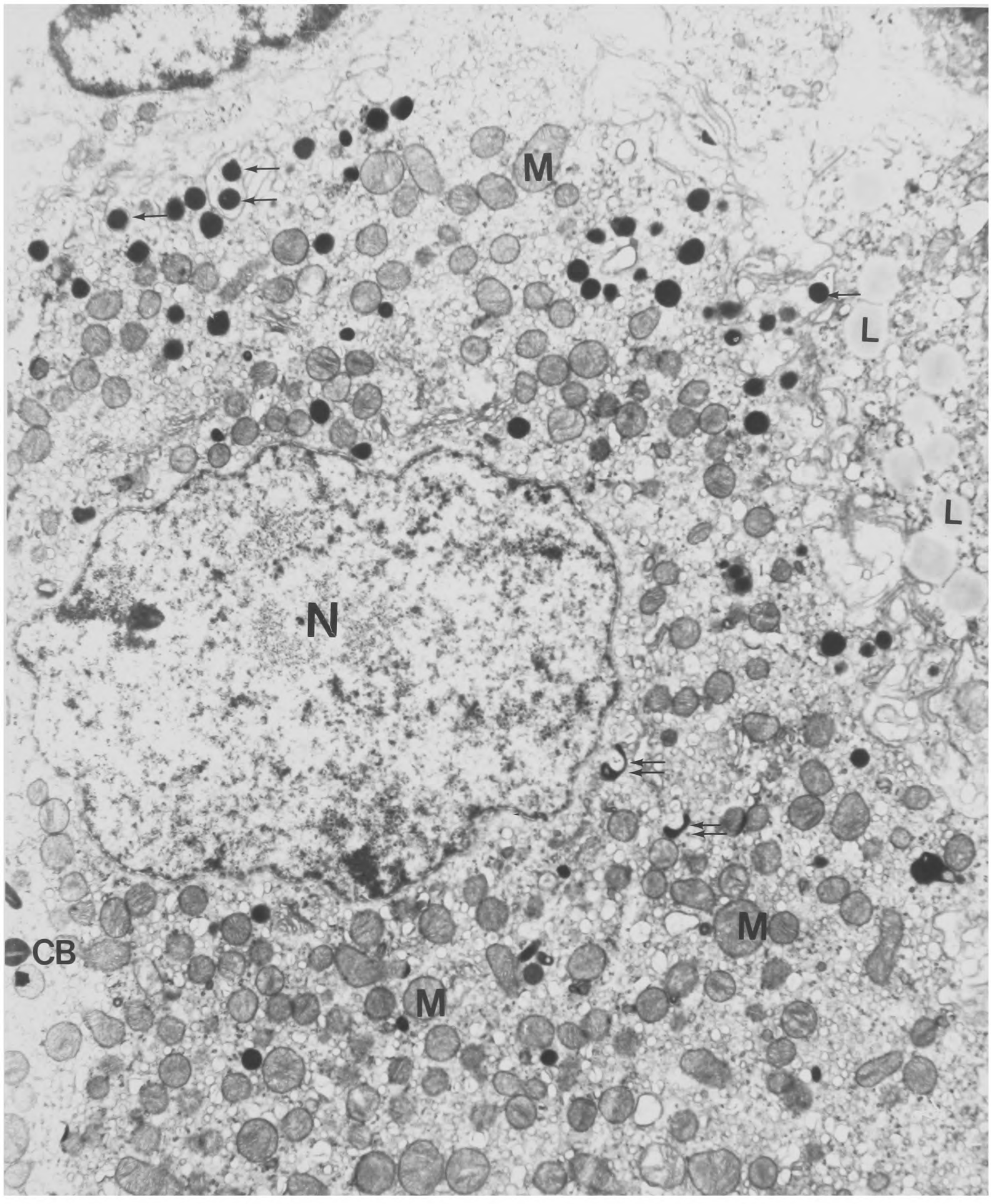

Ex
CB

Fig. 2-6: The distribution of electron-dense granules in the luteal cytoplasm of a cow at day 13 of the oestrus cycle

The electron-dense granules are distributed throughout the cytoplasm, but occur in greatest numbers near the edges of the cell. Some of the granules have undergone secretion from the cell and are located in the extracellular space (→). The granules are mostly spherical, but a "dumb-bell" variant is sometimes seen (↯). N = nucleus; M = mitochondrion; L = lipid droplet ; CB = crystalline body. X 9,380.


M
L
L
N
CB
M
M

and were also often found near the edge of the cell. These (and only these) electron-dense granules were observed in the extracellular space, just outside of the cell, which suggested that they had been secreted by exocytosis. Their extrusion from the cell was accompanied by fusion of their membranes with the cell membrane. Once outside of the cell, the contents of the granules appeared to disperse; this process was accompanied by a gradual diminishment in their electron-density (Figs. 2-5 and 2-7).

Microperoxisomes were indistinguishable from the secretory granules on morphological grounds, but were subsequently identified in large luteal cells using a specific cytochemical staining technique. Similarly, primary lysosomes were not readily identifiable by morphological examination alone, but occasionally secondary lysosomes were seen in the cytoplasm.

The only other granular organelles observed in the large luteal cells were "crystalline bodies", so called because they contained a central, elongated crystalloid structure which protruded from both sides (Fig. 2-8). The crystalline bodies did not undergo exocytosis.

2.3.2 Cytochemical Studies

2.3.2.1 Microperoxisome Cytochemistry

A typical day 15 section of large luteal cell cytoplasm stained cytochemically for peroxidase activity is shown in Fig. 2-9. Similar results were obtained for tissue sections from corpora lutea at days 6 and 17 of the bovine oestrus cycle.

Specific staining was localized over small granules called microperoxisomes distributed discretely throughout the cytoplasm. In general, the microperoxisomes occurred most

Fig. 2-7: Secretion of electron-dense granules from a luteal cell of a cow at day 13 of the oestrus cycle

Electron-dense granules (→) are located in folds of extracellular space (Ex) intercalating with the outer regions of the luteal cell. Secretion of the granules appears to be accompanied by loss of their surrounding membranes, followed by a diminishment in size and osmiophilicity of their contents. X 43,750.

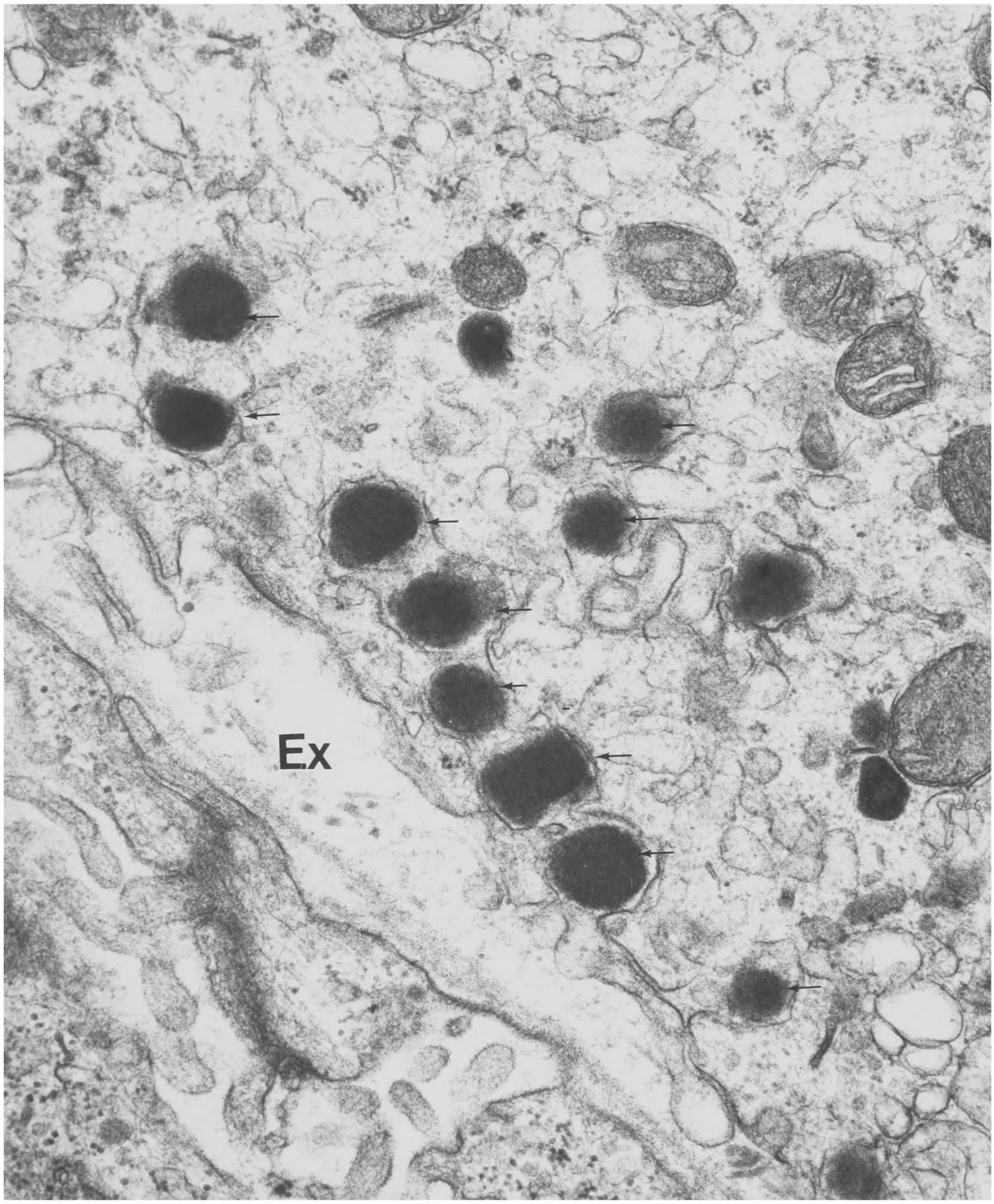

Ex

Fig. 2-8: Crystalline bodies in the cytoplasm of a large luteal cell from a cow at day 13 of the oestrus cycle

The crystalline bodies (CB) occur singly in small numbers throughout the cytoplasm. Two electron-dense granules (→) are located adjacent to crystalline bodies, and one electron-dense granule (‡) is found near a Golgi complex (G). M = mitochondrion; AER = agranular endoplasmic reticulum; Py = polysomes. X 43,750.

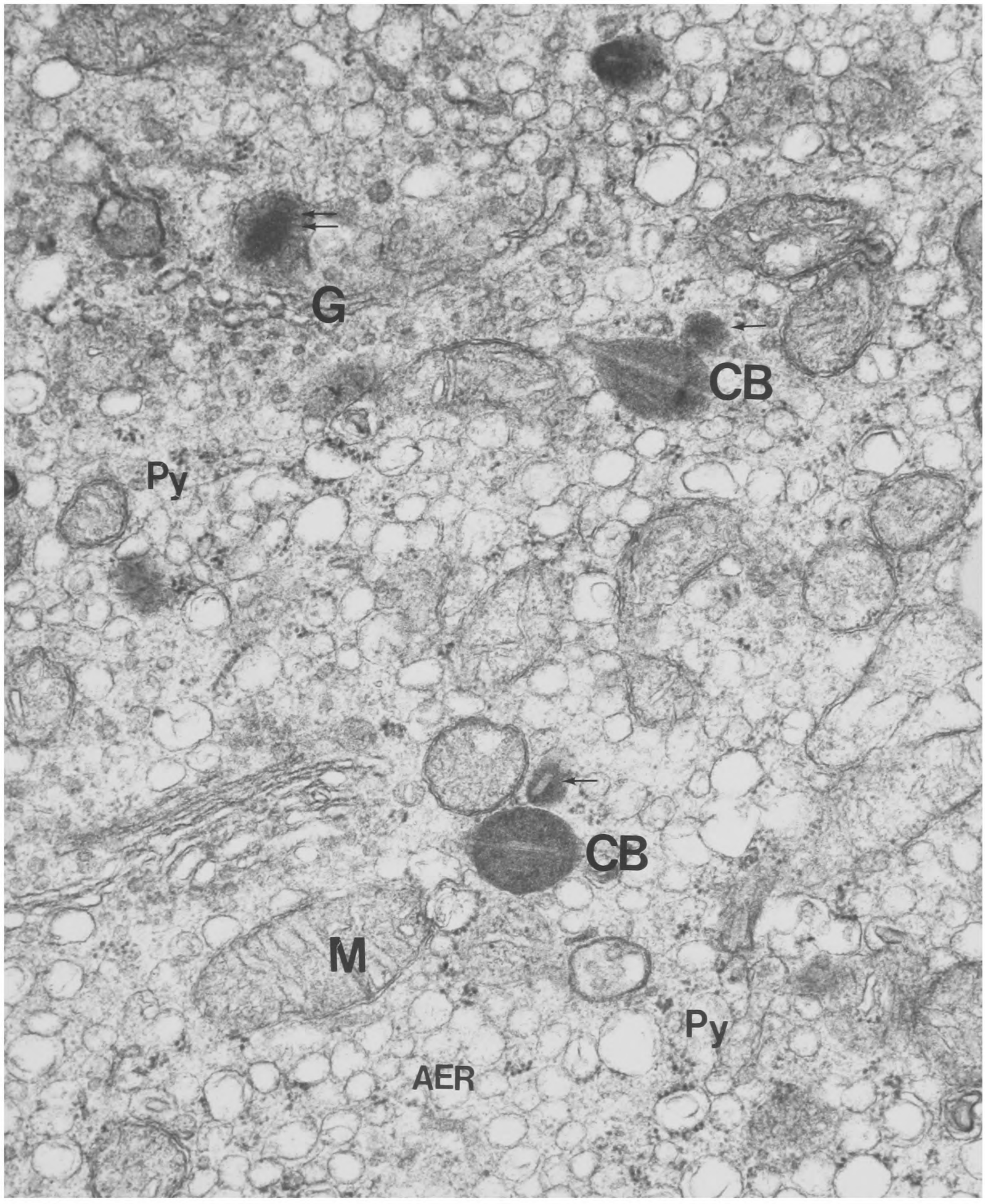
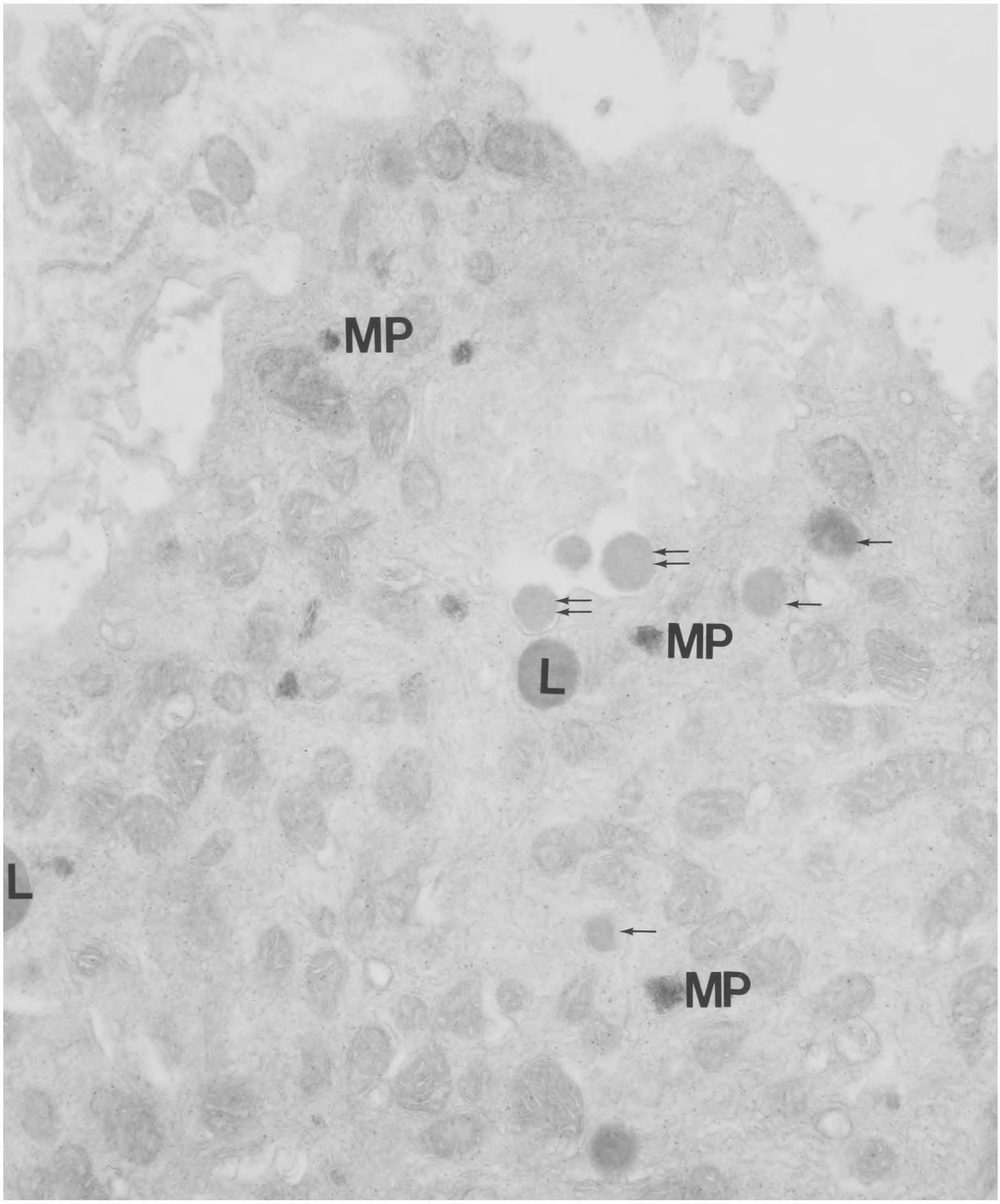

G
CB
Py
CB
M
Py
AER

Fig. 2-9: Portion of a luteal cell from a cow at day 13 of the oestrus cycle incubated with diaminobenzidine to demonstrate microperoxisomes

The section is not counterstained and the dense osmiophilic reaction product can be seen over microperoxisomes (MP). Non-reacting granules can also be seen (→) some of which have been secreted (↠). L = lipid droplet. X 17,250.


MP
L
MP
L
MP

abundantly in those areas of the cytoplasm which contained the greatest numbers of lipid droplets. The diameter of the microperoxisomes ranged from 0.15 to 0.25 µ. Larger osmophilic granules (0.2-0.4 µ in diameter) did not stain cytochemically for peroxidase activity and were morphologically identified as the putative secretory granules. In Fig. 2-9, three of the latter are located in the extracellular space, and are not stained, whereas microperoxisomes were never observed outside of the luteal cells. This suggests that microperoxisomes are not secreted from the luteal cell.

2.3.2.2 Lysosome Cytochemistry

The cytoplasm of large luteal cells from a day 17 bovine corpus luteum contained cytochemically detectable acid phosphatase activity (Fig. 2-10). The osmophilic reaction product was observed over secondary lysosomes and within some of the Golgi cisternae.

The putative secretory granules did not stain for acid phosphatase either in the luteal cell cytoplasm or in the extracellular spaces. These results were confirmed in tissue sections of corpora lutea obtained on days 6, 12, 15 and 20 of the bovine oestrus cycle.

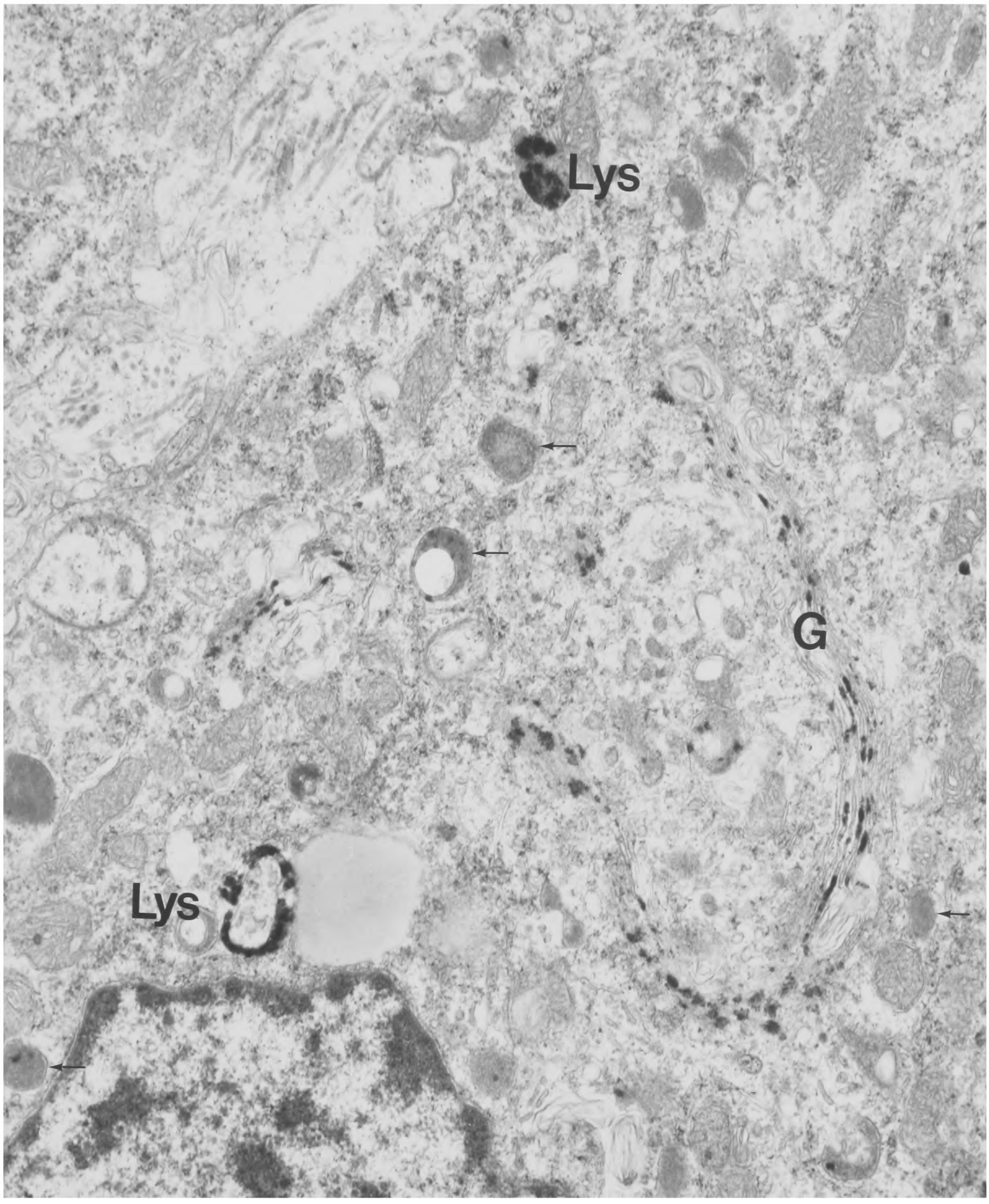
2.3.3 Morphometric Analysis of the Bovine Corpus Luteum Throughout the Oestrus Cycle

2.3.3.1 The Corpus Luteum

Low magnification micrographs were analyzed as described in the Methods section to obtain the proportions of luteal cells on days 6, 13, 15, 17 and 20 of the oestrus cycle (Table 2-1). One corpus luteum was examined for each stage of the cycle. The luteal cells comprised about 50% of the area fraction

Fig. 2-10: Portion of a large luteal cell from a cow at day 17 of the oestrus cycle incubated to demonstrate acid phosphatase activity

Reaction product can be seen in some of the Golgi cisternae (G) and in lysosomes (Lys). Other granules (→), can be seen which are not positively stained. X 26,000.


Lys
G
Lys

Category		Day of Oestrus Cycle				
		6	13	15	17	20
Luteal cells	No.	1045	2990	1663	1629	1578
	%	51.2	70.9	71.6	74.2	59.0
Blood vessels + Rbc	No.	249	308	173	156	174
	%	12.2	7.3	7.5	7.1	6.5
Extracellular space	No.	606	735	349	322	616
	%	29.7	17.4	15.0	14.7	23.1
Others	No.	142	183	139	89	305
	%	7.0	4.3	6.0	4.1	11.4
Numbers of observations		2042	4216	2324	2196	2673
Number of fields		17	36	19	20	24

Table 2-1: The proportion of luteal cells in the bovine corpus luteum throughout the oestrus cycle.

The tissue sections were prepared, photographed and analyzed as described in the text. The final magnification was 4,130 X.

(directly proportional to the volume fraction) of the corpus luteum on day 6, at which time the secretion rate of progesterone was still rising (Fig. 2-11). At day 12, progesterone secretion was maximal and the large luteal cells reached their greatest area fraction of around 70%. At day 20, when the corpus luteum ceased secreting significant amounts of progesterone (Fig. 2-11) and degenerative processes predominated, the proportion of large luteal cells decreased to represent about 60% of the corpus luteum.

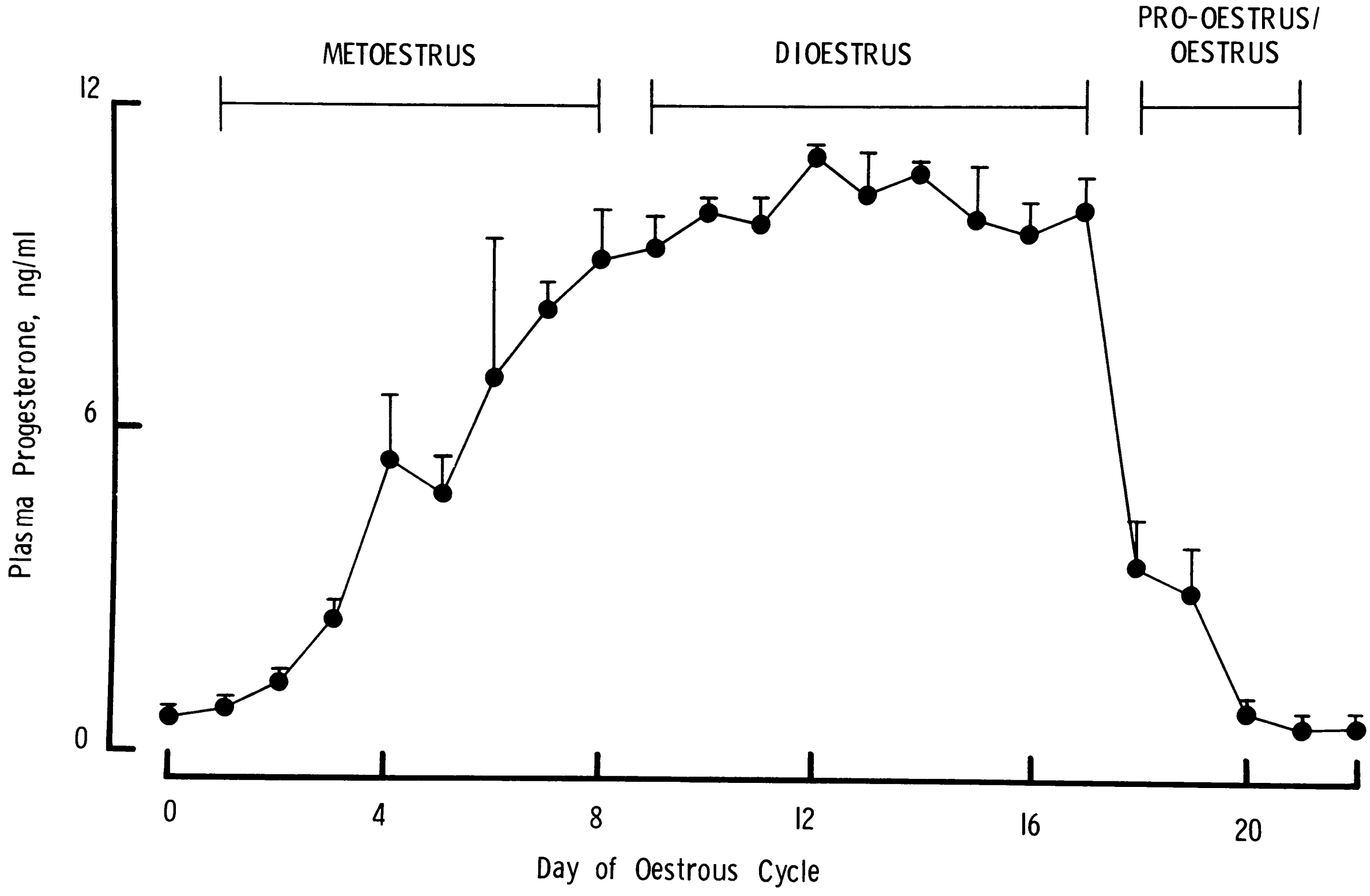
2.3.3.2 Cytoplasm of the Large Luteal Cell

Table 2-2 summarizes the results of the morphometric analysis of the cytoplasm of large luteal cells on five different days of the oestrus cycle, and Fig. 2-12 presents some of the data in histogram form. The proportion of mitochondria rose throughout the cycle. Lipid droplets fell in numbers as the rate of secretion of progesterone by the luteal cells increased, and rose in numbers by day 20, when progesterone secretion had ceased. The protein-synthesizing compartment, consisting of ger plus polysomes and ribosomes, steadily increased from day 6 to day 17 of the cycle, which suggested that increased protein synthesis accompanied the increased synthesis of progesterone.

The Golgi apparatus (cisternae plus vesicles) constituted a constant 5-7% of the luteal cell cytoplasm from days 6-17, but by day 20 its contribution had declined to around 2% of the luteal cell cytoplasm. Until day 20, aer-ground plasm were the most abundant cytoplasmic components, which would be expected in such a steroidogenically-active tissue (Christensen and Gillim, 1969). Primary lysosomes were often difficult to identify positively in the cytoplasm of the large luteal cells,

Fig. 2-11: The concentration of progesterone in peripheral plasma of cows throughout the oestrus cycle

Blood was obtained throughout five oestrus cycles from six animals. Values are plotted as means ± SEM. The data was kindly supplied by Dr. R. Heitzman of the Institute for Research on Animal Diseases, Agricultural Research Council, Compton, and was obtained from the herd which was the source of corpora lutea used in this study (see Methods, section 2.2.1).


METOESTRUS
DIOESTRUS
PRO-OESTRUS/
OESTRUS
12
6
0
Plasma Progesterone, ng/ml
0
4
8
12
16
20
Day of Oestrous Cycle

Category		Day of oestrus cycle				
		6	13	15	17	20
Mitochondria	No.	346	570	678	628	669
	%	15.6	25.6	31.0	25.8	34.0
Lipid droplets	No.	204	60	36	121	342
	%	9.2	3.2	1.6	5.0	17.4
Agranular endoplasmic reticulum + ground plasm	No.	1317	1215	937	1033	590
	%	59.4	54.5	42.8	42.4	30.0
Granular endoplasmic reticulum + ribosomes	No.	6	164	167	297	229
	%	0.3	7.4	7.6	12.2	11.7
Densely-staining bodies	No.	55	69	64	136	37
	%	2.5	3.1	2.9	5.6	1.9
Golgi vesicles	No.	19	72	111	96	32
	%	0.9	3.2	5.1	3.9	1.6
Golgi cisternae	No.	40	42	56	59	18
	%	1.8	1.9	2.6	2.4	0.9
Lysosomes	No.	2	1	7	30	37
	%	0.09	0.05	0.03	1.2	1.9
Open vesicles	No.	209	29	124	11	7
	%	9.5	1.3	5.7	0.5	0.4
Crystalline bodies	No.	2	2	3	0	0
	%	0.09	0.09	0.14	0	0
Unassigned	No.	12	4	6	23	5
	%	0.5	0.2	0.3	0.9	0.25
Number of observations		2212	2228	2189	2434	1966
Number of fields		21	22	22	22.5	22

Table 2-2: The proportion of subcellular components in the cytoplasm of the large luteal cells at different stages of the oestrus cycle.

The tissue sections were prepared, photographed and analyzed as described in the text. The final magnification was 14,030 X.

Fig. 2-12: The variation in the proportions of subcellular organelles in the cytoplasm of large luteal cells during the bovine oestrus cycle

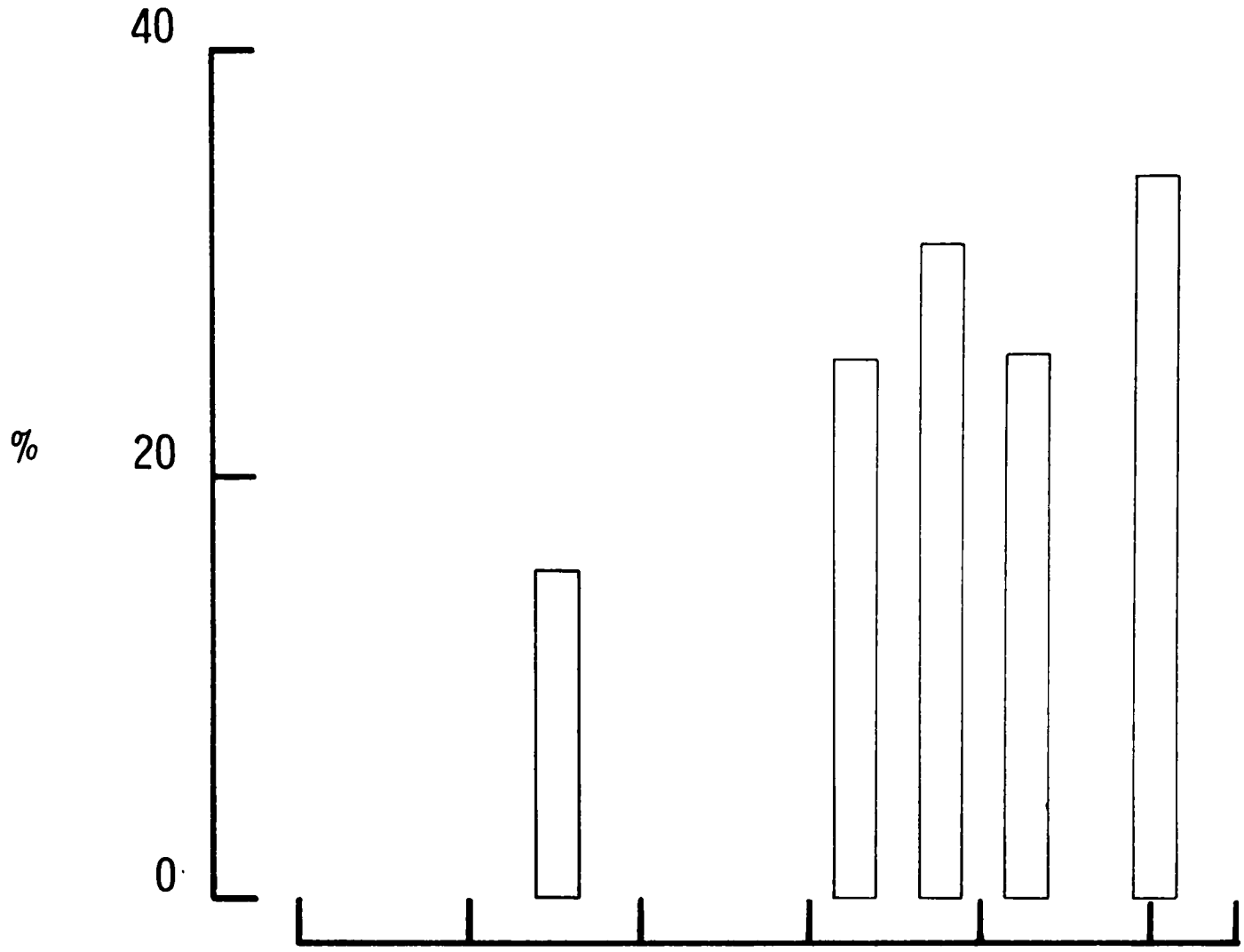
A) Mitochondria
Granular endoplasmic reticulum plus ribosomes

B) Golgi cisternae and vesicles
Lipid droplets

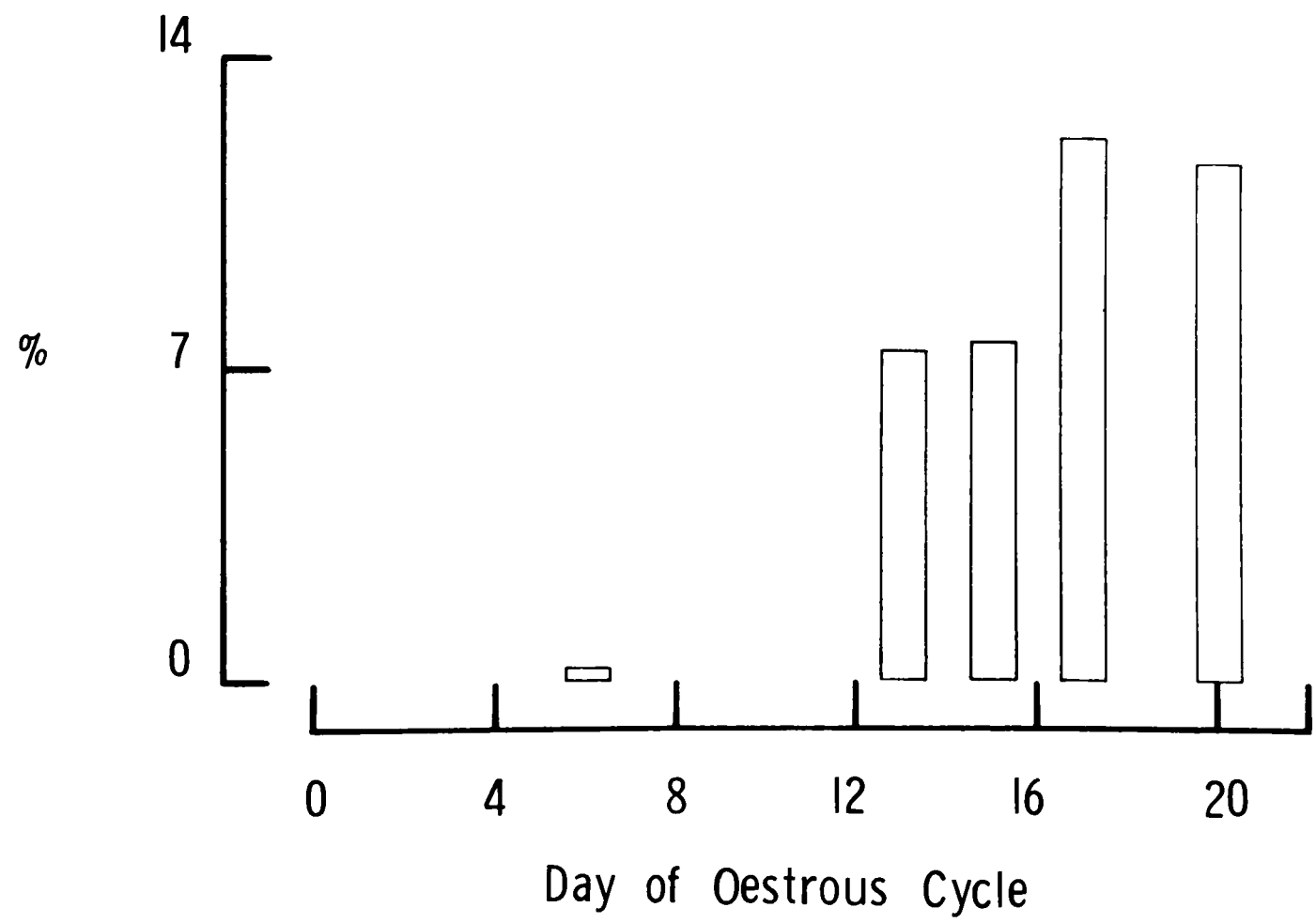
C) Ground plasm plus agranular endoplasmic reticulum
Lysosomes

D) Densely-staining bodies
The portion above the dashed lines represents the contribution by microperoxisomes to the total population of densely-staining bodies at days 6, 15 and 17 of the oestrus cycle.

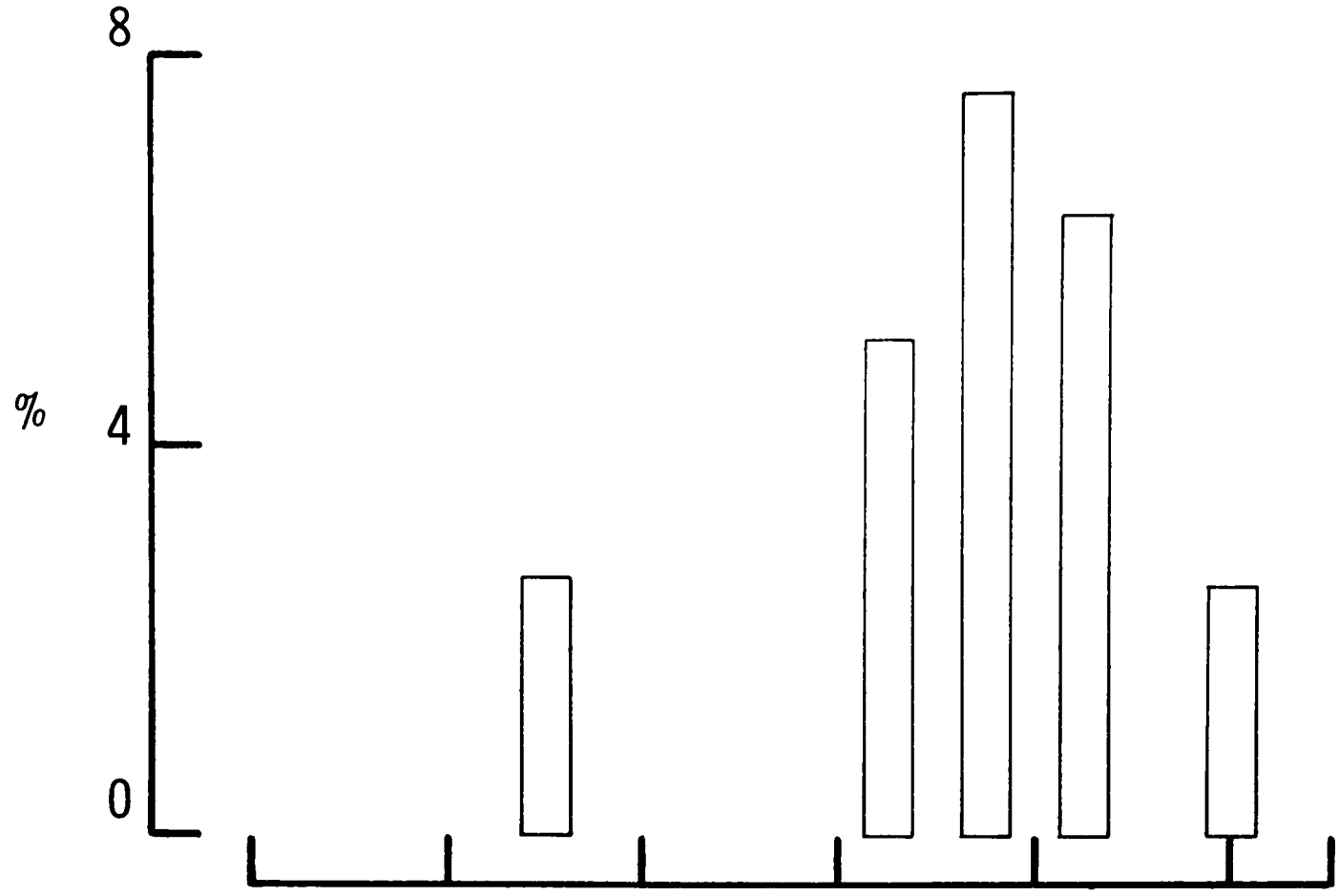
MITOCHONDRIA



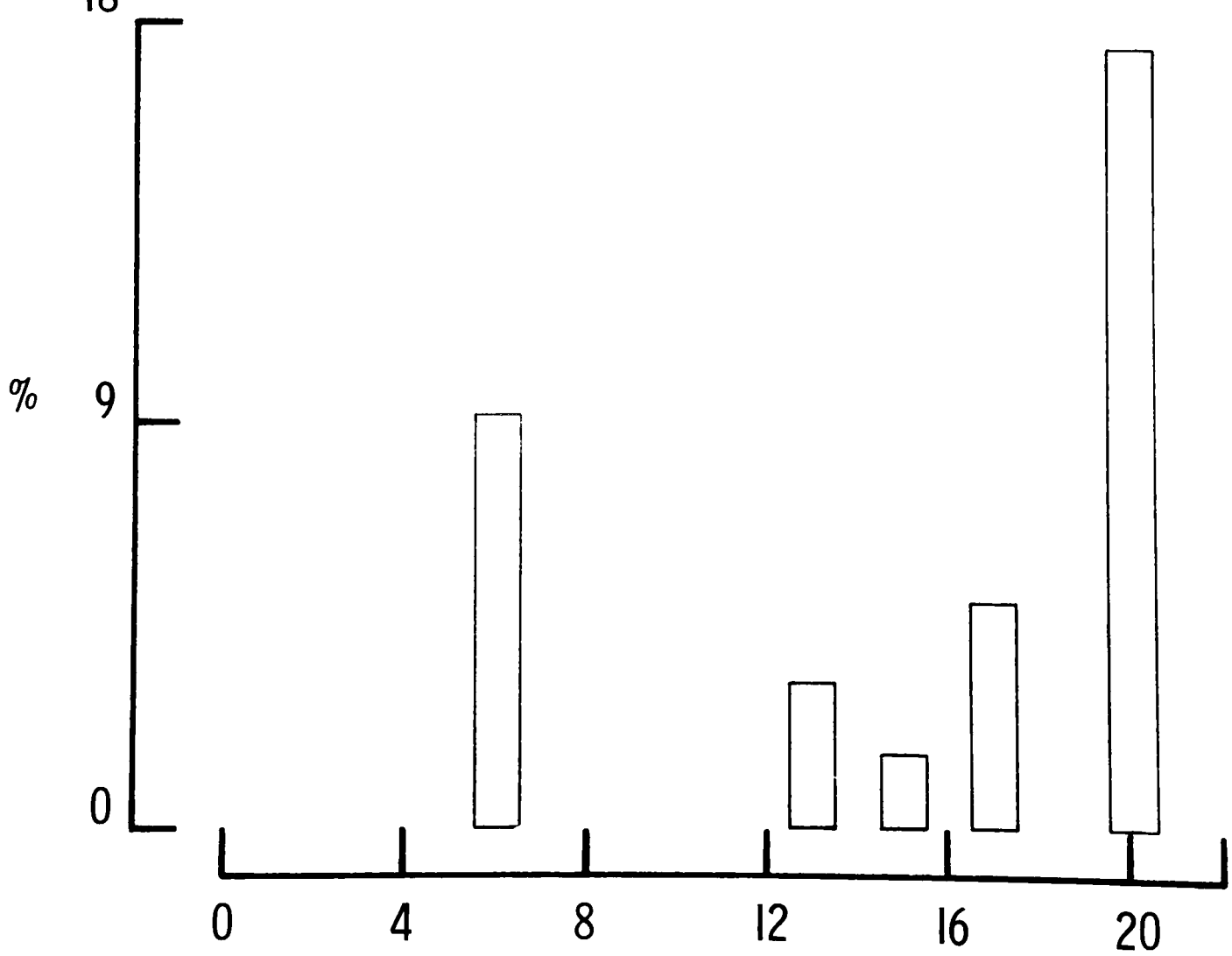
ENDOPLASMIC RETICULUM
PLUS RIBOSOMES



GOLGI CISTERNAE AND VESICLES

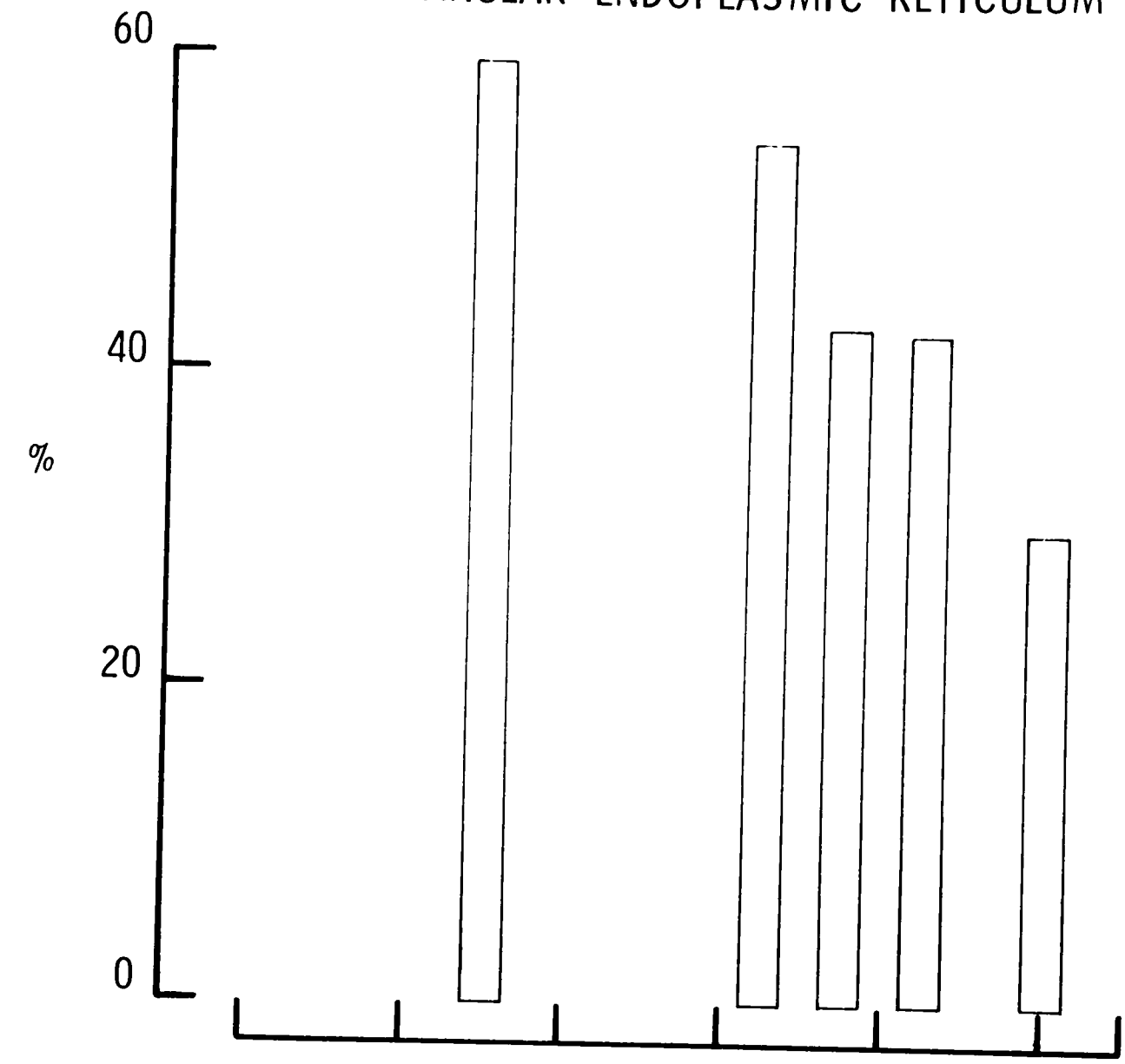


LIPID DROPLETS

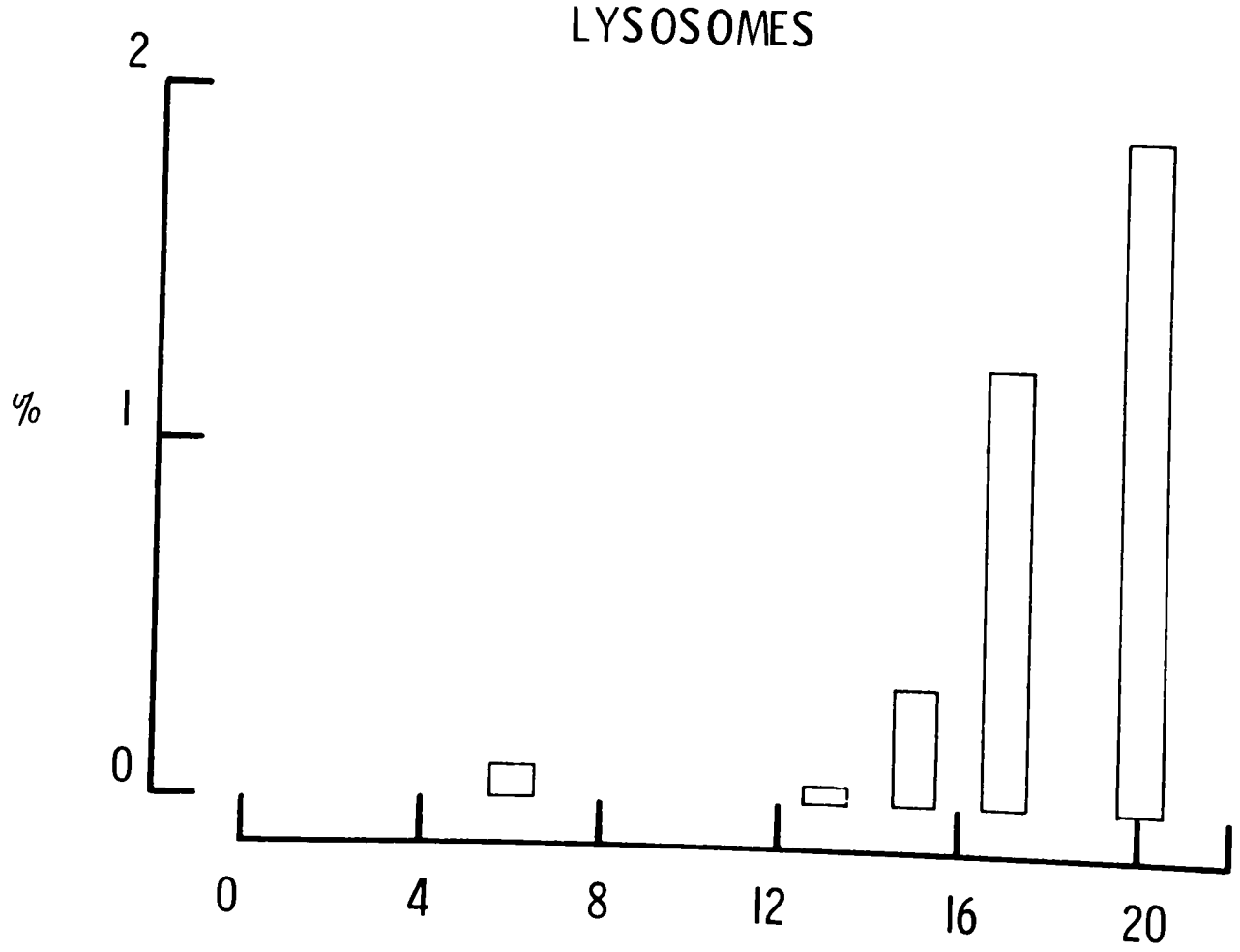


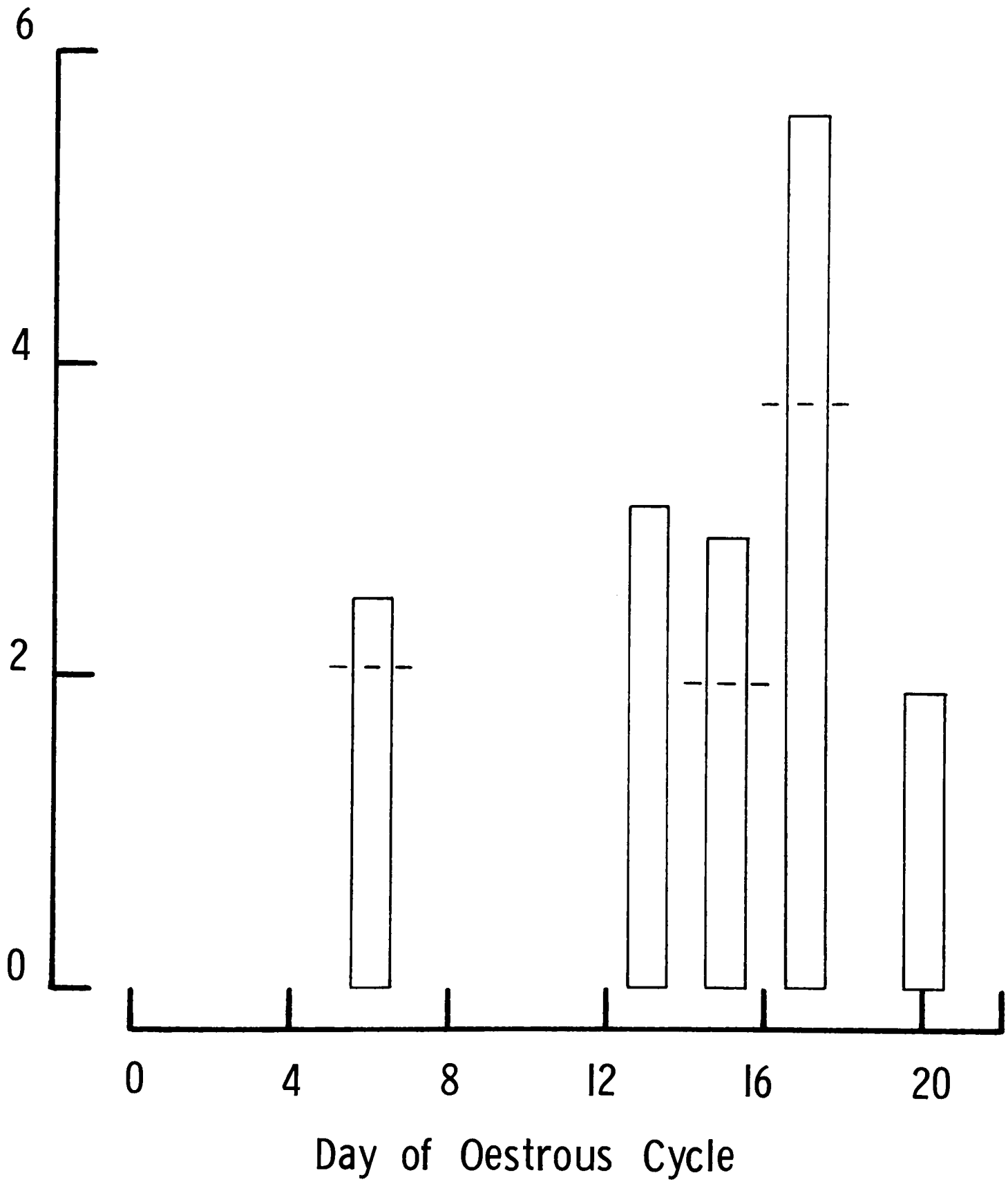
Day of Oestrous Cycle

GROUND PLASM PLUS
AGRANULAR ENDOPLASMIC RETICULUM



LYSOSOMES




DENSE-STAINING BODIES
6
4
2
0
%
0
4
8
12
16
20
Day of Oestrous Cycle

but when regression of the bovine corpus luteum commenced secondary lysosomes (containing inclusion bodies) were prevalent and readily identified.

The category of densely-staining bodies included not only the putative progesterone secretory granules, but also microperoxisomes (whose size and electron density overlapped with the former) and any other electron-dense, membrane-bound bodies (such as primary lysosomes) of 0.2-0.4 μ diameter. The general class of densely-staining bodies varied with the stage of the oestrus cycle: they constituted about 2-3% of the cytoplasm of the large luteal cells from days 6-15, and reached 5.5% on day 17, thereafter declining to about 2% on day 20.

In order to determine the proportion of microperoxisomes within the total population of densely-staining granules, cytochemistry was carried out on tissue sections from corpora lutea of days 6, 15 and 17 of the oestrus cycle. Micrographs were prepared of non-counterstained sections which were then analyzed morphometrically for microperoxisomes and other electron-dense granules (Table 2-3). The microperoxisomes were generally smaller than the putative secretory granules (0.15-0.25 μ and 0.2-0.4 μ diameter, respectively) and displayed a "mottled" appearance, due to deposition of osmophilic masses of insoluble DAB. Even so, the analysis was difficult to perform as the densely-staining bodies that were not microperoxisomes were difficult to identify in the non-counterstained sections where the degree of contrast was poor. Had the sections been counterstained, the microperoxisomes would not have been identifiable unequivocally, since they would have become as electron-dense as the other granules. Table 2-3

	Day of oestrus cycle		
	6	15	17
Number of microperoxisomes	42	76	72
Number of other densely-staining bodies	216	156	148
% of microperoxisomes	16.3	32.8	32.7
Number of fields	14	13	14

Table 2-3: The cytoplasmic proportion of microperixomes in the large luteal cells at three different times of the oestrus cycle.

The final magnification was 14,030 X.

shows that the content of microperoxisomes in the cytoplasm of the large luteal cells doubled between days 6 and 15 to constitute approximately one-third of the total population of electron-dense granules.

In summary, the morphological analysis showed that the proportion of large luteal cells in the bovine corpus luteum rose and fell in parallel with the pattern of progesterone secretion. Furthermore, during the phase of active steroidogenesis (approximately days 6-17 inclusive), the cytoplasm of the large luteal cells contained densely-staining granules which were neither lysosomes nor microperoxisomes as judged by quantitative morphological and cytochemical criteria. It was only this additional type of electron-dense granule which appeared to undergo secretion from the luteal cells.

2.4 DISCUSSION

2.4.1 Morphological and Cytochemical Considerations

The luteal cells of the bigger domestic animals and of man are very large, attaining up to 40 μ in diameter, in comparison with other types of steroid-secreting cells (Christensen and Gillim, 1969; Enders, 1973). Such extensive cellular hypertrophy by the luteal cells of the cow, sheep, pig, goat and human may reflect both the need for the rapid development of a functional corpus luteum and the need for synthesis of large amounts of progesterone (approximately 40 mg per day in the cow, Short (1959)). In this context, it may be significant that luteal cells produce only a restricted range of steroids compared with, for example, adrenocortical cells (Fawcett et al., 1969; Savard, 1973). The luteal cell margins were extensively convoluted, so providing a large surface area for adsorptive and secretory processes. Extensive refolding of luteal cells also occurs in the human (Adams and Hertig, 1969b), mouse and hamster (Enders, 1973). Densely-staining bodies (which had lost their unit membranes) were observed in the extracellular spaces adjacent to the large luteal cells of the bovine corpus luteum. The complex and extensive network of adjoining capillaries in luteal tissue probably facilitates the entry of steroid hormone into the ovarian circulation (Heap et al., 1973).

The morphological features of the large luteal cells of the cow were comparable to those described in different mammalian species for steroid-secreting cells generally, and for luteal cells in particular (Fawcett et al., 1969; Christensen and Gillim, 1969; Enders, 1973). Although bovine

luteal cells contain abundant aer, it was only rarely observed to be arranged in whorls, and even then these whorls were not as extensive as those observed in the luteal cells of the guinea pig (Crombie et al., 1971), pig (Bjersing, 1967b) and human (Crisp et al., 1970).

Osmiophilic inclusions were not a feature of bovine luteal mitochondria, in contrast to the mitochondria of the luteal cells of some other species (Enders, 1973) and the adrenal mitochondria of the pig (Fujioka et al., 1978). The cristae were predominantly tubular in the cells of the mid-cycle bovine corpus luteum. The transition from lamelliform to tubular cristae may be related to the changes in mitochondrial enzyme content which are known to occur as the granulosa cells of the follicle become luteinized and incorporated into the corpus luteum (Enders, 1973; Long, 1973). The mitochondria of the luteinized cells contain the cholesterol side chain cleavage enzyme complex, an additional cytochrome P450 (Savard, 1973), and possibly other steriodogenic enzymes for the first time. It is possible that these components require a different conformation of inner mitochondrial membranes. The highly tortuous tubular arrangement of cristae in luteal mitochondria greatly increases the area of the inner membrane exposed to the matrix compartment (Enders, 1973), and this may serve to promote greater areas of interaction between the substrates, intermediates and enzymes of steroidogenesis.

The "crystalline bodies" were always present in small numbers in the luteal cytoplasm and did not undergo exocytosis in either the cow (present study) or the pig (Bjersing, 1967b). Their function is unknown and their appearance may be artifact-

ual. It is possible that they are related to the "Reinke crystalloids" seen especially in the interstitial cells of the human testis (Fawcett and Burgo, 1960), although they closely resemble the insulin granules found in the beta cells of the pancreas of the dog and bat (Fawcett et al., 1969). It may be significant that the amino acid sequences and conformations of relaxin and insulin are very similar, (James et al., 1977; Bedarkar et al., 1977; Isaacs et al., 1978). In fact, it has been suggested that relaxin may be included in a family of insulin-like growth factors with both structural and functional similarities in modulating cell growth and activity. These include nerve growth factor, somatomedins A and C, as well insulin and non-suppressible insulin-like activity (James et al., 1977).

As Christensen and Gillim (1969) pointed out, electron micrographs of luteal cells reveal more than one type of electron-dense, membrane-bound granule; furthermore, the elucidation of their possible functions has not been helped by the bewildering range of names that have been ascribed to them. Cytochemical techniques have been developed for the identification *in situ* of two granular subcellular organelles, namely lysosomes (Smith and Farquhar, 1966) and microperoxisomes (Novikoff and Goldfischer, 1968). Previous cytochemical studies of steroidogenic cells have concentrated on the role of lysosomes in steroidogenesis (e.g., Elfont et al., 1977) and luteal cell regression (e.g., Bjersing et al., 1970a; Gemmell et al., 1976) and on the distribution of microperoxisomes among different cell types. Microperoxisomes have been found in the cells of the adrenal cortex (Beard, 1972), the testicular

interstitium (Reddy and Svoboda, 1972), and the primate corpus luteum (Gulyas and Yuan, 1975). Therefore, cytochemistry enabled the identification of lysosomes and microperoxisomes in the bovine corpus luteum, and showed the latter to be distinct from the secretory granules.

Microperoxisomes were identified cytochemically in an alkaline DAB medium by the peroxidatic component of their catalase activity[2]. Under these conditions, the peroxidase activity associated with ger and Golgi elements is destroyed so that the reaction product is exclusively localized over microperoxisomes (Herzog and Fahimi, 1976).

Microperoxisomes were smaller than the putative secretory granules in the bovine luteal cells and were frequently found in those areas of the cytoplasm containing the greatest numbers of lipid droplets. They were not observed within, or adjacent to, the Golgi membranes and were distinguished from primary lysosomes by the finding that only the latter gave a positive reaction in Gomori acid phosphatase medium in tissue sections prepared from the same corpus luteum.

The putative secretory granules were cytochemically negative with respect to both microperoxisomes and lysosome staining, and clearly represented a third, discrete population. In mid-cycle luteal cells, they were more numerous than microperoxisomes and primary lysosomes. Furthermore, only the granules from this third population appeared to be extruded from the cell, i.e., specific cytochemical staining for micro-

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2: Cytochemistry confirmed that catalase was an appropriate enzyme marker for microperoxisomes in the subcellular fractionation studies reported in Chapter 3.

peroxisomes and lysosomes was always intracellular.

Gulyas and Yuan (1975) also observed three distinct populations of granules in luteal cells of the pregnant rhesus monkey: large granules of irregular outline, medium-sized granules (0.5-1.2 μ diameter), and small granules (0.1-0.45 μ diameter) which they identified as microperoxisomes by cytochemistry. They were unable to suggest a function for the largest granule; they could have been lysosomes or they may have been analogous to the relaxin storage granules described in the luteal cells of pregnant pigs (Belt et al., 1971; Cavazos et al., 1969) and rats (Long, 1973). The medium-sized granules were not microperoxisomes (Gulyas and Yuan, 1975) and were morphologically very similar to the secretory granules described in the luteal cells of sheep (Gemmell et al., 1974) and cows (present study).

In the luteal cells of the pregnant rhesus monkey, "microperoxisomes were frequently observed contiguous with lipid droplets" and, in some instances, there appeared to be a fusing of the membranes of these organelles (Gulyas and Yuan, 1975). In another study of the pregnant rhesus monkey, these same authors reported that 106 out of 107 microperoxisomes (identified cytochemically) were "directly associated with the aer" (Gulyas and Yuan, 1977). A number of reports have actually suggested that microperoxisomes are derivatives of the endoplasmic reticulum (Novikoff and Novikoff, 1972, 1973; Reddy and Svoboda, 1973; Black and Bogart, 1973).

Although microperoxisomes probably occur ubiquitously in all cell types (Holmes and Masters, 1978), their identification is usually based solely on their catalase activity. In fact

they are known to also contain a range of oxidative enzymes, whose composition may vary in different cells according to their varying physiological functions (Novikoff, 1976). Currently, the role of microperoxisomes in the metabolism of luteal cells is unknown. The fact that they appear to be derived from the aer and that they are frequently found in association with (or at least adjacent to) lipid droplets in the cytoplasm of a number of different cells (Leuenberger and Novikoff, 1973; Novikoff and Novikoff, 1973; Novikoff et al., 1973), has lead to the suggestion that microperoxisomes are involved in steroidogenesis in luteal cells (Gulyas and Yuan, 1975). Cholesterol and its esters are stored in lipid droplets (Deane et al., 1962), so that the enzymes contained in microperoxisomes may participate in the release of these steroidogenic substrates from their sites of storage and may also facilitate the initial movement of these substrates through aer to the sites of their utilization in mitochondria (Gulyas and Yuan, 1975).

Lysosomes are known to contain various acid hydrolases, including acid phosphatase (de Duve et al., 1955; Deane et al., 1962). Previous cytochemical studies in rat liver and other tissues have shown that the Golgi apparatus displays acid phosphatase activity in a medium buffered at pH 5. This is thought to reflect the packaging of lysosomes by the Golgi elements, prior to their "budding off" from the latter, and has given rise to the concept of a functional entity called GERL made up of elements of the Golgi, endoplasmic reticulum and lysosomes (Novikoff et al., 1971). Not all of the Golgi cisternae showed positive staining for acid phosphatase in

either this study or those cited above. This suggests that this organelle has functions additional to the packaging of lysosomes and correlates with the observation that Golgi membranes are prominent in luteal cells of the mid-cycle cow, when lysosomes are not numerous.

Cytochemistry revealed no secondary lysosomes (or autophagosomes, McClellan et al., 1977) and very few primary lysosomes in sections of bovine luteal tissue obtained at mid-cycle. Once luteal cell regression had begun, large numbers of secondary lysosomes, frequently containing inclusions of other organelles, were evident. The participation of secondary lysosomes in the degeneration of luteal cells at the end of the luteal phase of the oestrus cycle has been previously documented in the rat (Deane et al., 1962) and sheep (Deane et al., 1966; Dingle et al., 1968; Bjersing et al., 1970a; Gemmell et al., 1976; McClellan et al., 1977). During the later stages of luteal regression in the sheep numerous secondary lysosomes appeared to be formed directly from Golgi or GERL membranes (McClellan et al., 1977). In sheep the secondary lysosomes which predominated during luteal cell regression were more fragile than the lesser numbers of primary lysosomes which occurred during the active steroidogenic phase (Dingle et al., 1968). However, gonadotrophin treatment of immature rats led to a decrease in large, labile lysosomes and an increase in lighter primary lysosomes (Dimino et al., 1977), which suggests that lysosomal function may be influenced by the pituitary.

Although lysosomes may participate in processes additional to the degenerative ones associated with luteal regression

(Moor et al., 1978), the precise function of the primary lysosomes that were occasionally observed in mid-cycle luteal cells of the cow is unknown. Dingle et al.(1968) reported that in sheep the specific activity of acid phosphatase was similar in active and regressing corpora lutea. Elfont et al. (1977) have observed acid phosphatase staining around the rim of lipid bodies in gonadotrophin-treated, immature rats. On the basis of these results, these authors have suggested that the lysosomal system is involved in making substrate available for steroid synthesis and/or in facilitating steroid secretion. However, a role for primary lysosomes, rather than microperoxisomes, in the steroidogenic process is unlikely for the following reasons. In the cow, microperoxisomes comprised at least four-fold more of the luteal cell cytoplasm than did lysosomes during the active luteal phase. Microperoxisomes were commonly observed adjacent to lipid droplets, whereas the lysosomes present at these times appeared to occur randomly throughout the cytoplasm. Acid phosphatase staining around the rim of lipid droplets was only observed during luteal regression, by which time secondary lysosomes were much in evidence and secretion of progesterone had practically ceased.

It is more likely that small numbers of primary lysosomes are required during the active luteal phase to degrade unwanted compounds including gonadotrophin and, possibly, steroid receptors. Polypeptide hormones such as insulin, prolactin and growth hormone have been localized within cells using immunofluorescent techniques (Kolata, 1978) and specific binding of LH to lysosomes has been demonstrated in ovine luteal cells (Chen et al., 1977). Also, prostaglandin and gonadotrophin

receptors have recently been found associated with lysosomal membranes in bovine luteal cells (Mitra and Rao, 1978). Finally, it was significant that in sections of bovine luteal cells stained for acid phosphatase activity, another class of membrane-bound granule was also associated with the cisternae of the Golgi apparatus. These granules were free of product resulting from acid phosphatase activity. Such an observation has also been made in the sheep (McClellan et al., 1977).

2.4.2 Ultrastructural Changes Throughout the Bovine Oestrus Cycle: Correlation of Luteal Structure and Function

"Little progress has been made so far in integrating structure and function toward a better understanding of functional organization within steroid-secreting cells" (Christensen and Gillim, 1969). This is in contrast to the understanding achieved of the secretory processes for protein hormones and polypeptide hormones (Fawcett et al., 1969). The mode of steroid secretion remains to be elucidated.

Therefore, with the recent proposal of a granular mode of steroid secretion in the corpus luteum (Gemmell et al., 1974) it was hoped that morphometric analysis of luteal cell cytoplasm throughout the bovine oestrus cycle would provide a correlation between variations in the proportions of the organelles and the pattern of steroidogenesis. A previous morphometric analysis of bovine luteal cells did not separately identify densely-staining granules and did not consider a granular route of progesterone secretion (Priedkalns and Weber, 1968b).

Analysis at low magnification provided a measure of the proportion of luteal cells in the corpus luteum at different

stages of the cycle. The luteal cells comprised 50% of all cell types at the beginning and end of the oestrus cycle and reached a maximum of 71-74% at mid-cycle, when progesterone synthesis was maximal. Using less reliable quantitative procedures, Niswender et al. (1976) estimated that the luteal cells comprised a constant 50-54% of the ovine corpus luteum from days 10-17 of the oestrus cycle. Somewhat surprisingly, Priedkalns and Weber (1968b) did not estimate the proportion of luteal cells comprising the bovine corpus luteum throughout the oestrus cycle in their study.

Analysis at higher magnification revealed changes in the relative proportions of a number of different organelles throughout the oestrus cycle. For example, the proportion of mitochondria rose throughout the cycle, indicating an increased need for the products either of the energy systems or of the steroidogenic enzymes of mitochondria (Savard, 1973), or both (Bjersing, 1967b). The enzyme complex which carries out the cleavage of the side chain of cholesterol requires reducing equivalents such as NADPH and an electron-transport system utilizing molecular oxygen and cytochrome P450 (Kimura and Ono, 1968; Savard, 1973). There is some evidence that this electron-transport system operates in the reverse sequence to the other, ubiquitous mitochondrial system which provides reducing equivalents and energy in the form of ATP in the corpus luteum and all other cell types (Green at al., 1957; McIntosh et al., 1971; Gower, 1975a). Therefore, as progesterone synthesis must proceed via side chain cleavage of cholesterol in the mitochondria, increased steroidogenesis by the luteal cells may necessitate increased numbers of mitochondria in these cells.

At this stage it may be helpful to reiterate those steps in the synthesis of progesterone that the different organelles of the bovine corpus luteum are known to participate. The synthesis of cholesterol from acetate involves enzymes that are either in the membranes of aer (microsomes) or in the surrounding ground plasm (cytosol fraction). The cholesterol so formed is then believed to undergo transport to mitochondria for cleavage of the side chain in a series of hydroxylation reactions. The resulting pregnenolone is acted upon by enzymes which also reside in the reticulum or in the matrix, leading to formation of progesterone.

The relative proportion of ground plasm-aer declines as the cycle progresses, but at all times remains the most abundant subcellular compartment of the luteal cells. This decline probably does not imply less participation in the overall metabolism of the cells by this compartment, but may simply reflect increasing amounts of other organelles, especially mitochondria, ribosomes-ger, Golgi membranes, and lipid droplets at day 20.

The aer increases rapidly in granulosa cells following luteinization in most species, including the cow (Priedkalns and Weber, 1968a) and pig (Bjersing, 1967b), and takes up a characteristic configuration, variously described as "branched tubules, tubular sheets and fenestrated cisternae" (Enders, 1962; Christensen and Gillim, 1969). Enders (1973) has suggested that this configuration (which is unique to steroid-secreting cells) would be the most suitable if the enzymes involved in the sequential steps of steroid synthesis were alternatively situated in the aer, the mitochondria and the

ground plasm. Indeed, many of the steroidogenic enzymes measurable in homogenates of adrenal and corpus luteum of a number of species are recovered after subcellular fractionation in either the mitochondrial, microsomal or soluble fractions (Savard, 1973; Gower, 1975b). Many of the enzymes involved in the synthesis of cholesterol from acetate also occur in the microsomal fraction (Enders, 1973).

However, the aer may be involved in functions besides steroidogenesis. For example, Fawcett et al. (1969) have noted that the Golgi cisternae frequently appear to be continuous at their margins with tubular elements of the aer in steroid-secreting cells. Such a relationship is also seen in protein-secreting cells such as those of the exocrine pancreas (Palade, 1975). As the Golgi apparatus appears to "bud off" small osmiophilic granules which are neither microperoxisomes nor lysosomes in bovine luteal cells (this study) and which are not lysosomes in ovine luteal cells (McClellan et al., 1977), the possibility exists that the aer also transports progesterone (and other steroids) from its site of synthesis in the mitochondria to its site of packaging into granules in the Golgi. This process would be comparable to that operating in cells which release catecholamines (Douglas, 1966, 1974b) and protein hormones (Fawcett et al., 1969; Palade, 1975). Such a mechanism in adrenocortical cells would provide a means of enabling steroid metabolism concomitantly with transport, and would provide a rational means for intermediates to reach mitochondria, where they undergo 11β-hydroxylation.

The Golgi cisternae and vesicles rise to a peak at day 15 of the bovine oestrus cycle, before declining rapidly by day 20.

The Golgi complex of bovine luteal cells is large and extensive. This is also a feature of the luteal cells of other species (Enders, 1973) and correlates with the large numbers of granules observed in these tissues. Various coated and smooth vesicles, coated tubules, and small granules have been observed with or very near to this organelle in the luteal cells of cow (this study), sheep (Gemmell et al., 1974; Corteel, 1975; McClellan et al., 1977), dog (Abel et al., 1975a,b), and human (Crisp et al., 1970). It has been suggested that the coated vesicles represent "circulating plasmalemma" (Palay, 1963). In the porcine luteal cell, a "shower of coated vesicles appeared at peak progesterone production" (days 8-12), and this influx of coated vesicles was accompanied by the formation of dense bodies in the cytoplasm (Cavazos et al., 1969).

The full array of functions carried out by the Golgi complex in luteal cells is presently unknown. Luteal Golgi fractions have not been isolated using biochemical subcellular fractionation techniques and, because of the difficulty of obtaining specific enzyme markers for this organelle, it is not known to what extent Golgi membranes contaminate microsomal fractions obtained from other tissues. It is significant that they appear to be in direct communication with the extensive aer in various endocrine cells (Fawcett et al., 1969). It is likely that the principal function of the Golgi apparatus in many cell-types is to condense and package primary lysosomes (Friend and Farquhar, 1967) and various secretory products (Palade, 1975), including progesterone (Long and Johns, 1974). In luteal cells, this organelle is probably the site of

formation of the secretory granules (Gemmell et al., 1974; this study). The content of the granules - progesterone and binding proteins - are presumably transported to the Golgi for packaging via the extensive tubular network of aer present in these cells.

Palade, Jamieson and coworkers have found that transfer of secretory product from the aer to the Golgi in pancreatic cells entails the exchange of a high permeability, aer membrane for a Golgi membrane of lower permeability and whose lipid composition is similar to that of the plasmalemma. This Golgi membrane is therefore more suitable for intracellular movement and subsequent exocytosis of zymogen granules from the beta-cell (Palade, 1975). Subsidiary functions of the Golgi complex in other types of cells include the condensation of sugar products and addition of terminal sugar groups to a secretion (Enders, 1973), participation in saccharide synthesis for glycoprotein cell coats (Whaley et al., 1973), and packaging of chromaffin granules (Douglas, 1966). In fact, Hand and Oliver (1977), in a cytochemical study of the lacrimal and salivary glands, and the exocrine pancreas of rats, guinea pigs and monkeys stated that their "observations supported the role of GERL in the processing of secretory material, and confirmed its intimate involvement with the information of secretory granules".

It appears that all eukaryotic cells produce secretory proteins, the basic general secretory functions being the production of cell wall components in plant cells and the production of lysosomes in animal cells (Palade, 1975). This explains the presence in all eukaryotic cells of Golgi and aer membranes, and correlates the extensive amounts of these

organelles in luteal and adrenocortical cells with their high rates of secretion of steroid (Fawcett et al., 1969) and protein (Laychock and Rubin, 1974; Abel et al., 1977). The secretion of highly specialized proteins, catecholamines and steroids by a variety of specialized cells (including luteal cells) is thought to be superimposed upon the general, basic level of secretory activity (Palade, 1975). However, the intimate involvement of the Golgi complex with steroid secretion by luteal cells is not proven. For example, Gillim et al., (1969) stated that that "the absence of secretory structures in human lutein cells thus suggests that steroids pass through the cell membranes into extracellular space by active transport or by diffusion". However, in another ultrastructural study of the human corpus luteum, Crisp et al. (1970) stated that "small coated vesicles, multivesicular bodies and large, membrane-bound, electron-opague granules are invariably associated with the Golgi complex". Long and Jones (1974) noted that the coated vesicles in opossum adrenocortical cells are similar to the coated invaginations at the cell surface, and this led them to suggest that these specialized membraneous bodies may be a means of secreting hormonal steroids. In the rat, Reese and Moon (1938) found that hypophysectomy produced a marked diminishment of the Golgi of the zona fasciculata cells of the adrenal, whereas injection of ACTH induced its hypertrophy.

In the cow, lipid droplets comprised 9% of the cytoplasm of the large luteal cells at day 6, declined to about 3% during the period of maximal secretion of progesterone and rose again to 17% by day 20. Lipid droplets contain cholest-

erol and cholesterol esters (Fienberg and Cohen, 1965) and are stores of steroid precursors (Deane et al., 1966; Enders, 1973). Lipid is a useful criterion of activity in some species (Fawcett et al., 1969; Heap et al., 1973; Enders, 1973). The cytoplasmic concentration of lipid droplets in bovine luteal cytoplasm varied inversely with the rate of progesterone synthesis. Thus, when regression was fully under way at day 20 of the oestrus cycle, and luteal steroidogenesis was minimal, the amount of lipid droplets rose sharply.

Individual cells within the granulosa cell population of the corpus luteum may have greater amounts of lipid than others (Enders, 1973). Such was the case in the bovine corpus luteum. Furthermore, there appeared to be an inverse relationship between the number of secretory granules and evidence of their exocytosis with the relative abundance of lipid droplets. This may indicate that the bovine luteal cells undergo a steroidogenic cycle such that, at any particular time, some are just beginning to secrete progesterone, some are secreting progesterone maximally, whilst some are declining in steroidogenic activity.

The enzymes necessary for synthesis of cholesterol and its esterification, and hydrolysis of cholesterol esters, are found, in part, in the microsomal fraction (Siekevitz, 1963; Schyamala et al., 1966; Savard, 1973). Transport of lipids inside the tubules of aer has also been reported (e.g., Porter et al., 1961). Furthermore, the membranes of the endoplasmic reticulum are rich in phospholipids and cholesterol (Siekevitz, 1963). These findings led Bjersing (1967b) to propose that aer is the site of synthesis of cholesterol from acetate in the

corpus luteum, and that lipid droplets are formed by this organelle. He pointed out that small osmiophilic inclusions similar in appearance to the contents of the lipid droplets were sometimes observed within the tubules of aer. Although inclusions within the aer were occasionally observed in micrographs of bovine luteal cells, their similarity in opagueness to the contents of lipid droplets was not obvious.

Lysosomes were difficult to identify at early stages of the bovine oestrus cycle but were more obvious by day 20 when they comprised 2% of the luteal cell cytoplasm. The role of secondary lysosomes in luteal regression has been well documented (Deane et al., 1966; Dingle et al., 1968; Bjersing et al., 1970a; Gemmell et al., 1976; Stacy et al., 1976; McClellan et al., 1977; Dimino et al., 1977; Elfont et al., 1977). For example, in the sheep the first structural signs of luteolysis were seen at day 12 of the oestrus cycle when a decrease in the secretion of progesterone and of densely-staining granules coincided with the emergence within the luteal cell of secondary lysosomes and autophagocytic bodies (Gemmell et al., 1976). In the anterior pituitary, lysosomes help regulate the secretory process by removing excess secretory granules (Smith and Farquhar, 1966).

It is interesting that infusion of $PGF_{2\alpha}$ into sheep on day 10 of the cycle induced structural regression of the corpus luteum within 24 h in a manner which was directly comparable to the morphological changes observed during natural luteolysis (Stacy et al., 1976; McClellan et al., 1977). This suggests that $PGF_{2\alpha}$ is the principal ovine luteolysin and that structural regression is a process which can take place rapidly

(after an appropriate stimulus) even during times of maximal progesterone secretion. In this context, it has been shown that the enzymes involved in steroidogenesis were not affected until after steroid synthesis had already decreased (Deane et al., 1966; Flint et al., 1974). Disruption of energy metabolism apparently was not a factor since isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase activities were unaltered in the regressing bovine corpus luteum, (Hansel et al., 1973) and cytochrome oxidase activity was unaltered in the ovine corpus luteum (McClellan et al., 1977). Therefore, it appears that a shutdown of the entire cellular energy and biosynthetic systems is not required to initiate regressive processes (McClellan et al., 1977).

The ger-ribosome compartment increased from negligible amounts at day 6 to between 7 and 12% of the luteal cytoplasm for the rest of the bovine oestrus cycle. The ger appeared as stacked cisternae and was localized in small patches throughout the cytoplasm. It also occurs in a similar fashion in the human corpus luteum (Adams and Hertig, 1969a; Crisp et al., 1970). Continuities between the cisternae of agranular and granular endoplasmic reticulum are commonly observed in ultrastructural studies of luteal cells (e.g., Enders, 1973). The presence of large nucleoli in bovine luteal cells during the active secretory phase, together with patches of well-developed ger and numberous ribosomes and/or polysomes scattered throughout the cytoplasm suggest that protein synthesis is an important feature of luteal metabolism (Cavazos et al., 1969; Gower, 1975b).

Secretion of protein together with steroid has been shown

to occur in the corpus luteum and adrenal (Rubin et al., 1974; Laychock and Rubin, 1974; Gemmell et al., 1977a; Abel et al., 1977). The bovine corpus luteum contains two binding proteins for progesterone (see Chapter 4), one or both of which is possibly involved in intercellular synthesis and/or transport of the hormone. It is likely that the presence of a progesterone binding protein in the secretory granules of the bovine corpus luteum renders the granules electron-dense. In the human, small membrane-bound granules (0.15-0.2 μ in diameter and containing a flocculent material) were closely associated with the terminal ends of the cisternae of the ger and near to lipid droplets, against a background of aer and mitochondria (Crisp et al., 1973).

Small densely-staining granules were found in the large cells of the bovine corpus luteum, analogous to those described in the sheep corpus luteum (Gemmell et al., 1974) and cat adrenal (Gemmell et al., 1977a), and observed in the human corpus luteum (Gillim et al., 1969; Adams and Hertig, 1969a,b; Crisp et al., 1970, 1973). From day 6, when they occupied 2.5% of the luteal cell volume, they gradually doubled in numbers to reach a maximum at around day 17. By day 20 of the oestrus cycle their volume had fallen to 2%. During mid-cycle when progesterone secretion is maximal, many of these granules were observed undergoing exocytosis whereas late in the cycle evidence of exocytosis was rare. These values include microperoxisomes, secretory granules, and any additional membrane-bound, electron-dense organelle of diameter less than 0.4 μ; they do not include lysosomes. In a separate analysis, microperoxisomes were identified cytochemically, and estimated to double in

numbers to comprise about 30% of the population of small osmiophilic granules from day 15 of the oestrus cycle. The latter finding suggests that there is an increased requirement for microperoxisomes during periods of intense progesterone synthesis, and is consistent with the proposals (see above) of a role of these organelles in the mobilization of the steroid precursors stored in lipid droplets.

Secretory granules are present throughout the most active luteal phase of the bovine oestrus cycle, i.e., from days 6-17. Although they comprise only some 3-4% of the luteal cell cytoplasm, they are probably packaged and released very rapidly so that at any instance very few granules would actually be in the luteal cytoplasm[3]. The concentration of secretory granules was maximal on day 17, at the onset of luteolysis. This is compatible with the finding that structural dislocation of the secretory process precedes the cessation of progesterone synthesis. Therefore, the numbers of secretory granules temporarily build up in the cytoplasm before being degraded by secondary lysosomes.

A previous quantitative ultrastructural study of the bovine corpus luteum did not specify densely-staining bodies as a separate counting category and did not use cytochemical techniques to aid the identification of granules. However, the overall trends in proportions of the major subcellular organelles (mitochondria, aer and Golgi membranes) at different stages of the oestrus cycle were similar to those reported here although a direct comparison is not possible

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3: See the final section of Chapter 3 for a more extensive discussion of this point.

since different counting criteria were used (Priedkalns and Weber, 1968b).

The possibility that the organelles of luteal cells are arranged in subcellular compartments has arisen from qualitative ultrastructural studies of the cannine corpus luteum (Abel et al., 1975a,b). High voltage electron microscopy (1000 kV) of half micron-thick sections gave increased "depths of field" compared with conventional electron microscopy (100 kV), so that the luteal cells of the dog appeared to be polarized with respect to the extracellular space. Thus, aer and the nucleus were confined to the basal regions of the cells, mitochondria were prevalent centrally, whilst microtubules, microfilaments and Golgi membranes were most abundant in the region of the cells that abutted the extracellular space. These authors suggested that such an arrangement of organelles was significant to both the synthesis and release of progesterone. However, they did not assign a role in these processes for the numerous densely-staining bodies found in the vicinity of the Golgi, even though they noted that they underwent exocytosis from the cells (Abel et al., 1975b). Although subcellular compartmentalization of luteal cells is observed in the dog (Abel et al., 1975a,b) and possibly in the human (Adams and Hertig, 1969a), it may not be a general feature of luteal cells since it is not evident in the rat (Enders, 1962), ewe (Bjersing et al., 1970; Gemmell et al., 1974; Corteel, 1975), pig (Bjersing, 1967b), or cow (Priedkalns and Weber, 1968 a,b; this study).

In summary, the work reported in this chapter shows that the large luteal cells comprise the bulk of the bovine corpus

luteum during the most active secretory period of the oestrus cycle.

At least three types of granules are present in mid-luteal cells; microperoxisomes, primary lysosomes and secretory granules. Only the latter appear to be exocytosed. The concentration of these secretory granules in the luteal cytoplasm correlates with the profile of progesterone secretion by these cells throughout the cycle. Changes in the cytoplasmic proportions of the other subcellular organelles are consistent with the need to: firstly, mobilize the steroid precursors stored in lipid droplets; secondly, to synthesize increased amounts of progesterone and protein; and thirdly, to package these products into discrete secretory granules.

CHAPTER 3

INTRACELLULAR LOCATION OF PROGESTERONE IN THE BOVINE CORPUS LUTEUM

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3.1.2 Subcellular Fractionation of Bovine Corpus Luteum

3.1.3 Experimental Approaches

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3.2.2.2 Analytical Ficoll Gradients

3.2.2.3 Preparative Zonal Gradients

3.2.3 Assays

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3.2.3.3 Marker Enzyme Assays

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2. Acid phosphatase
3. β-Glucuronidase
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7. Inosine diphosphatase
8. Lactate dehydrogenase

3.3 RESULTS

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3.4 DISCUSSION

3.1 INTRODUCTION

3.1.1 Subcellular Fractionation

Historically, subcellular fractionation has been a fruitful approach for the isolation and study of the composition of different subcellular organelles of the cell (de Duve, 1971; Steck, 1972; Amar-Costesec, 1974) and for the study of intracellular secretory processes, e.g., the secretion of zymogen granules from the pancreas (Jamieson and Palade, 1967a,b). Therefore, subcellular fractionation was undertaken in the investigation of the intracellular location of progesterone in the bovine corpus luteum. A general description of the principles that apply to all subcellular fractionation studies follows and then those aspects of the subject which are relevant to the fractionation of ovarian tissue will be discussed.

Tissue fractionation studies apply the chemist's analytical approach to an integrated study of cell structure and function, whereby fractionation is accompanied by analysis of the separated cellular constituents[1] and correlated with morphological examination of cellular components. The development of this integrated approach owes much to the pioneering work of Claude (1946), Schneider (1948), Palade (1951), and de Duve (1964).

All subcellular fractions proceed in the following sequence: homogenization, fractionation and analysis, i.e., they begin with an initial disruption of cellular organisation

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1: In accordance with the suggestion of de Duve (1964), morphological entities (mitochondria and other organelles) shall be designated "components" and biochemical entities (proteins, steroids and enzymes) shall be designated "constituents". This distinction avoids confusing intact cellular structural components with the properties of disrupted cells assessed by biochemical assay.

(usually by homogenization in an aqueous medium), followed by various manipulations which selectively redistribute the subcellular particles, and terminate with biochemical and sometimes morphological analysis of the resulting fractions. However, it is important to remember that interpretation proceeds in the reverse sequence, so that mental reconstruction of the structure-function relationships in the intact cell is based on the perilous jump from biochemical data of fractionated cell constituents (de Duve, 1964).

The initial homogenization of the tissue usually involves a compromise between complete disruption of the cells and maximal preservation of the structure and function of the released organelles. Early methods of reducing cells and tissues to a suspension of subcellular particles, such as grinding tissue in buffer with a pestle and mortar (Schneider, 1948), have given way to shearing of tissue minces in coaxial pestle homogenizers such as the Dounce and Potter-Elvehjem (Allfrey, 1959). The latter are most commonly used with soft tissue such as liver (Amar-Costesec, 1974) whereas harder tissues (e.g., muscle), which contain greater proportions of connective tissue, require harsher methods of tissue disruption such as machines with revolving stainless-steel blades. Examples are the Ultra-turax and Silverson tissue homogenizers. Other, less common, methods for disrupting cells include the use of rapid pressure changes (Wallach, 1967), percussive shock and extraction of cold, minced tissue with acetone (Steck, 1972). Even with the greatest care, homogenization inevitably causes some organelle damage; for instance, release of soluble material from damaged particles or its adsorption

onto the surface of particulate elements (de Duve, 1964). For example, the Potter-Elvehjem homogenizer, consisting of a teflon barrel in a close-fitting glass bore, is generally considered to provide the most gentle homogenization forces (more gentle than the glass-glass Dounce homogenizer). Even so, de Duve (1971) reports that its use with liver causes up to 15% breakdown of lysosomes so that their enzymatic contents are made soluble. Recently, molecular contamination has been investigated in fractionation studies of porcine pancreas, where exocrine proteins leaked from compartments damaged during tissue homogenization and were adsorbed to other sites. Correction for these leakage-adsorption artifacts enabled a description of the kinetics of intercellular transport derived from cell fractionation data to be as accurate as that achieved from quantitative autoradiographic studies (Scheele and Tartakoff, 1977; Scheele et al., 1978).

The choice of homogenizing medium, whether sucrose, polymerized sucrose (Ficoll), iodinated medium (Metrizamide) or polyvinylpyrrolidone (Diadrast, Hartley et al., 1960), and of buffer and ionic strength is largely empirical. It is nonetheless important since the choice can greatly affect both the nature of the tissue-disruptive process and the stability of the released organelles. Neville (1960) used 1 mM $NaHCO_3$ as homogenizing medium for the preparation of plasma membranes, but buffered sucrose has been the most popular choice of homogenizing medium with most investigators, ranging from hypertonic sucrose (0.88 M, Hodgeboom, Schneider and Palade, 1948) to isotonic sucrose (0.25 M, most studies since Schneider, 1948). There are therefore no rules which govern

the optimal homogenization and buffer conditions for subcellular fractionation of different tissues, and so most investigators use buffered 0.25 M sucrose simply because this medium proved satisfactory in the pioneering fractionation studies of rat and mouse liver. Perhaps this is the reason for the seemingly random inclusion of various metal ions, especially divalent cations, in the homogenization medium. Thus, up to 3 mM calcium ion has been used in the preparation of membrane-enriched fractions (Takeuchi and Terayama, 1965) where it is reported to have a stabilizing effect on enzymes (Giesow, 1978); on the other hand, 1-5 mM EDTA has been used in the disruption of hamster small intestine epithelial cells (Miller and Crane 1961). The presence of divalent calcium ion in the homogenization medium has also been reported necessary for nuclear preservation (Hunter and Commerford, 1961).

The initial homogenate thus obtained is the starting point for subsequent fractionation, and it is a complex mixture reflecting the intricate organization of the original cells. The situation becomes increasingly complex if the homogenate is derived from a tissue with more than one cell type. For example, the mid-luteal corpus luteum consists of theca interna as well as luteinized granulosa cells (Enders, 1973). Furthermore, the latter may be subdivided into large and small cells with differing proportions of mitochondria in their cytoplasm (Priedkalns and Weber, 1968a,b).

From here the subcellular fractionation procedure can go in one of two directions: analytical or preparative. In the analytical approach fractionation is made without attempt at immediate purification of the desired organelle, but instead

on the sole basis of the physical properties of the particles, e.g., equilibrium density or surface charge. On the other hand, the preparative approach aims at purifying maximal amounts of a particular organelle, accompanied by assessment of the purity of the final, enriched fraction. Ideally, the two aims are combined and fractionation yields a collection of purified fractions separated in a quantitative fashion. In practice, a compromise has to be made between purity and yield. In the subcellular fractionation of a tissue for the first time, as in the investigation reported here of the intracellular location of progesterone in the bovine corpus luteum, the analytical approach must take precedence over the preparative approach although without abandoning attempts to obtain maximal yields where appropriate within the analytical scheme (Beaufay et al., 1974; Steck, 1972). Morphological and cytochemical methods are complementary to the biochemical analysis, but must not, by themselves, be used as criteria of purity of fractionated subcellular particles unless special efforts are undertaken to make their application quantitative and statistically valid (Baudhuim et al., 1967).

Fractionation of subcellular organelles is most commonly achieved using centrifugation, where the particles are separated according to their differences in size and/or buoyant densities. Less common methods, which are based on the other physiochemical properties of the subcellular particles, include chromatographic separation on celite columns (Hymer and McShan, 1962), microfiltration (Perdue and McShan, 1962), electrophoresis and phase partition (de Duve, 1964; Steck, 1972). Affinity chromatography may be a method

for the future (Puca et al., 1972; Cheng et al., 1976).

The centrifugation techniques are overwhelmingly the most popular and separate subcellular particles according to their size (differential centrifugation or differential pelleting), buoyant density (sedimentation-equilibrium), or by a combination of both (rate-zonal). In addition, density gradient centrifugation can utilize either continuous or discontinuous gradients. The most powerful fractionation procedure for most tissues has been differential centrifugation followed by density gradient centrifugation in a continuous gradient (de Duve, 1964). Even with the latter, considerable overlap of one subcellular organelle with another occurs, so that centrifugation in continuous gradients does not usually enable the complete purification of one organelle from the others but, at best, provides an enriched fraction (de Duve, 1964; De Pierre and Karnovsky, 1973). The analytical advantages of density gradient centrifugation have in recent years been extended to the preparative scale by the introduction of zonal rotors, which allow large amounts of material to be fractionated (Connock et al., 1974; Cline and Ryel 1971).

The final stage in any subcellular fractionation procedure is the biochemical, morphological and cytochemical analysis of the constituents and components, respectively, of the separated fractions. Biochemical analysis usually involves the use of marker enzymes, i.e., enzymes whose activities are known to be found in a single class of organelles (single location) and whose activities remain constant throughout the course of fractionation (de Duve, 1964; Steck, 1972). A set of generally accepted markers has now been established. For

example, succinate dehydrogenase, which resides in the outer mitochondrial membrane, is a commonly used mitochondrial marker; the distribution of acid phosphatase is considered a reliable indicator of lysosomes although it seems advisable to assay for more than one lysosomal enzyme now that several subclasses of this organelle have been separated from each other by differential and density gradient centrifugation (Michell et al., 1970).

A marker enzyme may be unevenly distributed within one type of subcellular entity; e.g., Evans (1969, 1970) found that his light subfraction of plasma membrane contained most of the 5'-nucleotidase, leucine aminopeptidase, and Mg^{2+}-ATPase activities, whereas his heavy subfraction contained most of the Na^{+}, K^{+}-ATPase activity. Also, the location of a marker enzyme may vary in different tissues, so that glucose-6-phosphate dehydrogenase was incorrectly assigned as a microsomal marker, rather than as a soluble marker, in homogenates of bovine corpus luteum (Gospodarowicz, 1973) by analogy with its microsomal location in rat liver (Sottacasa et al., 1967; Amar-Costesec et al., 1974). For these reasons, it is advisable, wherever possible, to ensure the suitability of the marker enzymes used in the subcellular fractionation of a particular tissue for the first time. Organelle markers need not be enzymes. Thus, progesterone would be expected to be a suitable "biochemical handle" for progesterone-secreting granules in the corpus luteum. The use of enzyme and non-enzyme markers in the analysis of fractions from density gradients allows the biochemical identification of those subcellular constituents which have been separated on the basis of their sedimentation-

rate or density characteristics.

In addition to using enzyme and non-enzyme markers to identify the composition of subcellular fractions, measurement of the total and specific activity of an appropriate marker in the initial homogenate and in isolated fractions enables the recovery and enrichment of its subcellular constituent to be calculated; conversely, contamination may be quantified (de Duve, 1964). The biochemical assays then allow the construction of a "flow-sheet" or "balance-sheet", whereby the enzyme activities and masses of non-enzyme markers in each fraction are compared with the activities and masses in the starting homogenate, and any abnormal losses or gains are detected[2].

Finally, a cautionary comment should be made concerning the interpretation of the analytical data arising from a subcellular fractionation experiment: organelle damage or even breakdown can occur during the course of the experiment which may, for example, involve many hours of centrifugation. In the course of the studies reported here, the time taken to complete fractionation experiments was reduced to only 3.5 h by using sucrose gradients in a zonal rotor.

3.1.2 Subcellular Fractionation of the Bovine Corpus Luteum

Gospodarowicz (1973) has fractionated bovine corpus luteum to obtain a plasma membrane-enriched fraction and Azhar and Menon (1976) and Rao and Mitra (1977) used Gospodarowicz's fractionation procedure in their investigation of prostaglandin receptors in the plasma membrane fraction of bovine corpus luteum. In these fractionation studies of the bovine corpus

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2: Enzyme latency, i.e., the increase in activity associated with the release of an enzyme from disrupted organelles, compared with the activity of intact organelles, would be revealed at this stage.

luteum, and in the fractionation of tissue from hamster corpus luteum (Chatterjee and Greenwald, 1976), the preparative approach was followed so that little attention was directed toward the distributions of unwanted organelles, measurements of which are essential to any assessment of the enrichment of the organelle under study. Also, the limited number of marker enzymes that were used for characterization of the composition of isolated fractions were assayed under conditions that were known to be optimal for their assay in other tissues (principally rat liver) without prior determination of their suitability to homogenates of corpus luteum.

Bramley and Ryan (1978a,b) have recently reported the validation of a number of enzyme markers to the fractionation of superovulated rat ovary. They used the analytical approach to subcellular fractionation and reported isolation of two discrete fractions of plasma membrane, each containing different complements of marker enzymes. This was in contrast to the earlier isolations of plasma membrane from bovine corpus luteum of Gospodarowicz (1973), Azhar and Menon (1976) and Rao and Mitra (1977), who all obtained only one, apparently homogeneous, plasma membrane fraction. The latter studies also lacked a suitable microsomal (mainly endoplasmic reticulum) marker so that their estimation of the degree of purity of their plasma membrane fractions was probably not valid.

Subcellular fractionation of the bovine corpus luteum was undertaken here with the aim of obtaining analytical information on the intracellular location of progesterone, the primary secretory product of this tissue. The secretion of

progesterone inside membrane-bound secretory granules entails an important consequence, which is open to experimental test. For the hormone to be sequestered inside granules at higher concentration than the surrounding cytoplasm it must be bound in some way, possibly to protein; otherwise it would diffuse across the granule membrane. Therefore, intragranular progesterone, by being bound to macromolecules such as protein, is rendered particulate and will be sedimentable by centrifugation. Furthermore, if the progesterone remains in the granules after homogenization of the luteal cells then it is predicted that these progesterone-containing granules will have a characteristic buoyant density in sucrose density gradients, and may thus be resolvable from the other subcellular particles.

The selective enrichment of such granules was a secondary aim, bearing in mind that morphometric analysis and cytochemical studies of this tissue (Chapter 2) showed that they represented only 2-3% of the volume of the luteal cell cytoplasm. This is to be compared to the neural lobe of the rat pituitary, where Morris (1976) calculated that the neurosecretory granules occupied at least 6% of the volume of the lobe, and whose cells did not contain such high proportions of agranular endoplasmic reticulum. Purification of the neurosecretory granules of rabbit and bovine posterior pituitaries have been reported. In both cases, 80% of the activities of oxytocin and vasopression in the tissue were sedimentable after homogenization (Barer et al., 1963; Dean and Hope, 1968). The cells of the anterior pituitary also contain numerous hormone granules, so that their isolation has been possible from castrated male rats using subcellular fractionation techniques (Perdue and

McShan, 1962).

In studies of the sheep corpus luteum, differential centrifugation of mid-luteal homogenates resulted in recovery of up to one-third of the total progesterone in the 10,700 g pellet (Gemmell et al., 1974). The finding that a significant proportion of steroid hormone was sedimentable corroborated the ultrastructural evidence for progesterone secretory granules in the sheep corpus luteum.

Kramers et al. (1975) reported that the bovine corpus luteum also contained a particulate fraction of progesterone which was sedimented at 10,000 g and resolvable on sucrose density gradients. In another abstract, Abel et al. (1977) reported, from *in vitro* studies of sheep corpus luteum, that the production of progesterone was accompanied by the release of two proteins. These proteins co-migrated on polyacrylamide gells with protein extracted from granule-enriched fractions obtained from sucrose density gradients.

3.1.3 Experimental Approaches

The objectives of this study were to use subcellular fractionation techniques to confirm that the mid-luteal corpus luteum of the cow, like the sheep, contained a sedimentable fraction of progesterone and then to identify those subcellular components with which this sedimentable fraction of hormone was associated. The assignment of particulate progesterone to a particular subcellular organelle would provide evidence in support of the hypothesis that this hormone is localized in granules.

Therefore, the experimental approach was as follows:

1. Establish the proportion of progesterone in the mid-luteal

corpus luteum of the cow that is sedimentable at various g-forces.

2. Determine the centrifugation conditions that simultaneously provide maximal sedimentation of progesterone with minimal contamination of subcellular constituents.
3. Obtain progesterone- and granule-enriched fractions by separating subcellular constituents according to their differing buoyant densities in equilibrium density gradients.
4. Monitor the partitioning of progesterone and cell organelles by radioimmunoassay and marker enzyme analysis, respectively, throughout all fractionation steps.
5. Examine gradient fractions, both morphologically and cytochemically, as an adjunct to the biochemical analysis.
6. Subject the sedimentable fraction of progesterone to various disruptive treatments prior to fractionation on sucrose density gradients. The object was to determine the strength and nature of the binding interaction that renders progesterone particulate; e.g., to determine whether or not particulate hormone was localized in vesicles or in granules, which could be disrupted to release their contents.
7. Determine whether or not the occurrance of progesterone in a particulate fraction was a phenomenon specific to this steroid hormone.

3.2 METHODS

3.2.1 Tissue Manipulations

The following details the initial steps in the fractionation of bovine corpus luteum that was utilized in all experiments. Mid-luteal phase bovine corpora lutea were obtained from two local abattoirs. The material was placed on ice within 10 min of death of the cows. All subsequent manipulations were performed at 0-4^{o}C. In the laboratory, some 60-90 min later, the corpora lutea were decapsulated, minced using a Climpex tissue mincer (fitted with a stainless steel grid perforated by 1.7 mm diameter holes), and weighed. This procedure removed most of the connective tissue, and the theca externa surrounding the mass of luteinized granulosa and theca cells.

All sucrose solutions were buffered with either 0.01 M Trizma 7.0[3] (pH 7.5 at 5^{o}C) or 0.01 M HEPES, pH 7.4 (at 22^{o}C). A 10% w/v homogenate in buffered 0.25 M sucrose was obtained (H_1) with three to five strokes of a Potter-Elvehjem homogenizer (Thomas, Philadelphia, Pa., U.S.A. No. C44491, 0.177 mm clearance) driven by an MSE homogenizer at quarter-speed. The homogenate (H_1) was centrifuged at 600 x g_{max} for 5 min (MSE, Mistral 6L) to remove nuclei, large pieces of cell debris and incompletely homogenized and unbroken cells. After centrifugation the supernatant (S_1) was decanted and recentrifuged at 10,000 x g_{av} for 30 min (Beckman Spinco L2-65B). Floating lipid was removed with a clean dry glass rod. The supernatant (S_2) was decanted and the pellet resuspended in buffered 0.25 M sucrose (H_2, 10,000 g pellet) by 3 handstrokes

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3: 0.01 M Trizma 7.0 is equivalent to 0.01 M Tris-HCl, pH 7.5 at 5^{o}C.

of a Potter-Elvehjem homogenizer (No. A8427, 0.127 mm clearance) to a total volume in ml equivalent to the number of grams of original minced corpus luteum tissue (after allowing for sampling). Up to 1.5 ml of this 10,000 x g_{av} pellet (H_2) was loaded onto sucrose density gradients (Fig. 3-1 shows the fractionation scheme).

In some experiments radioactively-labelled steroids, plus and minus non-radioactive steroid, were equilibrated with H_2 for 1 h on ice before aliquots were loaded onto sucrose density gradients.

In one of the preliminary studies, separate aliquots of S_1 were used in a differential centrifugation experiment with average g-forces ranging from 2,000 to 40,000.

A microsomal fraction (H_3, 100,000 g pellet) was obtained by centrifuging supernatant S_2 at 96,300 x g_{av} for 30 min, and resuspending the pellet in buffered sucrose. The supernatant (S_3) was retained. In some experiments aliquots of the cytosol were loaded onto sucrose density gradients as described below.

3.2.2. Density Gradient Centrifugation

3.2.2.1 Analytical Sucrose Gradients

Initially, linear 26-45% w/w buffered sucrose gradients were formed by the "two-pump" method[4] over 0.5 ml or 2 ml cushions of 55% w/w sucrose in centrifuge tubes of the Beckman SW41 or SW27 rotors, respectively. After loading, they were centrifuged in either SW41 or SW27 rotors for 2.25 h and 2.5 h

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4: The "two-pump" method of forming linear sucrose gradients consists of pumping the heavy limit solution of sucrose into the centrifuge tube at exactly twice the rate that the light limit solution is pumped into the heavy solution. If equal volumes of each solution are used, a linear gradient is obtained.

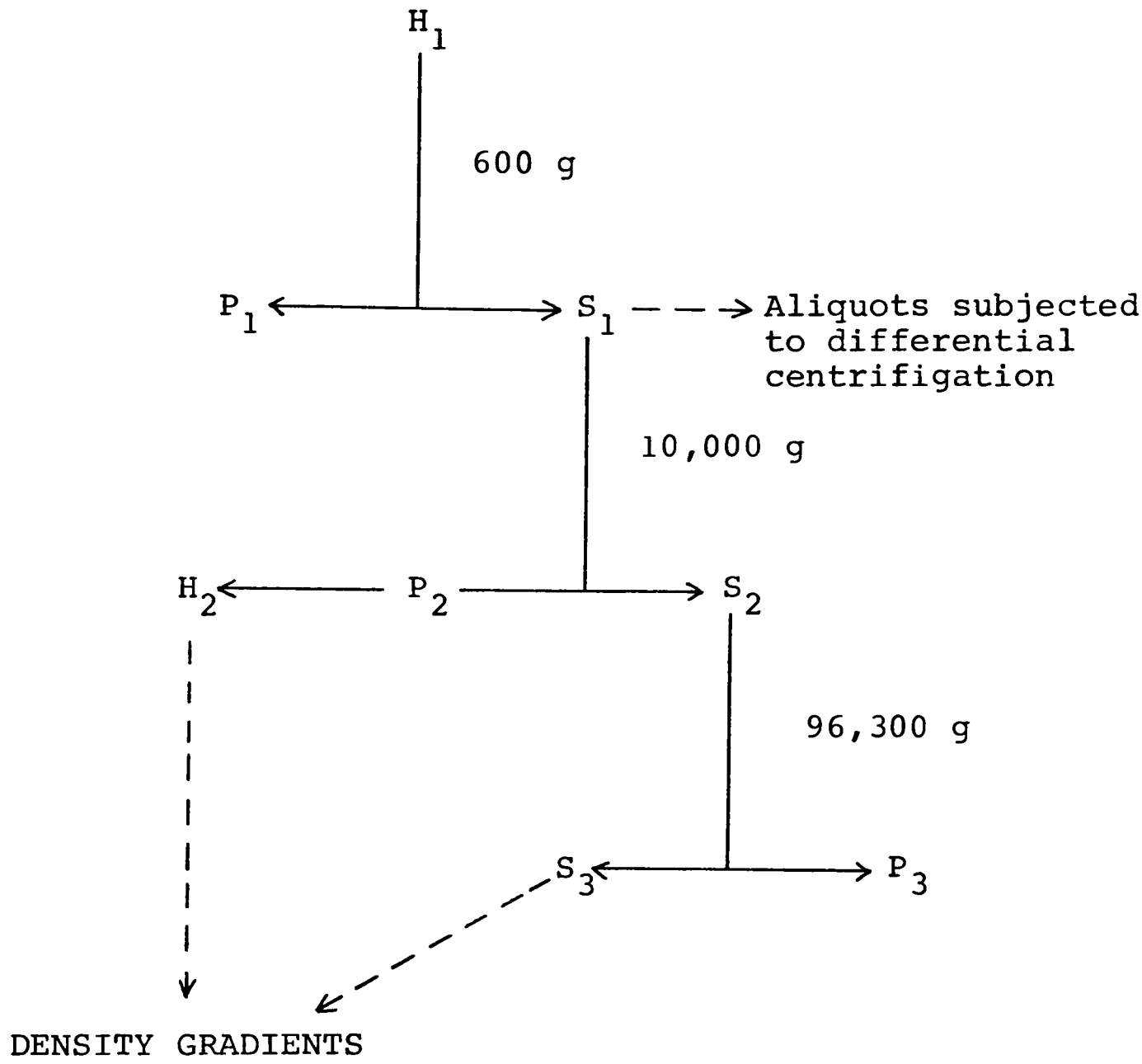


Fig. 3-1: Tissue manipulations of homogenates of pooled mid-luteal corpora lutea

at 25,000 rpm (82,500 g_{av}) and 4°C. Elution into 0.5 ml or 2 ml fractions, respectively, was performed by placing a long 16-gauge stainless-steel needle (centred by a loose-fitting cork) through the gradient and pumping out from the bottom.

Subsequently, linear 15-50% w/w buffered sucrose gradients were prepared using an ISCO Model 570 gradient former. The gradients were usually formed in polyallomer rather than cellulose nitrate tubes (to minimize non-specific adsorption of steroids) and were of 36 ml capacity with a 1.5 ml underlay of 50% w/w sucrose. After loading, the gradients were centrifuged in a Beckman SW27 rotor for 2.25 h as above. They were eluted into 2 ml fractions by upward displacement with 60% w/w sucrose containing 0.001% w/v bromophenol blue using an ISCO Model 184 tube-holding and piercing mechanism coupled to a non-peristaltic pump.

3.2.2.2 Analytical Ficoll Gradients

Linear buffered 0-20% w/w and 5-25% w/w Ficoll gradients in 0.25 M sucrose were prepared and eluted as above using the ISCO apparatus. Centrifugation was also performed as above but times varied between 2.25 h, 17.5 h and 22.5 h.

The sucrose concentration or density (20°/4°) of individual fractions was determined by refractometry (Bellingham and Stanley refractometer, Tunbridge Wells, U.K.). Fractions were stored, often in multiple aliquots, at -20°C until further analysis was carried out. In some experiments, fractions of identical density from more than one gradient were pooled and stored in multiple aliquots. This ensured that there were no more than one or two cycles of freezing and thawing prior to each enzymatic analysis.

When pooled gradient fractions were processed for morphological examination or cytochemical studies, they were fixed directly in 3% v/v glutaraldehyde, collected by centrifugation at either 5,000 or 10,000 x g_{av}, and treated as described in Chapter 2 for tissue sections.

3.2.2.3 Preparative Zonal Gradients

Zonal gradients using the Beckman Ti-14 rotor were prepared and run according to the company's instruction manual. The rotor was loaded with the following sequence of sucrose solutions, buffered by 10 mM HEPES, pH 7.4:

(i) 400 ml gradient (200 ml 15% w/w x 200 ml 40% w/w sucrose or 200 ml 15% w/w x 200 ml 50% w/w sucrose)

(ii) approximately 250 ml 55% w/w sucrose (as cushion, some of which was displaced to make room for sample and overlay)

(iii) 20 ml sample (H_2 in buffered 0.25 M sucrose)

(iv) 80 ml 5% w/w overlay (located nearest to the core of the rotor)

Centrifugation was performed at 12-5^{o}C and 30,000 rpm for exactly 1.5 h, timed from when the rotor achieved the set speed.

The gradient was eluted by displacement with 60% w/w sucrose - 0.001% w/v bromophenol blue into 10 ml fractions, some of which were processed for morphological examination using the electron microscope.

3.2.3 Assays

3.2.3.1 Progesterone Radioimmunoassay

Progesterone was measured by radioimmunoassay (Challis et al., 1973) using an antiserum (456/6) raised in rabbits against 11α-succinyl-progesterone conjugated with bovine

serum albumin. Pregnenolone, 17α-hydroxy-progesterone, 20α-hydroxy-pregn-4-en-3-one, cortisol and oestadiol-17β showed less than 1.5% cross reaction with the antiserum (Table 3-1). The solvent blanks were routinely equivalent to <5 $pg.ml^{-1}$. Recovery of progesterone after extraction was 83.6 ± 7.5% (mean ± SD, n=20). When assaying the progesterone content of subcellular fractions, the use of 0.1 M NaOH did not increase the efficiency of extraction of hormone, and was therefore routinely omitted. The progesterone level of a stock of corpus luteum S_1 supernatant pool was routinely determined as an assay control; its value was 21.1 ± 3.3 $ng.ml^{-1}$ (mean ± SD, n=51). The interassay coefficient of variation was 15.6%.

3.2.3.2 Protein Assays

Protein concentration was determined in sucrose solutions buffered by 0.01 M Trizma 7.0 according to Lowry et al. (1951). Before assay, the protein was precipitated with 5% w/v trichloroacetic acid and then dissolved in 0.5 M NaOH. When the sucrose solutions were buffered by 0.01 M HEPES, the fluorimetric method of Böhlen et al. (1973) was used for protein determination. In both assays bovine serum albumin was used as the protein standard. Over the concentration ranges 0-50 $\mu g.ml^{-1}$ and 50-500 $\mu g.ml^{-1}$, the two methods correlated to within 10% and 5%, respectively.

3.2.3.3 Marker Enzyme Assays

Enzyme activities of fractionation stages (H_1,S_1, etc.) and density gradient fractions were used as markers for subcellular constituents (Table 3-2).

All enzyme determinations of the fractionation stages were carried out in duplicate at least, whereas only single

Table 3-1: Cross reactions of steroids in the progesterone radioimmunoassay using antiserum 456/6.

The ability of different cross-reacting steroids to decrease the binding of a constant amount of ^{3}H-progesterone was measured. The data is expressed in two ways:

$$\text{Relative binding} = \frac{\%\text{ displacement by 1 ng steroid}}{\%\text{ displacement by 1 ng progesterone}} \times 100$$

$$\%\text{ Cross reaction} = \frac{\text{ng progesterone to give 50\% drop in cpm bound}}{\text{ng steroid to give 50\% drop in cpm bound}} \times 100$$

(Data obtained by Miss G. Pooley in the same laboratory).

	% Cross Reaction	% Relative Binding
Progesterone	100.0	100.0
11α-hydroxpregn-4-en-3, 20-dione	177.0	82.7
5α-pregnane-3,20-dione	35.3	87.6
11β-hydroxpregn-4-en-3, 20-dione	35.0	77.3
Deoxycorticosterone	12.5	62.9
Corticosterone	3.8	43.2
5β-pregnane-3,20-dione	2.5	45.2
Oestradiol-17β	0.7	34.5
16α-hydroxprogesterone	0.5	34.5
20β-hydroxypregn-4-en-3-one	0.2	35.7
5α-androstane-3,17-dione	<0.1	0
5β-androstane-3,17-dione	<0.1	17.2
Dihydroepiandrosterone	<0.1	2.3
Cholest-4-en-3-one	<0.1	2.5
Cholesterol	<0.1	24.1
Cortisone	<0.1	7.6
Oestriol	<0.1	5.0
Oestrone	<0.1	0
Oestradiol-17α	<0.1	0
3β,17α-dihydroxypregn-5-en-20-one	<0.1	17.9
17α-hydroxypregn-4-en-3-one	<0.1	41.0
20α-hydroxypregn-4-en-3-one	<0.1	31.0
Pregnenolone	<0.1	26.2
Testosterone	<0.1	17.9

Marker enzyme	Subcellular organelle
Succinate dehydrogenase	Mitochondrial inner membrane
Acid phosphatase	Lysosomes
β-Glucuronidase	Lysosomes
Alkaline phosphatase	Plasma membrane
Catalase	Microperoxisomes
NADH-cytochrome c reductase (rotenone-insensitive)	Endoplasmic reticulum (plus outer mitochondrial membrane)
NADPH-cytochrome c reductase (rotenone-insensitive)	Endoplasmic reticulum
Inosine diphosphatase	Endoplasmic reticulum
Lactate dehydrogenase	Soluble

Table 3-2: The marker enzymes used to identify various subcellular constituents of the cells of mid-luteal corpora lutea from cows.

The assignments are based on the work here as well as on the work of others.

References: De Duve, 1964, 1967; Sottocassa et al., 1967; Steck, 1972; and Beaufay et al., 1974.

enzyme determinations were possible for some gradients due to the number of different assays that were carried out on the 2 ml fractions. Appropriate substrate and sample blanks were included in all cases, and the assays were all validated using the initial homogenate (H_1) and/or supernatant (S_1).

Enzyme activities were calculated as Enzyme Units (E.U., µmoles of product formed per minute) except for succinate dehydrogenase and catalase activities which were Δ Absorbance at 490 nm per min and $k.sec^{-1}$ (the 1st order rate constant), respectively.

1. Succinate dehydrogenase (EC 1.3.99.1) was measured by the reduction of added succinate to form the red-coloured formazan succinate-2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium, according to Pennington (1961).

The 1 ml reaction mixture contained:

0.05 M potassium phosphate, pH 7.4

0.1% w/v INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium)

0.05 M sodium succinate.

The assay was started by the addition of substrate on ice and incubated at 37°C for 30 min. The reaction was stopped by placing the assay tubes on ice and immediately adding ice-cold 10% w/v trichloroacetic acid.

The reaction product was extracted into ethyl acetate and the phases were separated by gentle centrifugation (600 g). The absorbance of the upper phase at 490 nm was measured. Control reactions lacked substrate and reagent blanks lacked enzyme solution. The reaction was zero-order with respect to protein concentration up to 50 $A_{490}.min^{-1}.ml^{-1}$ and up to 60 min

of incubation.

2. Acid phosphatase (EC 3.1.3.2) was measured by modification of the methods of Bergmeyer et al. (1974) and Hübscher and West (1965) so that a direct colorimetric response was obtained. The 3 ml reaction mixture contained:

0.1 M sodium acetate buffer, pH 5.6

4 mM EDTA (disodium salt) (to inhibit alkaline phosphatase)

0.1% v/v Triton X-100

8 mM p-nitrophenol phosphate, sodium salt.

Control reactions lacked substrate and reagent blanks lacked enzyme solution. The reaction was stopped after 30 min at 37^{o}C by the addition of NaOH to a final concentration of 0.33 M, and the absorbance at 405 nm determined. The reaction was zero-order with respect to protein concentration up to 12×10^{-3} EU.ml^{-1} and up to 40 min of incubation.

3. β-Glucuronidase (EC 3.2.1.31) determination was modified from Fishman (1974) and Bergmeyer et al. (1974). The 1 ml reaction mixture contained:

0.095 M sodium acetate buffer, pH 4.5

0.1% v/v Triton X-100 (to prevent enzyme latency)

4 mM p-nitrophenol-β-D-glucuronide, sodium salt.

Control reactions lacked substrate and reagent blanks lacked enzyme solution. The reaction was stopped after 4 h at 37^{o}C by the addition of 3 ml of 0.2 M glycine, adjusted to pH 10.7 with NaOH, and the absorbance at 405 nm determined. The reaction displaced zero-order kinetics with respect to protein concentration up to 0.4 EU.ml^{-1} and up to 4 h of incubation.

4. Alkaline phosphatase (EC 3.1.3.1) determination was

based on the method of Hübscher and West (1965), but p-nitrophenol phosphate was used as substrate. The 1 ml reaction mixture contained:

0.04 M ethanolamine buffer, pH 10.5

5 mM $MgCl_2$

1 mM NaF (to inhibit acid phosphatase)

0.1% v/v Triton X-100

0.5 mM p-nitrophenol phosphate, sodium salt.

Control reactions lacked substrate and reagent blanks lacked enzyme solution. After incubation for 30 min at 37°C, the reaction was terminated by the addition of NaOH to a final concentration of 0.07 M and the absorbance at 405 nm determined. The reaction was zero-order to a protein concentration of at least 0.03 $EU.ml^{-1}$ and up to 2 h of incubation.

5. Catalase (EC 1.11.1.6) was determined without modification according to Aebi (1974).

The 3 ml reaction mixture contained:

0.05 M potassium phosphate buffer, pH 7.0

0.01 M H_2O_2.

The buffer and H_2O_2 solution were brought to 30°C (A_{240}= 0.4-0.6) and the reaction was started by the addition of enzyme solution. The reaction was followed under initial conditions at 240 nm. Immediately prior to assay, aliquots of the enzyme solution were treated with Triton X-100 to a final concentration of 1% w/v; in some cases centrifugation was necessary to remove insoluble material. The kinetics of the catalase reaction were first-order, so enzymatic activity was defined in terms of a rate constant,

$$k = 1/\Delta t(sec) \times 2.3 \log(A_1/A_2)$$

where A_1 = absorbance at 0 sec and A_2 = absorbance at 60 sec.

6. NADH-cytochrome c reductase (rotenone-insensitive) (EC 1.6.2.1) and NADPH-cytochrome c reductase (rotenone-insensitive) (EC 1.6.2.3) determinations were modified from Fleischer and Fleischer (1967) and Sottacasa et al. (1967).

The 1 ml reaction mixtures contained:

0.05 M potassium phosphate buffer, pH 7.4

0.1% w/v ferricytochrome c (oxidized form)

0.005 mg rotenone (freshly prepared in ethanol)

0.2 mM KCN (freshly prepared)

0.02% w/v NADH or NADPH, sodium salts.

Buffer, cytochrome c, rotenone and enzyme solution were incubated at 37°C for 10 min, KCN was added to inhibit mitochondrial cytochrome c oxidase, and the reaction was started by the addition of NADH or NADPH. The enzymatic activities were assayed at 37°C by following the reduction of cytochrome c, monitored as a decrease in absorbance at 550 nm. Control reactions lacked NADH or NADPH. The reactions were zero-order.

7. Inosine diphosphatase (EC 3.6.1.6) was determined without modification according to Beaufay et al. (1974), and the released inorganic phosphate was measured according to Weil-Malherbe and Green (1951).

The 1 ml reaction mixture contained:

0.05 M Tris-HCl buffer, pH 7.5

5 mM $MgCl_2$

0.05% w/v sodium deoxycholate

5 mM inosine diphosphate (sodium salt)

in a Henly plastic tube.

The reaction was initiated by the addition of substrate

and incubated for 30 min at 37°C; control reactions lacked inosine diphosphate. The reaction was terminated in an ice-water bath by the addition of 2 ml of cold 0.154 M perchloric acid.

For the phosphate ion determination the following reagents were added directly to the terminated reaction mixture:

3 ml isobutanol

0.5 ml 5% w/v ammonium molybdate dissolved in 2 M sulphuric acid.

The mixture was vortexed for exactly 15 sec., following which 2.5 ml of the upper (isobutanol) phase was removed into a second plastic Henly tube.

To the second tube, the following reagents were added:

2.5 ml of acidified ethanol (10 ml concentrated H_2SO_4: 490 ml ethanol)

0.1 ml diluted $SnCl_2$ (stored as a stock solution of 10 g $SnCl_2$ per 25 ml of concentrated HCl and diluted 1:40 v/v freshly before use).

The absorbance of the blue-coloured reaction product at 750 nm was then determined, and compared against a standard curve ranging from 0 to 0.35 µmoles phosphate ion (0.35 µmoles gave an absorbance at 750 nm of 1.06 under these conditions). Appropriate substrate and enzyme controls were included throughout the procedure.

8. Lactate dehydrogenase (EC 1.1.1.27) was determined according to Bailey and Wilson (1968) and Willcox (1975).

The 3 ml reaction mixture contained:

0.1 M potassium phosphate, pH 7.5

0.33 M sodium pyruvate

0.14 mM NADH.

The lactate dehydrogenase (LDH) activity was assayed at 25°C by following the oxidation of NADH, monitored as a decrease in absorbance at 340 nm. A reagent blank was used in the reference cuvette.

3.3 RESULTS

3.3.1 Preliminary Experiments

3.3.1.1 Evaluation of Homogenization and Buffer Conditions

The aim was to determine the optimal method of homogenizing minces of corpora lutea in 0.25 M sucrose with a tissue : buffer ratio of 1 gm : 10 ml. Then, using the best conditions for tissue disruption, the effects of various buffers on the process were evaluated. Phase contrast microscopy was used to monitor the degree of tissue disruption, with the state of the dispersed nuclei acting as an indication of gross organelle damage.

Table 3-3 summarizes the results of various methods of tissue disruption and of the results of homogenization in a variety of isotonic sucrose media. Thus, the Silverson and Buhler homogenizers gave non-uniform tissue disruption with definite nuclear damage. The Potter-Elvehjem homogenizers did not cause discernible nuclear damage and were therefore preferable. Three to five strokes using the Thomas Potter-Elvehjem homogenizer was the procedure of choice, because it gave extensive tissue disruption without damaging the nuclei. No advantage was obtained using 10 to 15 strokes of the latter, especially as progressively more damage to subcellular organelles was expected under these conditions. Therefore, 3-5 strokes of the Thomas Potter-Elvehjem homogenizer was chosen for homogenization in the evaluation of buffer conditions and in all subsequent experiments.

Phase contrast microscopy showed that isotonic sucrose, unbuffered or buffered by Tris-HCl or HEPES, was a suitable medium for homogenization (Table 3-3B). The inclusion of the

Conditions of Homogenization	Results
Silverson, 30 sec at 1/3 speed + 30 sec at 2/3 speed	Surface foaming; non-uniform disruption of tissue; damaged nuclei
Buhler, 30 sec at 1/5 speed + 30 sec at 1/2 speed	Very non-uniform disruption of tissue; damaged nuclei
5 strokes at 50 rpm of Jencons Potter-Elvehjem	Extensive disruption of tissue; large pieces of cytoplasm; nuclei undamaged
3-5 strokes at 50 rpm of Thomas Potter-Elvehjem	Extensive and uniform disruption of tissue; nuclei undamaged
10 strokes at 50 rpm of Thomas Potter-Elvehjem	Extensive and uniform disruption of tissue; nuclei undamaged
15 strokes at 50 rpm of Thomas Potter-Elvehjem	Extensive and uniform disruption of tissue; nuclei undamaged

Table 3-3A: The effect on tissue dispersion of different methods of homogenizing minces of bovine corpus luteum tissue.

The homogenizing medium was 0.25 M sucrose. The clearances of the Jencon and Thomas Potter-Elvehjem homogenizers were 0.198 mm and 0.177 mm, respectively.

Homogenizing Medium	Results
0.25 M sucrose	Extensive and uniform disruption of tissue; nuclei undamaged
0.25 M sucrose, 1-3 mM $CaCl_2$	Extensive disruption of tissue; nuclei undamaged; aggregation of cytoplasmic particles
0.25 M sucrose, 10 mM Tris-HCl, pH 8.0 ($22^{o}C$)	Extensive and uniform disruption of tissue; nuclei undamaged
0.25 M sucrose, 1-3 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0 ($22^{o}C$)	Extensive disruption of tissue; nuclei undamaged; aggregation of cytoplasmic particles
0.25 M sucrose, 10 mM HEPES, pH 7.4 ($22^{o}C$)	Extensive and uniform disruption of tissue; nuclei undamaged
0.25 M sucrose, 1-3 mM $CaCl_2$, 10 mM HEPES, pH 7.4 ($22^{o}C$)	Extensive disruption of tissue; nuclei undamaged; aggregation of cytoplasmic particles

Table 3-3B: The effect on tissue dispersion of varying the buffer and ionic composition of the homogenization medium, using 3-5 strokes at 50 rpm of the Thomas Potter-Elvehjem homogenizer.

non-physiological buffers Tris and HEPES in the homogenization medium was considered desirable for the preservation of marker enzyme activities. The fact that these buffers were non-physiological avoided possible complications in the assay of marker enzymes that can arise from the use of physiological buffers (e.g., phosphate buffers cannot be used when inosine diphosphatase activity is measured by the release of inorganic phosphate from the substrate, inosine diphosphate).

The presence of added 1-3 mM $CaCl_2$ caused extensive aggregation of disrupted cytoplasmic particles; without exogenous $CaCl_2$, the latter did not aggregate noticeably. The effect was irreversible and was not prevented by the subsequent addition of 3 mM EDTA as chelating agent. This aggregation phenomenon seemed specific to divalent calcium ion since $MgCl_2$ did not cause such aggregation. In tissue of other cell-types, the presence of divalent ions has caused aggregation of disrupted particles (see Steck, 1972). For example, the presence of either KCl or divalent magnesium prevents the separation on sucrose gradients of melanin granules from mitochondria (Menon and Haberman, 1974). Membrane permeability and enzyme stability have been shown to be sensitive to the concentration of both exogenous and endogenous divalent calcium ion (Heher and Milani, 1978; Geisow, 1978).

In conclusion, it was found that the best compromise between tissue disruption, satisfactory dispersion of cytoplasm, and minimal nuclear damage, was obtained using 3-5 strokes of the Thomas Potter-Elvehjem homogenizer in a medium of 0.25 M sucrose, buffered by either Tris or HEPES.

3.3.1.2 Morphological Examination of Tissue Fractions

As an additional check of the degree of tissue disruption and of the state of the disrupted subcellular components, pieces of the starting mince of corpus luteum tissue, plus aliquots of the different fractionation stages (obtained as shown in Fig. 3-1), were fixed by immersion in glutaraldehyde and subsequently viewed under the electron microscope.

Fig. 3-2 shows the result of this experiment: electron-dense granules were observed in minces of bovine corpus luteum analogous to those seen in intact tissue sections (Chapter 2). The initial homogenate (H_1) also contained electron-dense granules, indicating that at least some of them survived the process of homogenization. The electron micrographs of H_1 confirmed the observations made using the phase contrast microscope, namely, that the starting homogenate consisted of intact nuclei and organelles (except for mitochondria), separated from each other in those areas of the homogenate which did not contain either unbroken cells or large, intact fragments of cytoplasm. The mitochondria were altered in the presence of 0.25 M sucrose to become nearly spherical with dilated cristae so that they displayed a generally swollen appearance. This phenomenon has been found by various workers, in a variety of tissues, e.g., rat liver (de Duve, 1967).

The 600 g pellet (P_1) contained nuclei, large fragments of intact cytoplasm and some unbroken cells. In the latter intact electron-dense granules were evident. Thus, homogenization had not been complete and did not enable the recovery in the initial supernatant of the total complement of electron-dense granules from the corpora lutea used in these experiments.

Fig. 3-2: Electron micrographs of the different fractions obtained by fractionation of bovine corpus luteum

Material was fixed in Trizma-buffered 3% v/v glutaraldehyde for 1-2 h at 4°C, and embedded and sectioned as described in section 2.2.1 of Chapter 2. H_1, P_1 and H_2 were obtained using Trizma-buffered 0.25 M sucrose as homogenizing medium, as described in section 3.2.1 of this Chapter and outlined in Fig. 3-1.

A: Minced bovine corpus luteum, prior to homogenization

Mitochondria (M) and electron-dense bodies (→) appear morphologically-intact. The membraneous components (principally agranular endoplasmic reticulum) have become vesiculated (Mv), probably because fixation was not started until 90 min post mortem. M = mitochondrion; L = lipid droplet. X 17,250.

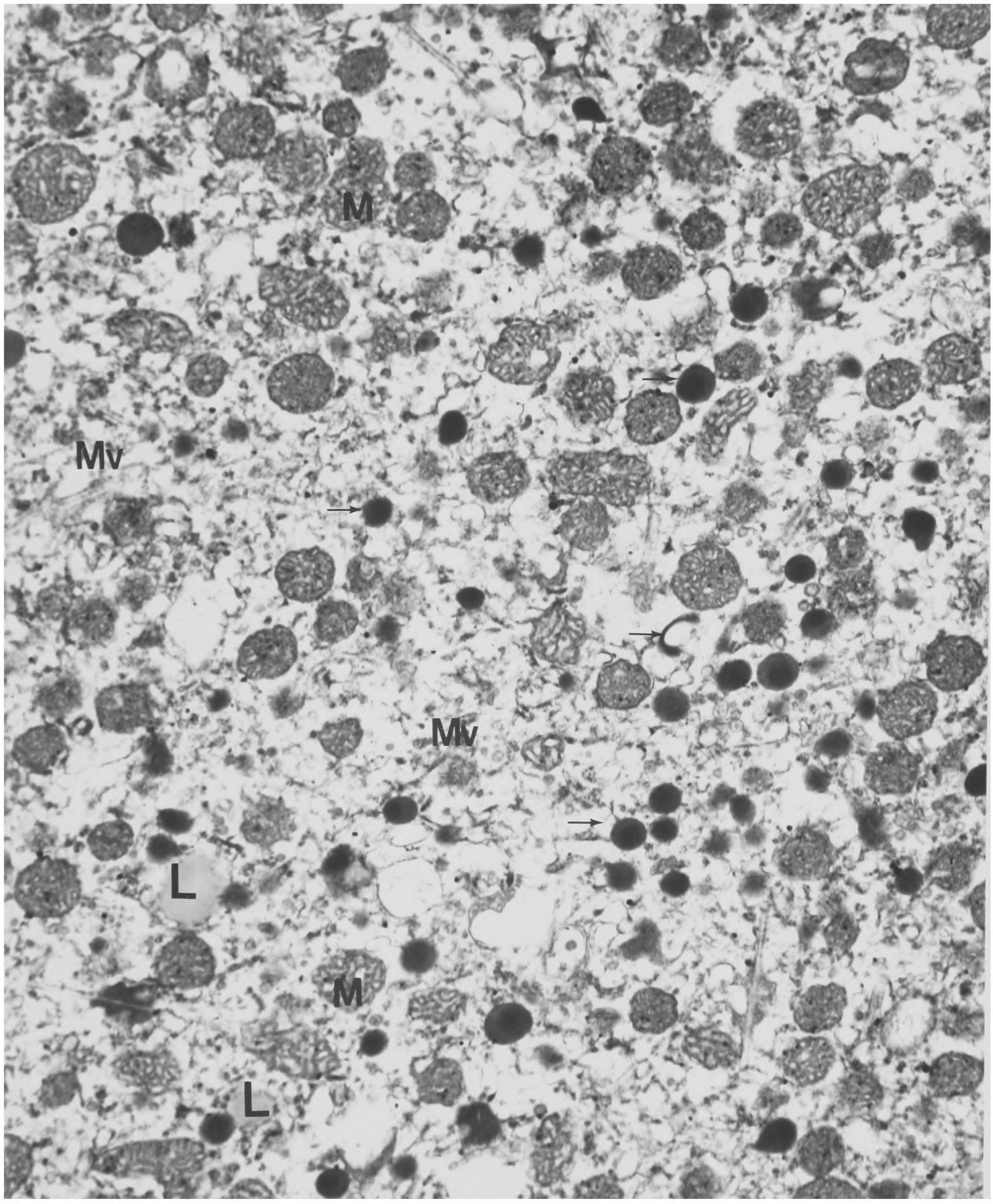

M
Mv
Mv
L
M
L

Fig. 3-2: Electron micrographs of the different fractions obtained by fractionation of bovine corpus luteum

B: The starting 10% w/v homogenate, H_1.

The homogenate principally consists of swollen mitochondria (M), dispersed membraneous components (Mv), disrupted Golgi membranes (G), and lipid droplets (L). Small numbers of electron-dense granules are also present (→), many of which appear morphologically-intact. X 33,750.

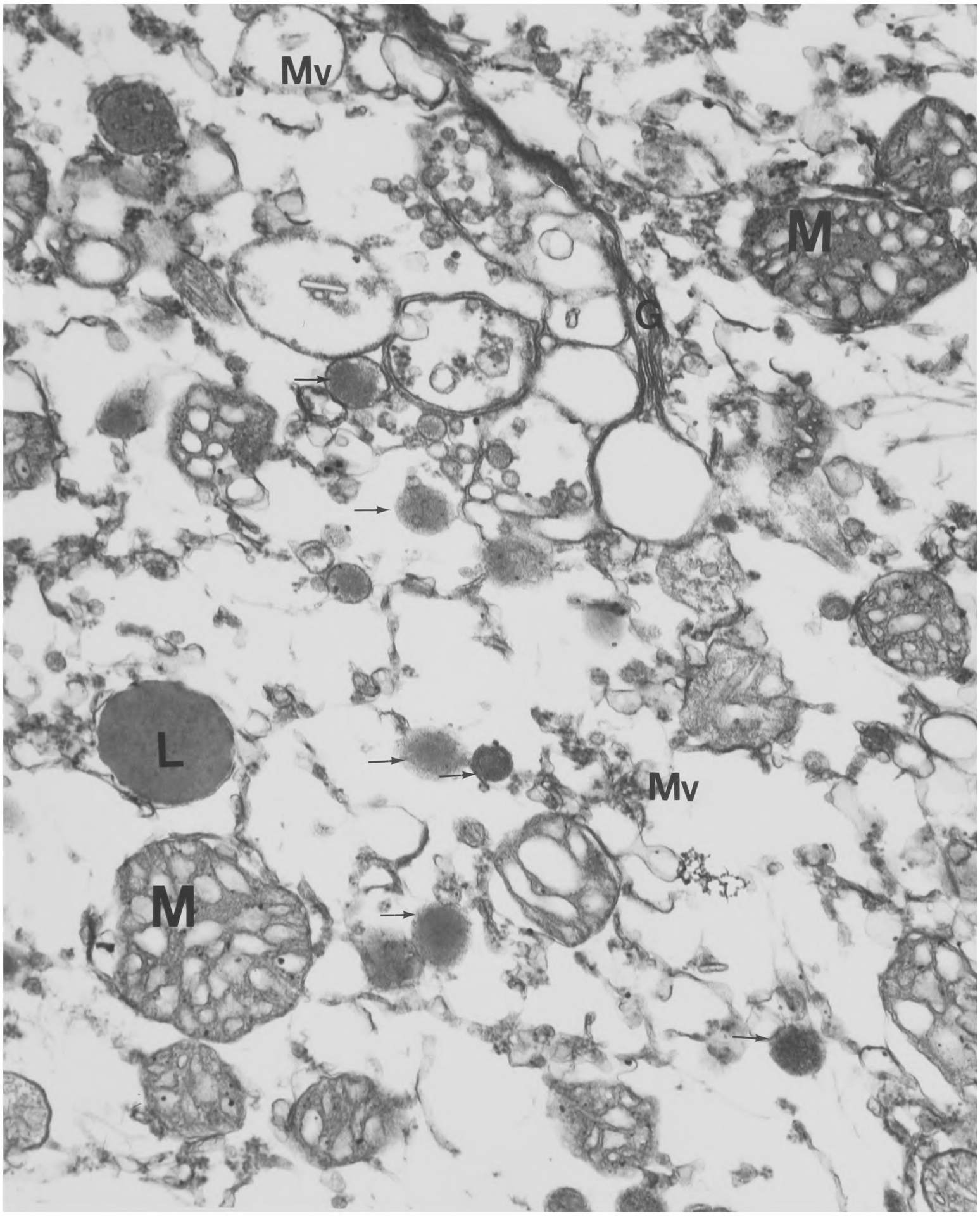

Mv
M
G
L
Mv
M

Fig. 3-2: Electron micrographs of the different fractions obtained by fractionation of bovine corpus luteum

C: The pellet, P_1, obtained by centrifuging H_1 for 5 min at 600 g.

The pellet consists of nuclei (N) and large fragments of cytoplasm. The latter contain lipid droplets (L), swollen mitochondria (M), disrupted endoplasmic reticulum and Golgi membranes (Mv) and electron-dense granules (→). X 8,600.

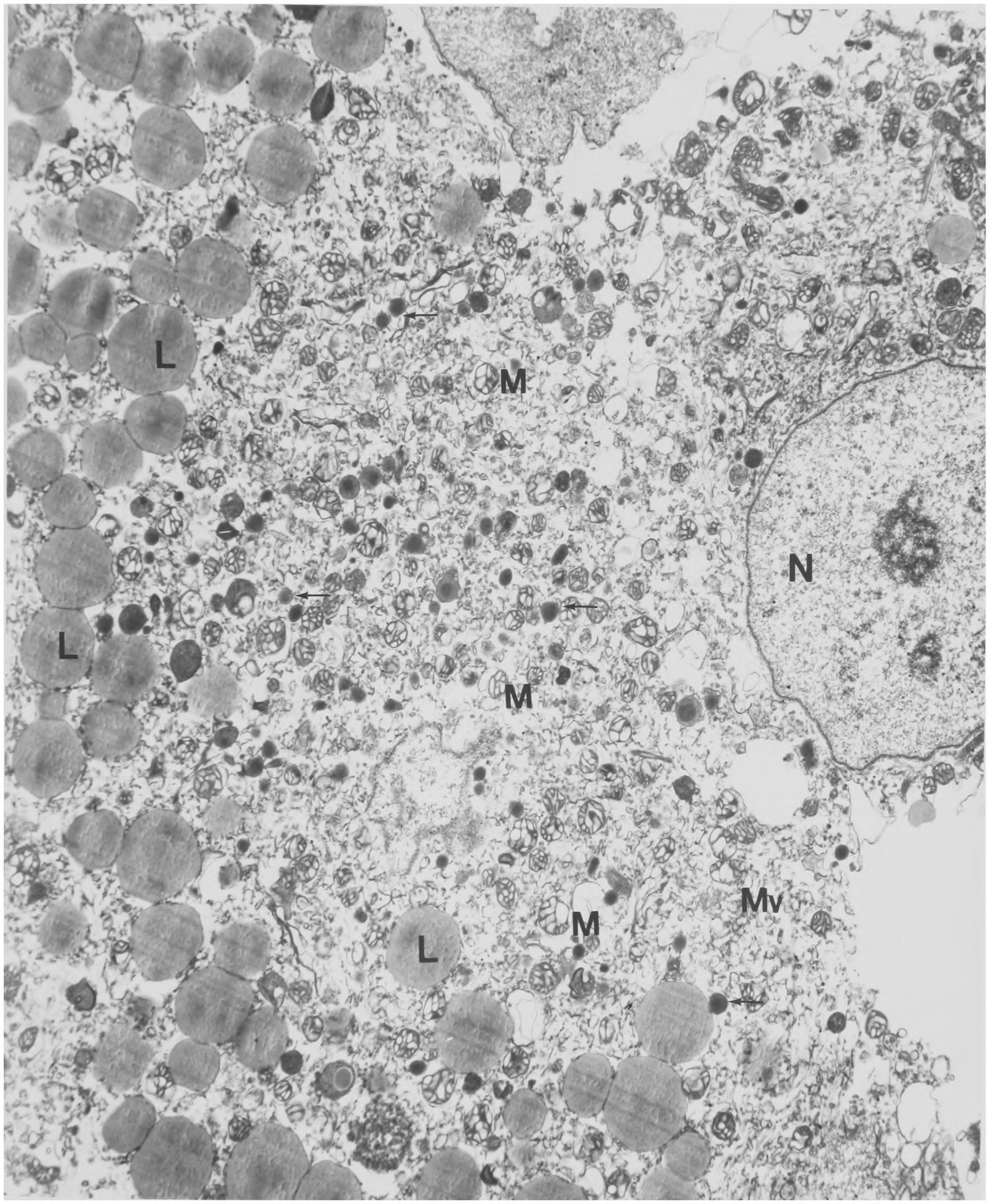
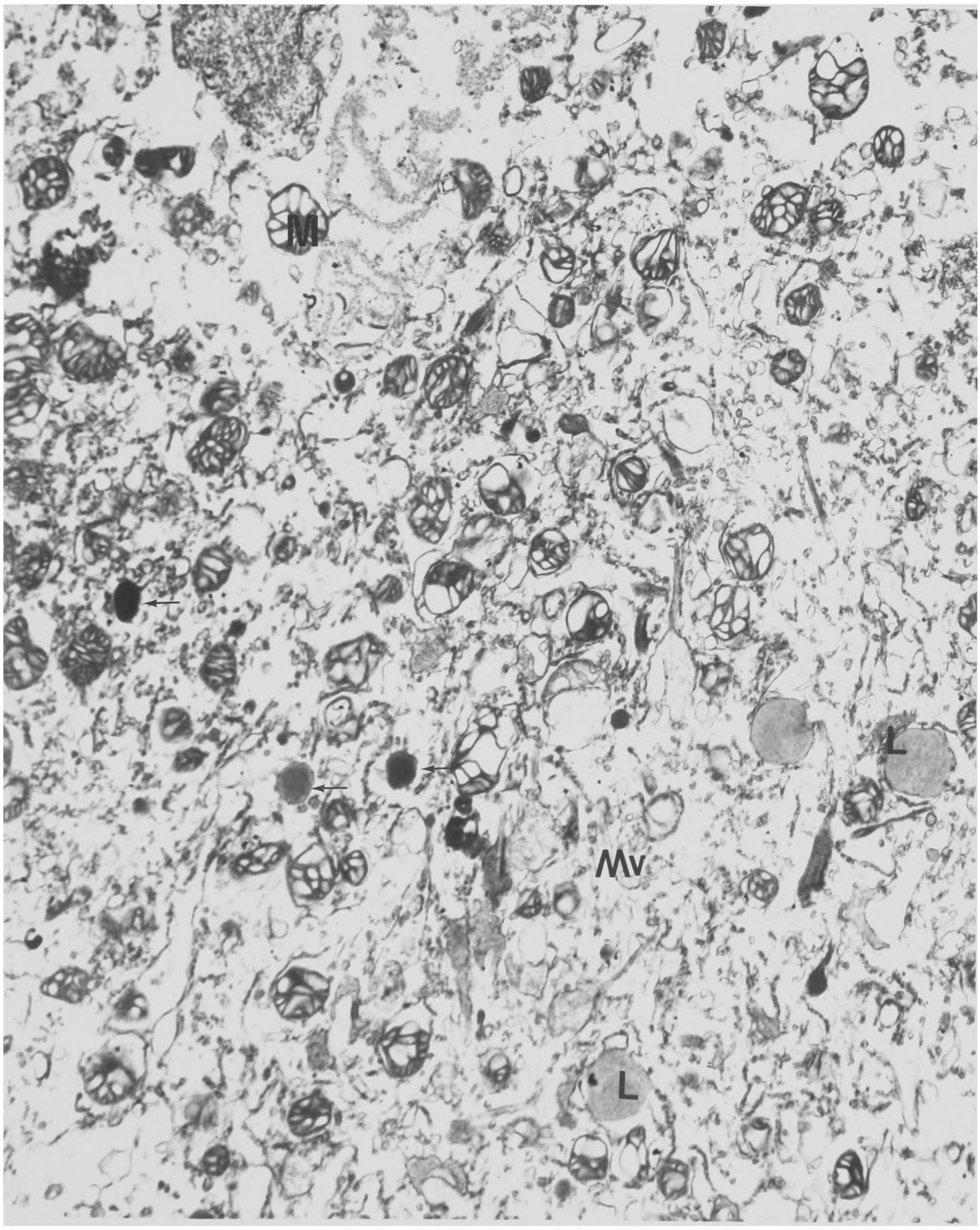

L
M
N
L
M
L
M
Mv

Fig. 3-2: Electron micrographs of the different fractions obtained by fractionation of bovine corpus luteum

D: The homogenate, H_2, obtained from resuspension of the 10,000 g pellet.

The homogenate principally consists of disrupted endoplasmic reticulum and Golgi membranes (Mv) and mitochondria (M). It also contains small numbers of lipid droplets (L) and electron-dense granules (→). The latter appear to be morphologically-intact. X 60,750.


M
L
Mv
L

Homogenate H_2 contained the material sedimented by centrifugation at 10,000 g, which was fractionated further on density gradients in subsequent experiments. It contained electron-dense granules with intact outer membranes, observed against a predominant background of other subcellular components, including mitochondria, lipid droplets, microperoxisomes and much vesiculated membraneous material. This morphological examination of the main fractions obtained during the course of fractionation of bovine corpus luteum minces indicated the following:

1. The electron-dense granules represented a very small proportion of the total of the subcellular particles observed in homogenates H_1 and H_2.
2. Homogenate H_2, the material which was later applied to density gradients, contained morphologically intact electron-dense granules.

3.3.1.3 Differential Centrifugation of Bovine Corpus Luteum

Previous differential centrifugation studies of homogenates of bovine corpus luteum did not use a wide range of marker enzymes and did not measure progesterone concentrations (Gospodarowicz, 1973; Azhar and Menon, 1976; Rao and Mitra, 1977). Gemmell et al. (1974) measured progesterone concentrations in a differential centrifugation experiment of homogenates of sheep corpus luteum but did not carry out marker enzyme analysis. Therefore, the objective of the present study was to determine the centrifugation conditions which gave the greatest selective enrichment of sedimentable progesterone with minimal recovery of subcellular organelles as judged by marker enzyme analysis (see Table 3-2 for the marker enzyme attributions).

Bovine corpus luteum homogenates were subjected to differential centrifugation as follows. Firstly, aliquots of the 600 g supernatant (S_1) were centrifuged at forces ranging between 2,000 g and 40,000 g, and marker enzyme analysis was performed on the supernatants and resuspended pellets. In subsequent experiments, the resuspended 10,000 g and 100,000 g pellets (H_2 and H_3, respectively) plus the 100,000 g supernatant (S_3) were subjected to more complete biochemical analysis as part of the routine monitoring of organelle damage during density gradient experiments. Table 3-4 shows the results from a differential centrifugation experiment. The cow corpus luteum contained a sedimentable fraction of progesterone, thus confirming the report of Kramers et al. (1975), and in agreement with the findings for the sheep corpus luteum (Gemmell et al., 1974). There was a progressive increase in the recovery of sedimentable progesterone in the pellet as the centrifugation forces were increased: at approximately 6,000 g about 25% of the hormone in S_1 was recovered in the pellet (or 33% relative to H_1), and at 15,000 g about 33% of the hormone in S_1 was recovered in the pellet (or 45% relative to H_1). These values are comparable to those found in the sheep corpus luteum where centrifugation of 10,700 g sedimented about 28% of the total progesterone (Gemmell et al., 1974).

A greater proportion of the denser subcellular organelles such as mitochondria and lysosomes were pelleted as the centrifugation forces were increased; however, considerable organelle breakdown occurred during the course of the experiment as revealed by the large amounts of marker enzyme activities for these organelles found in the last three supernants.

Table 3-4: Distribution of progesterone, protein and selected marker enzymes upon differential centrifugation of the 600 g-supernatant fraction of mid-luteal bovine corpora lutea.

The supernatant (S_1) was prepared from an homogenate of corpora lutea as described under Methods. The buffer was 10 mM Trizma 7.0 containing 0.25 M sucrose and 20 ml aliquots of S_1 were centrifuged in an SW41 rotor for 30 min at 4°C. The pellets were each resuspended to a total volume of 10 ml in the above medium and analyzed together with the supernatants for progesterone, protein and four marker enzymes.

The percent of progesterone and protein mass, succinate dehydrogenase, lactate dehydrogenase and acid phosphatase activities in each fraction are expressed relative to S_1, set at 100%. Alkaline phosphatase activity was more variable from fraction to fraction, and is therefore expressed relative to the total activity recovered at each centrifugation step, set at 100%.

ND = not determined

Average centrifugation force, g		Progesterone	Protein	SDH	Acid phosphatase	Alkaline phosphatase	LDH
1,960	Supernatant	95.1	81.9	66.7	86.6	98	83.7
	Pellet	14.4	14.2	37.1	13.1	2	20.5
4,000	Supernatant	99.6	81.3	55.0	91.2	84	84.9
	Pellet	19.3	17.1	53.1	20.3	16	8.6
6,000	Supernatant	81.7	82.3	43.7	79.7	76	85.6
	Pellet	25.8	18.9	61.7	27.7	24	ND
7,830	Supernatant	53.3	78.9	45.0	72.7	51	85.9
	Pellet	23.9	20.6	55.9	25.4	49	ND
9,900	Supernatant	73.9	73.6	45.8	69.1	84	90.3
	Pellet	26.1	23.9	74.0	31.0	14	ND
14,800	Supernatant	62.4	74.3	44.3	63.8	55	93.2
	Pellet	35.6	23.9	75.7	36.3	45	ND
20,700	Supernatant	68.3	74.2	47.6	41.2	39	93.0
	Pellet	27.1	24.2	76.2	55.8	61	ND
39,600	Supernatant	67.6	68.2	42.0	47.2	16	89.3
	Pellet	34.6	29.5	78.0	56.1	84	18.9

This was probably caused by the long period of time (5-7 h) which elapsed between the initial low speed centrifugation of H_1 to yield S_1 and the subsequent, higher speed centrifugation of aliquots of S_1 at >9,900 g. Progessively more plasma membrane and protein were sedimented as the centrifugation forces were increased.

At 40,000 g, recovery of sedimentable progesterone was greatest but so was contamination by mitochondria, lysosomes and plasma membrane enzyme markers. At the other end of the centrifugation range, namely 2,000 g and 4,000 g, contamination by the latter was much less but this was achieved at the expense of considerably lower recovery of sedimentable progesterone.

Therefore, 10,000 g was chosen as the centrifugation force for obtaining a pellet of sedimentable progesterone which could be investigated in subsequent density gradient experiments. At this g-force, about 25% of the tissue progesterone was sedimentable, accompanied by minimal contamination of membrane components such as plasma membrane and endoplasmic reticulum. Implicit in this decision was the expectation that sedimentable progesterone was not associated with either dense organelles such as mitochondria and lysosomes or with membrane components.

Two points should be made at this stage. Firstly resuspension and re-centrifugation of the 600 g pellet (P_1) did not significantly increase the amount of progesterone in the supernatant, indicating that the progesterone in P_1 was not loosely adsorbed to cellular components but was entrapped within undisrupted cells and large fragments of cytoplasm (The electron micrograph of P_1 in Fig. 3-2 supports this finding). Secondly, sequential re-centrifugation of the resuspended

10,000 g pellet (H_2) only gradually solubilized increasing amounts of steroid hormone. In one experiment, the first, second and third 10,000 g centrifugations yielded in their pellets 24.7%, 22.0% and 21.0%, respectively, of the total tissue progesterone. This finding showed that sedimentable progesterone was also particulate progesterone.

A more extensive analysis of marker enzyme distributions was carried out on the initial homogenate and 600 g supernatant, and on the supernatants and resuspended pellets from centrifugation at 10,000 g and 100,000 g (see Fig. 3-1 for fractionation scheme). The data from a number of experiments is presented in Table 3-5.

In agreement with the earlier differential centrifugation experiment (Table 3-4), between 20-30% of the total activities of acid phosphatase and β-glucuronidase (lysosomes), catalase (microperoxisomes), NADH-cytochrome c reductase and inosine diphosphatase (microsomes) and around 50% of the succinate dehydrogenase activity (mitochondria) were recovered in the 10,000 g pellet. The 100,000 g pellet, prepared from the 10,000 g supernatant, contained a further 13% of the total progesterone, indicating that at least 30% of the progesterone was particulate. A further 20% of the marker enzyme activities for microsomes and plasma membranes were also in this pellet, together with lesser amounts of the other marker enzymes.

About a third of the total tissue progesterone was not sedimented at 100,000 g but remained in the cytosol (Table 3-5). The presence of lysosomal and microperoxisomal marker enzymes in the cytosol fraction indicated that some organelle damage had occurred during fractionation. Thus, some of the

Table 3-5: The distribution of progesterone, protein and marker enzymes obtained upon differential centrifugation of homogenates of bovine corpora lutea.

The 10,000 g pellet, 100,000 g pellet and supernatant (cytosol) were prepared as described under Methods. The percent of progesterone, protein or enzyme activity in each fraction is expressed relative to the homogenate, set at 100% Values are given as the mean ± SD; the number of experiments is designated in parentheses.

Mass or Activity	Percent of Homogenate Mass or Activity		
	10,000 g Pellet	100,000 g Pellet	100,000 g Supernatant
Progesterone	19.1 ± 3.6 (12)	13.0 ± 5.7 (5)	34.0 ± 7.0 (5)
Protein	17.0 ± 5.6 (5)	10.4 ± 0.9 (3)	41.6 ± 5.8 (3)
Succinate dehydrogenase	46.7 ± 7.6 (6)	3.8 ± 0.7 (3)	0 (3)
Acid phosphatase	25.2 ± 9.7 (6)	13.8 ± 7.0 (3)	27.0 ± 3.2 (3)
β-glucuronidase	30.7 ± 6.1 (6)	13.4 ± 4.7 (3)	10.1 ± 1.8 (3)
Catalase	21.8 ± 5.1 (3)	16.3 (1)	20.3 (2)
Alkaline phosphatase	21.5 ± 7.9 (4)	17.2 (2)	6.7 (2)
NADH-cytochrome c reductase (rotenone-insensitive)	33.3 ± 5.0 (4)	20.4 (2)	1.7 (2)
Inosine diphosphatase	24.9 ± 7.1 (3)	19.5 (1)	8.4 (1)

progesterone found in the supernatant may well have been released from its particulate form by these procedures. In contrast, the marker enzymes for microsomes, plasma membranes and mitochondria, which are firmly membrane bound, were almost completely sedimented.

3.3.2 Density Gradient Centrifugation

In an attempt to obtain further information on the association of particulate progesterone with subcellular organelles, the 10,000 g homogenate (H_2) was fractionated analytically on continuous sucrose and Ficoll density gradients and preparatively on the zonal rotor using sucrose as density medium.

3.3.2.1 Initial Sucrose Density Gradients

Initially, linear 26-45% w/w and 15-50% w/w sucrose gradients were evaluated. Fig. 3-3 shows the progesterone, protein and marker enzyme profiles of a 26-45% w/w sucrose gradient. The amount of H_2 that was applied to this gradient was equivalent to 0.9 gm of starting mince of corpus luteum. It was found that loading of more than the equivalent of 1.5-2.0 gm of starting mince (usually in 1.5-2.0 ml of H_2) to any of the analytical gradients exceeded the capacity of the gradients so that the resolution of organelles obtained at lower sample loadings were lost.

The distribution of progesterone on the 26-45% w/w sucrose gradients shown in Fig. 3-3 was trimodal. There was a soluble hormone peak in the sample zone at the top of the gradient and a second peak of particulate hormone that had entered the gradient to band at between 30.5% w/w and 32% w/w

Fig. 3-3A: The distribution of progesterone, marker enzymes and protein on a 26-45% sucrose gradient.

An aliquot of the 10,000 g_{av} pellet (H_2), prepared from an homogenate of bovine corpora lutea, was loaded onto a 26-45% w/w linear sucrose gradient. The amount of material applied to the gradient was that derived from 0.9 gm of minced tissue. Centrifugation was carried out for 2.5 h at 82,500 g_{av}.

The abscissa denotes the tube number as eluted from the gradient. On the ordinate, the mass or activity in each tube is plotted as a percentage of the total mass or activity recovered from the gradient.

● = Progesterone
▲ = Acid phosphatase
○ = % Sucrose, w/w

A

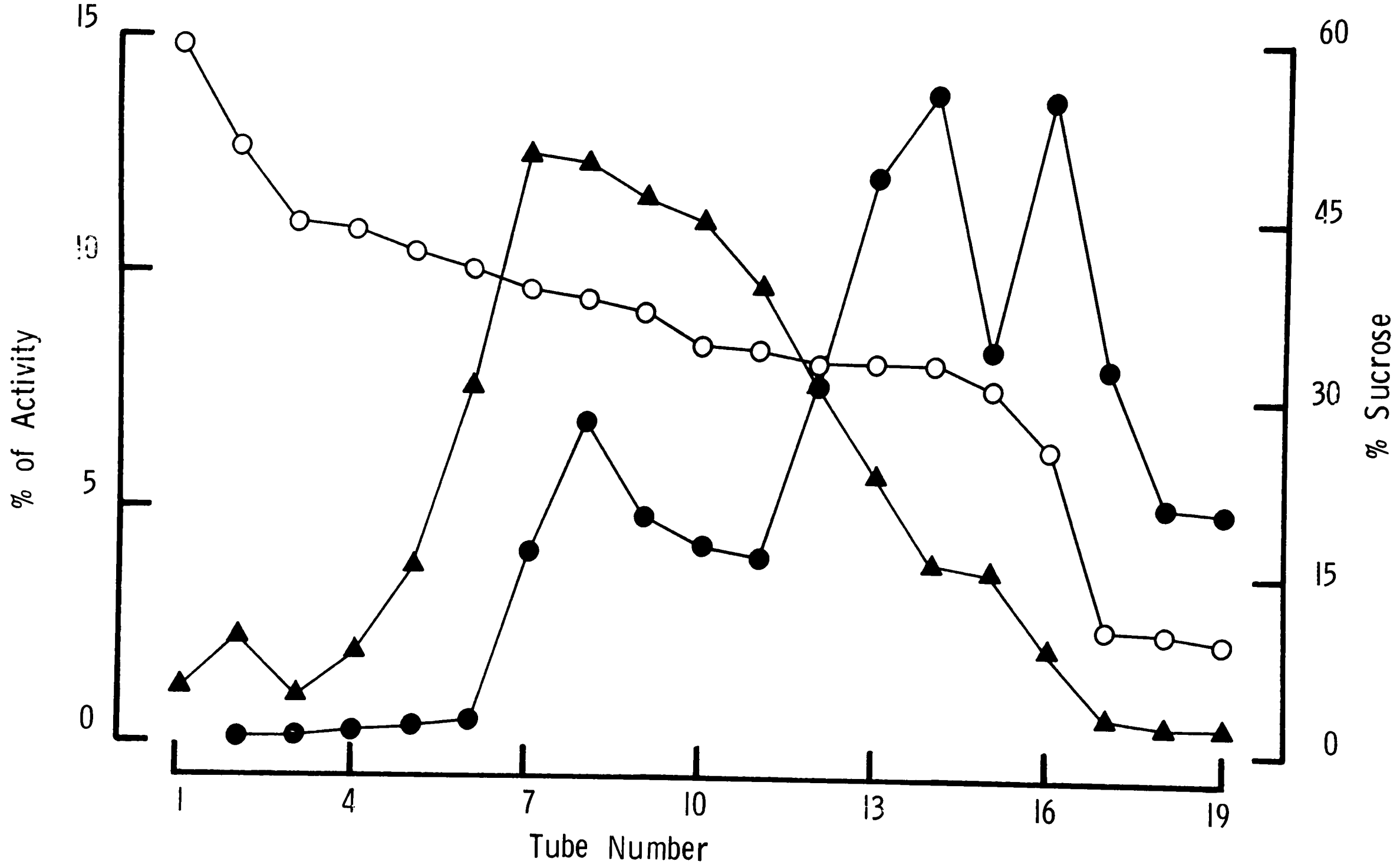

15
10
5
0
% of Activity
60
45
30
15
0
% Sucrose
1
4
7
10
13
16
19
Tube Number

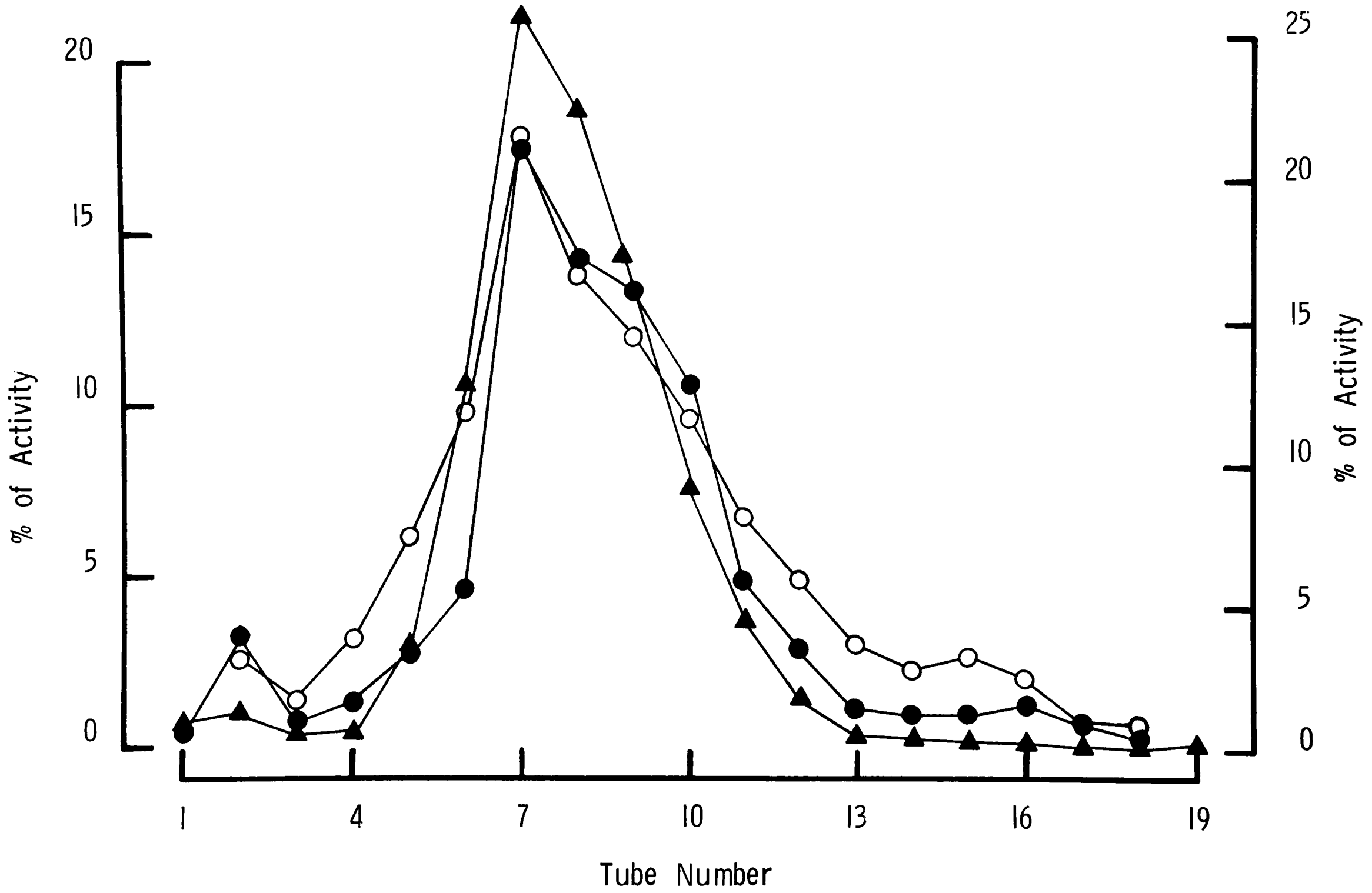
Fig. 3-3B: The distribution of progesterone, marker enzymes and protein on a 26-45% sucrose gradient.

● = Protein (0-25% activity)

○ = β-glucuronidase (0-20% activity)

▲ = Succinate dehydrogenase (0-25% activity)

B


20
15
10
5
0
% of Activity
25
20
15
10
5
0
% of Activity
1
4
7
10
13
16
19
Tube Number

sucrose, d=1.129-1.137[5]. The third peak of hormone (of variable size, and usually smaller than seen in this particular experiment) occurred at approximately 38% w/w sucrose (d=1.166), where marker enzyme analysis showed most of the dense organelles such as mitochondria and lysosomes were located.

It was the second, particulate peak of progesterone that was of primary interest in the investigation of the possible location of this hormone in membrane-bound granules. Its banding density was lower than that of the mitochondria and lysosomes, yet sufficiently great to suggest an association with other, less dense, subcellular organelles. However, very little protein was measureable underneath this second progesterone peak, suggesting that the subcellular components banding in this region of the gradient represented only a small proportion of the total mass of the luteal cells or, alternatively, that these components contained little protein. The lysosomal marker enzyme profiles of the 26-45% w/w sucrose gradient did not coincide: β-glucuronidase gave a more discrete peak than acid phosphatase (Fig. 3-3). This was a consistent feature throughout the density gradients, run under a variety of conditions, and clearly shows that the heterogeneity of this class of organelle observed in other tissues (e.g., aortal smooth muscle of rabbit, de Duve, 1971; Hinton and Dobrota, 1976) is also found in the bovine corpus luteum. These lysosomal enzyme markers display differing stabilities and/or

---

5: The concentrations and densities of sucrose quoted in this experiment are tentative, as refractive indices were obtained using a blood/urine refractometer, using an arbitrary scale which was calibrated by reference to sucrose solutions of known concentration.

locations in lysosomes.

In an effort to remove the overlap that occurred between the lysosomal enzyme markers and particulate progesterone obtained with the 26-45% w/w sucrose gradient (Fig. 3-3), a steeper gradient extending over a greater sucrose concentration range was evaluated. Fig. 3-4 shows the progesterone and lysosomal enzyme marker profiles of such a gradient, which extended from 15-50% w/w sucrose. It was found that the altered sucrose concentration profile of this gradient merely increased the width of the lysosome and particulate progesterone peaks without reducing the overlap between these subcellular constituents. In fact, the amount of overlap was greater, rather than less, using the 15-50% w/w sucrose gradient compared with the 26-45% w/w sucrose gradient. Alternatively centrifugation for 2.5 h may not have been sufficient for the steeper gradient to reach sedimentation-equilibrium; however, subsequent experiments using both sucrose and Ficoll density gradients discounted this possibility.

3.3.2.2 Subsequent Sucrose Density Gradients

The sucrose concentration range of the gradient was altered to 20-40% w/w sucrose to maintain particulate progesterone banding in the top one-third of the gradient. This also prevented mitochondrial and lysosomal overlap by reducing the maximum sucrose concentration so that the latter moved to the bottom of the gradient where the sucrose density was less than the buoyant densities of these organelles at sedimentation-equilibrium. Therefore, linear 20-40% w/w sucrose gradients were used in subsequent experiments where comprehensive biochemical, morphological and cytochemical analyses were

Fig. 3-4: The distribution of progesterone and marker enzymes on a 15-50% sucrose gradient

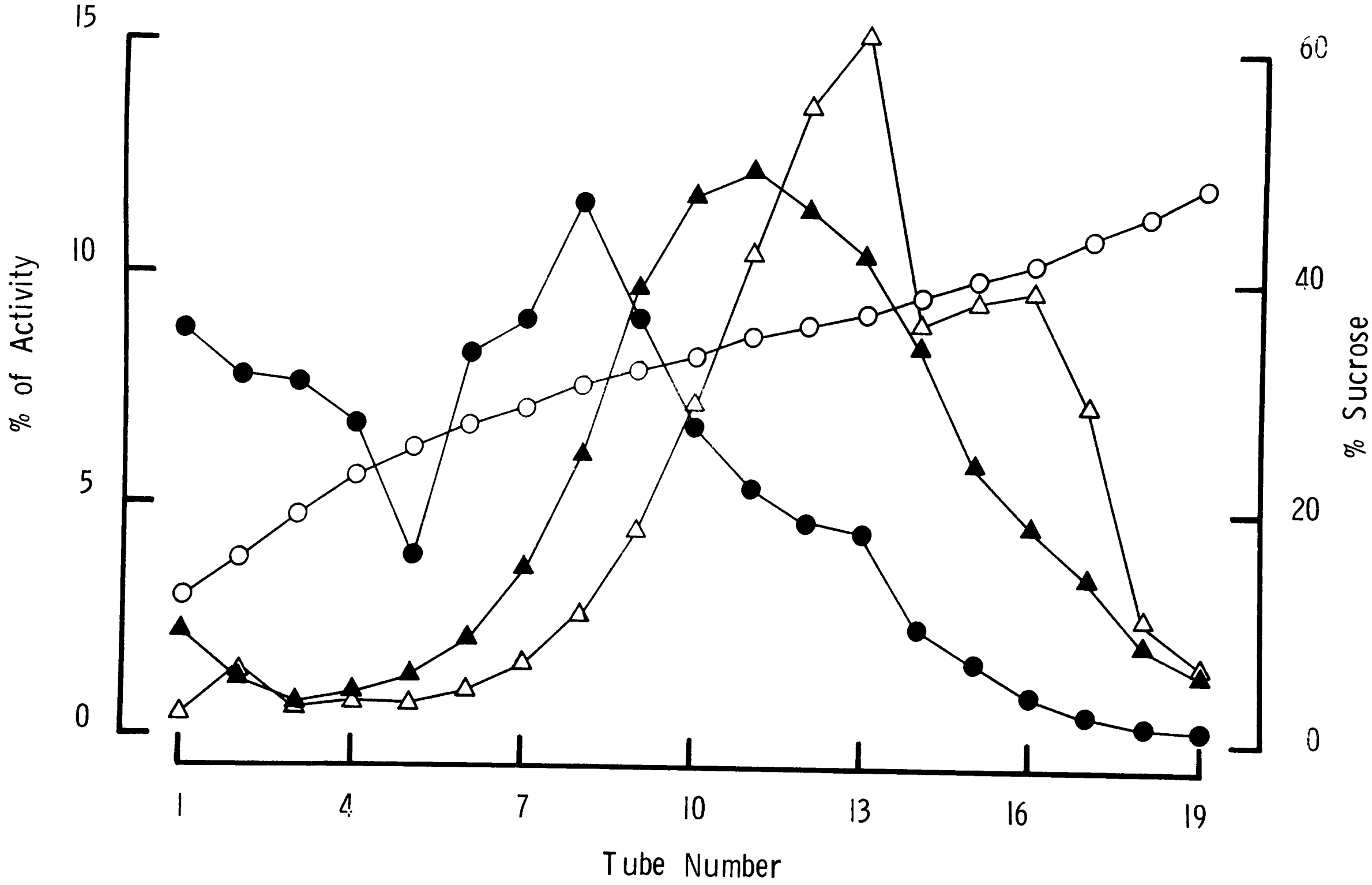
An aliquot of the 10,000 g_{av} pellet, prepared from an homogenate of bovine corpora lutea, was applied to a 15-50% w/w linear sucrose gradient. The material loaded onto the gradient was derived from 1.5 gm of minced tissue. Centrifugation was carried out for 2.5 h at 82,500 g_{av}.

The abscissa denotes the tube number as eluted from the gradient. On the ordinate, the mass or enzyme activity in each tube is plotted as a percentage of the total mass or enzyme recovered from the gradient.

Biochemical analysis of aliquots of the fractionation stages and of the gradient fractions revealed the following information:

	% of H_1 in H_2	Amount loaded onto gradient	% Recovery from gradient
Progesterone	17.1	17.1 ng	92.4
Acid phosphatase	13.9	1.43 EU	88.5
β-Glucuronidase	23.5	0.33 EU	90.4

● = Progesterone
▲ = Acid phosphatase
△ = β-Glucuronidase
○ = % Sucrose. w/w


15
10
5
0
% of Activity
60
40
20
0
% Sucrose
1
4
7
10
13
16
19
Tube Number

carried out.

The resolution of progesterone, protein and marker enzymes on such a gradient is shown in Figs. 3-5 and 3-6. Similar results were obtained in 7 other experiments for progesterone, and in at least 3 other experiments for each of the marker enzymes. Tubes 1-8 of the eluted gradient contained about 80% of the progesterone recovered from the gradient. Some 20% of the progesterone was found in tubes 1 and 2, and thus had remained in the sample zone and had not entered the gradient. The progesterone of a cytosol aliquot loaded onto an analogous gradient was also recovered in tubes 1 and 2 (Fig. 3-7). These tubes also contained about 5% of the acid phosphatase, β-glucuronidase and catalase activity, presumably as a result of some enzyme leaching from the organelles. Tubes 3-7 (22.5-27.3% w/w sucrose, density = 1.094-1.113), comprised about 50% of the recovered progesterone, negligible catalase and succinate dehydrogenase activity, and less than 5% of β-glucuronidase, acid phosphatase, alkaline phosphatase, NADH-cytochrome c reductase and inosine diphoshpatase activities. The latter three enzyme activities were spread through the gradient from tube 8 onwards (sucrose density >1.116). Progesterone levels were low from tube 8 onwards whereas acid phosphatase, β-glucuronidase, catalase and succinate dehydrogenase activities were highest from tube 14 onwards (sucrose density >1.146). Thus the lysosomes, mitochondria and microperoxisomes were completely resolved from the particulate progesterone peak, while a small proportion of the microsomes (mainly endoplasmic reticulum) and plasma membrane overlapped in this region.

The enrichment (relative specific activity) of

Fig. 3-5A: The distribution of progesterone, enzyme activities and protein on 20-40% w/w sucrose density gradients.

Aliquots of the resuspended 10,000 g_{av} pellet, prepared from an homogenate of bovine corpora lutea, were loaded onto 20-40% w/w sucrose gradients. Centrifugation was carried out for 2.25 h at 82,500 g_{av}. The fractions of three gradients were pooled. The abscissa denotes the tube number as eluted from the gradient. On the ordinate, the mass or enzyme activity in each tube is plotted as a percentage of the total mass or activity recovered from the gradient.

● = Progesterone
▲ = Protein
○ = % Sucrose, w/w

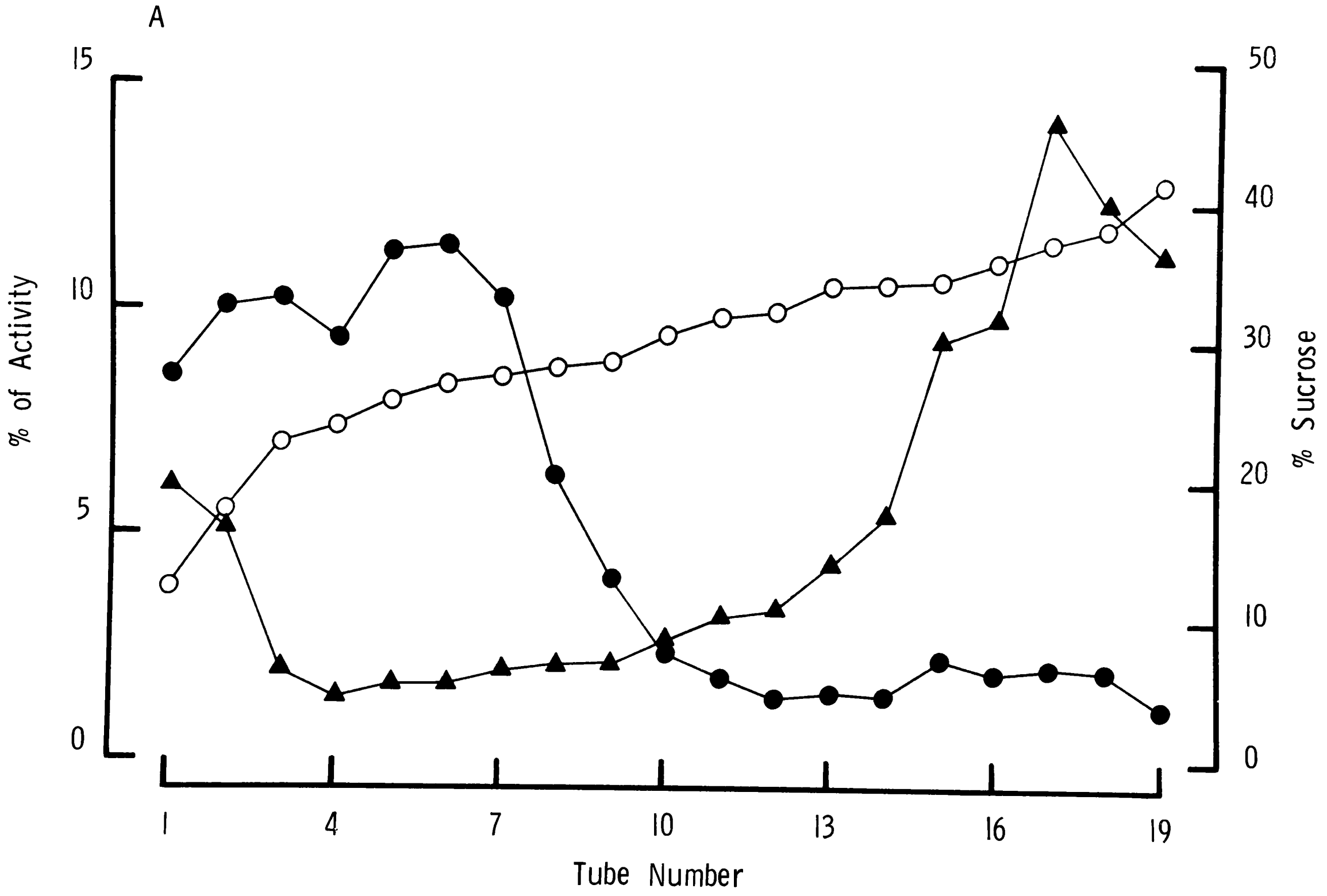

A
15
10
5
0
% of Activity
50
40
30
20
10
0
% Sucrose
1
4
7
10
13
16
19
Tube Number

Fig. 3-5B: The distribution of progesterone, enzyme activities and protein on 20-40% w/w sucrose density gradients.

Biochemical analysis of aliquots of the fractionation stages and of the gradient fractions revealed the following information:

	% of H_1 in H_2	Amount loaded onto gradient	% Recovery from gradient
Progesterone	26.3	6.06 µg	77.9
Protein	21.4	20.05 mg	88.2
Acid phosphatase	29.8	0.819 EU	93.6
β-Glucuronidase	39.3	0.206 EU	114.5
Catalase	19.2	0.767 EU	127.0
Succinate dehydrogenase	58.8	25.0 EU	97.8
Inosine diphosphatase	28.5	0.534 EU	102.5
NADH-cytochrome c reductase	40.8	7.64 EU	79.4
Alkaline phosphatase	23.3	0.319 EU	82.0

● = Acid phosphatase
○ = β-Glucuronidase
▲ = Catalase
△ = Succinate dehydrogenase

B

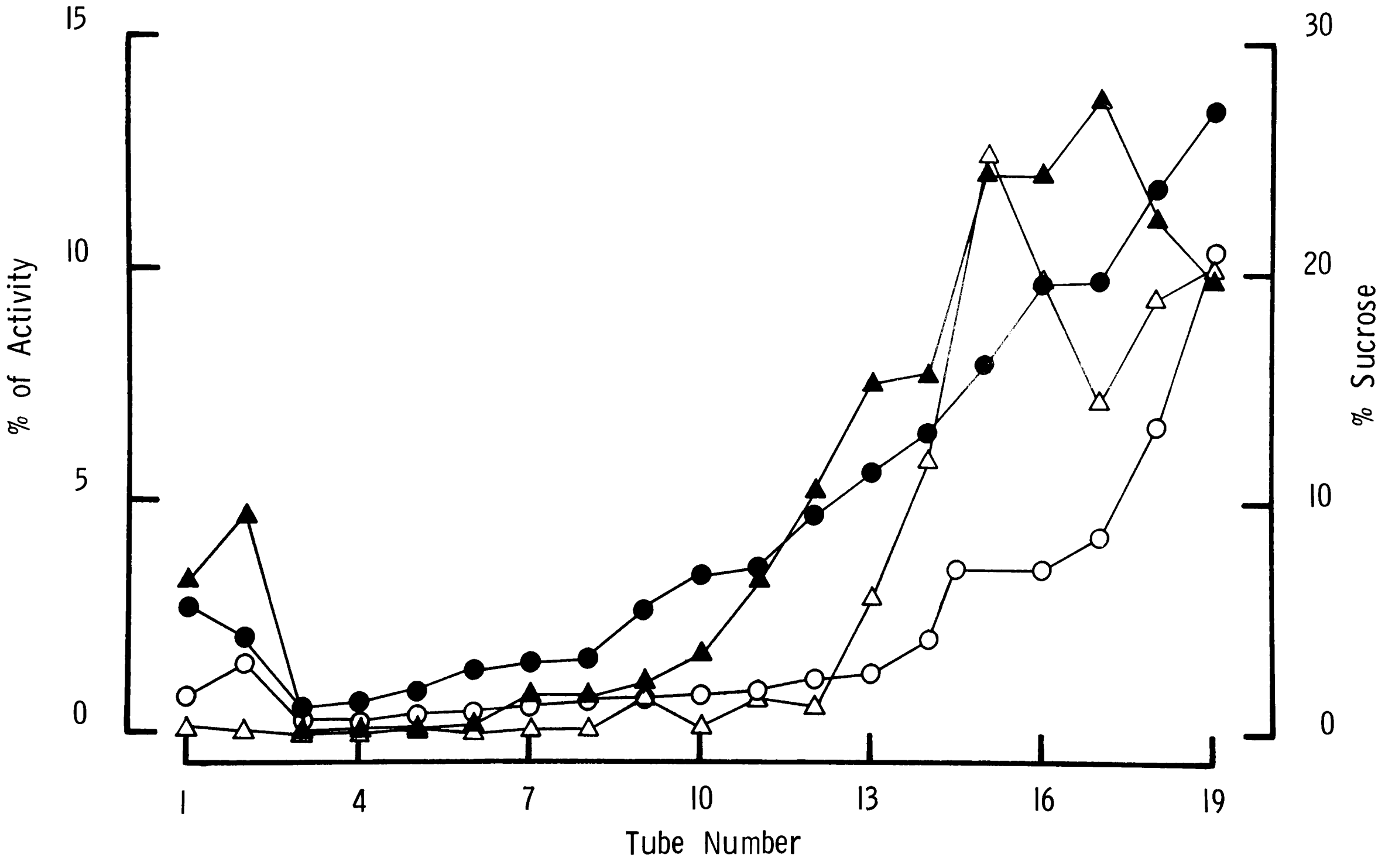

15
10
5
0
% of Activity
30
20
10
0
% Sucrose
1
4
7
10
13
16
19
Tube Number

Fig. 3-5C: The distribution of progesterone, enzyme activities and protein on 20-40% w/w sucrose density gradients.

● = Inosine diphosphatase

○ = NADH-cytochrome c reductase

▲ = Alkaline phosphatase

C

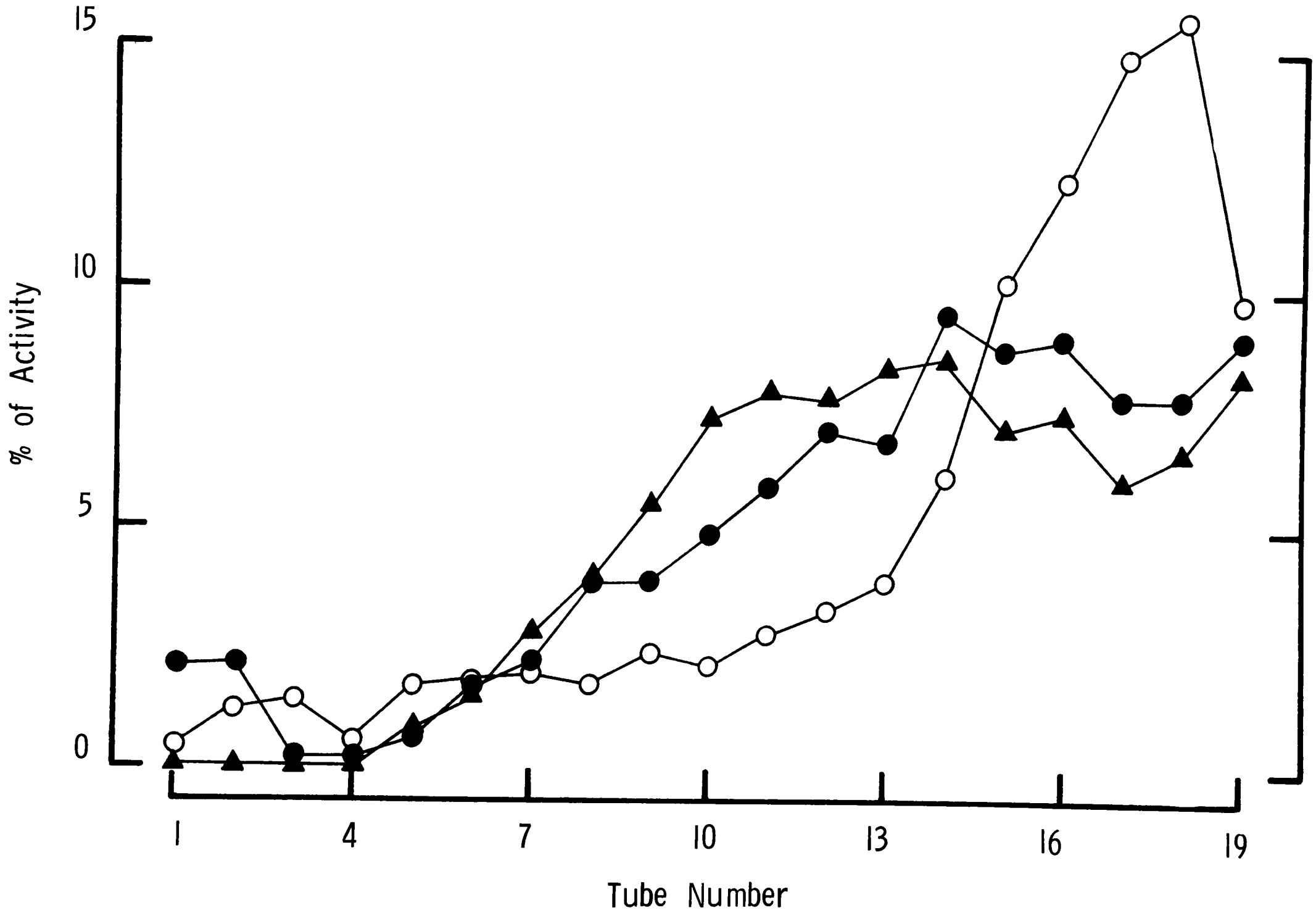


Fig. 3-6A: The relative specific activity of progesterone and marker enzymes on 20-40% w/w sucrose density gradients.

Details of the experimental procedures are given in the Methodology section and in the legend to Fig. 3-6. The results shown are from the same representative experiment.

The abscissa denotes the tube number as eluted from the gradient; the ordinate value is the relative specific activity or enrichment.

$$\text{Relative specific activity, RSA} = \frac{\%\ \text{mass or activity recovered in tube}}{\%\ \text{protein recovered in tube}}$$

where H_1 = 100% RSA.

● = Progesterone

○ = % Sucrose, w/w

A

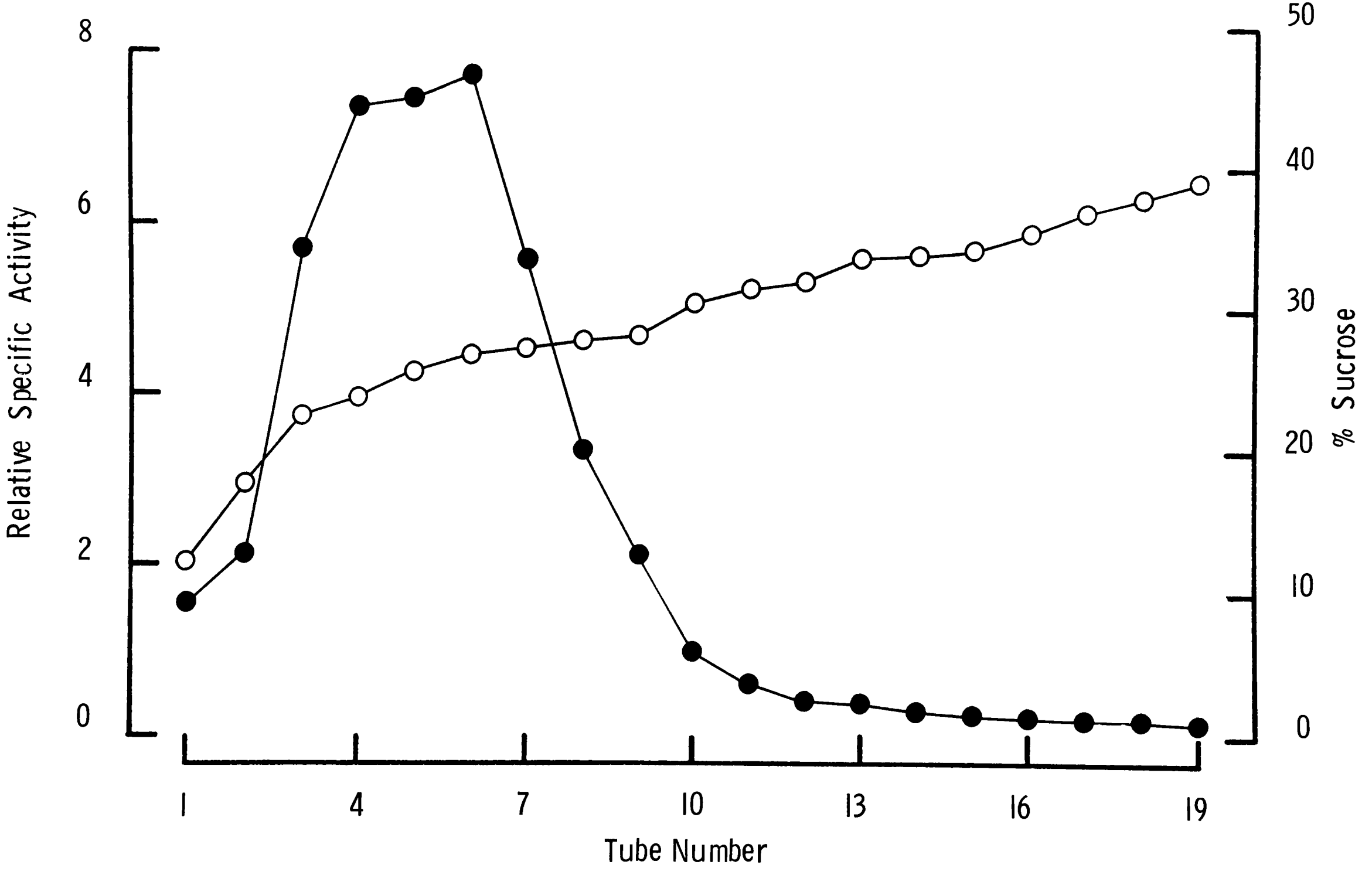

Relative Specific Activity
0
2
4
6
8
% Sucrose
0
10
20
30
40
50
1
4
7
10
13
16
19
Tube Number

Fig. 3-6B: The relative specific activity of progesterone and marker enzymes on 20-40% w/w sucrose density gradients.

△ = Acid phosphatase

○ = β-glucuronidase

▲ = Catalase

● = Succinate dehydrogenase

B

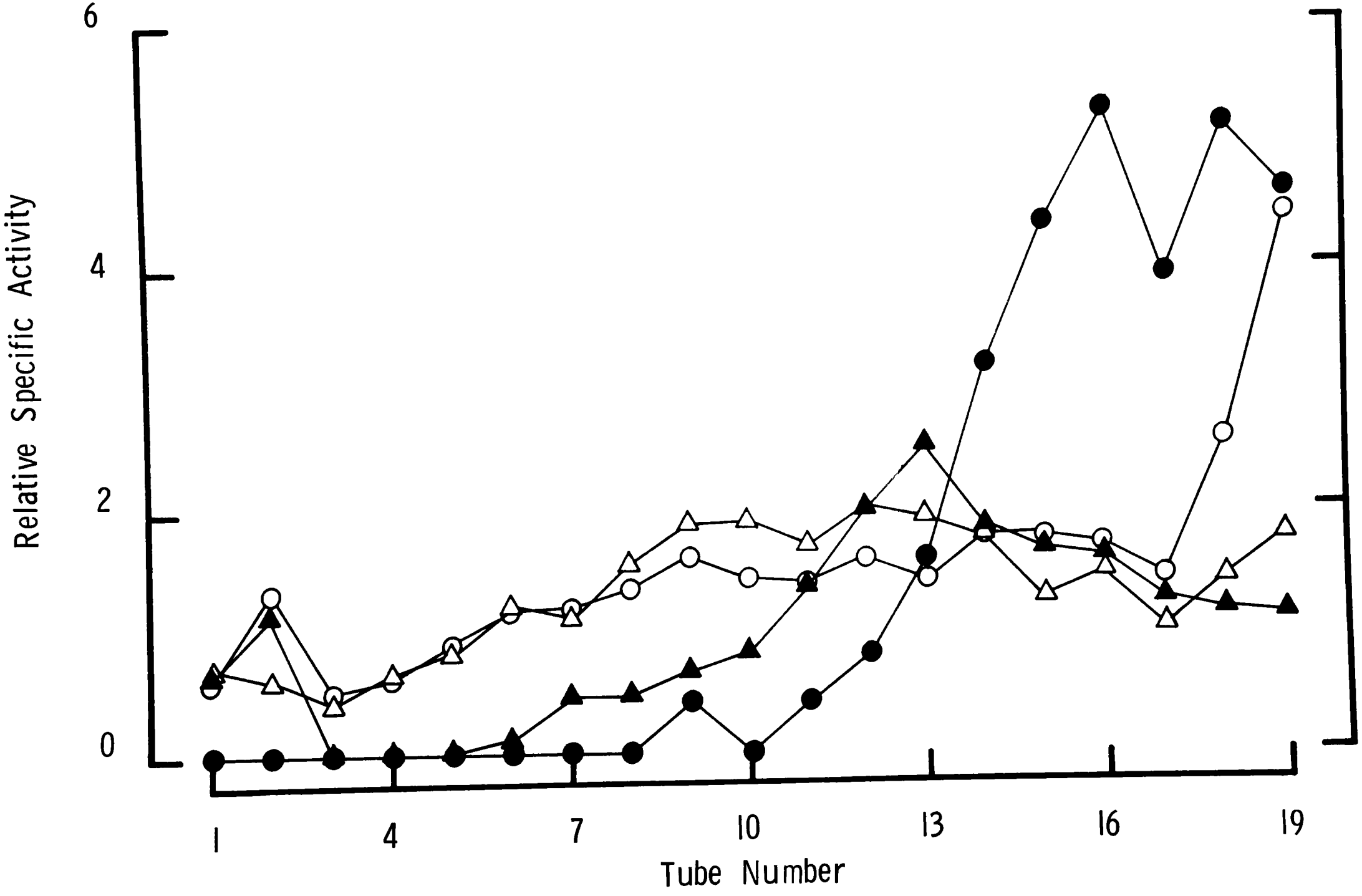


Fig. 3-6C: The relative specific activity of progesterone and marker enzymes on 20-40% w/w sucrose density gradients.

● = Inosine diphosphatase
○ = NADH-cytochrome c reductase
▲ = Alkaline phosphatase

C

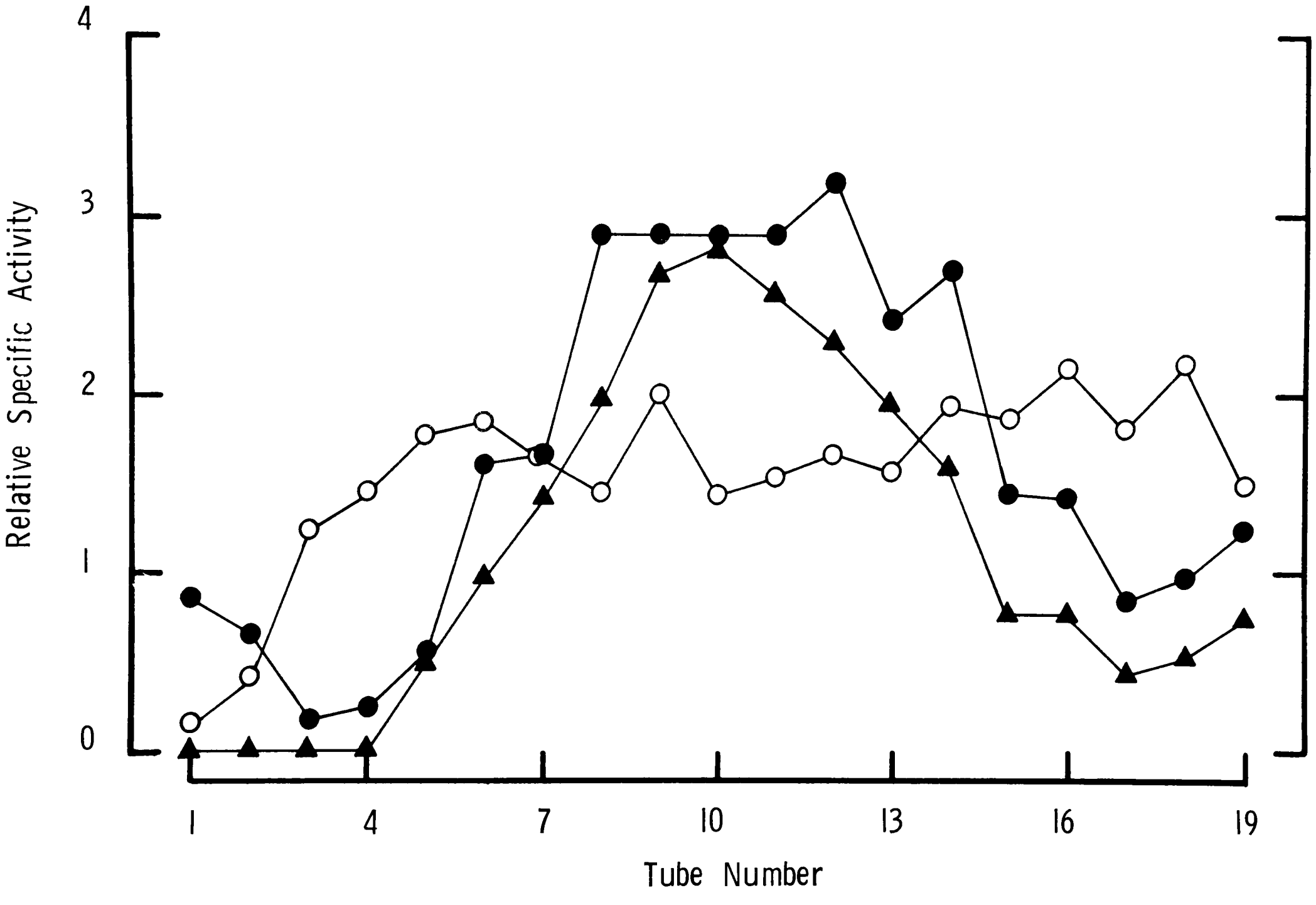

Relative Specific Activity
4
3
2
1
0
1
4
7
10
13
16
19
Tube Number

Fig. 3-7: Resolution of the cytosolic fraction of bovine corpus luteum on a 20-40% sucrose gradient.

A cytosolic fraction (S_3) was obtained by centrifugation of an homogenate of bovine corpora lutea at 110,000 g_{av} for 1 h in the SW41 rotor. A 2.5 ml aliquot of S_3 was applied to a linear 20-40% w/w sucrose gradient, which was centrifuged for 3 h at 82,500 g_{av}.

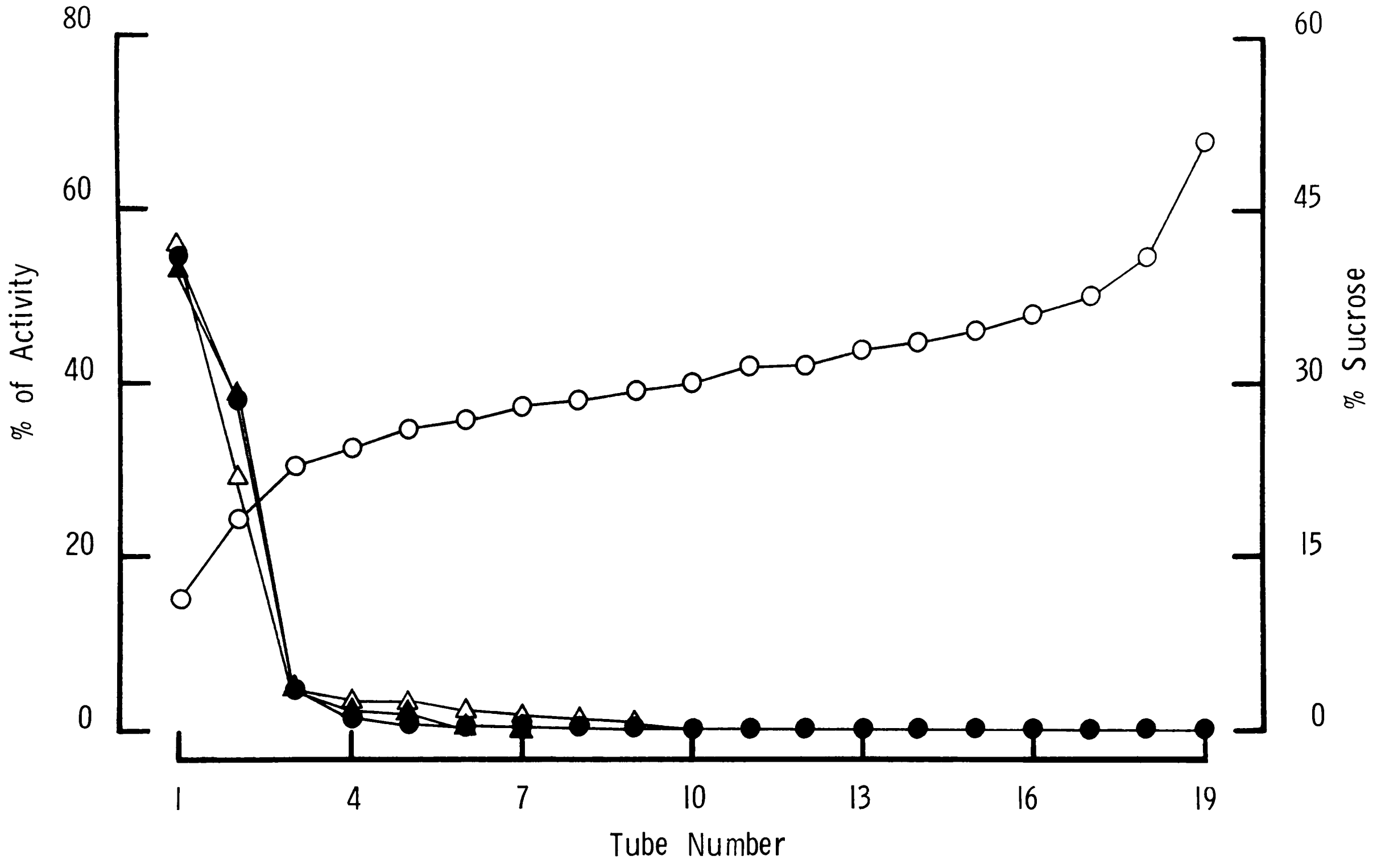
The abscissa denotes the tube number as eluted from the gradient. On the ordinate, progesterone mass and catalase activity is plotted as a percentage of the total mass or activity recovered from the gradient.

Biochemical analysis of aliquots of the fractionation stages and of the gradient fractions revealed the following information:

	% of H_1 in H_2	Amount loaded onto gradient	% Recovery from gradient
Progesterone	30.0	5.99 ng	103.0
Succinate dehydrogenase	6.6	0.225 EU	97.8
Acid phosphatase	30.0	0.296 EU	104.5
β-Glucuronidase	11.5	0.021 EU	85.0

● = Progesterone
▲ = Succinate dehydrogenase
△ = Acid phosphatase
○ = % Sucrose, w/w

NB: β-Glucuronidase activities are not plotted for clarity. Its profile closely paralleled that of acid phosphatase.


80
60
40
20
0
% of Activity
60
45
30
15
0
% Sucrose
1
4
7
10
13
16
19
Tube Number

progesterone compared with the starting homogenate was greatest in tubes 3-8. Maximum enrichment of 7- to 8-fold occurred in tubes 4-6 in the experiment shown in Fig. 3-6. In four other experiments the enrichment in this region ranged from 7- 17-fold so that mean enrichment was 13.5 ± 4.2 (SD) -fold (n=5). The only other significant enrichments in this region were obtained for NADH-cytochrome c reductase and inosine diphosphatase, the microsomal marker enzymes. This enrichment was, however, less than 2-fold. The reductase enzyme is also located on the outer membrane of mitochondria. Tubes 1 and 2, which contained progesterone that had not entered the gradient, showed a much lower enrichment owing to the presence of soluble protein which did not enter the gradient.

All marker enzymes showed higher enrichments at densities greater than that of the progesterone peak, and the position of maximal enrichment usually corresponded to the position of maximal enzyme activity. The endoplasmic reticulum and plasma membrane markers were spread throughout the gradient and thus not heavily enriched in any fraction. This distribution occurs because of the variation in vesicle size and density which arises during homogenization of these membraneous elements, and has been reported in homogenates made from other tissues (de Duve, 1971). No attempt was made to separate lysosomes, mitochondria and microperoxisomes, so that the enrichment obtained in the denser regions of the gradient (2- to 4-fold) was less than if the gradient had been extended to effect a separation.

Prolonged centrifugation (17.5 h and 22.5 h) of 20-40% w/w sucrose gradients gave maximal enrichments of progesterone

and other marker enzymes at the same densities as found for the shorter centrifugation time. Thus, by 2.5 h in a 20-40% w/w sucrose gradient, equilibrium had been attained.

3.3.2.3 Ficoll Density Gradients

Polymerized sucrose (Ficoll) is the most commonly used alternative fractionation medium to sucrose. Both substances provide solutions of high density suitable for density gradient experiments, but Ficoll solutions, unlike sucrose solutions, are low in osmotic pressure. In addition, Ficoll has much lower permeability towards cell membranes due to its high molecular weight, so that subcellular particles generally band at lower densities in Ficoll gradients compared with sucrose gradients (Pharmacia Handbook, 1975).

Therefore, it was decided to apply H_2 to linear gradients of Ficoll to determine whether or not this medium would provide better separation than sucrose of particulate progesterone from the endoplasmic reticulum and plasma membrane constituents. Buffered sucrose was retained as the homogenization medium and, in fact, the Ficoll gradients contained buffered 0.25 M sucrose (equivalent to 8% w/w) throughout. Fig. 3-8 shows the progesterone and catalase profiles obtained using a linear gradient of 0-20% w/w Ficoll. Both profiles were similar to those observed using 20-40% w/w sucrose gradients. Sedimentation equilibrium with respect to progesterone was achieved in this gradient, which was centrifuged for 2.25 h, since centrifugation of a duplicate gradient overnight yielded an unchanged hormone profile. In other experiments, 5-25% w/w Ficoll gradients were prepared and run. Fig. 3-9 shows the result of hormone, protein and marker enzyme analysis of such

Fig. 3-8: The distribution of progesterone and catalase on a 0-20% Ficoll gradient.

An aliquot of the 10,000 g_{av} homogenate, H_2, prepared from an homogenate of bovine corpora lutea, was loaded onto a 0-20% w/w Ficoll gradient, containing buffered 0.25 M sucrose throughout. Centrifugation was carried out for 2.25 h at 82,500 g_{av}. The gradient was eluted into 2 ml fractions.

The abscissa denotes the tube number as eluted from the gradient. On the ordinate, progesterone mass and catalase activity is plotted as a percentage of the total mass or activity recovered from the gradient.

Biochemical analysis of aliquots of the fractionation stages and of the gradient fractions provided the following information:

	% of H_1 in H_2	Amount loaded onto gradient	% Recovery from gradient
Progesterone	21.1	7.78 µg	64.5
Catalase	27.7	1.36 EU	80.3
Protein	21.9	27.23 mg	

● = Progesterone
▲ = Catalase
○ = Sucrose, w/w

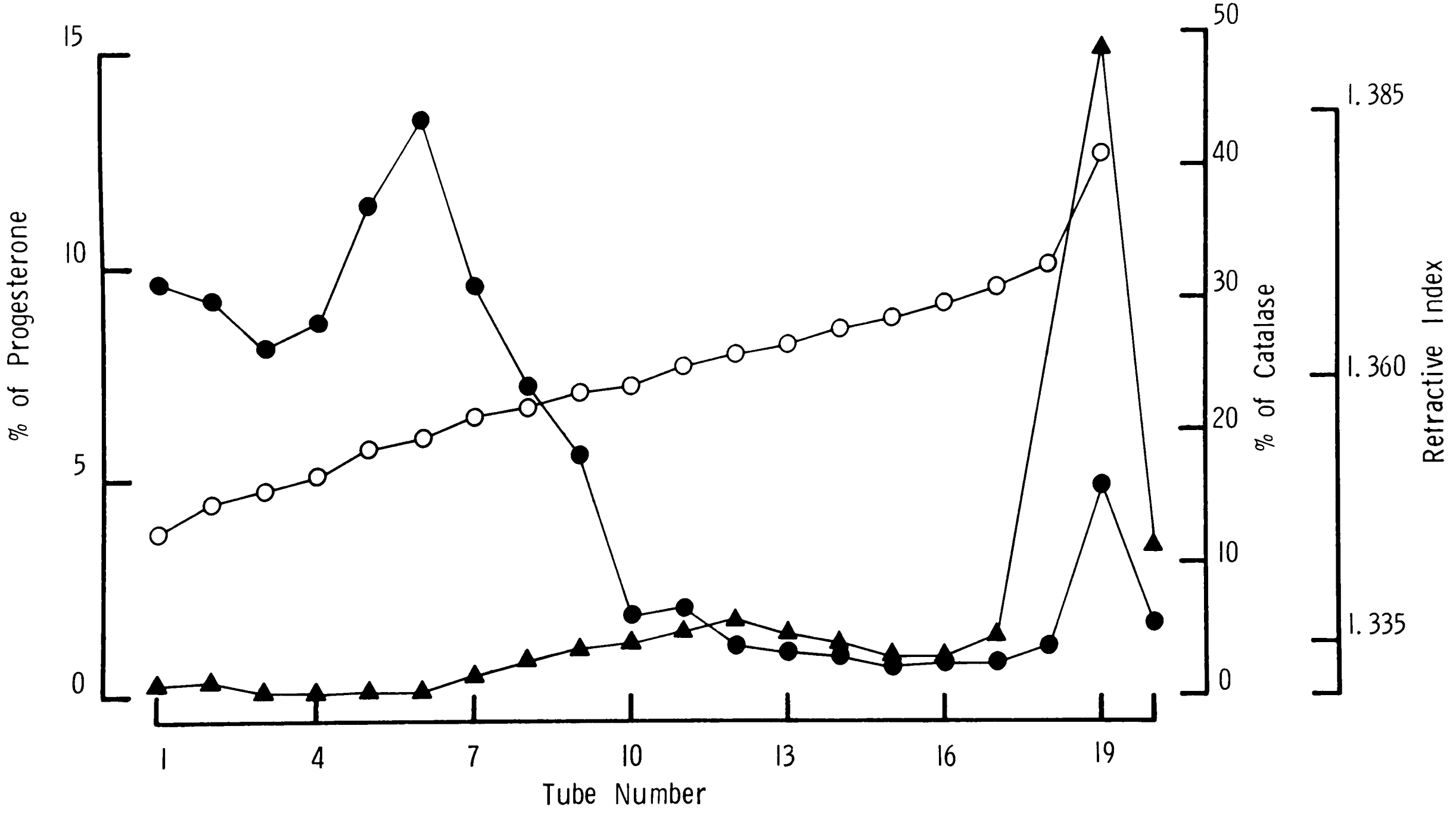

15
10
5
0
% of Progesterone
50
40
30
20
10
0
% of Catalase
1.385
1.360
1.335
Retractive Index
1
4
7
10
13
16
19
Tube Number

Fig. 3-9A: The distribution of progesterone, protein and marker enzymes on a 5-25% w/w Ficoll gradient.

An aliquot of the 10,000 g_{av} homogenate, H_2, prepared from an homogenate of bovine corpora lutea, was loaded onto a 5-25% w/w Ficoll gradient, containing buffered 0.25 M sucrose throughout. Centrifigation was carried out for 2.25 h at 82,500 g_{av}. The gradient was eluted into 2 ml fractions.

The abscissa denotes the tube number as eluted from the gradient. On the ordinate, the mass or enzyme activity is plotted as a percentage of the total mass or activity recovered from the gradient.

● = Progesterone
▲ = Protein
○ = Refractive Index

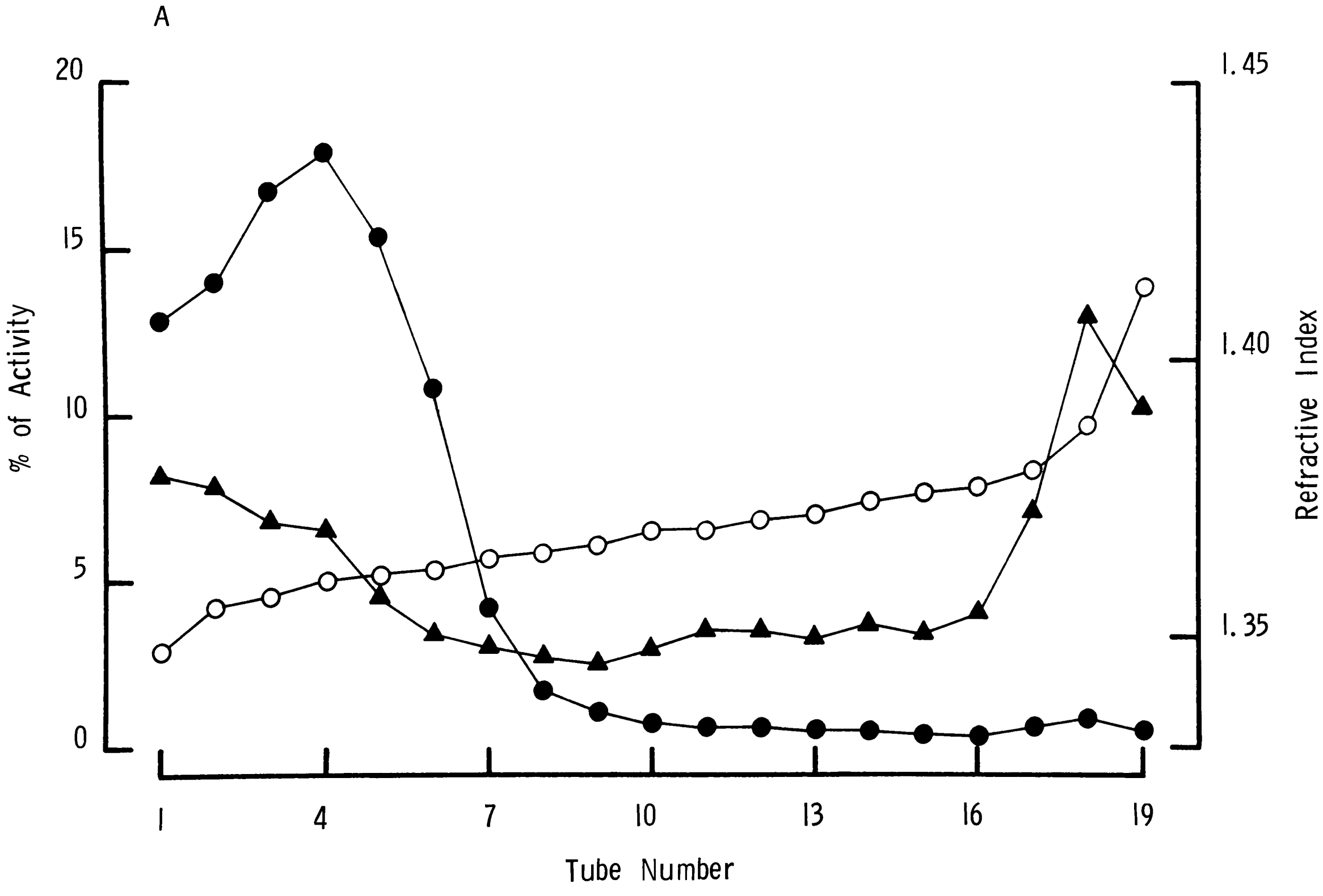
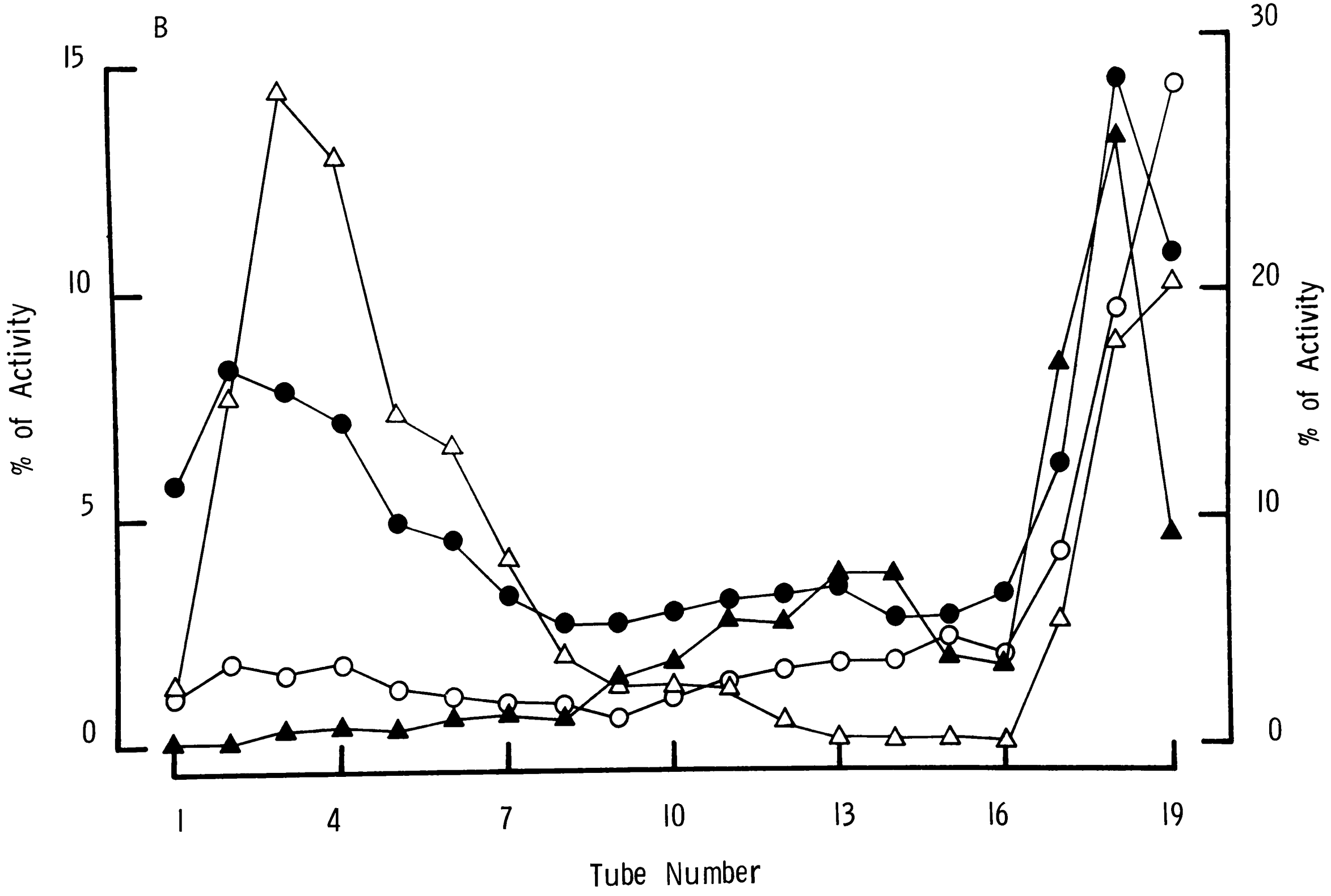

A
20
15
10
5
0
% of Activity
1.45
1.40
1.35
Refractive Index
1
4
7
10
13
16
19
Tube Number

Fig. 3-9B: The distribution of progesterone, protein and marker enzymes on a 5-25% Ficoll gradient.

Biochemical analysis of aliquots of the fractionation stages and of the gradient fractions provided the following information:

	% of H_1 in H_2	Amount loaded onto gradient	% Recovery from gradient
Progesterone	26.3	6.07 µg	82.2
Acid phosphatase	29.8	0.819 EU	96.3
β-Glucuronidase	39.3	0.206 EU	113.6
Succinate dehydrogenase	58.8	25.0 EU	76.0
Alkaline phosphatase	23.3	0.319 EU	87.0
Protein	21.4	20.05 mg	75.4

● = Acid phosphatase (0-15% acvitity)
○ = β-Glucuronidase (0-30% activity)
▲ = Succinate dehydrogenase (0-15% activity)
△ = Alkaline phosphatase (0-15% activity)


B
15
10
5
0
% of Activity
30
20
10
0
% of Activity
1
4
7
10
13
16
19
Tube Number

a gradient, centrifuged for 2.25 h. Once again, centrifugation of duplicate gradients overnight (18 h and 22.5 h) gave profiles of the subcellular constituents unchanged from those observed after centrifugation for only 2.25 h.

Although the sedimentation profiles of particulate and "mitochondrial" progesterone on both 0-20% w/w and 5-25% w/w Ficoll gradients were analogous to those obtained using sucrose gradients, the amount of hormone that was released into the soluble fraction (remaining at the top of the gradient) was slightly reduced. However, lysosomol enzyme markers banded bimodally in Ficoll such that at least 10-fold more activity was located at the top of the 15-50% w/w gradient. Thus, lysosomes were less dense and more fragile in Ficoll. Plasma membrane constituents also displayed much lower buoyant densities in this medium to band with particulate progesterone. Therefore, Ficoll was not as suitable a density medium as sucrose because in Ficoll gradients, unlike sucrose gradients, the peak of particulate progesterone was significantly contaminated with lysosomal and plasma membrane constituents.

3.3.2.4 Sucrose Gradients in the Zonal Rotor

The use of a zonal rotor in sucrose density centrifigation enables up to 20-fold greater sample loads, and is reported to provide faster and superior resolution of subcellular organelles than is obtained using conventional linear gradients run in large-capacity swinging-bucket rotors (Schneider and Smith, 1977; Chapter 1 of Hinton and Debrota, 1976). Therefore, aliquots of H_2 were applied to 400 ml sucrose gradients in the Ti-14 zonal rotor; the objectives were to evaluate zonal density gradient fractionation of particulate progesterone and to

process larger amounts of it so that morphological examination of fixed gradient fractions could be carried out on greater amounts of material.

Two different gradient conditions were tested: 15-40% w/w and 15-50% w/w sucrose. Fig. 3-10 shows the result of the 15-40% w/w zonal gradient: it was heavily overloaded with respect to progesterone and so resolved the latter poorly into one very broad band of hormone which extended some two-thirds of the way into the gradient.

The experiment was repeated, loading less sample onto a steeper gradient ranging from 15-50% w/w sucrose (Fig. 3-11). Progesterone was now resolved into a similar overall profile to that obtained with the 39 ml, linear 20-40% w/w sucrose gradients. One major peak of hormone was obtained, extending from the soluble, sample zone into the particulate region of the gradient to end at approximately 27% w/w sucrose (d=1.113), plus a smaller peak banding with the mitochondrial enzyme marker, succinate dehydrogenase. The latter, "mitochondrial" progesterone peak was larger in this experiment, relative to the major hormone peak at lower density, than observed with the linear 20-40% w/w gradients. The major particulate progesterone peak was maximal over the density range of 20-27% w/w sucrose (d=1.081-1.113), which was directly comparable to its position on linear 20-40% w/w sucrose gradients, where it banded maximally over the density range 22.5-27% w/w sucrose (d=1.094-1.113). Thus, the radial sucrose gradient of the zonal rotor provided resolution of sedimentable progesterone comparable to, but not superior to, the resolution obtained using linear, analytical sucrose gradients.

Fig. 3-10: The distribution of progesterone on a 15-40% w/w zonal sucrose gradient.

An aliquot of the 10,000 g_{av} homogenate, H_2, prepared from an homogenate of bovine corpora lutea, was loaded onto a 15-40% w/w sucrose gradient of 400 ml formed in the Ti-14 zonal rotor. Centrifugation was carried out for 90 min at 30,000 rpm, after which the gradient was eluted into 10 ml fractions.

The proportion of the original homogenate progesterone in H_2 was 21.0% and 171.3 µg (in 47.6 ml) of hormone was applied to the gradient.

● = Progesterone, $ng.ml^{-1}$

○ = % Sucrose, w/w

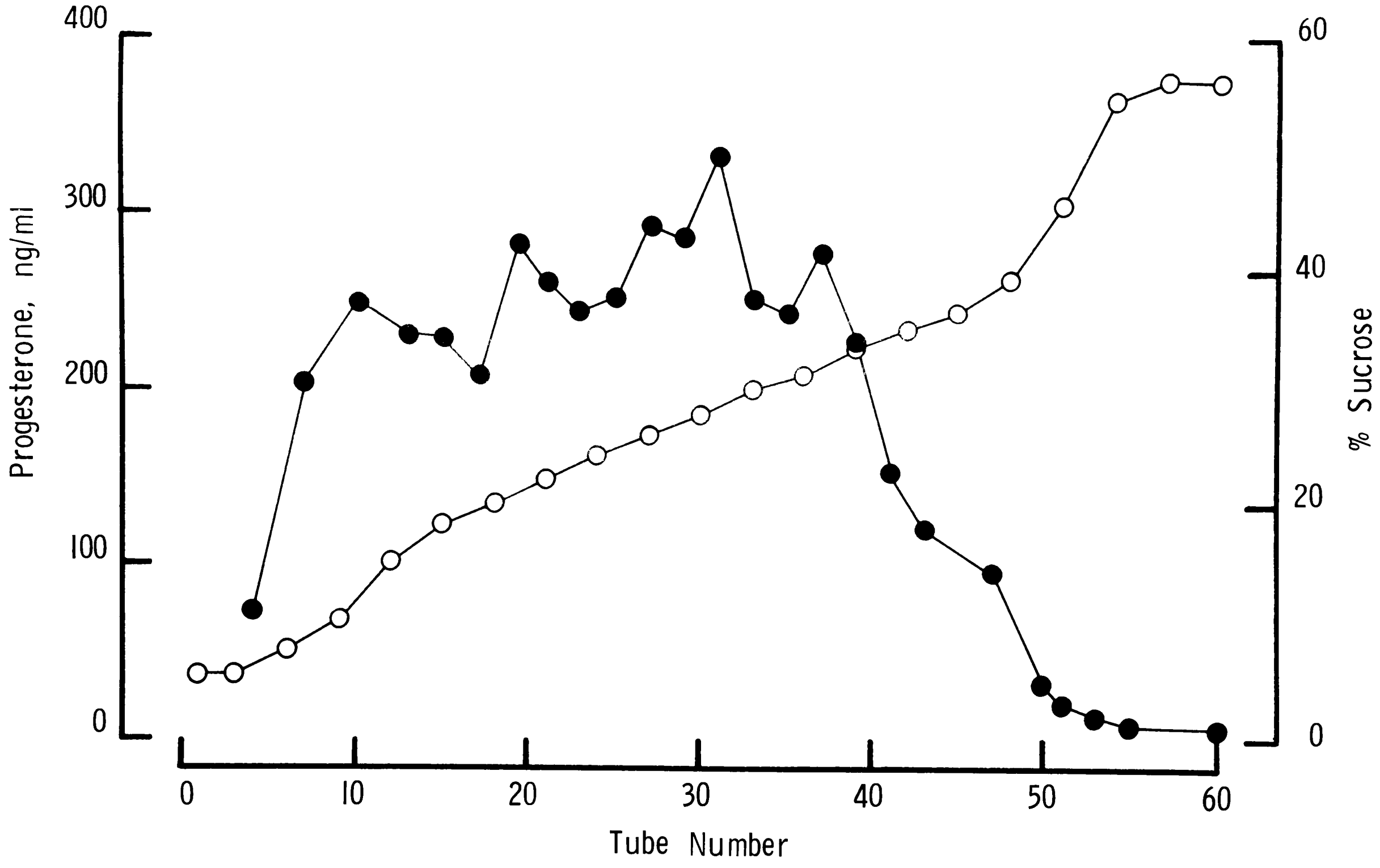

400
300
200
100
0
Progesterone, ng/ml
60
40
20
0
% Sucrose
0
10
20
30
40
50
60
Tube Number

Fig. 3-11A: The distribution of progesterone, protein and succinate dehydrogenase activity on a 15-50% zonal sucrose gradient.

An aliquot of the 10,000 g_{av} homogenate, H_2, prepared from an homogenate of bovine corpora lutea, was loaded onto a 15-50% w/w sucrose gradient of 400 ml formed in the Ti-14 zonal rotor. Centrifugation was carried out for 90 min at 30,000 rpm, after which the gradient was eluted into 10 ml fractions.

The proportion of the original homogenate progesterone in H_2 was 22.3% and 85.5 µg (in 20.0 ml) of hormone was applied to the gradient.

● = Progesterone, $ng.ml^{-1}$

○ = % Sucrose, w/w

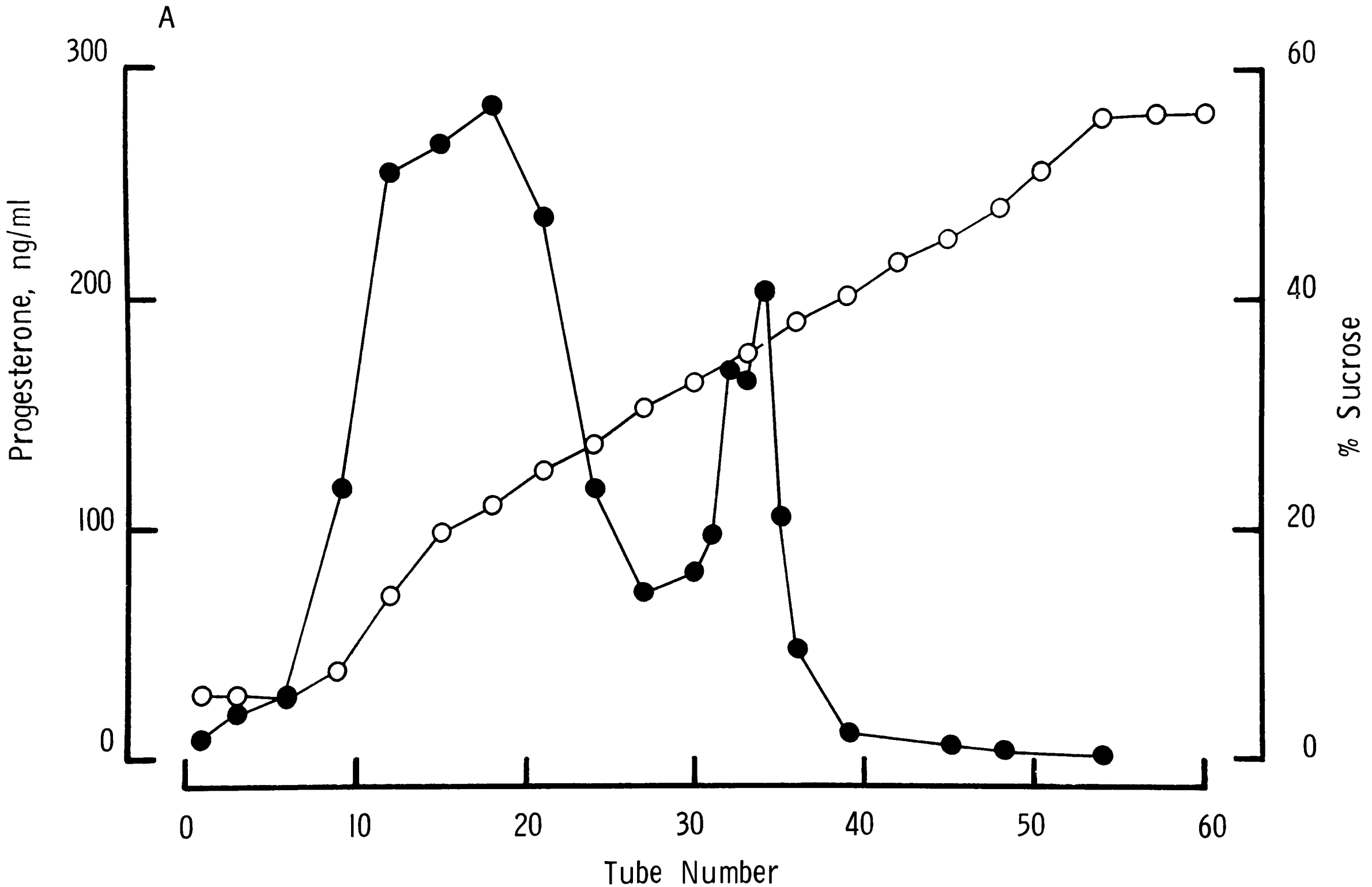
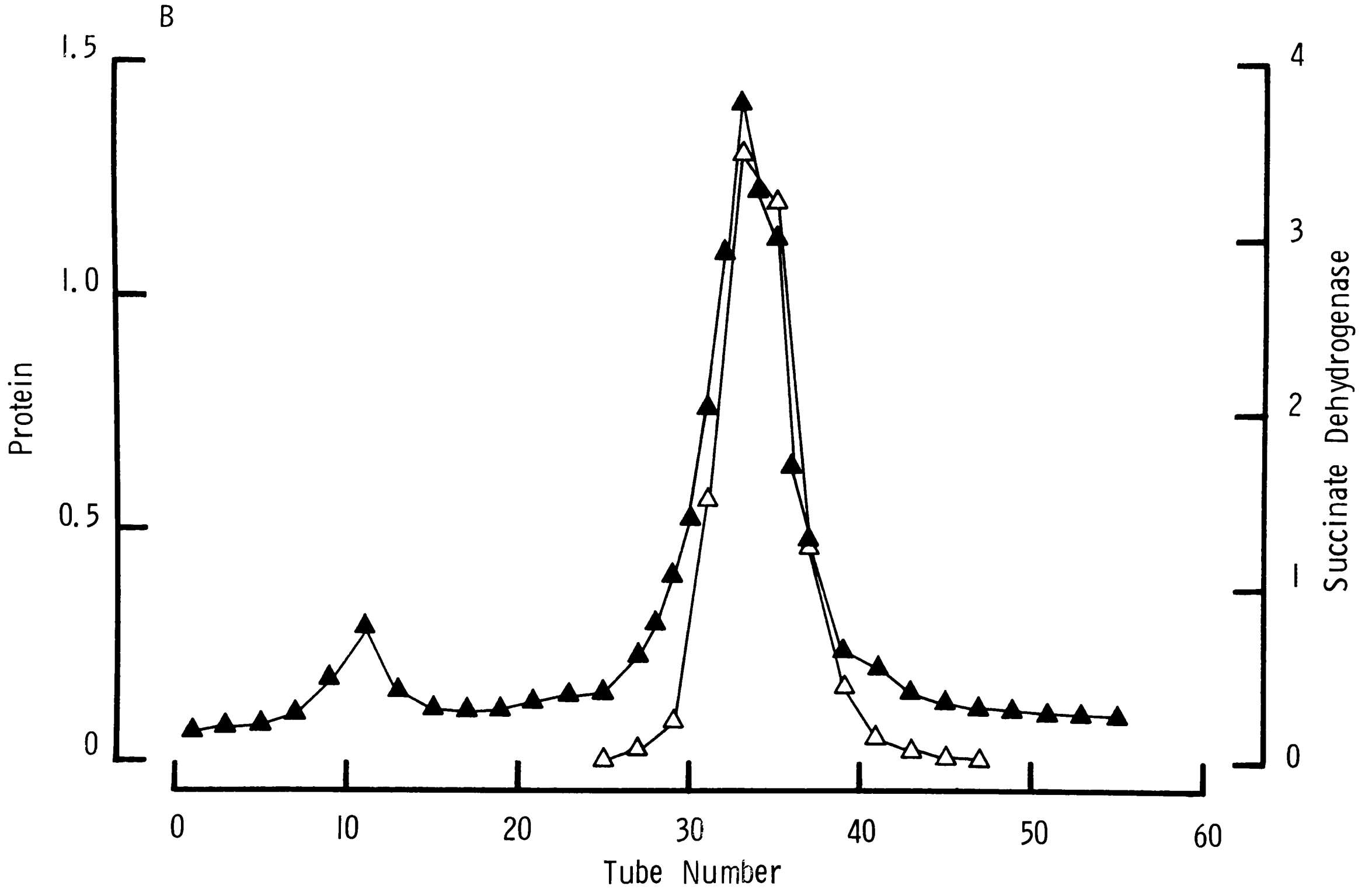

A
300
200
100
0
Progesterone, ng/ml
60
40
20
0
% Sucrose
0
10
20
30
40
50
60
Tube Number

Fig. 3-11B: The distribution of progesterone, protein and succinate dehydrogenase activity on a 15-50% zonal sucrose gradient.

▲ = Protein, $mg.ml^{-1}$

△ = Succinate dehydrogenase, $EU.ml^{-1}$


B
1.5
1.0
0.5
0
Protein
4
3
2
1
0
Succinate Dehydrogenase
0
10
20
30
40
50
60
Tube Number

3.3.3 Morphology and Cytochemistry of Gradient Fractions

Fixed gradient fractions were investigated morphologically and cytochemically in an attempt to correlate marker enzyme data with the morphological appearance of fractionated subcellular components. Concommitantly, the aim was to compare the observations made using intact tissue sections with those made using fractionated components. Morphological examination of fixed fractions from the particulate hormone region of the sucrose gradients was undertaken in order to determine whether or not densely-staining granules were also present, and to thus link particulate progesterone with the presence of electron-dense granules. Subsequent acid phosphatase and peroxidase cytochemistry of fixed gradient fractions allowed lysosomes and microperoxisomes, respectively, to be differentiated from the secretory granules.

3.3.3.1 Morphology

Examination of fixed pooled fractions from three 20-40% w/w sucrose density gradients showed that electron-dense granules were present in tubes 5+6 where progesterone concentration and enrichment were maximal (Fig. 3-12A). These fractions also contained many membrane vesicles of various sizes. In contrast to tissue sections, the shape of granules from the gradient was usually irregular. The size range was consistent with that found in tissue sections. The increase in pleomorphism may have been caused by the experimental manipulations. Electron-dense granules were observed in material from tubes 11+12 and tubes 17+18 of the gradient. These granules were not associated with the presence of progesterone, and were subsequently shown by cytochemical

Fig. 3-12A: Morphological examination of fixed fractions from linear sucrose gradients

An homogenate of mid-cycle corpora lutea from cows was prepared and centrifuged on linear 20-40% w/w sucrose gradients as described under Methods. Tubes of identical sucrose density from three gradients were pooled. One-third of the fixed pellet from pooled tubes 5+6 was examined under the electron microscope. Electron-dense granules (→) are present, together with vesicles of various sizes and other membraneous components. X 28,000

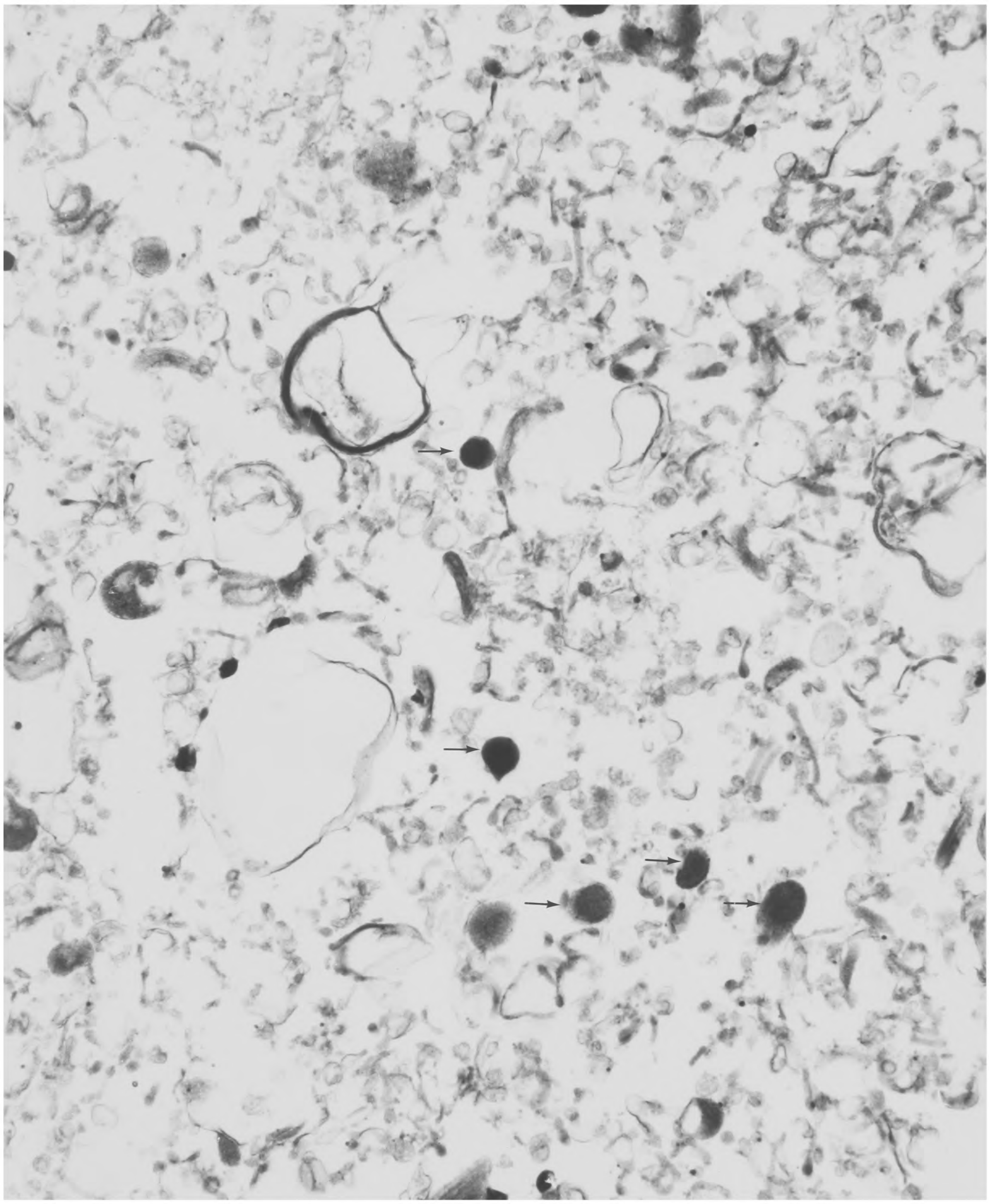
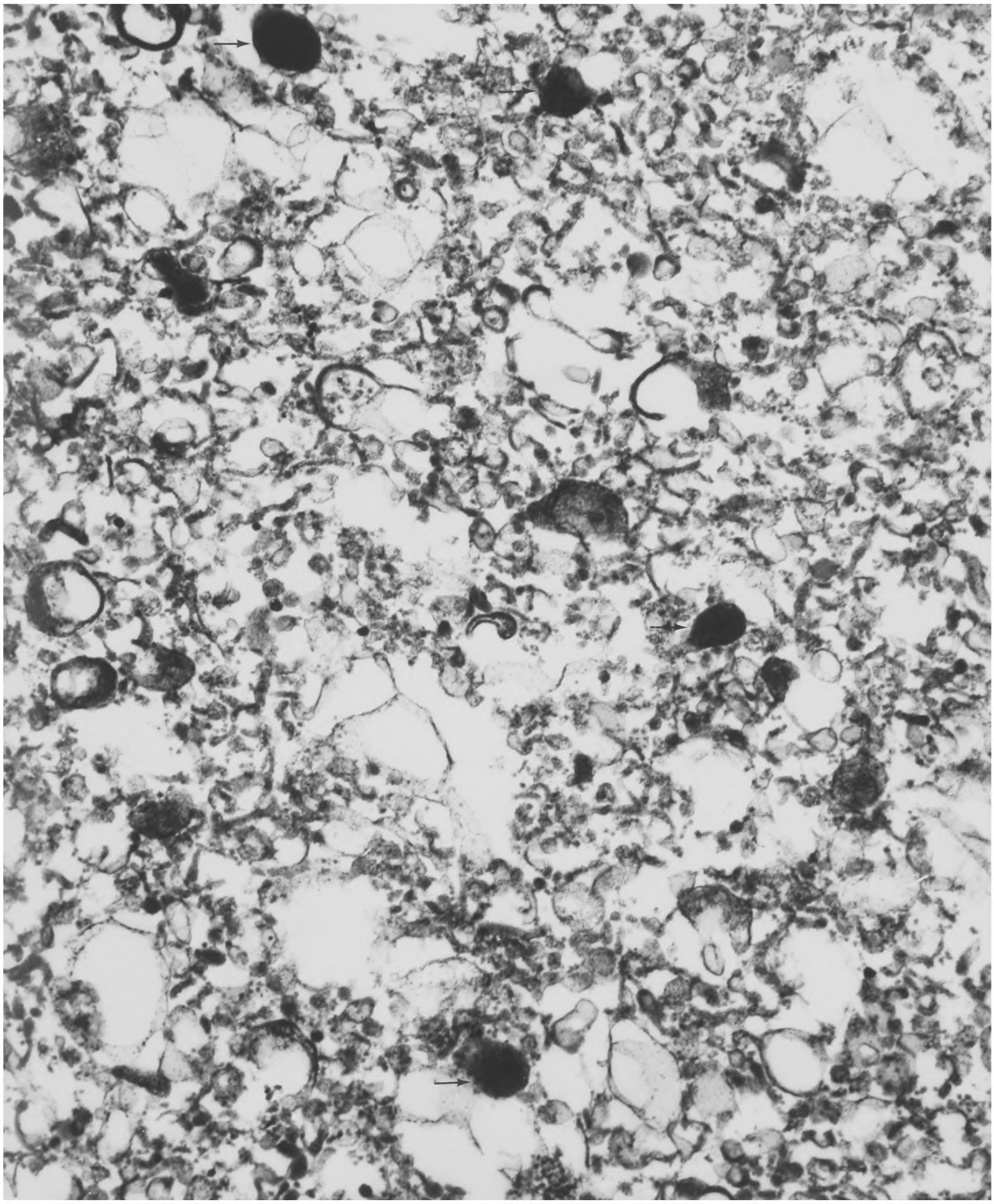


Fig. 3-12B: Morphological examination of fixed fractions from a zonal sucrose gradient

An homogenate of mid-cycle corpora lutea from cows was prepared and centrifuged on a zonal 15-50% w/w sucrose gradient as described under Methods. The fixed pellet from tube 18 was examined under the electron microscope. Electron-dense granules (→) are present, together with vesicles of various sizes and other membraneous components. X 33,750.



techniques to be mainly microperoxisomes or lysosomes. Tubes 11+12 also contained many membrane vesicles consistent with the presence of plasma membrane and agranular endoplasmic reticulum (and this was where the enzyme markers for these subcellular components were maximally enriched). Tubes 17+18 consisted mainly of mitochondria plus some lysosomes and microperoxisomes, which was also in accordance with their respective enzyme marker activities.

Examination of fixed fractions from a 15-50% w/w sucrose zonal gradient identical to that described above (see Fig. 3-11) gave analogous results to those obtained from fixed fractions of the analytical, 20-40% w/w sucrose gradients. Thus, six fractions (tube numbers 14-18 and 20) from the particulate region of the major progesterone peak of the zonal gradient (ranging from 17.7-24.5% w/w sucrose) contained electron-dense granules (Fig. 3-12B). The contamination by other subcellular components was comparable to that observed with the analytical gradient fractions. The electron-dense granules observed in micrographs of the zonal gradient fractions showed less pleomorphism that those granules observed from the corresponding region of the analytical gradients (Fig. 3-12B). A possible explanation resides in consideration of the times taken to complete the two types of gradient experiment: 3.5 h for the zonal gradient experiment compared with 8 h for the analytical gradient experiment. Therefore, in the former case, secretory granules and other subcellular organelles would have less time to become damaged before glutaraldehyde fixation than in the experiments utilizing analytical gradients.

3.3.3.2 Microperoxisome and Lysosome Cytochemistry

Cytochemical studies of fixed fractions from 20-40% w/w sucrose gradients showed that very few (if any) of the granules in tubes 5+6 stained positively with alkaline DAB (not shown). In tubes 11+12 (which contained substantial catalase activity) many positively-staining granules, and only an occasional non-staining granule, were observed (Fig. 3-13). In tubes 17+18 less microperoxisomes were observed per field, probably because of the vast number of mitochondria present in this part of the gradient. Acid phosphatase activity was only observed in material obtained from the densest region of the gradient (tubes 17+18, Fig. 3-14). Tubes 5+6, from the region where particulate progesterone enrichment was maximal (see Fig. 3-6), contained electron-dense granules which cytochemistry showed were neither lysosomes nor microperoxisomes. This correlated with marker enzyme distributions for these latter organelles, which showed negligible activities in tubes 5 and 6.

In the middle of the gradient, at tubes 11 and 12, progesterone content and also progesterone enrichment were minimal, which correlated with the cytochemical finding that the electron-dense granules in this region of the gradient were nearly all microperoxisomes. At the bottom of the gradient, progesterone content was also low but marker enzyme activities for the denser organelles were maximal. Thus, mitochondria dominated the morphological pictures of tubes 17+18, but cytochemistry revealed the presence of small numbers of both microperoxisomes and lysosomes. Few, if any, electron-dense granules were observed in tubes 17+18 that could not be identified as either lysosomes or microperoxisomes. Thus, the

Fig. 3-13: Peroxidase cytochemistry of fixed fractions from a sucrose density gradient

An homogenate of mid-cycle corpora lutea from cows was prepared and centrifuged on linear 20-40% w/w sucrose gradients as described under Methods. Tubes of identical sucrose density from three gradients were pooled. One-third of the fixed pellet from pooled tubes 11+12 was incubated in an alkaline diaminobenzidine medium to demonstrate microperoxisomes. The section is not counter-stained and the dense osmiophilic reaction product can be seen over microperoxisomes (MP). An occasional non-stained granule (→) can be seen. Membrane vesicles constitute the remainder of the field. X 28,000.

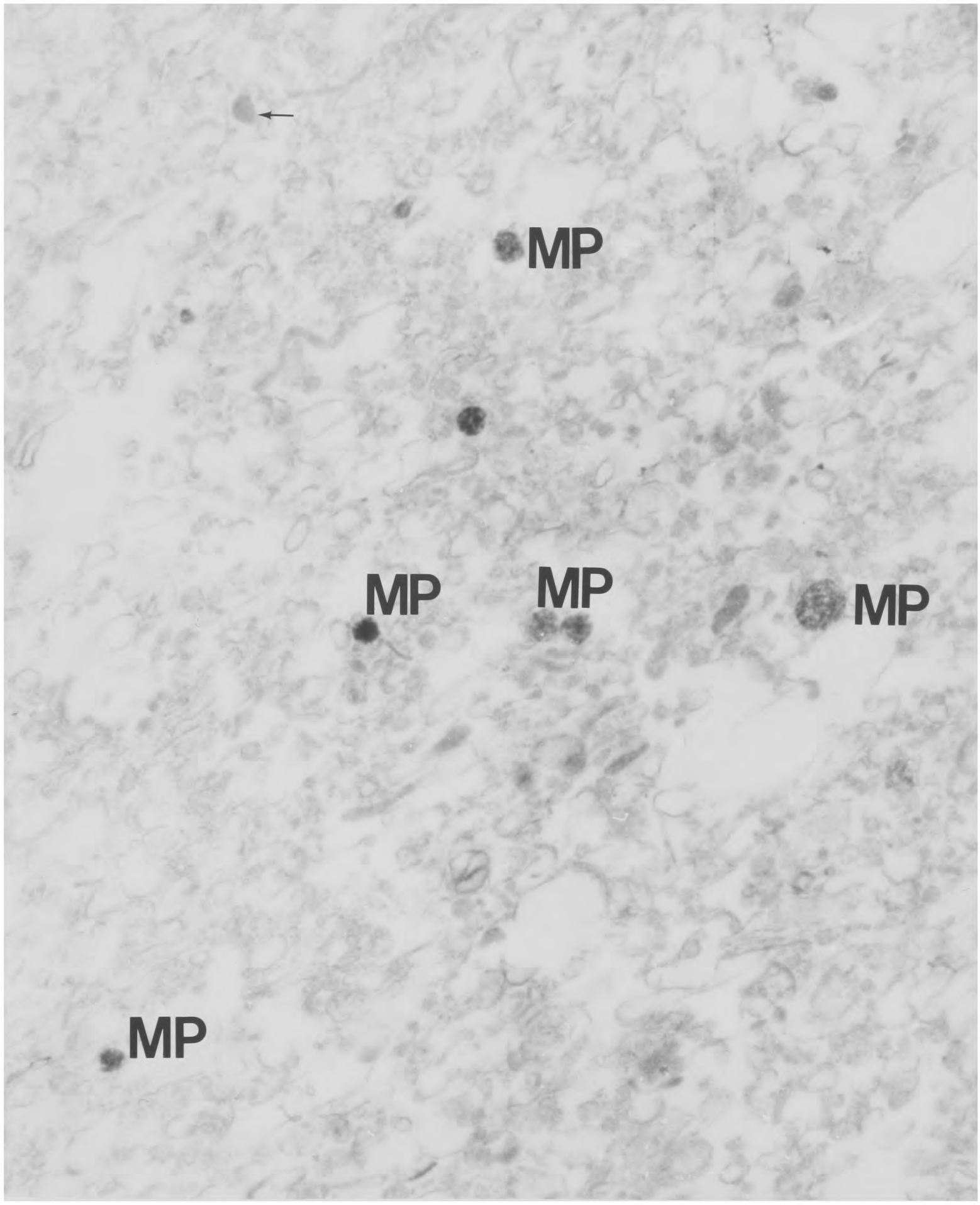
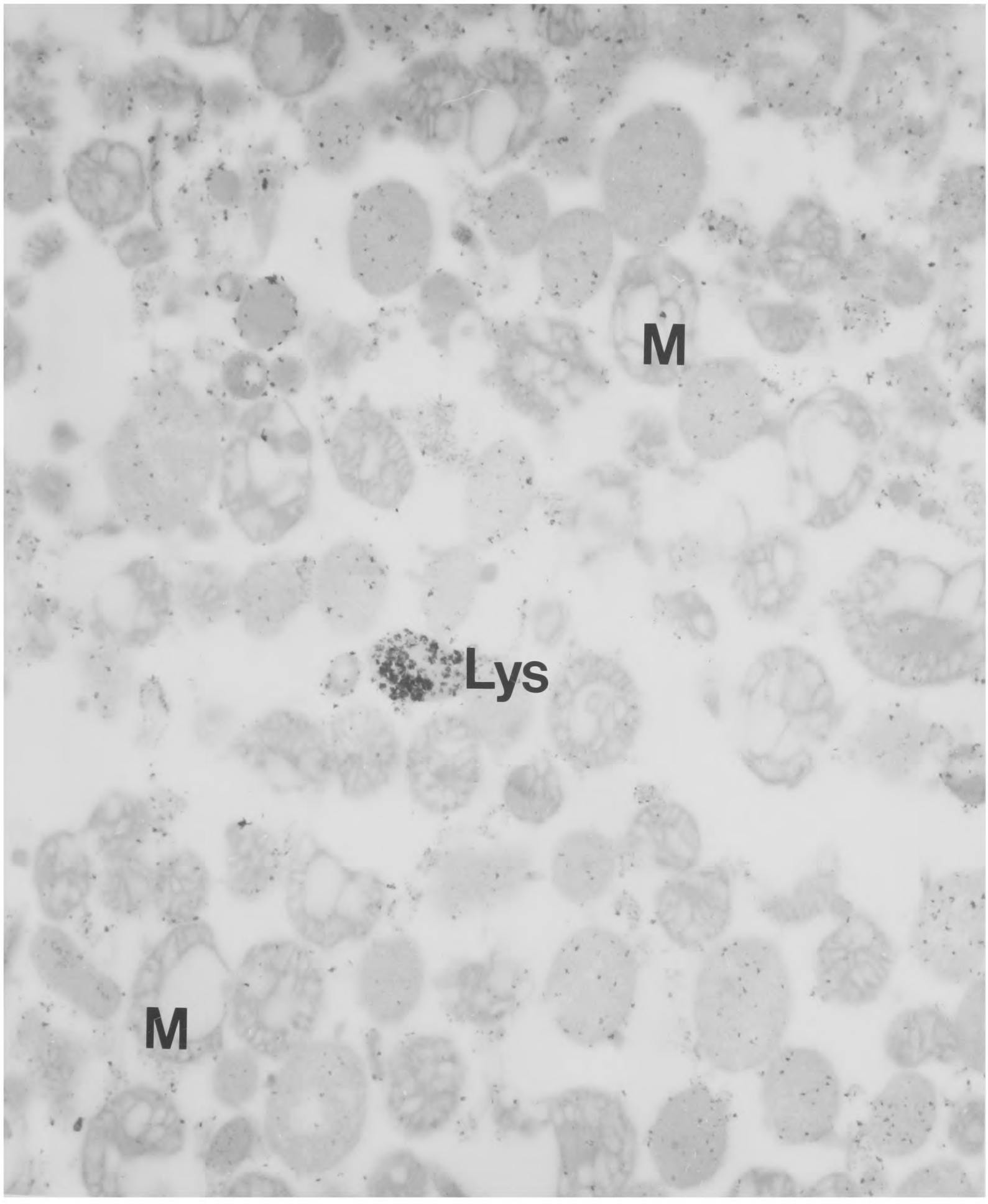

MP
MP
MP
MP
MP

Fig. 3-14: Acid phosphatase cytochemistry of fixed fractions from a sucrose density gradient

An homogenate of mid-cycle corpora lutea from cows was prepared and centrifuged on linear 20-40% w/w sucrose gradients as described under Methods. Tubes of identical sucrose density from three gradients were pooled. One-third of the fixed pellet from pooled tubes 17+18 was incubated to demonstrate the presence of acid phosphatase activity. The dense reaction product identifies a lysosome (Lys); some background stain is observed. The rest of the field consists principally of mitochondria (M). X 26,000.


M
Lys
M

morphological and cytochemical findings confirmed the marker enzyme distributions.

3.3.4 Treatments of Homogenate H_2 Before Sucrose Gradient Centrifugation

If particulate progesterone was localized inside a membrane-bound organelle such as the secretory organelle described in ultrastructural studies of sheep and cow corpora lutea, then treatments which disrupt membrane-bound organelles in other tissues (de Duve, 1971; Steck, 1972) might also disrupt the secretory granules and render soluble their particulate progesterone. Four treatments of the resuspended 10,000 g pellet (H_2) were carried out prior to centrifuging aliquots of it on 20-40% w/w sucrose gradients in the SW27 rotor. The objective of the first three treatments - repeated freezing and thawing, partial sonication, or exposure to Triton X-100 detergent - was to test whether disruption of subcellular organelles (measureable by enzyme markers) would alter the banding characteristics of particulate progesterone. In the fourth treatment, a range of radioactively-labelled steroids were equilibrated with H_2 prior to its fractionation on sucrose gradients. The aim here was to determine if the sedimentable progesterone would equilibrate with exogenous steroid, and to determine if the penetration of progesterone into the gradient was specific to this steroid hormone.

1. Effect of Repeated Freezing and Thawing: Fig. 3-15 shows the effect that five rapid cycles of freezing and thawing of H_2 had on the subcellular constituent profiles of the 20-40% w/w sucrose gradient compared with the control gradient for this experiment (Fig. 3-16). The progesterone profile was unaltered

Fig. 3-15A: The effect of freezing and thawing of H_2 on the distribution of progesterone and marker enzymes on a 20-40% sucrose gradient.

An aliquot (1.5 ml) of H_2 was rapidly subjected to five cycles of freezing and thawing using a dry ice/acetone bath and 22°C waterbath, respectively. It was then loaded onto a linear 20-40% w/w sucrose gradient, which was centrifuged and eluted as described in the Methodology section and in Fig. 3-5.

The abscissa denotes the tube number as eluted from the gradient. On the ordinate, the mass or enzyme activity in each tube is plotted as a percentage of the total mass or activity recovered from the gradient.

● = Progesterone
▲ = Acid phosphatase
△ = Alkaline phosphatase
○ = NADPH-cytochrome c reductase

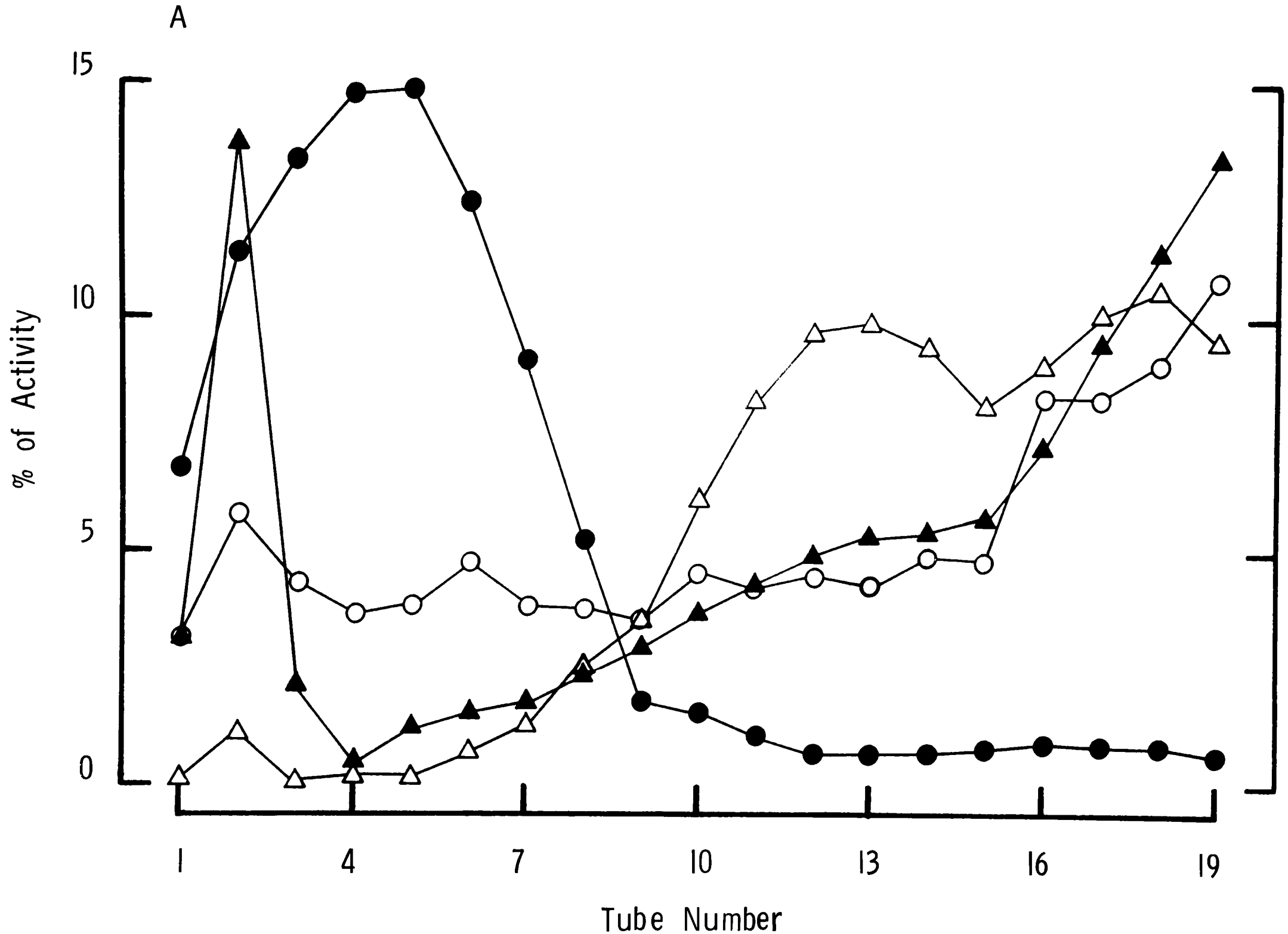

A
15
10
5
0
% of Activity
1
4
7
10
13
16
19
Tube Number

Fig. 3-15B: The effect of freezing and thawing of H_2 on the distribution of progesterone and marker enzymes on a 20-40% sucrose gradient.

Biochemical analysis of aliquots of the fractionation stages and of the gradient fractions revealed the following information:

	% of H_1 in H_2	Amount loaded onto gradient	% Recovery from gradient
Progesterone	16.4	4.49 µg	99.6
Acid phosphatase	21.0	0.583 EU	114.4
β-Glucuronidase	30.4	0.188 EU	83.7
Succinate dehydrogenase	52.3	7.23 EU	83.0
Alkaline phosphatase	31.4	1.085 EU	70.0
NADH-cytochrome c reductase	29.9	3.21 EU	81.0
NADPH-cytochrome c reductase	19.4	0.156 EU	97.4
Protein	34.0	13.98 mg	-

● = β-Glucuronidase
○ = % Sucrose, w/w
▲ = NADH-cytochrome c reductase
△ = Succinate dehydrogenase

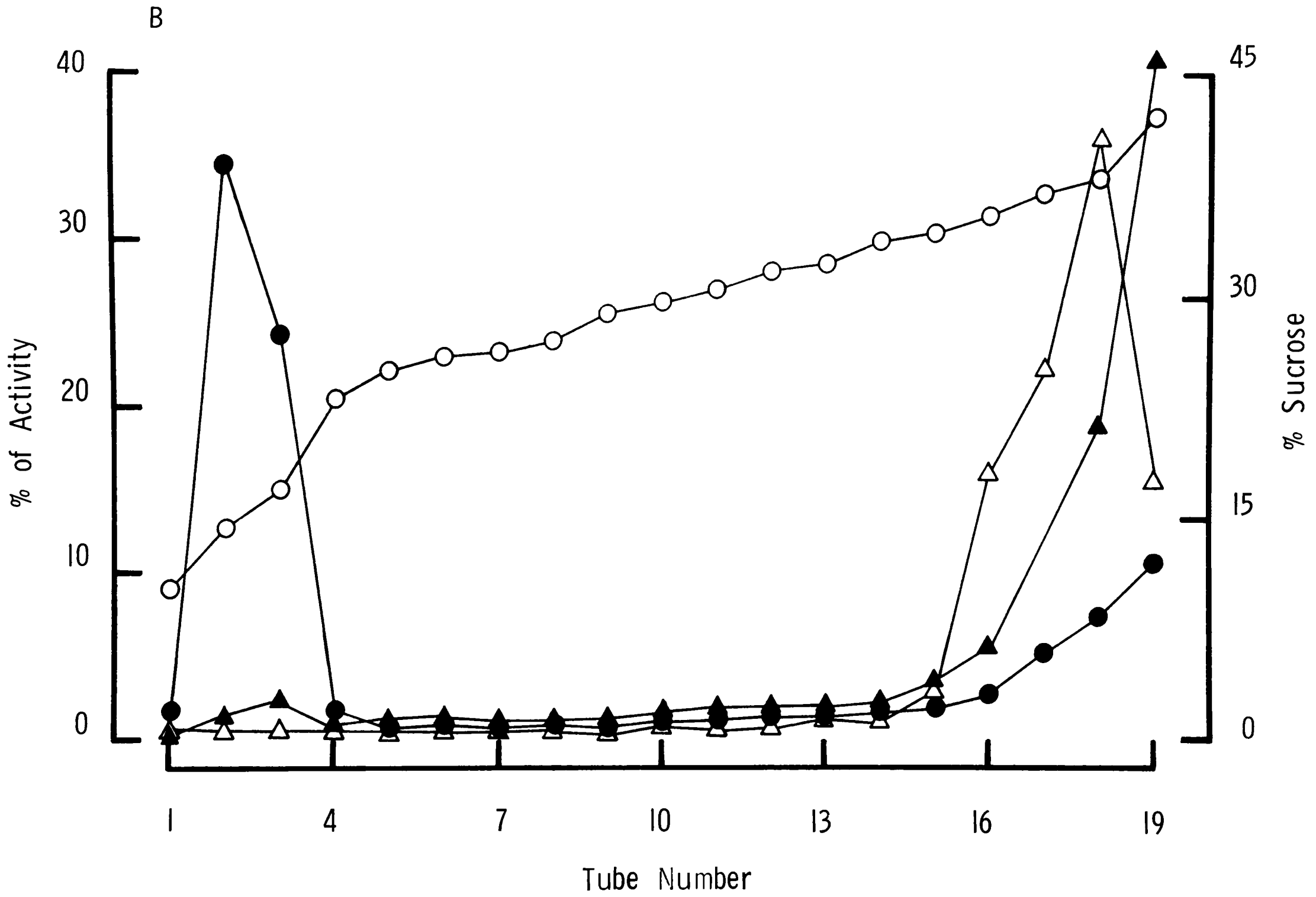

B
40
30
20
10
0
% of Activity
45
30
15
0
% Sucrose
1
4
7
10
13
16
19
Tube Number

Fig. 3-16A: The distribution of progesterone and marker enzymes on a 20-40% sucrose gradient.

An aliquot of the resuspended 10,000 g_{av} pellet (H_2), prepared from an homogenate of bovine corpora lutea, was loaded onto a 20-40% w/w sucrose gradient. The material applied to the gradient was derived from 0.68 gm of minced tissue. Centrifugation was carried out for 2.5 h at 82,500 g_{av}.

The abscissa denotes the tube number as eluted from the gradient. On the ordinate, the mass or enzyme activity in each tube is plotted as a percentage of the total mass or activity recovered from the gradient.

● = Progesterone (0-15% activity)
△ = NADPH-cytochrome c reductase (0-15% activity)
○ = Acid phosphatase (0-25% activity)
▲ = Alkaline phosphatase (0-25% activity)

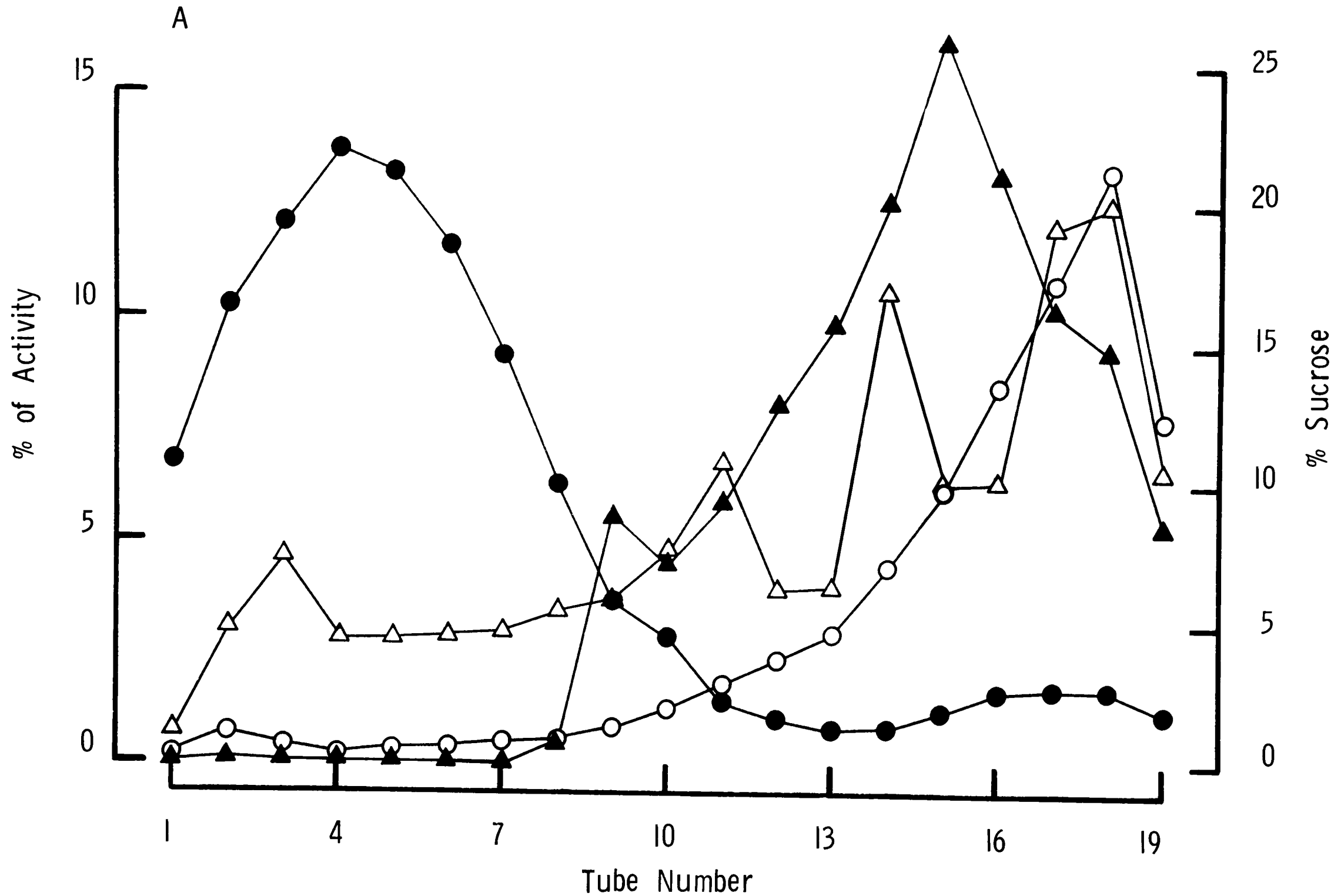

A
15
10
5
0
% of Activity
25
20
15
10
5
0
% Sucrose
1
4
7
10
13
16
19
Tube Number

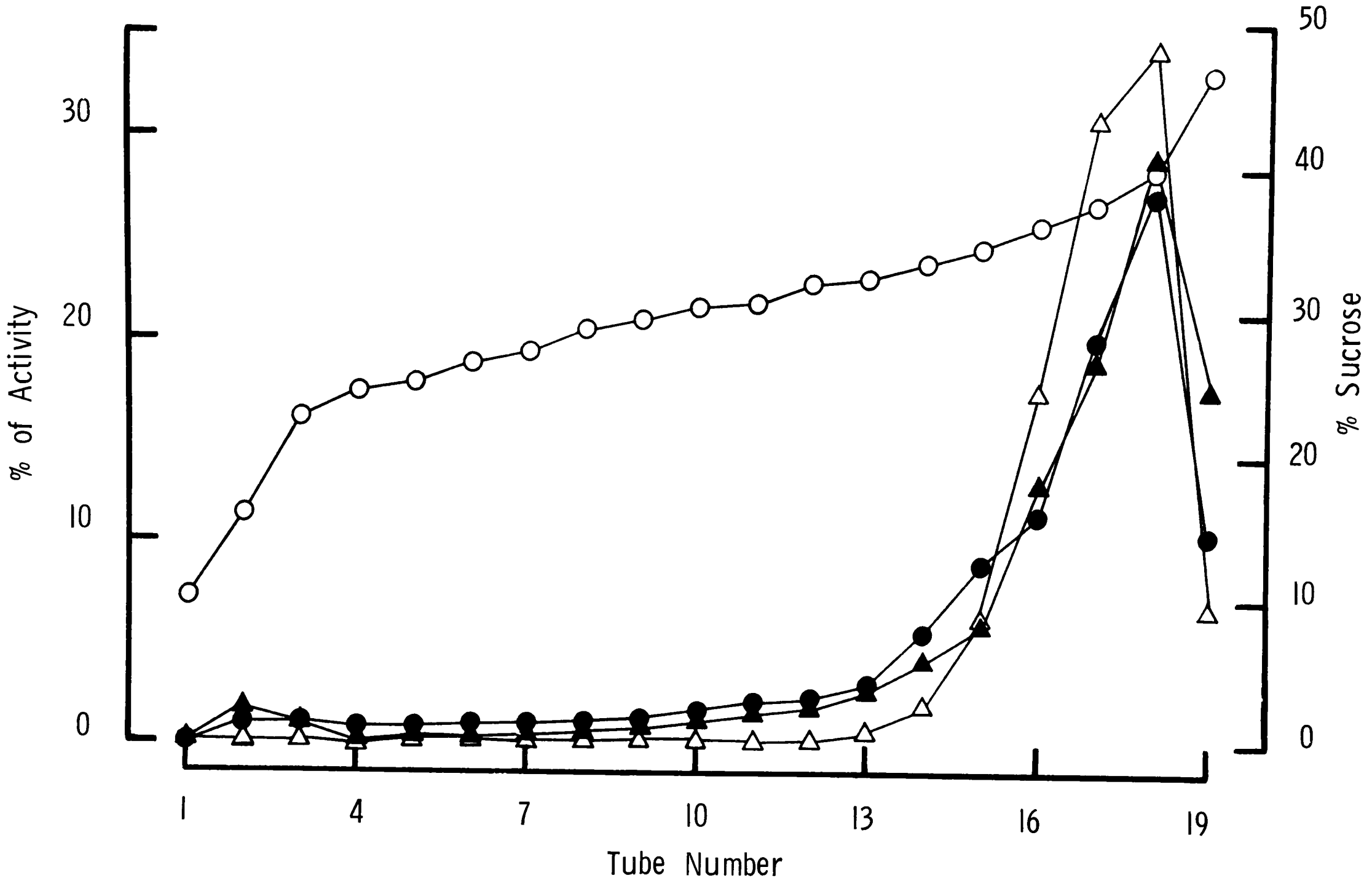
Fig. 3-16B: The distribution of progesterone and marker enzymes on a 20-40% sucrose gradient.

Biochemical analysis of aliquots of the fractionation stages and of the gradient fractions revealed the following information:

	% of H_1 in H_2	Amount loaded onto gradient	% Recovery from gradient
Progesterone	16.4	4.49 µg	116.2
Protein	34.0	13.98 mg	-
Acid phosphatase	21.0	0.583 EU	114.2
β-Glucuronidase	30.4	0.188 EU	93.4
Succinate dehydrogenase	52.3	7.23	97.2
Alkaline phosphatase	31.4	1.085 EU	79.8
NADH-cytochrome c reductase	29.9	3.21 EU	77.6
NADPH-cytochrome c reductase	19.4	0.156 EU	89.5

▲ = β-Glucuronidase
△ = Succinate dehydrogenase
● = NADH-cytochrome c reductase
○ = % Sucrose, w/w

B


% of Activity
30
20
10
0
50
40
30
20
10
0
% Sucrose
1
4
7
10
13
16
19
Tube Number

although most of the β-glucuronidase and about half of the acid phosphatase activities were rendered soluble. The separation of acid phosphatase upon repeated freezing and thawing into soluble and particulate fractions has been reported previously (Mahadevan and Tappel, 1968), and attributed to the presence of isozymes (Sloat and Allen, 1969). On the other hand, the banding positions of mitochondrial, plasma membrane and endoplasmic reticulum constituents (identified by their succinate dehydrogenase, alkaline phosphatase and NADH/NADPH-dependent cytochrome c reductase activities, respectively), were not altered by repeated freezing and thawing.

2. Effect of Triton X-100: Fig. 3-17 shows the effect of pre-treating H_2 with the non-ionic detergent, Triton X-100, to a final concentration of 0.5% w/v on the sucrose gradient profiles of progesterone and marker enzymes. Particulate progesterone was almost completely solubilized and no "mitochondrial" progesterone was found. The detergent also shifted the banding positions of some of the marker enzyme-identifiable organelles relative to the control gradient upon which untreated H_2 was fractionated (Fig. 3-17). Thus, the lysosomes were completely disrupted and their marker enzyme activities (acid phosphatase and β-glucuronidase) solubilized. Succinate dehydrogenase activity showed that the mitochondria were rendered less dense, and that they banded over a wider density range than mitochondria not exposed to detergent. Furthermore, the membraneous components had been significantly solubilized so that 30% of the alkaline phosphatase and 61% and 48% of the NADH- and NADPH-dependent cytochrome c reductase activities, respectively, were located in the first 3 fractions of the gradient.

Fig. 3-17A: The effect of treating H_2 with Triton X-100 on the distribution of progesterone and marker enzymes on a 20-40% sucrose gradient.

A 1.5 ml aliquot of the resuspended 10,000 g_{av} pellet (H_2) was made 0.5% w/v in Triton X-100 by the addition of 0.075 ml of 10% w/v detergent (dissolved in buffered 0.25 M sucrose) and kept on ice, with intermittent shaking, for 30 min. The mixture was then loaded onto a 20-40% w/w sucrose gradient, which was centrifuged for 2.25 h at 82,500 g_{av}.

The abscissa denotes the tube number as eluted from the gradient. On the ordinate, the mass or enzyme activity in each tube is plotted as a percentage of the total mass or activity recovered from the gradient.

● = Progesterone
▲ = Acid phosphatase
△ = β-Glucuronidase
○ = % Sucrose, w/w

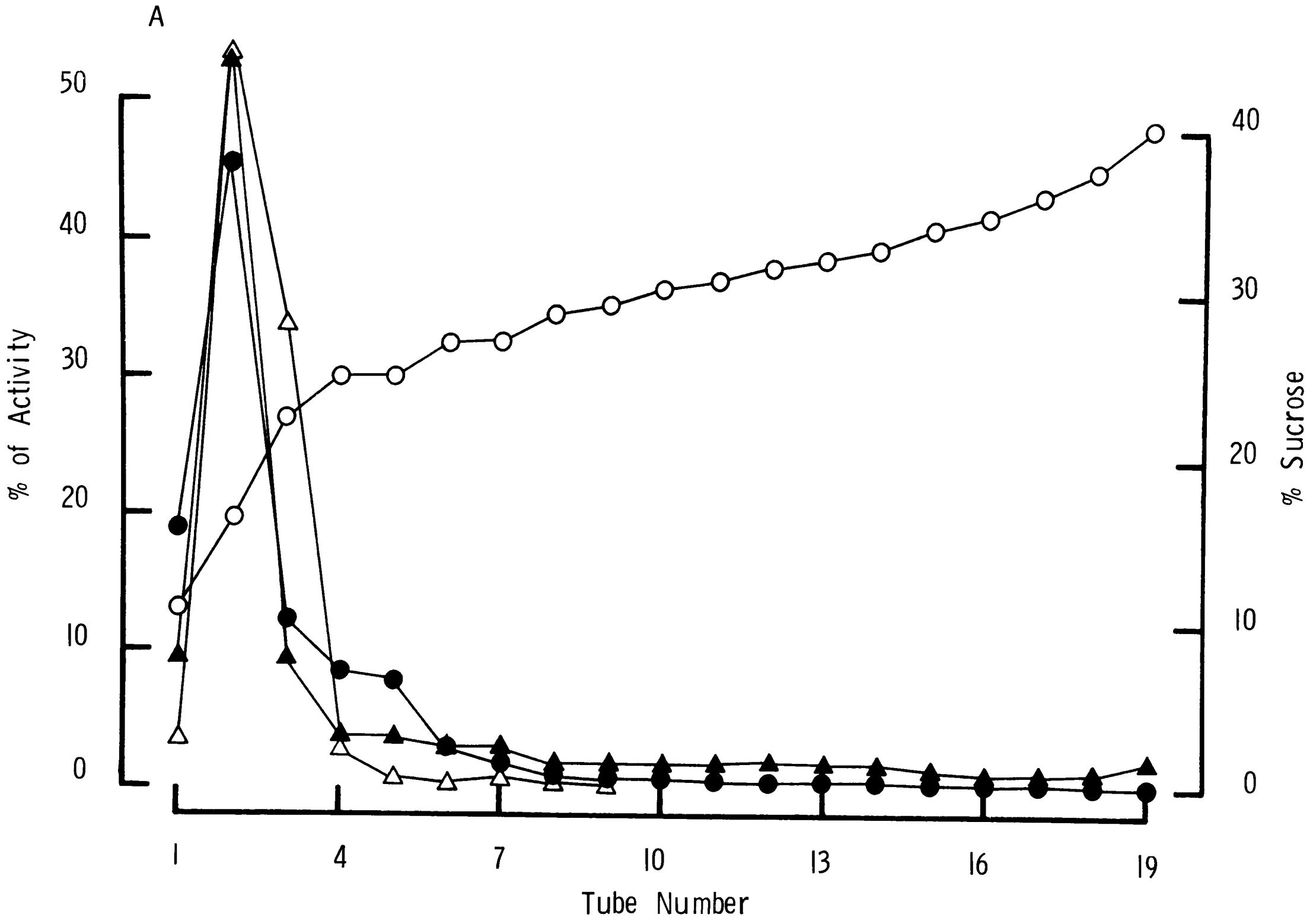

A
50
40
30
20
10
0
% of Activity
40
30
20
10
0
% Sucrose
1
4
7
10
13
16
19
Tube Number

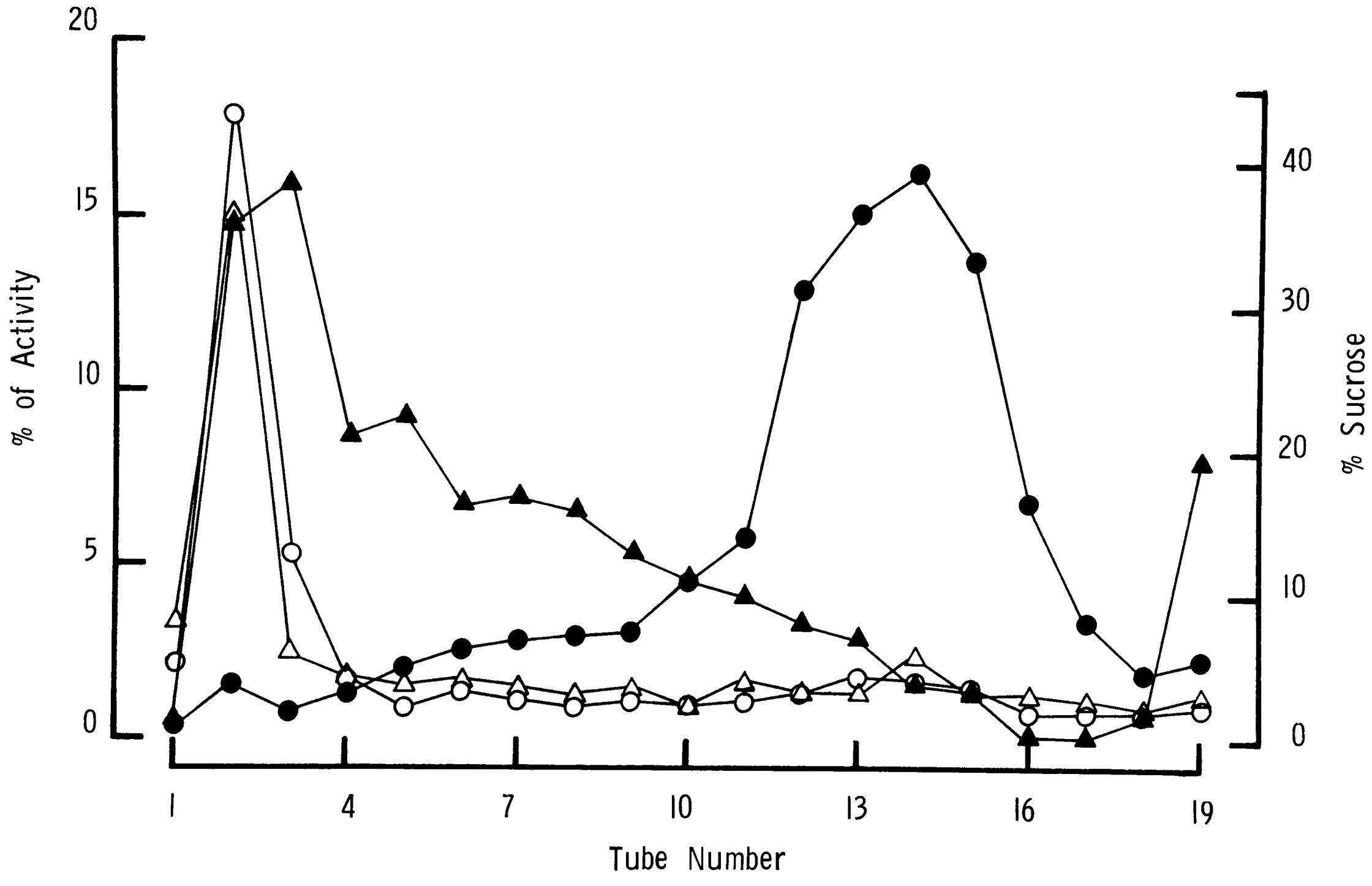
Fig. 3-17B: The effect of treating H_2 with Triton X-100 on the distribution of progesterone and marker enzymes on a 20-40% sucrose gradient.

Biochemical analysis of aliquots of the fractionation stages and of the gradient fractions revealed the following information:

	% of H_1 in H_2	Amount loaded onto gradient	% Recovery from gradient
Progesterone	16.4	4.99 µg	102.2
Acid phosphatase	21.0	0.583 EU	106.6
β-Glucuronidase	30.4	0.188 EU	63.4
Succinate dehydrogenase	52.3	7.23 EU	56.3
Alkaline phosphatase	31.4	1.085 EU	29.9
NADH-cytochrome c reductase	29.9	3.21 EU	25.5
NADPH-cytochrome c reductase	19.4	0.156 EU	96.0

● = Succinate dehydrogenase (0-20% activity)
▲ = Alkaline phosphatase (0-20% activity)
○ = NADH-cytochrome c reductase (0-45% activity)
△ = NADPH-cytochrome c reductase (0-45% activity)

B


20
15
10
5
0
% of Activity
40
30
20
10
0
% Sucrose
1
4
7
10
13
16
19
Tube Number

It should be remembered that NADH-dependent cytochrome c reductase has a dual location in the cells of the bovine corpus luteum (see Fig. 3-5), being associated with both the endoplasmic reticulum as well as with the outer mitochondrial membrane. Such a dual location for this enzyme has been reported in other tissues, for example mouse liver (de Duve et al., 1955), although this is not always the case since it is only associated with the microsomal fraction in rat liver (Amar-Costesec et al., 1974).

3. Effect of Partial Sonication: In this experiment, incomplete sonication (incomplete for technical reasons) was performed on the resuspended 10,000 g pellet, H_2. The resulting gradient profiles (not shown) for progesterone and marker enzymes revealed that lysosomal disruption and a shift in the hormone profile toward greater solubility was achieved, analogous to that obtained by detergent pre-treatment.

4. Effect of Equilibrating with Radioactive Steroids: The resuspended 10,000 g pellet (H_2) was incubated with added radioactive progesterone, cortisol or R5020 before being loaded onto sucrose density gradients. Fig. 3-18 shows the ^{3}H-cortisol and ^{14}C-progesterone profiles of a 20-40% w/w sucrose gradient when H_2 had been equilibrated with labelled steroids before fractionation. Exogenous, radioactive progesterone traced an analogous sedimentation profile to that of endogenous progesterone, determined in the control gradient (not shown) by radioimmunoassay. However, exogenous cortisol remained in the soluble region at the top of the gradient. The profiles were unaffected by the use of tracer amounts of cortisol or by the use of exogenous cortisol in amounts equimolar to that of

Fig. 3-18: The distribution of radioactively-labelled progesterone and cortisol on a 20-40% sucrose gradient

The resuspended 10,000 g_{av} pellet (AH_2) was prepared in the usual manner from an homogenate of bovine corpora lutea (see Methods, section 3.2.1 and Fig. 3-1). It was then incubated with ^{3}H-cortisol, ^{14}C-progesterone and unlabelled cortisol for 1 h on ice, and centrifuged again at 10,000 g_{av} for 30 min. An aliquot (1.5 ml) of the second resuspended pellet (BH_2) was loaded onto a 20-40% w/w sucrose gradient, which was centrifuged at 82,500 g_{av} for 2.5 h.

Two ml fractions from the gradient were collected, 0.5 ml aliquots of which were counted in 10 ml of Cocktail N scintillant. Counting efficiencies were calculated by reference to appropriate quench curves.

Liquid scintillation counting of aliquots of the fractionation stages and gradient fractions provided the following additional information:

Fractionation Stage	% Distribution	
	Cortisol	Progesterone
First 10,000 g resuspended pellet (AH_2)	100	100
Second 10,000 g resuspended pellet (BH_2)	22.2	63.8
Second 10,000 g supernatant (BS_2)	74.0	26.2

	Cortisol	Progesterone
Radioactive steroid applied to gradient	33,134 dpm	60,190 dpm
Recovery of radioactive steroid from gradient	94.8%	82.3%
Total steroid applied to gradient	3.76 nmol	20 nmol (by RIA)

● = ^{14}C-progesterone, % of total recovered per fraction

▲ = ^{3}H-cortisol, % of total recovered per fraction

○ = % Sucrose, w/w

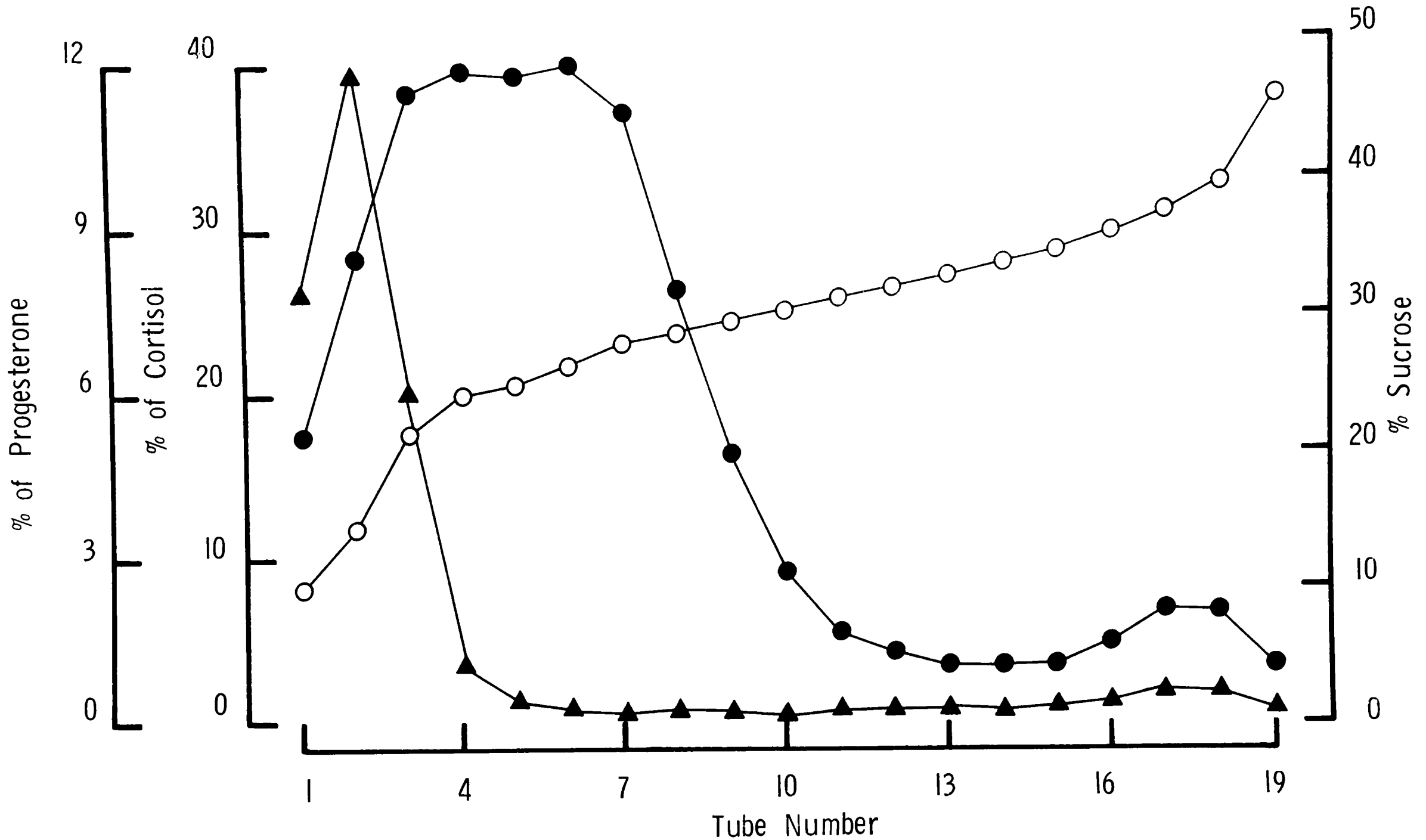

% of Progesterone
12
9
6
3
0
% of Cortisol
40
30
20
10
0
% Sucrose
50
40
30
20
10
0
1
4
7
10
13
16
19
Tube Number

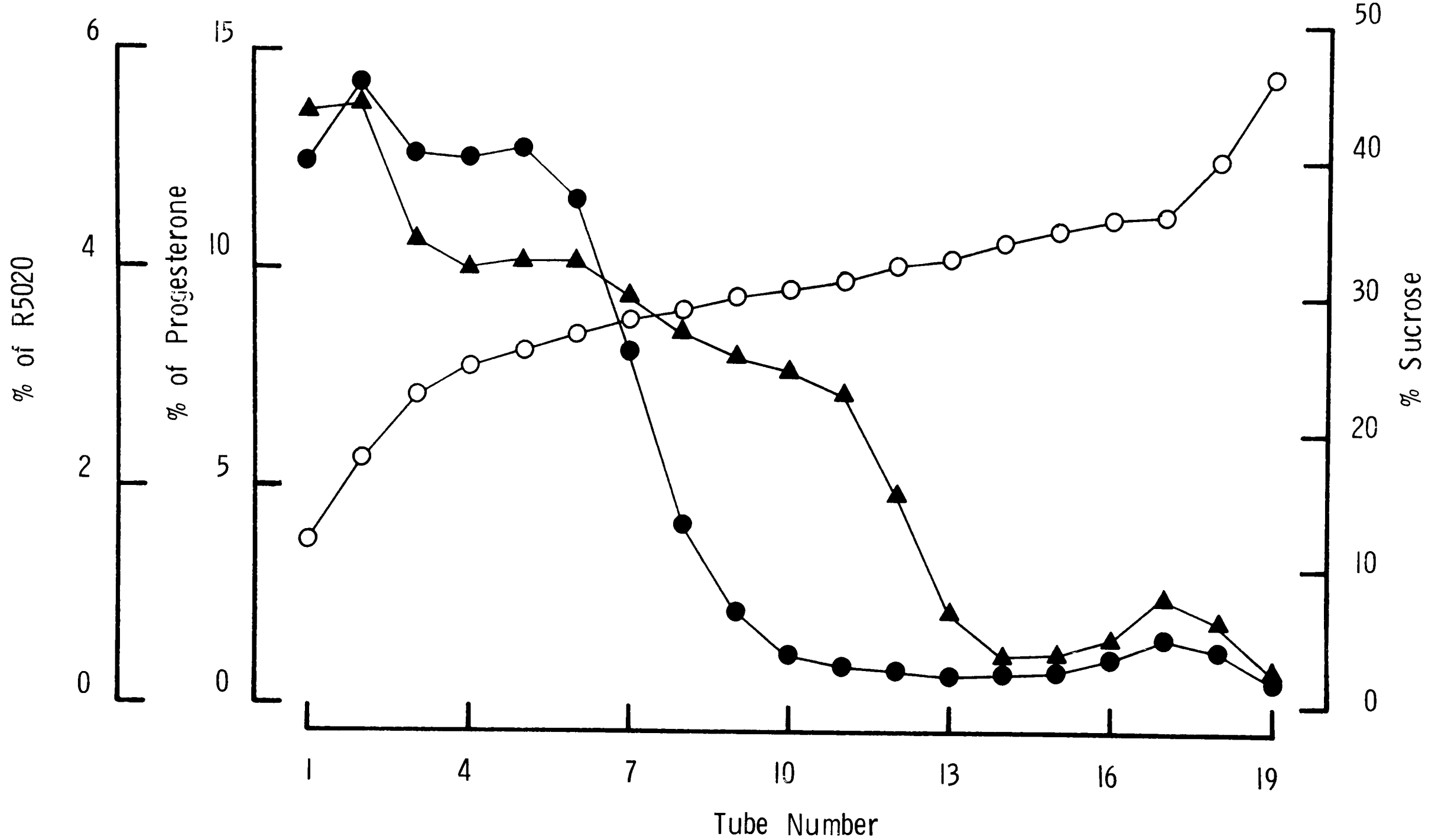
Fig. 3-19: The distribution of radioactively-labelled progesterone and R5020 in a 20-40% sucrose gradient.

The resuspended 10,000 g_{av} pellet (H_2) was prepared from an homogenate of bovine corpora lutea. It was incubated with ^{3}H-R5020 and ^{14}C-progesterone for 1.25 h on ice, after which a 1.0 ml aliquot was applied to a 20-40% w/w sucrose gradient. The gradient was centrifuged for 2.25 h at 82,500 g_{av}, eluted, and its radioactivity was counted as described for Fig. 3-18.

	R5020	Progesterone
Radioactive steroid applied to gradient	282,967 dpm	192,753 dpm
Recovery of radioactive steroid from gradient	73.5%	77.5%
Total steroid applied to gradient	1.5 pmol	16.36 nmol[a]

[a]Endogenous progesterone was measured by RIA.

● = ^{14}C-progesterone, % of total recovered per fraction
▲ = ^{3}H-R5020, % of total recovered per fraction
○ = % Sucrose, w/w


% of R5020
% of Progesterone
% Sucrose
6
4
2
0
15
10
5
0
50
40
30
20
10
0
1
4
7
10
13
16
19
Tube Number

endogenous progesterone.

Furthermore, the two steroids were differentially taken up by H_2 (see legend to Fig. 3-18): the re-centrifuged 10,000 g pellet bound approximately two-thirds of the added progesterone, whilst it bound only one-third of the added cortisol. Thus, some 3-fold more progesterone than cortisol was bound by H_2, and these steroid hormones were resolved differently on sucrose density gradients.

In a separate experiment, radioactive progesterone and R5020 were incubated with H_2: Fig. 3-19 shows that R5020 penetrated further into the subsequent sucrose gradient than did progesterone. Table 3-6 summarizes the results of additional experiments where a range of radioactive steroids were equilibrated with homogenate H_2 and their distribution on sucrose gradients determined. The steroid profiles fell into two distinct groups - those whose profiles mimiced that of progesterone and those whose profiles were of the "cortisol-type" whereby the particular steroid did not enter the gradient but, rather, remained in the soluble sample zone at the top of the gradient. The exception was the synthetic progestogen, R5020, which penetrated further into the gradient than did progesterone itself.

Enters the gradient:	Does not enter the gradient:
Progesterone	Cortisol
Pregnenolone (as progesterone)[a]	Aldosterone
R5020[b]	Androstenedione
Oestradiol-17β	Dehydroepiandrosterone
Oestradiol-17α	Testosterone
Oestrone	Cholesterol

Table 3-6: Penetration of steroids into 20-40% w/w sucrose gradients.

The results were obtained from several different experiments. Aliquots of the resuspended 10,000 g pellet (H_2), obtained from homogenates of bovine lutea, were equilibrated with tracer quantities (2.5-5 pmol) of ^{14}C-progesterone and ^{3}H-steroid for 30 min on ice with occasional stirring. Then 1.5 ml of each mixture was loaded onto 20-40% w/w sucrose gradients and centrifuged in the SW27 rotor for 2.25 h at 82,500 g at 4^{o}C. Fractions of 2 ml were collected, and 0.5 ml aliquots were counted by liquid scintillation in 10 ml of Cocktail N Scintillant.

For cortisol, oestradiol-17β, oestrone and androstenedione, separate experiments were carried out using exogenous steroid concentrations which were approximately equimolar to that of the endogenous progesterone concentration (about 16 nmol/ml), but their gradient profiles were unchanged from those using only tracer quantities.

a, TLC showed that pregnenolone was quantitatively converted to progesterone during the course of the experiment.

b, R5020 penetrated further into the gradient than any of the other steroids (see Fig. 3-19).

3.4 DISCUSSION

The subcellular fractionation studies reported here have confirmed the observation of Kramers et al. (1975) that the bovine corpus luteum contains some of its progesterone in a form sedimentable by centrifugation. Fractionation of this sedimentable portion of progesterone using sucrose gradients resolved it into a peak which banded discretely from most of the known organelles of luteal cells. Biochemical analysis of gradient fractions, coupled with their morphological and cytochemical examination, showed that electron-dense granules banded together with the peak of particulate hormone and that these granules were neither lysosomes nor microperoxisomes. Therefore, it is suggested that the particulate progesterone was sequestered inside these electron-dense granules.

Mid-cycle corpora lutea were used in these experiments because at this stage of the oestrus cycle their production of progesterone and concentration of electron-dense granules were greatest. The interval between death at the abbatoir and the start of an experiment in the laboratory was some 60 to 90 min. During this time, the intracellular location of progesterone, activity of steroidogenic enzymes, and integrity of subcellular organelles were unlikely to have been affected since Armstrong and Black (1966) have shown that there are no morphological or steroidogenic differences between cells isolated from bovine corpora lutea immediately after death or some 2 h post mortem.

The homogenization of corpus luteum tissue was simultaneously required to be as complete and as mild as possible. Therefore, the tissue was minced prior to homogenization to

ensure that homogenization was performed on pieces of tissue of uniform size. Together with the standardization of the homogenization conditions, this enabled the starting homogenate of bovine corpus luteum to be as constant as possible from one experiment to the next, bearing in mind that corpora lutea were judged to be mid-cycle from their gross anatomical appearance. Although the homogenization of luteal cells was not complete, some organelle damage resulted during the process since some of the lysosomal and microperoxisomal marker enzyme activities were recovered in the cytosol (Table 3-5).

Differential centrifugation of the 600 g supernatant derived from the starting homogenate shows that the cow corpus luteum, like that of sheep corpus luteum (Gemmell et al., 1974), contains one-third of its total progesterone in particulate form, since it can be sedimented by centrifugation. This figure is probably an underestimate since the 600 g centrifugation step removed unbroken cells and large cytoplasmic fragments, which would contain some progesterone. In fact, 30% of the total progesterone is lost in the 600 g pellet. If the hormone in this pellet is distributed similarly to the hormone released from the homogenized cells, then particulate progesterone would constitute one-third of the hormone entrapped in this pellet. This implies that the sedimentable fraction of progesterone in the bovine corpus luteum is at least 40%. In this context, it should be recalled that electron micrographs of the 600 g pellet revealed the presence of morphologically intact, electron-dense granules in both unbroken cells and large cytoplasmic fragments. Sedimentable steroid hormones,

consistent with particulate localizations, also appear to be freatures of other steroidogenic tissues such as the placenta and adrenal cortex. In preliminary experiments (data not shown), about 20% of the total progesterone was sedimented at 5,000 g from an homogenate of ovine placenta, and 22% of added radioactive cortisol was recovered in the 10,000 g pellet after centrifugation of an homogenate of bovine adrenal cortex.

The description of "sedimentable" progesterone as also "particulate" hormone is justified from the finding that once progesterone was sedimented at 10,000 g, two further re-suspension and re-centrifugation cycles of the pellet released only minor amounts of progesterone.

The morphometric studies of Chapter 2 showed that the electron-dense granules represented only 2-3% of the cytoplasm of the luteal cells of the mid-cycle corpus luteum of the cow. Comparable values are unavailable for the corpora lutea of other species. Furthermore, the population of electron-dense granules of the bovine corpus luteum includes microperoxisomes, lysosomes and possibly other types of granules in addition to the putative, progesterone-secretory granules. Therefore, the isolation of the latter free from other subcellular organelles was not a realistic objective using currently available techniques, and justified the analytical approach to the subcellular fractionation of the corpus luteum adopted in these studies.

Steroid hormones have generally been considered to leave endocrine cells by passive or facilitated diffusion (e.g., Vogt, 1944; Fawcett et al., 1969; Gillim et al., 1969; Enders, 1973). If diffusion is the means by which progesterone leaves luteal

cells, the process must be rapid since the corpus luteum produces large amounts of the hormone. Short (1959) calculated that the pregnant cow produces 36 mg of the hormone per 24 h. Heap et al. (1973) have calculated from the data of Erb et al. (1968) that the corpus luteum of the pregnant cow secretes as much as 27 µg of progesterone per min., equivalent to 39 mg per 24 h (or 124 µmole per day), in close agreement with the figure of Short (1959). The secretion rate of the corpus luteum of the oestrus cycle is directly comparable since in the cow progesterone is secreted only from the corpus luteum during pregnancy (Short, 1959; Gomes and Erb, 1965).

It has recently been suggested that steroid hormones do not diffuse from endocrine cells but are secreted in granules (Gemmell et al., 1974; 1977a). Such a proposal requires that the bovine corpus luteum produce and secrete large numbers of granules. The low concentrations of secretory granules in bovine mid-luteal cells at any instant does not preclude their involvement in the secretory process since the corpus luteum does not store steroid hormones but, rather, releases them as soon as they are formed (Christensen and Gillim, 1969; Enders, 1973; Savard, 1973; Heap et al., 1973; Baird, 1977).

Electron micrographs of luteal cells obtained from mid-luteal animals show many electron-dense granules in the peri-cellular space. They have been extruded from the cell, probably by exocytosis (see Chapter 2). The rate of granule secretion must be very high in order to see evidence of such extensive exocytosis in electron micrographs of bovine luteal cells. Douglas (1974b) has calculated that an exocytotic event takes about 6 msec in the mast cell and Morris (1976) has used

this value to calculate that the maximally-stimulated neural lobe of the rat released about 6×10^6 granules per sec. Visualization of exocytotic figures in the neural lobe was rare since this secretion rate corresponded to only about two exocytotic profiles for every 10^6 neurosecretory granules studied by transmission electron microscopy, even assuming that each granule and its membrane could be visualized perfectly (Morris, 1976). Furthermore, it appears that fixation of tissues obscures the exocytotic process (Daikoku et al., 1973).

The cells of the anterior pituitary (Farquhar and Rinehart, 1954; Parry et al., 1978) contain considerably greater cytoplasmic concentrations of granules than do the luteal cells of the cow (or sheep, Gemmell et al., 1974). Exocytosis of pituitary granules is only seen in micrographs of cells obtained from animals whose rate of hormone secretion has been greatly stimulated (Charlton, 1975); even then exocytotic figures are less common than in the bovine corpus luteum. The foregoing suggests, firstly, that the rate of secretion of granules from the luteal cells must be considerably in excess of 6×10^6 per sec and, secondly, that the granules are not stored, as in the anterior pituitary, but are released as soon as they are formed. That is, synthesis of progesterone and formation of granules may be obligatorily-linked processes.

The resolution of particulate progesterone and subcellular organelles was investigated in a variety of density gradients. Most of the particulate progesterone entered continuous gradients of both sucrose and Ficoll. The portion found in the sample zone at the top of the gradient, along with some of the lysosomal and microperoxisomal marker enzyme activities,

was presumably leached out during resuspension of the 10,000 g pellet or during centrifugation. It was established that cytosolic progesterone also remained at the top of the gradient. As the cytosolic fraction contained protein-bound as well as soluble hormone (see Chapter 4), the progesterone which did enter density gradients must have been localized in entities whose densities were greater than those of individual protein molecules. Thus, any progesterone entering the density gradients must have been sequestered within an organelle or membrane.

Linear gradients of 20-40% w/w sucrose provided the greatest enrichment of particulate progesterone, with contamination mainly by endoplasmic reticulum and plasma membrane. The microsomal enzyme markers were spread throughout the gradient, so that although the total microsomal contamination was low in the progesterone-enriched region, the large volume of agranular endoplasmic reticulum in the luteal cells caused considerable vesicle contamination of the progesterone-enriched peak. In fact, the enzyme markers for endoplasmic reticulum were present in greater amounts and showed maximal enrichments at greater densities. Some 5% of the activity of the plasma membrane marker, alkaline phosphatase, extended into the peak of particulate progesterone, although maximal enrichment of this enzyme was obtained at 30.6% w/w sucrose (d=1.129), some 4-5 tubes away from the position of maximal hormone enrichment. Steck (1972) reported that in vesiculated form it banded between 28-34% w/w sucrose (d=1.117-1.146) in a number of studies using a variety of tissues and Gospodarowicz (1973) found that plasma membrane fractions derived from bovine corpora lutea

banded at 33% w/w sucrose (d=1.141) on discontinous gradients.

The assignment of an enzyme activity as a marker for a particular organelle can vary among different tissues. For example, Bramley and Ryan (1978a) were unable to detect catalase activity (as a marker for microperoxisomes) in homogenates of superovulated rat ovary, whereas this enzyme was found to be active in homogenates and gradient fractions of bovine corpora lutea in this study. Bramley and Ryan confirmed the findings made here using homogenates of the bovine corpus luteum that NADPH-cytochrome c reductase was a suitable marker for endoplasmic reticulum, although the enzyme was not very active. In contrast to the bovine corpus luteum, NADH-cytochrome c reductase was not a suitable marker for aer in the superovulated rat ovary, possibly because its assay in this tissue was complicated by the presence of an agent which rapidly and nonenzymically reduced cytochrome c (Bramley and Ryan, 1978a). As aer greatly predominates over ger in luteal cells (see Chapter 2), these enzyme activities mainly reflect the distribution of aer in homogenates of bovine corpora lutea. However, ger may also contain the enzymes.

The lysosomal markers, β-glucuronidase and acid phosphatase, vary slightly in their distribution upon tissue fractionation (de Duve, 1964; Bramley and Ryan, 1978a). In contrast to the findings for the super-ovulated rat ovary (Bramley and Ryan, 1978a), β-glucuronidase consistently sedimented at slightly greater density than acid phosphatase activity in these experiments, and the latter was more easily solubilized by freezing and thawing. Thus, in the fractionation of homogenates of bovine corpora lutea, acid phosphatase behaved as a soluble

intralysosomal enzyme whereas β-glucuronidase behaved like a membrane-associated enzyme.

Although glucose-6-phosphatase has been widely used as a marker for aer in liver fractionation studies (De Pierre and Karnovsky, 1973), the level of activity of this enzyme was very low in the bovine corpus luteum and so it could not be used routinely as an aer marker in this study. Gospodarowicz (1973) has also reported that the bovine corpus luteum contains very little glucose-6-phosphatase activity. Luteal cells possibly lack the enzyme competely since Bramley and Ryan (1978a) report that the meagre activity that is detected in the superovulated rat ovary seems to be due solely to the hydrolysis of glucose-6-phosphatase by alkaline phosphatase. Gospodarowicz (1973) has used glucose-6-phosphate dehydrogenase instead as an aer marker in the bovine corpus luteum, but Savard (1973) and Rao and Mitra (1977) claim that in the cow this enzyme is located exclusively in the soluble cytosolic fraction. This is also its location in the hamster corpus luteum (Chatterjee and Greenwald, 1976). This enzyme was not used routinely as a marker in the present study.

It was important to identify endoplasmic reticulum unequivocally in fractions of bovine corpus luteum, since preliminary studies showed that this organelle was the principal contaminant of particulate progesterone on density gradients. Inosine diphosphatase was used as a marker for endoplasmic reticulum in addition to NADH- and NADPH-cytochrome c reductase activities. The activity profiles of all three enzymes were comparable on sucrose gradients (see Fig. 3-5), except that NADH-cytochrome c reductase was also very active in

the mitochondrial region due to its additional location on the outer membrane of this organelle.

All the density gradient experiments were designed to reach sedimentation-equilibrium. The fact that prolonged centrifugation of up to 22.5 h did not alter either the progesterone or any of the marker enzyme profiles, compared with centrifugation for only 2.5 h, clearly demonstrates that sedimentation-equilibrium had been achieved within the shorter centrifugation period. Bramley and Ryan (1978b) centrifuged homogenates of superovulated rat ovary for 3.5 h in 20-55% w/w sucrose gradients, by which time sedimentation-equilibrium was achieved. The rapid attainment of equilibrium in these linear gradient experiments was aided by the inclusion of a discrete 50% w/w sucrose cushion at the bottom of the centrifuge tube, so that the continuous gradient was sitting on top of a sudden "step" increase in sucrose concentration. Therefore those subcellular organelles which banded at sucrose concentrations ranging beyond 40% w/w rapidly moved through the gradient and "banked-up" at the gradient-cushion interface. The latter accommodated large numbers of organelles, undamaged because they were not compacted on the bottom of the centrifuge tube.

Sucrose gradients in the zonal rotor were run with loadings of homogenate H_2 equivalent to approximately 15 linear sucrose gradients. Such gradients had the additional advantage of requiring much shorter centrifugation times than conventional gradients run in swinging-bucket rotors. This type of rotor has been successfully utilized in the preparation of granular structures such as the zymogen granules of the rat pancreas (Schneider and Smith, 1977) and microperoxisomes from the small

intestine of the guinea pig (Connock et al., 1974). In both cases the desired organelle occupied a greater proportion of the cytoplasm of the source cells than was the case with the secretory granules of the corpus luteum. Even with zonal gradients, the purification of secretory granules from the bovine corpus luteum was not possible. The progesterone profiles obtained using zonal gradients of 15-50% w/w sucrose were directly comparable to those obtained using linear 20-40% w/w sucrose gradients centrifuged either for 2.25 h or overnight. As the zonal gradient experiments were completed in half the time it took to complete the shorter linear gradient experiments, this suggests that progressive changes in the nature of particulate hormone did not occur during centrifugation of the gradients.

In all of the sucrose density gradients, some progesterone was recovered from near the bottom of the gradient; furthermore, the profile of this progesterone paralleled the profile of succinate dehydrogenase, the mitochondrial enzyme marker, suggesting that it was associated with this organelle. Mitochondria are known to contain the enzymes for converting cholesterol to progesterone via pregnenolone in the bovine corpus luteum (McIntosh et al., 1971). In the case of the zonal gradients, this "mitochondrial" progesterone peak was proportionally greater than it was in the linear gradients, which indicates that at the shorter centrifugation times less of this hormone was solubilized. It is unknown if the "mitochondrial" progesterone is bound to either carrier or enzyme molecules, or if it is included within one of the mitochondrial membranes. In this context, it is interesting that a cytosolic pregnenol-

one binding protein has recently been isolated from severely-homogenized adrenal cortex of the guinea pig, and a mitochondrial association for this protein was not ruled out (Strott, 1977). The bovine corpus luteum contains two progesterone binding proteins, one of which may be associated with specific organelles (Chapter 4). Although radioimmunoassay of gradient fractions detected the "mitochondrial" peak of hormone, when homogenate H_2 was incubated with radioactively-labelled progesterone prior to loading onto zonal gradients, the "mitochondrial" hormone peak was not detected in the radioactivity profile of the gradient fractions. Perhaps the mitochondrial-associated progesterone did not exchange with exogenous, labelled hormone because it was bound within the mitochondria and was inaccessible.

From the above, it appears that the interaction which renders progesterone particulate at sucrose concentrations of 23-27% w/w is different from the interaction which causes progesterone to co-migrate with mitochondria on density gradients. Alternatively, the mitochondrial-associated hormone may be adsorbed non-specifically to the organelle such that this adsorption has less opportunity to break-down with the shorter centrifugation times involved in running zonal compared with linear gradients. Indeed, recent fractionation studies of the porcine pancreas have shown that homogenization can result in leakage-adsorption artifacts, and it was suggested that such phenomena may well bedevil all tissue fractionation studies (Scheele et al., 1978). However, adsorption would not explain the difference between the progesterone profiles of gradient fractions assessed by radioimmunoassay and radioactivity.

The location of steroid hormones in small electron-dense granules is not confined to the bovine corpus luteum. Such granules are prevalent in the luteal cells of sheep examined at the mid-luteal phase of the oestrus cycle (Gemmell et al., 1974). Slices of ovine corpus luteum which are actively secreting progesterone *in vitro*, also secrete granules into the extracellular space (Parry, Quirk and Willcox, unpublished observations). Recently Abel et al. (1977) have reported that in similar preparations from the ewe, the number of granules exocytosed rose and fell with increased or decreased progesterone secretion, and that hormone secretion was accompanied by the release of a protein into the incubate. In the perfused cat adrenal, ACTH stimulation caused an increase in the number of granules within the tissue, along with an increased production of glucocorticoid and the release of protein into the perfusate (Rubin et al., 1974; Laychock and Rubin, 1974; Gemmell et al., 1977a). These authors also suggested that glucorticoids were secreted with a protein in granule form. It is likely that the secretory granules of the bovine corpus luteum contain protein which, in turn, renders them osmiophilic under the electron microscope. This protein may be one of the two progesterone binding proteins found in bovine luteal cells (Chapter 4), and it is possibly analogous to the protein which is secreted together with progesterone by slices of ovine luteal tissue *in vitro* (Abel et al., 1977).

It has also been suggested that electron-dense granules in the pregnant sow store relaxin (Cavazos et al., 1969, 1972; Belt et al., 1970, 1971). Relaxin is measureable in extracts of corpus luteum from pregnant sows and humans (Steinetz et

al., 1960; Weiss et al., 1977), but it is not detectable during the oestrus and menstrual cycles, respectively, of the sheep (Chamley et al., 1975) or of the human O'Byrne et al., 1978). Thus, relaxin may not be produced in the corpus luteum of cycling cows (see Chapter 2 for a more comprehensive discussion of this point).

Morphological examination of pooled fractions from 20-40% w/w sucrose gradients showed that electron-dense granules were only present in those fractions where enrichment of particulate progesterone was maximal. Although the granules resolved on linear sucrose gradients were usually somewhat distorted and irregular in shape compared with those observed in tissue fractions, they were less pleomorphic in fixed fractions from zonal gradients. This is probably because in the latter experiments the granules were exposed to high concentrations of sucrose for less time before glutaraldehyde fixation, so that they were subject overall to less osmotic trauma.

The density of the progesterone-enriched granular fraction was low compared with that of other cell organelles and was compatible with the sequestration of the hormone within these granules. Granules which contain polypeptide hormones band at greater density in sucrose gradients. For example, the gastrin-containing granules of the rat intestinal mucosa band between 39-41% w/w sucrose (d=1.17-1.18) (Trotman et al., 1976) and the neurosecretory granules of the bovine posterior pituitary band at 48% w/w (d=1.219) on non-linear sucrose gradients (Hope and Pickup, 1974). The chromaffin granules of the adrenal medulla and the granules contained in mast cells are sufficiently dense for their purification to be accomplished simply by differential

centrifugation of tissue homogenates (Bartlett and Smith, 1974; Uvnas, 1974; respectively). On hypertonic sucrose gradients, chromaffin granules band at greater densities than mitochondria (Lagercrantz et al., 1970).

In fixed fractions from pooled 20-40% sucrose gradients, microperoxisomes and lysosomes were cytochemically located in parts of the gradient separate from the peak of particulate progesterone. Their location corresponded to that observed for their respective enzyme markers. In contrast, the fractions which contained particulate hormone, and which also contained small electron-dense granules, did not contain microperoxisomes or lysosomes. Therefore, the cytochemical data is consistent with an intragranular location for particulate progesterone.

Preliminary investigations were made of the nature of the interaction which rendered about 40% of the progesterone in the bovine corpus luteum particulate. The stability of the association on 20-40% w/w sucrose gradients was unaltered by five cycles of freezing and thawing. However, addition of Triton X-100 detergent to homogenate H_2, or partial sonication of H_2, caused a large increase in the proportion of soluble relative to particulate hormone, compared with control gradients. Detergent also caused complete disruption of lysosomes and shifted the peaks of mitochondria, plasma membrane and endoplasmic reticulum to lower densities. On the basis of these preliminary, disruptive-type experiments it is suggested that particulate progesterone is not made soluble as easily as β-glucuronidase and acid phosphatase enzymes in lysosomes. Furthermore, treatment with detergent severe enough to alter the banding positions of all organelles and resulting in the solubilization of some

of them, also caused extensive, but not total, solubilization of particulate progesterone.

Exogenous, radioactive progesterone exchanged readily with the endogenous progesterone of H_2 since the hormone profiles on sucrose gradients determined by radioactivity and radioimmunoassay were identical. However, progesterone was not the only steroid to sediment at 10,000 g or to enter sucrose density gradients. When homogenate H_2 was centrifuged at 10,000 g following incubation with radioactively-labelled cortisol and progesterone, one-third of the cortisol and two-thirds of the progesterone was recovered in the pellet. Therefore, the binding and/or adsorption capacity of H_2 for cortisol (and possibly other steroids) is less than its binding capacity for progesterone. This effect is separate from the binding affinity that H_2 displays for a particular steroid on sucrose gradients. Added cortisol, androstenedione, dihydroepiandrosterone, testosterone, aldosterone and cholesterol did not enter the gradient but remained in the soluble sample zone. On the other hand, oestradiol-17β, oestradiol-17α and oestrone entered the gradient to give sedimentation profiles comparable to progesterone. The synthetic progestogen, R5020, behaved anomalously to penetrate the sucrose gradient to greater density than did progesterone (see Table 3-6). The reason for this is unclear, especially as the cytosolic fraction of the bovine corpus luteum bound R5020 less well than it bound progesterone (Chapter 4). Conversely, in the rat uterus, R5020 is bound more strongly by progesterone receptor proteins than progesterone (Vu Hai and Milgrom, 1978).

It was significant that although the bulk of cholesterol

did not enter the gradient to give a peak of particulate progesterone, the "mitochondrial" peak of cholesterol was larger (23% of the total cholesterol recovered from the gradient) than progesterone, R5020 or any of the oestrogens (5% of the total recovered from separate gradients). The cholesterol side chain cleavage enzyme complex is known to be located in the mitochondria (Sulimovici and Boyd, 1969), so that it is possible that cholesterol and the small amounts of progesterone and oestrogens that band with the mitochondria on sucrose gradients are actually associated (loosely or otherwise) with this organelle. Those steroids (other than cholesterol) which did not enter the gradient to give peaks of particulate hormone, did not of course give mitochondrial peaks.

The finding that oestrogens entered the gradient was not unexpected since Kimball and Hansell (1974) have shown that the bovine corpus luteum contains an oestrogen receptor which is probably involved in both naturally occurring and $PGF_{2\alpha}$-induced regression of the corpus luteum (Hansel, 1975). However, the fact that the oestrogen profiles on gradients were directly comparable to the progesterone profile in the particulate region of the gradient (23-27% w/w sucrose) is more difficult to understand. The binding specificity of the bovine luteal and endometrial cytosolic receptors was reported by Kimball and Hansel (1974) to be greatest for oestradiol-17β, whereas Korenman et al. (1970) reported that the bovine uterine receptor showed greatest specificity for diethylstilbestrol; both studies found that oestrone ranked next in specificity, and neither study tested the specificity of binding for oestradiol-17α. Progesterone was not bound by the receptor

proteins in either study. Therefore, it is possible that the material sedimented by centrifugation at 10,000 g contains oestrogen receptor protein analogous to, or identical to, the oestrogen receptor protein recovered in the cytosolic fraction of the corpus luteum. If this is the case, then the penetration of picogram amounts of oestradiol-17β, oestradiol-17α and oestrone into sucrose gradients may be via binding to this oestrogen receptor protein.

Biochemical and morphological analysis showed that the particulate steroid region of the gradient (23-27% w/w sucrose), contained principally endoplasmic reticulum and electron-dense granules. The oestrogen profiles parallel that of progesterone and so by the same argument as presented above for particulate progesterone, the oestrogen receptor protein is either not associated with the endoplasmic reticulum or (less probably) is associated with a subfraction of it which bands over a restricted range of sucrose density. Alternatively, oestrogens in picogram quantities are bound to, or loosely adsorbed to, the electron-dense granules.

Westphal (1971) pointed out that those steroids with the least number of polar constituents bind most strongly to proteins. Application of the "polarity" rule to the steroids which were applied with H_2 onto sucrose gradients revealed no consistent pattern: whilst all of the steroids which specifically entered the gradients were bi-polar, those which did not enter the gradients were mono-polar (cholesterol), bi-polar (androster-enedione, dehydroepiandrosterone and testoterone) and penta-polar (cortisol and aldosterone).

An alternative explanation for the differential entry

of steroids into the sucrose gradient is derived from studies of their solubilities in aqueous medium and in lipid bilayers carried out by Heap et al. (1970, 1971). That the differences in gradient behaviour of the steroids was not a solubility effect is indicated by the following. These steroids were all used in concentrations of 2.5-5.0 $pmol.ml^{-1}$, which was well within their limits of aqueous solubility (Westphal, 1971), even without taking into account the solubilizing influence of the protein present[6]. When cortisol, androstenedione, oestradiol-17β and oestrone were applied to gradients at concentrations in excess of their aqueous solubilities, their gradient profiles were unchanged. However, model studies showed that the entry of oestradiol into phospholipid bilayer vesicles ("liposomes") was greatly enhanced in the presence of progesterone (Heap et al., 1971). Therefore, if this effect is specific to oestrogenic steroids, then it is possible that the progesterone localized in the electron-dense granules of homogenate H_2 promoted the uptake of oestrogens into the granules. This may explain why the sedimentation profile of oestrogens so closely paralleled the sedimentation profile of progesterone on sucrose gradients. Conversely, if the non-oestrogenic steroids are not subject to this progesterone-mediated solubility effect, then they would not be expected to enter the sucrose gradients.

In conclusion, the biochemical, morphological and cytochemical studies carried out on homogenates of bovine corpus luteum showed that possibly as much as 40% of the

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6: For example, the solubility of progesterone in water at 37.5°C is 0.4 $\mu mol.ml^{-1}$, which is increased 12-times by the addition of 3% w/v human serum albumin (Westphal, 1971).

progesterone in this tissue is particulate, and that this property is due to association neither with the discrete organelles - mitochondria, lysosomes and microperoxisomes - nor with the vesiculated membraneous components such as plasma membrane and endoplasmic reticulum. In addition, the morphological and cytochemical results obtained using fixed gradient fractions, when considered in conjunction with the biochemical data, provided evidence that progesterone is localized in electron-dense granules. Therefore, these results support the concept that steroid hormones are packaged into secretory granules, which undergo exocytosis in a manner analogous to that described for catecholamines and protein hormones (Douglas, 1966; Fawcett et al., 1969).

Further confirmation of the hypothesis must await the development of subcellular fractionation techniques which will enable the purification of granules in quantities sufficient to investigate their composition more fully.

CHAPTER 4

PROGESTERONE BINDING PROTEINS

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4.4 DISCUSSION

4.1 INTRODUCTION

4.1.1 The Concept of Protein Binding

The binding of steroid hormones to plasma and intracellular proteins affects their activity, distribution, and metabolism (Germain et al., 1978). The first observations of binding of ligands to proteins were made 75 years ago, long before the chemical structure of steroids was elucidated. Heidenhain (1903) described the dialysis of acid azo dyes through parchment tubing and its inhibition by the addition of serum albumin. Bennhold investigated the binding of small molecules such as dyes, natural pigments and other, non-steroidal compounds to serum proteins (Bennhold,1924, 1966). In 1934, Brunelli published the first paper on the binding of a steroid hormone to serum proteins. His interpretation that oestrogens were transported in plasma by carrier proteins was influenced by the concept of a vehicle function for the serum proteins which had been developed by Bennhold (1932).

Throughout the years, different sites in steroid hormone-responsive cells have been especially studied in an attempt to elucidate the mechanisms of action of steroid hormones. During the 1920's and 1930's attention was focused on the selective permeability of the plasmalemma, whilst the 1940's and 1950's were characterized by investigation of the energy-yielding process of the cytoplasm. In the 1960's, the emphasis shifted to the nucleus and the way in which steroid hormones affect genetic events (Szego, 1976). Recently attention has reverted to events at the cell surface and in the cytoplasm of target cells. The transport of steroids in the body fluids and their movement out of steroidogenic tissues has received little

attention throughout the years even though the concept of noncovalent protein binding of steroids is common to all these interactions, whether they are operating at the level of tissues and cells, or subcellular compartments (Szego, 1976).

Serum albumins were originally thought to be the primary transport proteins for steroids (Westphal, 1971; Westphal, 1975) and to account for all the sex steroid binding capacity of serum (Eik-Nes et al., 1954; Slaunwhite and Sandberg, 1959). However, other studies showed that the β_1-lipoproteins contained practically all of the oestrogen in human plasma (Roberts and Szego, 1946), and it is now known that this fraction contains a specific binding protein for oestrogens and androgens (sex hormone binding globulin, SHBG: Mercier et al., 1966). Also, corticosteroid binding globulin (CBG), which binds cortisol, corticosterone and progesterone with high affinity, is an α_1-globulin (Seal and Doe, 1962).

The advent of ^{14}C-radioactively-labelled steroids of high specific activity allowed Jensen and Jacobson (1962) to demonstrate for the first time that target tissues such as the uterus could accumulate and retain oestradiol. However, the concept of intracellular protein binding of oestradiol was explicitly denied by these workers (Jensen and Jacobson, 1962). The definitive work on the protein nature of the macromolecules interacting with oestrogen, leading to its retention by target tissues, was carried out by Gorski, Toft and coworkers (Noteboom and Gorski, 1965; Toft and Gorski, 1966) and was confirmed and extended by Jensen et al. (1967, 1968). Jungblut et al. (1967) demonstrated that oestradiol binding in the nuclear fraction of the uterus was due to a macromolecular

complex and that this nuclear receptor was smaller than the cytoplasmic receptor.

The discovery of oestrogen receptors in oestrogen-responsive tissues stimulated numerous groups to search for similar receptors in the target cells of other steroid hormones. For example, it has been shown that there are receptors for androgens, especially testosterone and dihydrotestosterone, in the cytoplasm and nucleus of prostate cells (King and Mainwaring, 1974). A nuclear accumulation of progesterone in the uterus was first recognized autoradiographically by Sar and Stumpf (1973, 1974) and specific receptors for progesterone have also been discovered (Sherman et al., 1970). A genomic role for the progesterone receptor of oestrogen-primed chick oviduct has been clearly established (O'Malley et al., 1976). Thus, selective interaction with protein underlies the entire range of events from extra- and intra-cellular steroid hormone transport to molecular activity in specific target cells.

Steroid hormones interact with a range of different proteins during the course of their biosynthesis, distribution, degradation and modulation of target cell metabolism. Enzyme proteins recognize individual steroid structures and catalyze their transformation to other steroids. In addition, non-catalyzing specific binding proteins are found in the blood and in the cytoplasm of certain target and steroidogenic cells. Their concentration is similar to that of the steroids they bind, unlike receptor proteins and enzymes (Baulieu, 1975). Therefore, in the course of their natural history, steroid molecules may interact successively with enzymes, transport

proteins, intracellular receptor proteins and nuclear proteins.

4.1.2 Receptor Proteins

Although receptor proteins bind steroids, they are usually considered to be functionally distinct from the steroid binding proteins found in blood and in the cytoplasm of some cells. The physiological role of all steroid receptor proteins is thought to involve the following sequence of events:

$$\text{Steroid} + \text{Receptor} \rightleftharpoons \underset{\text{complex}}{\text{Steroid-receptor}} \longrightarrow \text{Nucleus} \longrightarrow \text{Response(s)}$$

The steroid hormone is complexed with the receptor protein(s), often at or near the plasmalemma, following which part or all of the complex is translocated through the cytoplasm to the nucleus, where part or all of the complex interacts with two or more components of the chromatin to give increased synthesis of specific mRNA sequences (Schulster et al., 1976). These in turn are translated on polysomes in the cytoplasm to produce specific enzymes and/or other proteins which manifest the physiological responses to the steroid hormone (Schulster et al., 1976).

There are many proposed variations upon this general "two-step" theme (Gorski and Gannon, 1976; Agarwal, 1978). For example, it is not known whether the transcriptional events in the nucleus require the presence of steroid, or whether the latter merely acts to promote the translocation of the cytoplasmic receptor to the nucleus (O'Malley et al., 1976). Also undefined is the mechanism by which selective parts of the genome are activated in accordance with the overall steroid stimulus. The progesterone receptor of the chick oviduct dissociates into two small proteins, one of which binds to nonhistone proteins and appears to have a regulatory function,

and the other which binds directly to DNA (Schrader et al., 1972, 1974; Kuhn et al., 1977; Schrader et al., 1977). It is not known if the oestrogen receptor of the uterus acts similarly (Janne et al., 1978).

Other models of receptor action suggest that they act simply as intracellular transport and storage proteins (Wagner, 1978) or that the receptor protein itself induces physiological changes without the assistance of the steroid hormone once it has been "activated" by the latter (Jungblut et al., 1976). It has even been speculated that steroid hormones can induce responses without involving cytoplasmic receptors (Sheridan, 1975), although this is most unlikely, since it contradicts most of the accumulated experimental evidence.

The hormonal response to a particular steroid is only manifested by those target tissues which possess specific receptors for the steroid. Target tissues contain only minute amounts of receptor, the concentrations being in the order of 0.1 to 1 $mg.kg^{-1}$ (Wagner, 1978). Additional evidence that the attachment of steroid to specific cytoplasmic receptors is a prerequisite for the expression of subsequent biological action is the correlation between hormone-receptor interaction and biological activity of the hormone in all target tissues studied so far and the requirement of the hormone-receptor complex in order to get an *in vitro* effect of the hormone (King and Mainwaring, 1974). However, the occurence of specific progesterone receptors in all progesterone-sensitive cells has not been demonstrated (King and Mainwaring, 1974).

The intracellular location of receptor proteins implies

that steroid hormones penetrate into their target cells. This is in contrast to the protein hormones which exert their effect primarily through specific receptors in the cell membrane. (Menon, 1973; Laychock and Rubin, 1977; Dorrington, 1977). For example, ACTH stimulates adrenocortical cells by the receptor-mediated stimulation of cyclic AMP in target cells which, in turn, activate protein kinases. The latter activate metabolic enzymes which promote the cytoplasmic responses to the original hormonal stimulus (Baulieu, 1975). However, steroids elicit responses directly and independently of cyclic AMP (although, the latter may modulate the intracellular processes initiated by steroid hormones).

4.1.3 Specificity and Binding Theory

There are both qualitative and quantitative aspects to the binding of steroids by plasma proteins and target cell receptors. Qualitatively, a target tissue receptor or intracellular binding protein is organ-specific since it binds only those steroids to which the tissue is hormonally responsive. The plasma proteins such as CBG and SHBG are qualitatively specific in that they bind only a limited number of steroids (Baulieu et al., 1970). These proteins are quantitatively-specific in that they are saturated by low amounts of steroid which are near to their physiological concentrations. At these concentrations non-specific binding proteins such as albumin are not saturated due to their large concentration and to the limited solubility of steroids in aqueous media (Baulieu et al., 1970). Specific proteins, unlike non-specific proteins, are characterized by high association constants which enable them to bind steroids even when the latter are present in low concentration.

The above considerations have given rise to the terms "high affinity, low capacity" binding (e.g., CBG and receptor proteins) and "low affinity, high capacity" binding (e.g., albumins). These concepts are defined mathematically from a consideration of the binding reaction:

$$S + P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} SP$$

Steroid (S) reacts with a protein molecule (P) to form the complex SP at a rate determined by the velocity constant k_1. SP dissociates at a rate determined by the backward velocity constant k_2. At equilibrium, the rates of both reactions are the same and the system can be described by the law of mass action where

$$K_A = \frac{k_1}{k_2} = \frac{SP}{SxP}$$ (Moore, 1963).

The reciprocal of this expression gives the dissociation constant, K_D. Both of these terms are in common use, and can be defined separately; K_D, usually expressed in units of moles.litre^{-1} gives a measure of the concentration of reactants required to form half maximal amounts of complex. When the concentration of P is unknown (as in the case of cytosolic fractions), K_D gives a measure of the concentration of steroid required to half saturate P. On the other hand, K_A can be considered as a dilution factor (expressed in litres.mole^{-1}; it is a measure of the number of litres to which one gram.mole of either receptor or steroid must be diluted to get 50% maximal binding (King and Mainwaring, 1974).

4.1.4 Circulating Steroid Binding Proteins

Plasma proteins reversibly bind greater than 95-97% of the steroids contained in blood so that steroids circulate for relatively long periods of up to several days in the plasma of blood (Baulieu, 1975). Only a small fraction appears to be associated with erythrocytes (Eik-Nes et al., 1953; Sandberg and Slaunwhite, 1958), although the *in vitro* uptake of steroids by erythrocytes has been reported (Brinkman et al., 1970). The main binding proteins of mammalian plasma are thought to be albumin, CBG, SHBG, α_1-acid glycoprotein (AAG) and, in pregnant guinea pigs, progesterone binding globlin (PBG).

4.1.4.1 Albumin

This protein comprises 60% of the plasma proteins (Wagner, 1978), and it binds most steroids with a K_A of 10^4-10^5 M^{-1} (Rosenthal et al., 1969; Westphal, 1971). Conjugated steroids, especially sulphates, are bound with slightly higher affinity (Crepy and Gueriguian, 1970). Although albumin has only a weak affinity for steroids it has at least two binding sites (Rosenthal et al., 1969). It has been reported to polymerize to dimers and trimers which simultaneously bind and/or adsorb three or more molecules (Westphal and Harding, 1973), so that it has almost unlimited binding capacity (Wagner, 1978). This capacity is so great that it is practically never saturated even at concentrations of steroid far in excess of the physiological range (Crepy and Gueriguian, 1970). Thus, interpretation of binding phenomena between steroids and high affinity binding proteins must not overlook the contribution made by albumin to the total sequestration of circulating steroid. In the human foetus, the concentration of SHBG is

low so that albumin is an important binder of oestrogens and androgens (Abramovich and Towler, 1978). Binding of cortisol to albumin rather than to CBG may be important to controlling the rate of transfer of cortisol to the foetus since albumin retarded the clearance of cortisol whereas CBG did not (Dancis et al., 1978). Nonetheless, the combination of steroids with albumin does not seem to interfere either with their biological activity or with metabolic transformation and rates of clearance in adults (Crepy and Gueriguian, 1970).

4.1.4.2 Corticosteroid Binding Globulin

Transcortin or CBG is an α_1-globulin (Daughaday, 1958) with a molecular weight of 52,000 in the human (Muldoon and Westphal, 1967; Bernutz et al., 1978). It is a glycoprotein, e.g., human CBG contains 26% carbohydrate, and is stable at 45^{o}C for 60 min (Westphal, 1971), unlike most receptor proteins. It appears to be present throughout the vertebrates (Seal and Doe, 1965; Freeman and Idler, 1971), and in the human its concentration ranges between 23 and 46 mg.litre^{-1} (Doe et al., 1964), irrespective of sex or age (De Moor and Heyns, 1968). It binds glucocorticoids and progesterone with relative affinities that vary somewhat according to species. For example, human CBG binds cortisol preferentially at 4^{o}C (K_A = 5 x 10^{8} M^{-1}), but at the physiological temperature (37^{o}C) affinity for progesterone (K_A = 8 x 10^{7} M^{-1}) is even higher than that for cortisol (K_A = 3 x 10^{7} M^{-1}) Seal and Doe, 1966b; Westphal, 1971). However, in women plasma cortisol concentrations are higher than those of progesterone during pregnancy and in the luteal phase. This, plus the fact that progesterone is more readily bound to albumin, may explain why a higher proportion of

cortisol than progesterone is bound to CBG (Milgrom, 1978). It is remarkable that CBG binds progesterone and glucocorticoids at the same site, despite their structural dissimilarity (Daughaday, 1958; Westphal, 1971; King and Mainwaring, 1974; Chan and Slaunwhite, 1977). Unlike most glucocorticoid and progestogen receptor proteins, CBG does not bind synthetic glucocorticoids (triamcinolone acetate or dexamethasone) or synthetic progesterone (R5020) (Kolanowski and Pizzaro, 1969; Raynaud, 1977).

CBG is probably synthesized in the liver (Rosenthal et al., 1969; Booth and Colas 1975), under the control of thyroid stimulating hormone (TSH) (Gala and Westphal, 1966). Oestrogen can stimulate the synthesis of CBG, possibly via TSH (Labrie et al., 1967) in man and some primates but not in ruminants such as the sheep and cow (Seal and Doe, 1966a).

4.1.4.3 Sex Hormone Binding Globulin

SHBG is also known as sex steroid binding protein (SBP; Mercier-Bodard and Baulieu, 1968), steroid binding β-globulin (SBβG; Westphal, 1971), testosterone binding globulin (TBG: Corvol and Bardin, 1973), testosterone-oestradiol binding globulin (TeBG; Crepy and Gueriguian, 1970), proteine liant la testosterone (PLT: Mercier, 1966) and oestradiol binding protein (EBP; Germain et al., 1978). It is the other major high-affinity steroid binding macromolecule of mammalian plasma, although it is not as ubiquitous as CBG (Corvol and Bardin, 1973). It is a β-globulin (Gueriguian and Pearlman, 1968) and has been reported to have a molecular weight of 52,000 (Mercier-Bodard et al., 1970) or 98,000-115,000 (Gueriguian and Pearlman, 1968; Iqbal et al., 1978). It is a

glycoprotein (Van Baelen et al., 1969) and, also like CBG, may polymerize readily (Wagner, 1978).

SHBG exhibits a range of steroid specificity depending on the species, which accounts for its different names. For example, the protein from human plasma binds both androgens and oestrogens, whereas a similar protein in rabbit is much more specific for androgens (Rosner and Darmstadt, 1973), and that of rat serum has greatest affinity for oestrogens (Soloff et al., 1971). It appears that the 17β-hydroxyl group is mandatory for the binding of steroids to SHBG (King and Mainwaring, 1974, and it does not bind steroid conjugates (Mercier-Bodard et al., 1970). The association constant at 37°C for most of the steroids which bind to SHBG is in the range of 10^7 to 10^8 M^{-1} (Pearlman et al., 1967; Hansson et al., 1973). Norgestrel, a synthetic steroid used in hormonal contraception, has recently been shown to bind to SHBG (Victor et al., 1976).

4.1.4.4 α_1-Acid Glycoprotein

AAG (or orosomucoid) is found in human serum and the serum of many other species. It is a glycoprotein (40% carbohydrate) and has a molecular weight of 41,000 daltons (Westphal et al., 1977). The biological function of this protein is unknown (Westphal, 1971), but it binds progesterone (K_A = 5.6 x 10^5 M^{-1} at 4°C) with an affinity intermediate between that of albumin and CBG (Ganguly et al., 1967). Its concentration (750 mg. l^{-1}) is more than 20-fold greater than that of CBG but 50-fold less than that of albumin (Kerley and Westphal, 1969). Apparently, AAG binds only a small portion of plasma progesterone and does not play a major role in the function and metabolism of this steroid (Crepy and Gueriguian, 1970). A protein tentatively

identified as AAG binds norgestrel in human plasma (Uniyal and Laumas, 1976). Therefore, the distribution of progesterone and cortisol in human plasma pregnancy is as follows (Westphal et al., 1977; Rosenthal et al., 1969):

Protein	% Progesterone	% Cortisol
Corticosteroid binding globulin	43-48	60-80
Human serum albumin	50-54	13-32
α_1-Acid glycoprotein	ca.1	-
Unbound	1-2	7-8

4.1.4.5 Alpha-fetoprotein

Alpha-fetoprotein (AFP) is the first α-globulin to appear in mammalian sera during embryonic development and is the dominant serum protein in early embryonic life (Uriel et al., 1976). Rat and mouse AFP possess high oestrogen-binding affinity (Uriel et al., 1972; Germain et al., 1978) whereas human AFP displays low (Uriel et al., 1975) or no (Swartz and Soloff, 1974) oestrogen-binding activity. The levels of AFP decline to adult levels by the end of the second year, so AFP is unlikely to feature significantly in the control of tissue oestrogen concentrations during puberty in the human female (Germain et al., 1978).

4.1.4.6 Progesterone Binding Globulin

PBG occurs in high concentration (more than 1 $mg.ml^{-1}$) in the serum of pregnant guinea pigs (Burton et al., 1974). Like CBG, SHBG and AAG, PBG is a glycoprotein (70% carbohydrate) and it has been purified by conventional chromatographic techniques (Milgrom et al., 1973) and by affinity chromatography (Cheng et al., 1976). It binds progesterone with highest affinity (K_A = 2.2 x 10^9 M^{-1} at 4°C), but it also binds dexycorticoster-

one and testosterone moderately well *in vitro* (Lea, 1973; Westphal, 1978).

PBG appears to be restricted to pregnant hystericomorphs (Heap and Illingworth, 1974), although circulating progesterone is also bound to CBG and albumin in these species (Westphal, 1971). It is restricted to the maternal circulation (Milgrom, 1978), and its physiological function is not known. It has been suggested that it preserves maternal progesterone from metabolic clearance and that it protects the foetus from exposure to a high concentration of the hormone (Westphal, 1977; Milgrom, 1978). It does not cross the placenta even though other maternal serum proteins of similar size (e.g., albumin and CBG) do so (Milgrom, 1978).

4.1.4.7 Physiological Significance of Circulating Steroid Binding Proteins

The physiologically-active fractions of steroid hormones are the 1-3% of their circulating concentrations that are soluble and unbound (Slaunwhite and Sandberg, 1959). The unbound fractions, and the total circulating concentration of steroids, are determined by the balance between their rates of secretion from endocrine cells, the amounts bound to protein, and their rates of clearance from the blood (Tate and Burstein, 1965). Since steroids are not considered to be stored in endocrine cells, their rates of synthesis and secretion are similar (Heap et al., 1973). The removal of steroids from the circulation is accomplished by metabolic degradation, principally in the liver (Tate and Burstein, 1965). It is usually quantified by the parameter known as the metabolic clearance rate (MCR), which is defined as the volume of blood that is

completely and irreversibly cleared of a compound in unit time (Heap, 1972). The production rate of a hormone under steady-state conditions can be calculated by multiplying the MCR by the blood concentration.

Steroid binding proteins function primarily by setting the total concentrations, rather than unbound concentrations, of circulating steroids (Tate and Burstein, 1965). Thus, the glucocorticoid concentrations in plasma throughout the vertebrates seems to vary directly as the concentration of CBG varies. For example, women and rhesus monkeys have higher plasma concentrations of glucocorticoids and CBG than either dogs and cats or ruminants such as cows, sheep and goats (Seal and Doe, 1965). It is the low plasma concentration of CBG in ewes that causes these animals to have circulating mid-luteal concentrations of cortisol and progesterone that are 10- to 15-fold lower than those of women (Lindner, 1964a; Thorburn et al., 1969; Guillebaud et al., 1977). The sheep has a higher MCR for cortisol than man, which Lindner (1964a) interpreted as being due to the lack of CBG-binding activity in sheep. However, subsequent studies have demonstrated that CBG is present in low concentrations in sheep plasma (Seal and Doe, 1965; Paterson and Hills, 1967). This confirms the generalization that the characteristic plasma concentrations and biological half-lives of cortisol (and other steroids) in different animal species is directly related to their plasma protein-binding capacities.

From studies of cortisol-binding to CBG (Nugent et al., 1961; Tate and Burstein, 1965), it appears that the fundamental difference between albumin-bound steroid and steroid bound to high-affinity proteins is their respective fates in the liver.

Unbound and albumin-bound steroids are metabolized in one passage through the liver, whereas CBG-bound cortisol and progesterone are not (Tate and Burstein, 1965). Therefore, the total concentration of circulating steroids is directly proportional to the concentration of high-affinity steroid binding proteins under steady-state conditions. Conversely, the proportion of albumin-bound steroid is independent of the unbound steroid concentration and of binding by other proteins (Tate and Burstein, 1965).

The high-affinity binding proteins influence the rate of change in plasma and tissue steroid concentration when secretion rates are altered. The capacity of CBG for cortisol is only 2-3 times the total amount of this hormone in plasma (Mills, 1962) and its binding affinity does not alter under different physiological conditions (Rosenthal et al., 1969). When the rate of cortisol secretion is increased, the total circulating concentration of cortisol increases beyond the total binding capacity of CBG so that the MCR rises as well (Tate and Burstein, 1965; Paterson and Hills, 1967; Paterson and Harrison, 1968). Such increased binding of cortisol by CBG under conditions of increased hormone secretion, or of retention of cortisol when the secretion rate of the hormone falls, provides a reduction in the concentration fluctuations in plasma cortisol (Tate and Burstein, 1965). Thus, an important function of CBG and the other high-affinity circulating binding proteins is to provide minimum constant concentrations of steroid hormones. If CBG had unlimited capacities for cortisol and progesterone, then tissues would be exposed to greater concentrations of hormone under conditions where secretion was increased since hepatic clearance would not be as rapid (Tate and Burstein, 1965).

Thus, the high-affinity of circulating steroid binding proteins provide hormones with protection from hepatic clearance but their low capacities for steroid enables them to maintain minimal concentrations of the latter whilst still making available to tissues high concentrations of hormones when rates of secretion are temporarily elevated.

If increased total steroid hormone concentrations are required then increased synthesis of binding protein is necessary. For example, during human pregnancy, an increased concentration of circulating progesterone is provided by an increased concentration of CBG and displacement of some of the cortisol from the protein by progesterone (Seal and Doe 1966a). The pregnant guinea pig synthesizes a specific high-affinity protein (PBG) which enables elevated plasma concentrations of progesterone to be maintained (Illingworth et al., 1970; Heap and Illingworth, 1974). In both cases, the MCR for progesterone is decreased during pregnancy.

Steroid binding proteins in blood also provide a means of transporting steroid hormones from their sites of synthesis to target cells where their sequestration may be transferred to intracellular receptor proteins. The latter generally show slightly higher affinities for steroids than do the binding proteins in blood. They may also protect steroid hormones against enzymatic attack, especially by the liver and erythrocytes. The latter contain 17β-and 20α-hydroxysteroid dehydrogenase activities (Van der Molen and Groen, 1968; Brinkman et al., 1970). Binding of steroids to protein also prevents non-specific adsorption to blood and lymph vessels (Westphal, 1961), lessens renal clearance (Sandberg and Slaunwhite, 1963),

and prevents undue build-up in the amounts of free hormone in extracellular spaces, where their physiological effects may be deleterious (Heap et al., 1973).

The following evidence supports a regulatory role for CBG and SHBG in women: 1. The plasma concentrations of corticosteroid and CBG are greatly increased during pregnancy and after oestrogen therapy (Crepy and Gueriguian, 1970) but hypercorticism does not occur (Sandberg, 1965). 2. The plasma concentration of androgen and SHBG are also elevated during pregnancy (Demisch et al., 1968) but virilization is avoided (Pearlman et al., 1967). 3. The increased concentrations of CBG that accompany oestrogen therapy depress the clearance rate of cortisol (Linder, 1964a).

The concept that CBG-bound cortisol represents an inactive hormone reservoir which is able to be released rapidly under conditions of sudden stress may not be valid in the rhesus monkey. De Moor and Steeno (1965) showed that the concentration of CBG was considerably less than the concentration of cortisol in this species (although the collection of blood may not have been achieved at basal, resting conditions). However, the CBG of *Macaca mulatta* (Murphy, 1969) and *Macaca nemestrina* binds corticosterone with 5-fold greater affinity than cortisol and corticosterone is present in higher concentrations than endogenous cortisol (Schiller et al., 1977).

Whole CBG-cortisol complexes can penetrate liver cells (Yates and Urquart, 1962; Rosenthal et al., 1974) where catabolic processes are known to occur although most steroid bound to CBG in the blood survives passage through this organ (Tate and Burstein, 1965). However, it is not yet clear if only

the free hormone fraction penetrates the cells of target tissues (Wagner, 1978). MacLaughlin and Westphal (1974) reported that the uterine cytosol of the pregnant guinea pig contains a progesterone-binding protein whose physiochemical properties resembled those of PBG from serum and were distinct from those of the uterine oestrogen and progesterone receptors of oestrogen-treated ovariectomized animals. In addition to specific progesterone receptors, CBG-like proteins have also been found in human colostrum and milk (Payne et al., 1976), the anterior pituitary of the rat (de Kloet et al., 1977) and in the uterine cytosols of rat (Milgrom and Baulieu, 1970; Booth and Colas, 1977), hamster (Do and Leavitt, 1978), rabbit (McGuire and Bariso, 1972) and man (McGuire et al., 1974). They have also been reported to occur in human mammary carcinoma (Pichon and Milgrom, 1977) and bovine corpus luteum (Leymarie and Gueriguian, 1970). Gueriguian et al. (1974) found that in women the apparent CBG content of uterine soluble protein was almost as high as the CBG content of serum protein. They suggested that CBG may accumulate in interstitial fluid of the myometrium, regulating the local concentration of bound and unbound progesterone and cortisol, and thereby exert hormonal influence on muscle and fibroblast, respectively.

CBG occurred in follicular fluid from pigs, sheep and cows and SHBG occurred in follicular fluid from sheep and cows. However, the bulk of the steroid in follicular fluid in those animals was bound to albumin with low affinity (Cook et al., 1977; Mahajan and Little, 1978). It is not known whether lymph draining the uterus contains CBG, but Sass (1964) found that lymph contained more globulin and less albumin than serum.

The follicular fluid of women with polycystic ovaries also contains an androgen-binding protein analogous to SHBG (Vigersky et al., 1978).

If some of the progesterone-binding activity in various mammalian uterine cytosols is due to CBG, its source is unknown. Recently, it has been reported that all of the CBG present in the cytosolic fraction of human endometrium is due to plasma contamination (Kreitmann et al., 1978). This may prove to be the case for all tissues where CBG has been reported to occur intracellularly. However, the situation remains unresolved, since Gueriguian et al. (1974) estimated the extent of blood contamination of human uterine cytosol by assaying for iron. They concluded that blood contamination was unlikely to account for all of the CBG-like binding. In addition, saline perfusion of rat uterus *in situ* did not decrease the observed CBG-like binding for progesterone (Booth and Colas, 1975). Examination of the data presented by various investigators, and of the methods and buffers used, suggests that there are two specific high-affinity progesterone-binding components in the uterus of various species. One protein sediments as a 6-8S form on sucrose gradients in the presence of glycerol or as a 7S form if glycerol is absent. A second 4S protein, usually in higher concentration, binds both corticosteroids and progesterone. This latter is either analogous to plasma CBG or is a closely-related protein.

Less information is available that would suggest an intracellular location for SHBG or AAG. However, Dickson et al. (1978) have reported that the liver cytosol of male rats contains a protein, distinguishable from the androgen receptor,

which bound both oestradiol and dihydrotestosterone.

4.1.4.8 Changes in the Concentration of Circulating Steroid Binding Proteins

During human pregnancy (and after oestrogen administration) the concentrations of CBG (Bernutz et al., 1978) and SHBG increase whilst those of AAG and albumin remain constant or decrease slightly (Musa et al., 1967). Sandberg et al. (1965) used iodinated CBG to demonstrate that the rise in this protein was due to increased synthesis, so that the concentration of CBG in pregnant women was 3-fold greater than in non-pregnant women. The plasma concentration of cortisol also rises during pregnancy but the unbound fraction does not (Rosenthal et al., 1969). Progesterone is not entirely available for competition with cortisol for binding to CBG because of the presence of large amounts of albumin, for which progesterone has greater affinity (Crepy and Gueriguian, 1970). This phenomenon may be restricted to species where placentation is of the haemochorial type (Seal and Doe, 1966a; Crepy and Gueriguian, 1970) since the concentration of CBG remains unchanged during pregnancy in the sheep and declines by 40% in the pregnant cow (Seal and Doe, 1966a). In addition, administration of oestrogen to sheep and dogs does not cause an increase in their plasma concentrations of CBG (Linder, 1964a), which may indicate that CBG synthesis is controlled differently in these animals (Tate and Burstein, 1965).

SHBG rises more steeply during human pregnancy than CBG, so that at term its level is 2-3 times that of the non-pregnant state, before declining rapidly after delivery (Pearlman et al., 1967). Higher concentrations of SHBG are obtained for twin

pregnancies (Rivarola et al., 1968). Since the protein is also elevated in men receiving oestrogen therapy for prostate cancer, Pearlman et al. (1967) have suggested that the concentration of SHBG is directly related to endogenous sex steroid production. Subnormal amounts of SHBG were found in hirsutic women and in women with polycystic ovaries whereas CBG concentrations were unaffected. Conditions which elevated CBG concentrations, such as adrenal insufficiency and lung cancer, did not alter SHBG concentrations (Westphal, 1971). In patients with liver cirrhosis CBG concentrations were low but those of SHBG were elevated (Wagner, 1978). Therefore, the synthesis and/or clearance of these two proteins may be regulated independently. Such a conclusion has also been reached from a study of plasma steroid binding proteins in the monkey, *Macaca nemestrina*, where the plasma concentration of SHBG rose by 28 days of pregnancy, fell through mid-pregnancy and then declined dramatically just prior to parturition. In contrast, the concentrations of CBG and albumin throughout pregnancy were almost unchanged from non-pregnant values (Schiller et al., 1978). These changes are different from those of women. In the male baboon, the serum concentrations of SHBG are greater than, whilst that of CBG are less than, those for men (Karr et al., 1978).

The maternal concentration of AAG, which binds small amounts of progesterone, rises during pregnancy to reach maximal levels at term. However, its concentration in the foetus remains low whilst that of albumin doubles, suggesting that more steroid is bound to the latter (Abramovich and Towler, 1978).

4.1.5 Intracellular Steroid Binding Proteins

There are a number of intracellular steroid binding proteins in target and endocrine cells, as distinct from receptor proteins, which are not translocated to the nucleus.

Recent evidence suggests that the biosynthesis of cholesterol is mediated by a multi-carrier system of at least three specific soluble proteins (Scallen et al., 1975), rather than by just a single sterol carrier protein (Ritter and Demsey, 1971,1973). Whilst not strictly steroid binding proteins, the cholesterol carrier proteins are nonetheless important intermediaries of steroidogenesis. For example, soluble thermostable proteins which bind cholesterol non-covalently have been identified in rat liver (de Anick and Saez, 1977; Erickson et al., 1978), bovine adrenal cortex (Teicher et al., 1978), guinea pig adrenal cortex (Strott and Lyons, 1978), rat adrenal cortex (Kan et al., 1972), sheep adrenal cortex (Lefevre et al., 1978), bovine corpus luteum (Kan et al., 1974) and sheep ovary and testis (Lefevre et al., 1978). Most of these proteins also augment specific enzymatic steps in cholesterol biosynthesis so that Scallen et al. (1975) have suggested that these proteins are soluble constituents of microsomal enzyme complexes, and that they function both as carriers of water-insoluble substrates and as essential enzyme components.

The hormonal control of steroidogenesis in the adrenal cortex (Garren, 1968; Laychock and Rubin, 1974) and corpus luteum (Abel et al., 1977) involves protein synthesis. Discrete pregnenolone- and pregnenolone sulphate-binding proteins have been described in the adrenocortical cytosols of guinea pig (Strott, 1977; Strott and Lyons, 1978) and rat (Kream and

Sauer, 1977). They are thought to be intracellular transport proteins and/or involved in the regulation of steroidogenesis. The adrenocortical cholesterol binding protein of guinea pig did not bind either pregnenolone or its sulphate, but it did bind cholesterol sulphate (Strott and Lyons, 1978). Therefore, these three protein factors may be part of the cholesterol side chain cleavage system and participate in regulation of the rate-limiting step of steroidogenesis since one binds the substrate (cholesterol) whilst the others separately bind the products (pregnenolone or its sulphate; Strott, 1977).

An androgen binding protein (ABP), which has high affinity for testosterone and dihydrotestosterone, has been described in the rat testis (French and Ritzen, 1973; King and Mainwaring, 1974) and in the seminal fluids of various mammalian species (Jegou and Le Gac-Jegou, 1978). It is not found in blood (Ritzen et al., 1971), but is produced by the Leydig cells and secreted with testosterone into the interstitial fluid (King and Mainwaring, 1974). ABP appears to be a genuine androgen carrier protein, although its precise fate after secretion into the interstitial fluid is unknown; it may be adsorbed by the epididymal cells together with testosterone (French and Ritzen, 1973). Another ABP has been described in the rat prostate (Heyns and De Moor, 1977). It is secreted by the prostate and is distinct from the ABP of rat testis (Heyns et al., 1978).

The uteri of early-pregnant rabbits and of animals treated with oestradiol and progesterone secrete large amounts of a small molecular weight protein called uteroglobin (Beier, 1968) or blastokinin (Krishman and Daniel, 1967). The physiological role of this protein is unknown and, although it binds progesterone and other progestogens (Beato and Baier, 1975), this

property is not necessarily relevant to its true function. It has been suggested that progesterone induces its synthesis, is bound to the protein and that the complex "affects" the blastocyst (Beato and Beier, 1975). However, uteroglobin is found in the rabbit lung (Beato and Beier, 1978). Rabbit uteroglobin may fulfill a role analogous to that of PBG in the guinea pig whereby the foetus is protected from exposure to high concentrations of progesterone. In this context, it is interesting that a pregnancy-specific protein has been found in the uterus of the cow, but it is not known if it binds steroid hormones specifically (Laster, 1977).

4.1.6 Bovine and Ovine Steroid Binding Proteins

The blood of sheep and cows of both sexes contains CBG and SHBG (Lindner, 1964a; Seal and Doe, 1965; Murphy, 1968; Corvol and Bardin, 1973), although it probably does not contain AAG (Martin et al., 1976). The follicular fluids of these animals also contain CBG and SHBG (Cook et al., 1977). Lindner (1964a) found that the plasma concentration of cortisol was low in sheep and cows compared to men and women. He concluded from protein-binding studies that plasma cortisol in ruminants was primarily bound to albumin rather than to CBG, both during pregnancy and non-pregnancy. However, ruminants were subsequently shown to contain CBG and SHBG (Seal and Doe, 1965; Paterson and Hills, 1967). Fairclough and Liggins (1975) also found that cortisol was principally bound to CBG rather than to albumin in foetal lambs, and that an increased capacity of CBG for the steroid was partly responsible for the prepartum increase in corticosteroid concentrations in normal foetal lambs.

Little has been published on the steroid specificities of the circulating binding proteins of sheep and cows, although

there is no reason to suspect that they will be substantially different from man, primates and other mammals. Martin et al. (1976) reported that testosterone was bound to both a CBG-like protein and SHBG in bulls and cows, but that the SHBG of cows had a greater capacity for testosterone, even though the concentration of the latter was 50 times greater in bulls. Furthermore, there was no difference between the SHBG concentrations of early-pregnant and non-pregnant females, so that the increased secretion of progesterone during pregnancy did not effect the concentration of SHBG (Martin et al., 1976). The protein has been purified (sex unspecified) and shown to be similar to SHBG of guinea pig (Lea, 1973) and women (Mercier-Bodard et al., 1970). It is a glycoprotein of molecular weight 89,000, contains 17% carbohydrate, and binds dihydrotestosterone with greater affinity than testosterone (Suzuki et al., 1977).

A soluble glucocorticoid binding protein has been found in the bovine adrenal cortex. The protein showed a high affinity for dexamethasone (K_D ca. 10^{-7} M) and was neither an enzyme nor a receptor by the usual criteria (Cochet et al., 1977). The authors speculated that it was an intracellular transport protein.

Of particular relevance to the present study was the finding of progesterone-binding activities in the cytosol of the bovine corpus luteum (Leymarie and Gueriguian, 1969, 1970). The binding affinities were measured using the equilibrium batch Sephadex technique described by Pearlman and Crepy (1967). The cytosol showed an affinity for progesterone that was variable but consistently higher than the affinities of either bovine

serum albumin or serum from a pregnant animal (Leymarie and Gueriguian, 1970). When the cytosolic fraction was fractionated by hydroxylapatite chromatography, added progesterone was bound by four different protein peaks. One of these peaks also bound added cortisol, and was indistinguishable from serum CBG, although its presence could not be explained by blood contamination. The second peak was thermostable and not saturable by progesterone and was classified as "albumin-like". However, it could not have been BSA because it did not bind added cortisol. The third peak exhibited high affinities for both steroids and was classified as "CBG-like" because of its low capacity, high affinity and thermostability at $60^{o}C$. The fourth peak, eluted at the highest concentration of buffer (0.65 M phosphate), had unique binding properties. Its equilibrium association constant for progesterone was determined as $2.8 \times 10^{6}\ M^{-1}$; added cortisol was not bound (Leymarie and Gueriguian, 1970). The authors speculated that the binding protein contained in this fourth fraction from the hydroxylapatite column had a role in the regulation of progesterone synthesis or its transport.

4.1.7 Measurement of Steroid-Protein Interactions

There are a variety of methods available for measuring steroid-protein interactions based on a number of different principles. Most of them require the physical separation of the bound from unbound ligand, although a few techniques (solubility increase, spectrophotometry, fluorimetry) do not (Westphal, 1971). Quantitative assays of steroid binding proteins (and receptors) have four objectives:

1. Determination of binding affinity for a given steroid in blood, serum, cytosolic fraction, etc., expressed as the

amount bound or unbound (% of total, micrograms per ml).

2. Determination of the binding capacity of a particular protein in blood or cytosolic fraction, expressed as concentration of binding sites, n(M) (= concentration of steroid maximally bound) in mole.litre^{-1}. If the molecular weight and the number of binding sites are known, the binding capacity may be given as grams per litre of blood or cytosol.

3. Determination of the number of binding sites in a protein.

4. Determination of the association constant(s) of a steroid-protein complex.

The presence of CBG-like proteins in cytosolic fractions of tissue raises the problem of distinguishing progesterone- or cortisol-binding to this high affinity protein from its binding to receptors or intracellular binding proteins. This difficulty has been circumvented in two ways. One approach is to use synthetic steroids which do not bind to serum proteins such as CBG or CBG-like proteins (e.g., R5020, Philibert and Raynaud, 1974 and R1881, Cowan et al., 1977). (However, the use of synthetic steroids cannot replace natural steroids in the identification of receptors or intracellular binding proteins). Alternatively, it is possible to inhibit CBG-like protein by adding excess radioinert cortisol (Milgrom, 1978).

The methods used in the measurement of steroid-protein interactions generally involve determination of the concentration of total bound ligand at equilibrium or the determination of the concentration of ligand bound to a specific protein. The former approach includes those techniques in which equilibrium is maintained, such as equilibrium dialysis (Westphal, 1971), the "batchwise" use of Sephadex G-25 (Pearlman and Crepy, 1967) and certain forms of ultrafiltration (Westphal,

1955; Keller et al., 1966). Techniques in which equilibrium is not maintained include the removal of bound steroid by small columns of Sephadex G-25, Sephadex LH-20, etc. (de Moor et al., 1962; Doe et al., 1964; Basset and Hinks, 1969; Ginsberg et al., 1974), precipitation by ammonium sulphate (Mayes and Nugent, 1968) or hydroxylapatite (Erdos et al., 1970), and filtration through Millipore (Batra, 1976) or DEAE-cellulose (Baxter et al., 1975). A classical non-equilibrium technique is that of sucrose gradient centrifugation (Toft and Sherman, 1975) but this method, like those of polyacrylamide gel electrophoresis (Hansson et al., 1977) and agar electrophoresis (Wagner, 1972), suffers from the difficulty of quantifying the degree of dissociation that occurs due to the length of time required to complete it. The non-equilibrium removal of free, rather than bound, steroid can be achieved by the use of adsorbents such as charcoal, Florisil, Fuller's earth and talcum powder (Westphal, 1971).

All those techniques which perturb and destroy the equilibrium set up between steroids and their binding complexes should ideally separate bound from unbound steroid instantaneously. In practice this is never achieved so that a certain amount of dissociation of bound steroid from its complex is inevitable (Baulieu et al., 1970). Westphal (1971) stated that equilibrium dialysis provided the least opportunity for dissociation and was the method by which all the others must be judged.

4.1.8 Experimental Approaches

The objectives of the work reported in this chapter were to confirm the presence of specific progesterone-binding

protein(s) in the bovine corpus luteum and to compare it with the progesterone-binding components of blood and lymph. The characterization of the steroid-binding properties of the binding protein of the corpus luteum is necessary before its projected role in the biosynthesis and/or secretion of progesterone can be investigated.

Therefore, the experimental approach was as follows:

1. Confirm the presence of a specific progesterone-binding activity in the cytosolic fraction of bovine corpus luteum using sucrose gradient centrifugation and equilibrium dialysis.
2. Investigate the possible heterogeneity of the progesterone-binding components of the bovine corpus luteum by fractionating its cytosol using techniques such as adsorption chromatography on hydroxylapatite, and monitor the binding activities of the separated components by sucrose gradient centrifugation and equilibrium dialysis.
3. Compare the progesterone-binding activity (or activities) of bovine corpus luteum with those of ovine corpus luteum and various bloods and lymph obtained from both species.
4. Measure the binding parameters of the cytosolic fraction of bovine corpus luteum for progesterone and determine the amount of cross reaction by other steroids for the binding component(s).
5. Purify the progesterone binding protein(s) in order to facilitate their physiochemical description and confirm that the cytosolic binding activity or activities resides in the purified protein(s).

4.2 METHODS

4.2.1 Collection of Material

Corpora lutea were obtained from mid-luteal and pregnant cows at two local abbatoirs. Jugular venous blood was collected in citrate at the same time. Additional bovine corpora lutea and jugular venous blood was provided by the Agricultural Research Council Laboratories at Compton, Berkshire. Samples of ovarian lymph, utero-ovarian venous (UOV) blood, and external jugular vein (JV) blood were collected from two ewes with indwelling catheters. The catheters were inserted by Dr. J.S. Robinson essentially as described by Lindner et al. (1964b), Morris and Sass (1966) and Robinson et al. (1976). In these sheep, the day of the oestrus cycle was known from synchronization with sponges of progestogen (Searle) and determination of peripheral plasma concentrations of progesterone. Ovine corpora lutea were collected either from animals known to be mid-luteal as above, or at death and estimated to be mid-luteal by gross anatomical appearance.

Human blood was taken from an antecubital vein by a research sister from patients in the second trimester of pregnancy.

4.2.2 Manipulation of Material

All non-abbatoir blood samples were collected in chilled, heparinized tubes and were centrifuged at 2,500 x g_{max} (Mistral 4L or 6L) for 15 min at 4^{o}C within 15 min of collection.

Cytosolic fractions were obtained as described in Chapter 3, except for the following modifications: (i) tissue homogenates were sometimes 1:5 w/v rather than 1:10 w/v, (ii) homogenization was carried out using 5-10 strokes of the Thomas Potter- Elvehjem, (iii) the buffer was either 5 mM or 40 mM potassium phosphate (KPB), pH 6.8 or pH 7.4, respectively,

and (iv) the 10,000 x g_{av} centrifugation step was frequently omitted so that a cytosolic fraction of either bovine or ovine corpora lutea was prepared directly from the 600 x g_{max} supernatant. All plasma samples, and those cytosolic fractions not used directly, were stored at -20°C and thawed once.

4.2.3 Sucrose Density Gradients

Linear gradients, ranging from approximately 5-20% w/w sucrose were formed in polyallomer centrifuge tubes using a Beckman Linear Gradient Former. The samples and gradients were buffered with 40 mM KPB, pH 7.4. The gradient samples were routinely equilibrated with exogenous steroids without prior removal of endogeneous steroids by dextran-coated charcoal.

Radioactively-labelled steroids were dried down onto the bottom of small glass test tubes and taken up immediately in aqueous sample. The steroids were allowed to equilibrate for 1.25-1.5 h on ice, with occasional shaking, after which 0.5 ml of the equilibrated mixture was loaded onto the top of cooled gradients. Bovine serum albumin (BSA) and/or alcohol dehydrogenase (ADH) (0.5 ml, 2 mg) were applied to duplicate gradients to provide standards of known sedimentation coefficient ($S_{20,w}$).

Centrifugation was carried out at 4°C in a Beckman L2-65B ultracentrifuge using an SW41 rotor for 16-21 h at 40,000 rpm (195,700 x g_{av}). The gradients were eluted into 0.5 ml fractions, and their sucrose density determined, as described in Chapter 3. KPB (0.5 ml) was added to the fractions and the absorbance at 280 nm determined spectrophotometrically. The radioactivity of 0.5 ml of the diluted fractions and of aliquots of the samples applied to the gradients were determined by liquid scintillation in 8 ml of Cocktail N scintillant on

either single or double label settings in a Packard Model 3380 counter.

4.2.4 Column Chromatography

4.2.4.1 Hydroxylapatite Column Chromatography

One part of hydroxylapatite (Sigma type 1) was mixed with two parts of pre-swollen Sephadex G-25[1], and equilibrated against 5 x 10 volumes of 5 mM KPB, pH 6.8. The slurry was poured into glass or perspex columns of varying dimensions and flushed with approximately three column volumes of 5 mM KPB, pH 6.8 (base buffer). Samples of up to one column volume were applied to the columns, which were flushed with 1.5-2.5 column volumes of base buffer in order to completely elute unadsorbed material. The columns were then developed in steps consisting of 1-1.5 column volumes of KPB, pH 6.8 at the following concentrations: 0.04 M, 0.06 M, 0.10 M, 0.15 M, 0.20 M and 0.65 M. The flow of buffer was assisted by fitting an LKB peristaltic pump to the outlet at the bottom of the columns. After use, the mixture of hydroxylapatite and Sephadex G-25 was discarded.

4.2.4.2 Sephadex G-200 Column Chromatography

Sephadex G-200 was allowed to equilibrate with 3 x 10 volumes of 40 mM KPB, pH 7.4 for at least four days at room temperature. The proportion of buffer was reduced and the slurry cooled to 4^{o}C. It was then poured into large glass columns and allowed to settle for 2-3 days under unit gravity.

Sample volumes were 1-2% of column volumes, and the samples were applied to the top of the resin with as little disturbance to the latter as possible. The columns were developed with 40

1: This mixture will be referred to as hydroxylapatite or "HAP".

mM KPB, pH 7.4 and allowed to flow under unit gravity.

An LKB Ultrarac 7000 fraction collector was used to collect fractions from both types of column. All operations were performed at 4°C, and the absorbance at 280 nm of fractions were determined using a Pye-Unicam SP 500 spectrophotometer. The columns were stored in buffer containing 0.01% w/v sodium azide.

4.2.5 Multiple Equilibrium Dialysis

The "knotted bag" technique of multiple equilibrium dialysis (MED) was used as described by Davies (1973). The method involved dialyzing varying concentrations of progesterone against a constant amount of protein. The experiments were carried out at constant ionic strength and pH (40 mM KPB, pH 7.4) and temperature (4°C). The purity of radioactively-labelled progesterone, pregnenolone, R5020 (and the other steroids used in the gradient experiments reported in Chapter 3) were checked by TLC and the steroids were repurified where necessary. It was assumed that the specific activities of radioactively-labelled steroids remained constant upon repurification. Each Scatchard analysis was performed in duplicate or triplicate. The following description of the technique sets out the conditions that were adopted for the majority of the study. Details of where the early MED experiments varied from these conditions are given in the text.

Preparation of dialysis vials: Stock solutions of radioinert progesterone and of {1,2,6,7(n)-^{3}H} progesterone (specific activity of 80-110 Ci.mmol^{-1}) were prepared in ethanol. The concentrations of radioinert steroid in preliminary experiments were such that addition of 0.1 ml of radioinert steroid

solution to 20 ml glass scintillation vials, together with 10 pmol of ^{3}H-progesterone in 0.1 or 0.2 ml, provided total amounts of steroid ranging from 10 to 3000 pmol. In subsequent experiments, the mass range of steroids was extended to 2.5 to 9000 pmol and consisted of 13 data-points.

The ethanol was evaporated under nitrogen at 35^{o}C, and the steroid taken up in 15 ml of KPB, pH 7.4 at 4^{o}C overnight. "Total count" polypropylene vials were also prepared by dissolving the residue from 0.1 ml aliquots of ^{3}H-progesterone solution in 8 ml scintillant with and without 0.5 ml of KPB.

Preparation of samples: Cytosolic fractions of bovine corpora lutea were prepared as described above. Equal volumes were treated with concentrated dextran-coated charcoal suspension (DCC: 6.25 gm activated charcoal + 0.625 gm dextran T-70 per 100 ml KPB, pH 7.4) for 20 min on ice and then centrifuged twice at 2,000 x g_{max} for 10 min. The supernatant was filtered through Metrical filters (0.45 µ pores). It was diluted to a protein concentration of approximately 1 mg.ml^{-1}, or accurately diluted to this concentration after protein determination according to Lowry et al. (1951) or Böhlen et al. (1973). In the former case, accurate determination of the protein concentration was carried out at the end of the experiment.

Plasma samples were thawed and centrifuged at 2000 x g_{max} for 15 min in order to precipitate any flocculent material (no discernible pellet usually resulted from this step). They were then treated with concentrated DCC solution and diluted as above.

Those pooled fractions from HAP chromatography which were subjected to MED were treated with concentrated DCC solution in

ratios of sample: DCC varying from 1:1 to 5:1, depending on the concentration of protein in the samples.

Dialysis tubing: Visking tubing (5/32 inch in diameter) was soaked in 40 mM KPB, pH 7.4 for 10 min before use.

Multiple Equilibrium Dialysis: 1 ml aliquots of DCC-treated and diluted sample were added to segments of dialysis tubing, knotted at one end. The bags were sealed by knotting the open ends and placed in the glass scintillation vials containing progesterone and KPB. The vials were kept on ice during this procedure.

The vials were capped, laid horizontally, and shaken for 36-44 h at $4^{o}C$. In later experiments, the period of dialysis was reduced to 18-24 h. Following dialysis, the protein solutions in the dialysis bags were emptied into glass test tubes. Once the inside and outside compartments had warmed to room temperature, 0.5 ml aliquots of each were dispersed into plastic vials containing 8 ml of cocktail N scintillant. Radioactivity was measured at $4^{o}C$.

The glass vials were soaked in a surface-active detergent (Decon 90), rinsed thoroughly in glass-distilled water, dried in an oven and re-used. The screw-caps were discarded.

Validation experiments: The time required for progesterone to fully equilibrate with the binding components inside the dialysis bag was measured. Ten vials (in triplicate) containing a constant amount of progesterone were dialyzed for different periods up to 72 h. In the same experiment, the time taken for remaining endogenous progesterone to exchange with exogenous progesterone was determined. A large amount of radioactive

progesterone was equilibrated with diluted cytosol for 3.33 h, treated with 0.2 parts of concentrated DCC solution to remove unbound progesterone, and aliquoted into dialysis bags as described above. Ten vials (in triplicate) were dialyzed for periods ranging between 0.5 and 46 h.

Analysis of data: The derivation of the equilibrium association constant and the total number of high-affinity binding sites was based on data analysis according to Scatchard (1949), as described by Davies (1973) and Rodbard and Feldman (1975). The following abbreviations were used:

B = Total bound progesterone concentration, $pmol.ml^{-1}$

Bspec = Progesterone bound by high-affinity component (e.g., binding protein)

K_a = Equilibrium association constant

n(M) = Binding site concentration (number of binding sites per protein molecule x concentration of binding protein), $pmol.mg^{-1}$

P = Progesterone-binding protein concentration, $mg.ml^{-1}$

S = Total progesterone concentration, $pmol.ml^{-1}$

S-P = Progesterone-protein complex concentration

U = Unbound progesterone concentration, $pmol.ml^{-1}$

The parameters to be plotted were calculated as follows (Davies, 1973; Rodbard and Feldman, 1975):

$$\frac{B}{U} = \frac{\text{(dpm inside)} - \text{(dpm outside)}}{\text{(dpm outside)}}$$

$$B = \frac{\{(\text{dpm inside}) - (\text{dpm outside})\} \times \text{total progesterone added}}{\text{total dpm added}}$$

$$\text{At equilibrium, } Ka = \frac{(\text{Progesterone} - \text{Protein})}{(\text{Progesterone})\ (\text{Protein})}$$

$$Ka = \frac{B}{U\ (\text{free binding sites})}$$

$$\frac{B}{U} = Ka\ (\text{free binding sites})$$

$$\frac{B}{U} = Ka\ \{(\text{total sites}) - (\text{occupied sites})\}$$

$$\frac{B}{U} = Ka\ \{n(M) - B\}\ \text{(Scatchard equation)}$$

In a system with only one binding component, the latter is a straight line with a slope of -Ka; at the intercept on the ordinate, B=O and:

$$\frac{B}{U} = Ka.n(M)$$

At the intercept on the abscissa, B/U = 0 and:

$$B = n(M)$$

In these experiments, there were at least two binding components so that a plot of B/U vs B yielded a curve. Subtraction of the non-specific binding from the specific, high-affinity binding component(s) to give a plot of Bspec/U vs Bspec was achieved according to Chamness and McGuire (1975a). The limiting B/U ratio was used and multiplied by the free progesterone concentration (U) at each point to determine the non-specific binding (in pmol) at that point. This was then subtracted from the measured total binding (B) to find the specific binding (Bspec) at that point:

$$\text{Bspec} = B - U \left(\lim_{B \to \infty} \frac{B}{U}\right)$$

Bspec is plotted against Bspec/U to produce the corrected Scatchard binding line for the high-affinity component(s). This line was fitted to the corrected data points by least-squares regression analysis (Snedecor and Cochran, 1971).

The slope of the line yields the negative of K_a; and at the intercept on the abscissa,

$$\text{Bspec}/U = 0 \text{ so that Bspec} = n(M).$$

Calculation of binding parameters initially used radioactivity in the form of cpm, but in the majority of experiments radioactivity was converted from cpm to dpm by reference to appropriate quench curves. The method of quench analysis utilized the "automatic external standardization" facility of the counter. The radioactivity of the protein (inside) compartment was detected with approximately 5% lower efficiency than the radioactivity of the buffer (outside) compartment. The endogenous progesterone concentrations of samples were determined by RIA and included in calculations of binding parameters. The correction for the contribution of non-specific binding to the total binding, and the fitting of a line through the corrected Scatchard data points by regression analysis were performed on a minicomputer (Data Dynamics).

Sources of error: As volumetric errors were potentially greater than all other sources of variation (Westphal, 1971), efforts were directed at minimizing them. The progesterone solutions were dispensed in 0.1 ml aliquots using an Oxford pipette (±1.5%), whereas tritiated progesterone was aliquoted by means of an Oxford Model S dispensor (±1%). The buffer solution was

most difficult to dispense accurately and reproducibly: pipetting by mouth (±2.5%) or using a Zipette (±5-10%) were not as satisfactory as using an Oxford Model L dispensor (+0.33%, ±0.5%). After MED, 0.5 ml aliquots of both the inside and outside compartments were added to scintillant using an Oxford pipette (±1%). All errors were estimated by weight, assuming a density of water of 1 gm.cc^{-1}. Thus, the cumulative volumetric error was initially between 6 and 13.5%, but this was subsequently reduced to 4% for the majority of the experiments. Radioactivities were estimated to within a standard deviation of ±1.5% (equivalent to 95% confidence limits at the radioactivities used) and were therefore less significant than the volumetric errors. It was found (also by weight) that the volume inside the dialysis bag was unchanged after dialysis at the protein concentrations used. Retention of radioactivity by the dialysis membrane at the smallest mass points amounted to ⩽1%, which was within the counting error.

4.2.6 Steroid Cross Reaction Studies

Estimation of the ability of different steroids to compete with progesterone for binding to the cytosolic components of bovine corpus luteum was carried out by MED. Different steroids, ranging in amounts from 10 pmol to 5 μmol, were added to vials containing 10 ± 0.5 pmol of progesterone, some of which was radioactive. Homologous or control analyses of progesterone, extending over the same range of concentration, were also included.

The level of binding achieved with 10 pmol of progesterone alone was set at 100% and the binding of progesterone in the presence of differing amounts of competing steroid was compared

to this value, i.e.,

$$\% \text{ Cross reaction} = \frac{\text{amount of progesterone causing 50\% decrease in binding}}{\text{amount of other steroid causing 50\% decrease in binding}} \times 100$$

4.2.7 Other Methods of Measuring Progesterone Binding

4.2.7.1 Batch Sephadex Technique

This is an equilibrium technique in which the semi-permeable membrane of MED is replaced by cross-linked dextran gel, and was first described by Pearlman and Crepy (1967) and Pearlman (1970). The basis of the method is as follows. Swollen Sephadex G-25 is shaken with diluted cytosol and a mixture of radioinert and radioactive steroid; when equilibrium is reached, the gel is allowed to settle, and protein (P) and bound steroid (B) are excluded from the gel to remain in the external (upper) phase. On the other hand, unbound steroid (U) partitions freely between the external and internal phases of the system. Sampling of the external phase permits estimation of B, after allowing for the fraction of U remaining in the external phase and correcting for non-ideal partitioning of U between gel and excluded phases.

Assay procedure: The assays were performed at room temperature ($22^{o}C$) or at $4^{o}C$; 200 mg aliquots of Sephadex G-25 (fine) were accurately weighed into glass-stoppered test tubes and allowed to swell overnight in 1 ml of 40 mM KPB, pH 7.4. The next day, 0.1 ml of KPB, pH 7.4 was added containing a constant amount of tritiated steroid (0.1 pmol, ca.6000 cpm) and varying amounts of radioinert steroid. Protein solution (0.9 ml of DCC-treated and diluted cytosol or plasma) was added to the assay tubes. "Blank" tubes contained 0.9 ml of buffer in place of protein

solution and "total count" tubes contained 0.1 ml of the tritiated steroid solution plus 1.9 ml of buffer.

The tubes were stoppered and revolved end-over-end at 25 rpm for 1 h; they were placed upright, unstoppered and allowed to stand for 1 h. A 0.2 ml aliquot of the clear supernatant solution was withdrawn from each tube and counted in 8 ml of Cocktail N scintillant at $4^{o}C$ with an efficiency of 33%.

Calculations: The equations used are derived in full in the original references (Pearlman and Crepy, 1967; Pearlman, 1970). Briefly, the mean value of radioactivity (cpm) added per tube (a) was obtained from the set of tubes which lacked Sephadex ("total count" tubes). The partition coefficient (C) for steroid in the absence of protein was obtained from the tubes containing only Sephadex and buffer ("blank" tubes). C was determined for each assay from the equation:

$$C = \frac{x}{a-x}$$ where "x" is the radioactivity remaining in the external phase upon equilibration; "a" is defined above.

The B/U ratio was derived as follows: In the absence of protein, x equals U in the external phase; hence $U = C\ (a-x)$. In the presence of protein $x = B + U$ in the external phase after equilibration; hence $B = x - U = x - C\ (a-x)$.

$$\text{Thus, } \frac{B}{U} = \frac{x - C\ (a-x)}{C\ (a-x)}$$

When equal volumes of fractions from a chromatographic column were used in the same assay, the variation in the B/U ratios of the different fractions provided an indication of their relative binding activities for a particular steroid (Gueriguian and Pearlman, 1968).

4.2.7.2 Fluorescent Progesterone-Binding Assay

The method of Stroupe et al. (1975) was used without modification. A Perkin-Elmer (model 124) double-beam spectrophotometer, operating at 25°C, was used to obtain the difference spectrum of partially-purified binding protein in the presence and absence of progesterone. The baseline was set using the same protein solution in both the reference and sample cells, after which progesterone was added to the sample cell dissolved in ethanol and an equal volume of ethanol was added to the reference cell.

Fluorescence was measured in an Aminco-Bowman spectrofluorometer equipped with a xenon lamp and a circulating water-bath set at 25°C. Activation and emission spectra were corrected only for blank fluorescence. The protein solutions were prepared in 40 mM KPB, pH 7.4 and endogenous steroid was removed by treatment with 0.2 parts of DCC solution as above. The fluorescence titrations were performed by adding progressively up to 150 µl of progesterone (1 mM) in ethanol to 2.5 ml of the binding protein solution. The effect of dilution on the fluorescence was allowed for in all calculations.

The affinity constant was determined from the expression:

$$K_a = \frac{\alpha}{(1-\alpha)^2 P}$$

where α is the degree of association as given by the ratio of quenching at the equivalence point over total quenching, and P is the binding site concentration at the equivalence point. The degree of quenching at the equivalence point was obtained by drawing a smooth curve through all data points between the two linear portions of the titration curve (Stroupe et al., 1975).

4.2.7.3 Non-Equilibrium Methods

A variety of methods of separating protein-bound from unbound progesterone and R5020 were evaluated.

1. Charcoal: Adsorption of unbound steroid to DCC followed by centrifugation to leave protein-bound steroid in the supernatant was attempted. Cytosolic fractions were prepared from 1:5 w/v homogenates of bovine corpus luteum. Endogenous steroid was removed with concentrated DCC solution as described above. In order to determine the concentration of cytosolic protein which provided maximal binding of steroid, a series of 0- to 20-fold diluted cytosolic solutions was prepared. Aliquots (0.5 ml) of these solutions were added to tubes in which ^{3}H-progesterone (0.25 pmol, ca. 20,000 cpm; total binding) or ^{3}H-progesterone in the presence of a large excess of radioinert steroid (ca. 100 pmol; non-specific binding) had been previously dried down under nitrogen and taken up in 0.2 ml KPB.

The tubes were incubated in duplicate for 2 h, 4 h, 8 h or 18 h at 4^{o}C and then treated with 0.2 ml of 1/10 - 1/100 DCC suspension for 20 min with periodic agitation on ice. The charcoal was pelleted by centrifugation at 2000 x g_{max} for 5 min, and the supernatants were decanted into 8 ml of Cocktail N scintillant and counted. Tubes containing buffer in place of protein solution were included in the assay for estimation of the effectiveness of charcoal adsorption. The efficiency of charcoal adsorption was calculated from the difference between the counts of ^{3}H-progesterone in KPB (total counts) and the counts of ^{3}H-progesterone in KPB after treatment with DCC (background counts). Specific binding was calculated from total binding less non-specific binding.

2. Filters: Different dilutions of cytosol were prepared as above and incubated for 2 h at 4°C. Retention of protein-bound steroid was attempted by filtration of the incubated cytosol solutions through filters of glass-fibre (Whatman GF/F and GF/C), diethylaminoethyl-cellulose (Whatman DE-81) or cellulose ester (Millipore PSED and PTGC). Retention of unbound steroid was attempted by filtration through filters of fibrous cellulose ester (Millipore HAWP, 0.45 μ pore size). Filtration was performed at 4°C.

3. Minicolumns: Separation of protein-bound from unbound steroid was attempted by gel filtration through small columns of Sephadex G-25, Sephadex LH-20 and Bigel P-10, and by adsorption of the protein-bound fraction to columns of amorphous HAP (Sigma) and spheroidal HAP (BDH). The columns were constructed from perspex of dimensions 0.6 (i.d.) x 15 cm, with an outlet of 0.2 cm in diameter. They were filled either to a height of 8 cm or with a known amount (0.5 or 0.7 g) of resin. The resin was constrained by means of a small disc of natural silk.

Different dilutions of cytosol or human plasma of pregnancy were prepared as above and incubated for 2 h, 4 h, or 18 h at 4°C. The volumes of protein solutions applied to the mini-columns and the nature of their elution are described in the text.

4.2.8 Polyacrylamide Gel Electrophoresis

4.2.8.1 SDS-Polyacrylamide Cylindrical Gels

The technique described by Weber and Osborn (1969) was used. Gels consisting of 10% w/v acrylamide and 0.1% w/v sodium dodecyl sulphate (SDS) were poured in glass tubes

(0.6 (i.d.) x 10 cm). The protein samples were diluted with, and sometimes dialyzed overnight against, a buffer consisting of 6 M urea, 1% w/v SDS, 1% w/v 2-mercaptoethanol and 0.01 M sodium phosphate buffer, pH 7.0. Bromophenol blue and glycerol were mixed with the dialyzed samples prior to their application to the top of the gels. The electrophoresis buffer consisted of 0.033 M sodium phosphate, pH 7.0 containing 0.1% w/v SDS. Electrophoresis was performed for varying times at constant current, and in no case exceeded 4 mA per gel.

The gels were removed from the tubes with a water-filled syringe and stained for 2 h at room temperature (ca. 22^{o}C) in 0.25% w/v Coomassie brilliant blue (R250) dissolved in methanol: water: glacial acetic (45: 45: 10); they were destained in the same solution minus the dye.

4.1.8.2 SDS-Polyacrylamide Slab Gels

For the estimation of protein molecular weights, a vertical slab gel apparatus was constructed and used according to Maizel (1971). The separating gel (8.5 x 12.5 cm) was poured first and a sample or stacking gel (1.5 x 12.5 cm) was poured on top of the separating gel once the latter had polymerized. The stacking gel was formed into 20 sample wells using a perspex comb. The gel solutions consisted of the following (volumes in ml):

	7.5% Separating gel	3% Sample gel
30% w/v Acrylamide	7.5	2.0
1% w/v Bisacrylamide	5.8	2.6
1.5 M Tris-HCl, pH 8.7	7.5	
1.0 M Tris-HCl, pH 6.8		2.5
Distilled water	8.6	8.0
20% w/v SDS	0.15	0.1
10% w/v Ammonium persulphate	0.20	0.2
Glycerol		2.0
TEMED	0.01	0.01

The electrophoresis buffer was 0.38 M glycine, 0.05 M Tris and 0.1% w/v SDS and electrophoresis was performed for up to 8 h at 40 V and 14 - 6 mA. Staining and destaining was accomplished using the same procedure as used for the cylindrical gels.

4.3 RESULTS

4.3.1 Preliminary Experiments

Evidence for specific progesterone-binding activity in the soluble fraction of bovine corpora lutea was sought. Sucrose gradient analysis provided a qualitative indication of the specificity of binding of a limited range of steroids and a crude estimate of the molecular weight of the binding component(s). MED was used to quantitate the progesterone-binding activity of cytosol and its fractionation on HAP allowed the binding activity to be distinguished from that of circulating proteins.

4.3.1.1 Sucrose Density Gradients

The cytosolic fraction of corpora lutea from mid-luteal cows was equilibrated with ^{3}H-progesterone and resolved on 5-20% w/w sucrose density gradients. Cytosolic ^{3}H-progesterone entered the gradient to band maximally at approximately the same density as bovine serum albumin (BSA,4.3S) and at lesser density than alcohol dehydrogenase (ADH,7.4S), run in duplicate gradients. Progesterone alone did not enter the gradient. In another experiment, cytosol from bovine corpora lutea was equilibrated with four different steroids (Fig. 4-1). Exogenous ^{3}H-progesterone entered the gradient to give a discrete peak in the 4S region, as did ^{3}H-R5020 and ^{3}H-danazol. In all three cases, most of the labelled steroid was taken equally into the gradient so that the trailing edge of the peaks merged smoothly with the 15-22% of the steroid remaining in the soluble region. Progesterone and the synthetic progestogen, R5020, were maximally bound in the same region of the gradient, suggesting that both were bound to the same component. In contrast, cortisol was not taken into the gradient specifically and was not protein-

Fig. 4-1A,B: Sucrose density gradient centrifugation of the cytosolic fraction of bovine corpus luteum

Aliquots (0.5 ml) of cytosol, prepared from mid cycle corpora lutea, were incubated with tritiated steroids and resolved on 5-20% w/w sucrose gradients, and analyzed as described under Methods. The gradients were formed in cellulose nitrate tubes.

The abscissa denotes the tube number as eluted from the gradient. On the ordinate, the radioactivity (cpm or dpm) recovered in each diluted fraction is plotted.

Details of the gradients were as follows:

Gradient	Exogenous Steroid	Radioactivity applied	Mass applied, pmol	% Recovery of radioactivity[a] from gradient
A	^{3}H-Progesterone	10,608 cpm	0.16	75
C	^{3}H-Cortisol	13,835 cpm	0.26	77
D	^{3}H-R5020	9,045 cpm	0.16	45
E	^{3}H-Danazol	2,620 cpm	0.15	91
F	^{14}C-Progesterone	23,355 dpm	0.20	57
	^{3}H-R5020	28,640 dpm	0.15	54
	Marker			
B	BSA		2 mg	

a: Assumed a loss of 7.5% upon reading of refractive indices.

A) ● = Progesterone, cpm.0.5 ml^{-1}
△ = Protein, A_{280}
○ = % Sucrose, w/w

B) ● = BSA, A_{280}
○ = % Sucrose, w/w

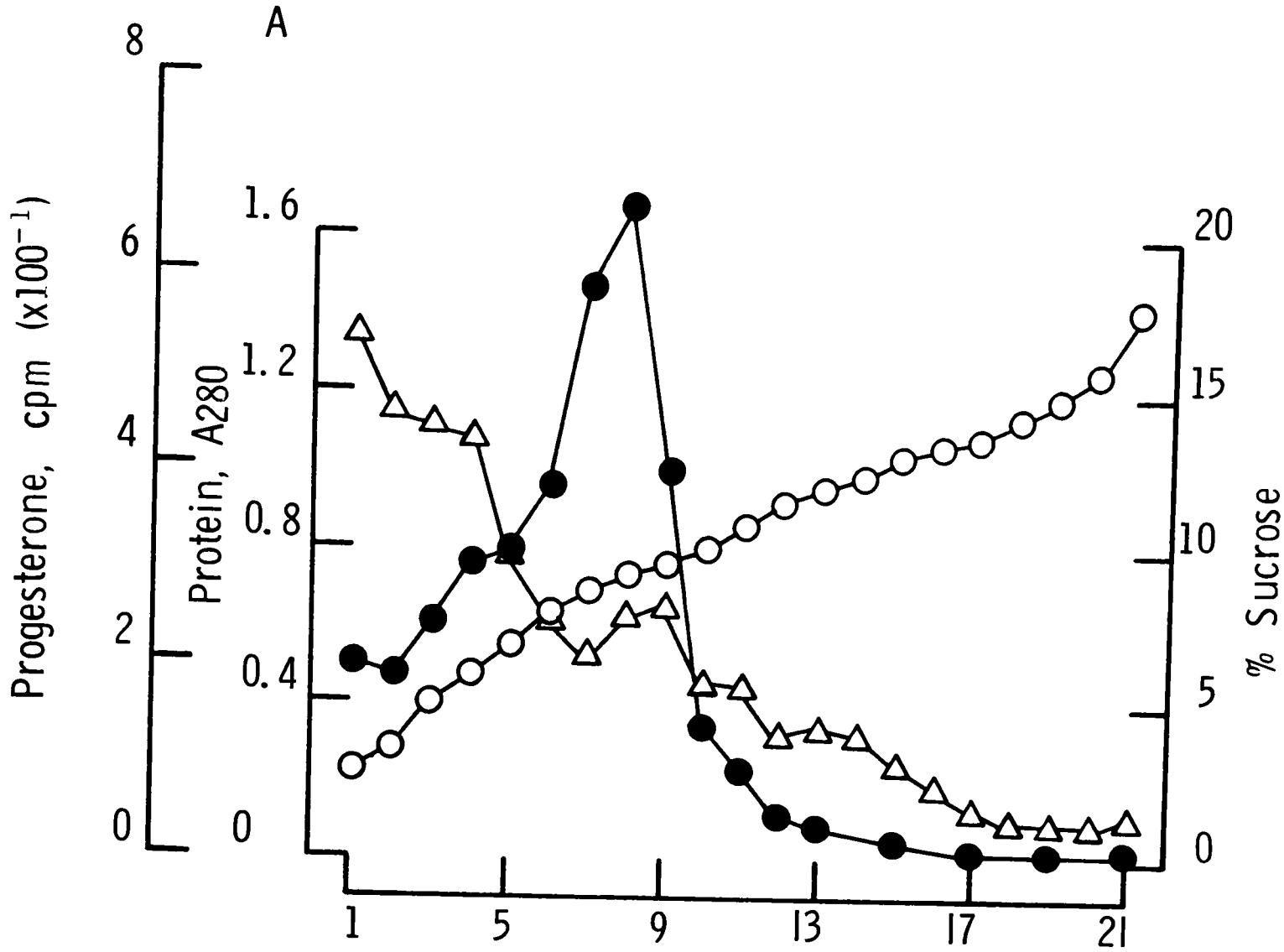

A
Progesterone, cpm (x100⁻¹)
Protein, A280
% Sucrose
Tube Number
B
BSA, A280
% Sucrose
Tube Number

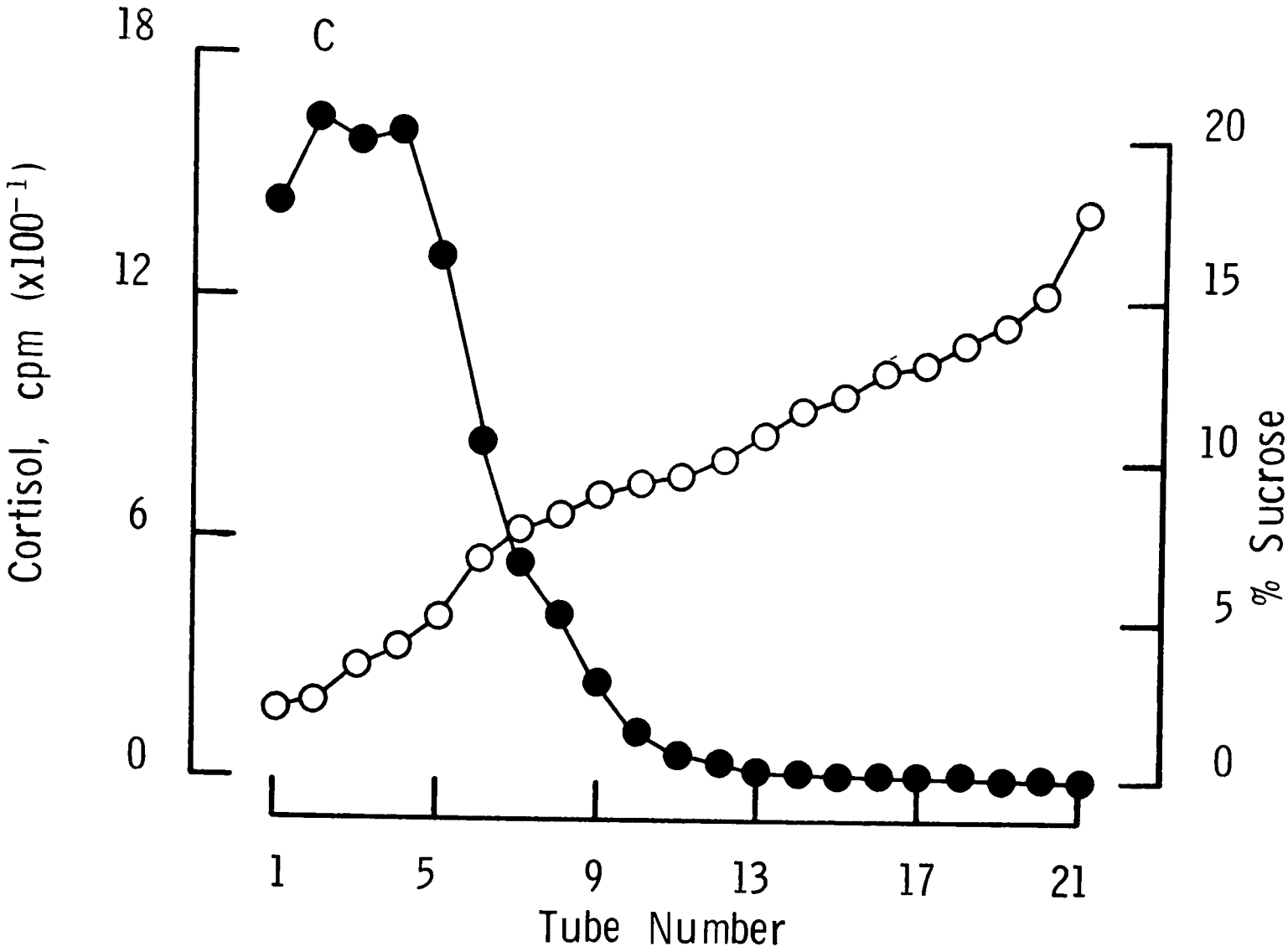
Fig. 4-1C,D: Sucrose density gradient centrifugation of the cytosolic fraction of bovine corpus luteum

C) ● = Cortisol, cpm.0.5 ml^{-1}

○ = % Sucrose, w/w

D) ● = Danazol, cpm.0.5 ml^{-1}

○ = % Sucrose, w/w


C
18
12
6
0
Cortisol, cpm (x100⁻¹)
20
15
10
5
0
% Sucrose
1
5
9
13
17
21
Tube Number

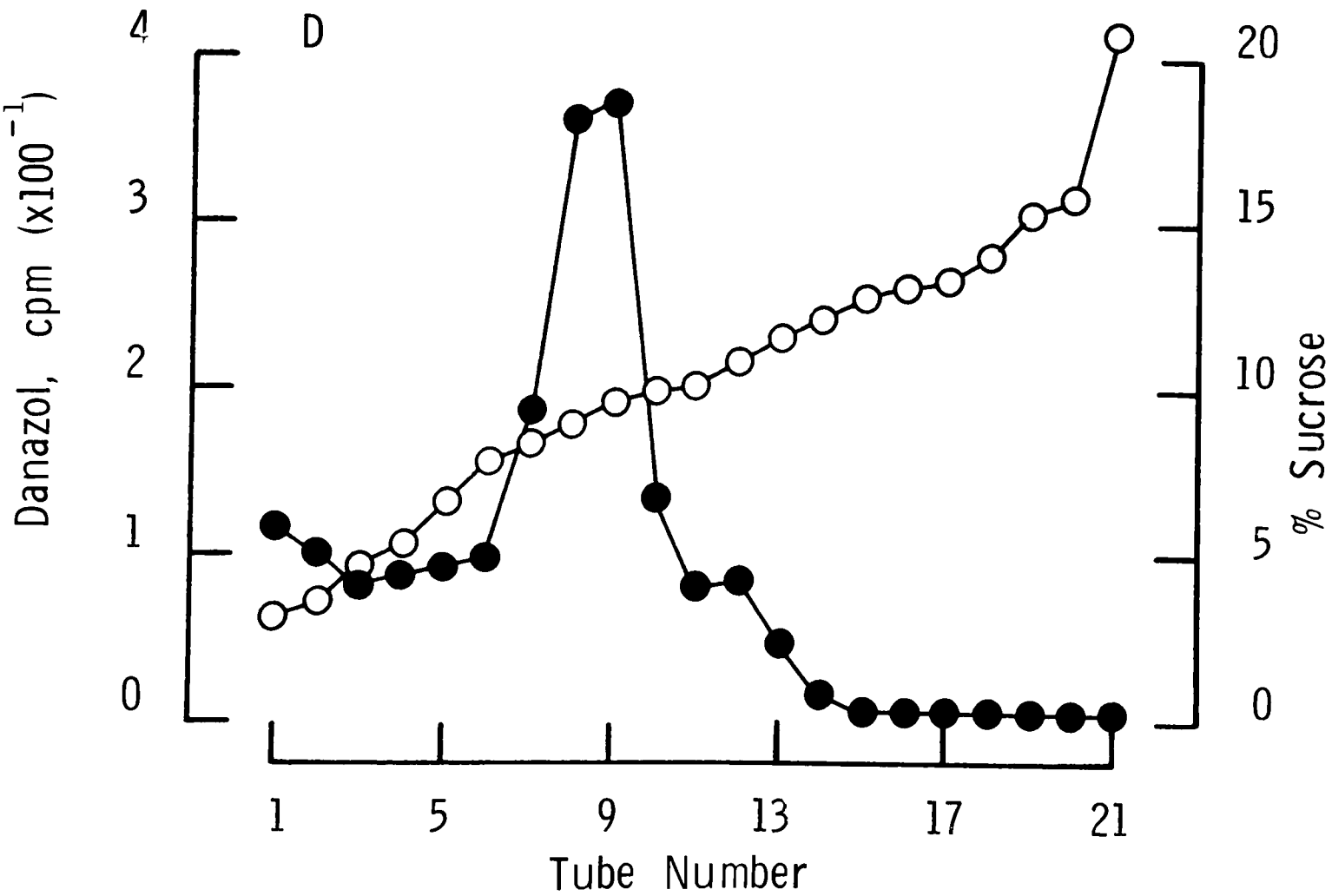
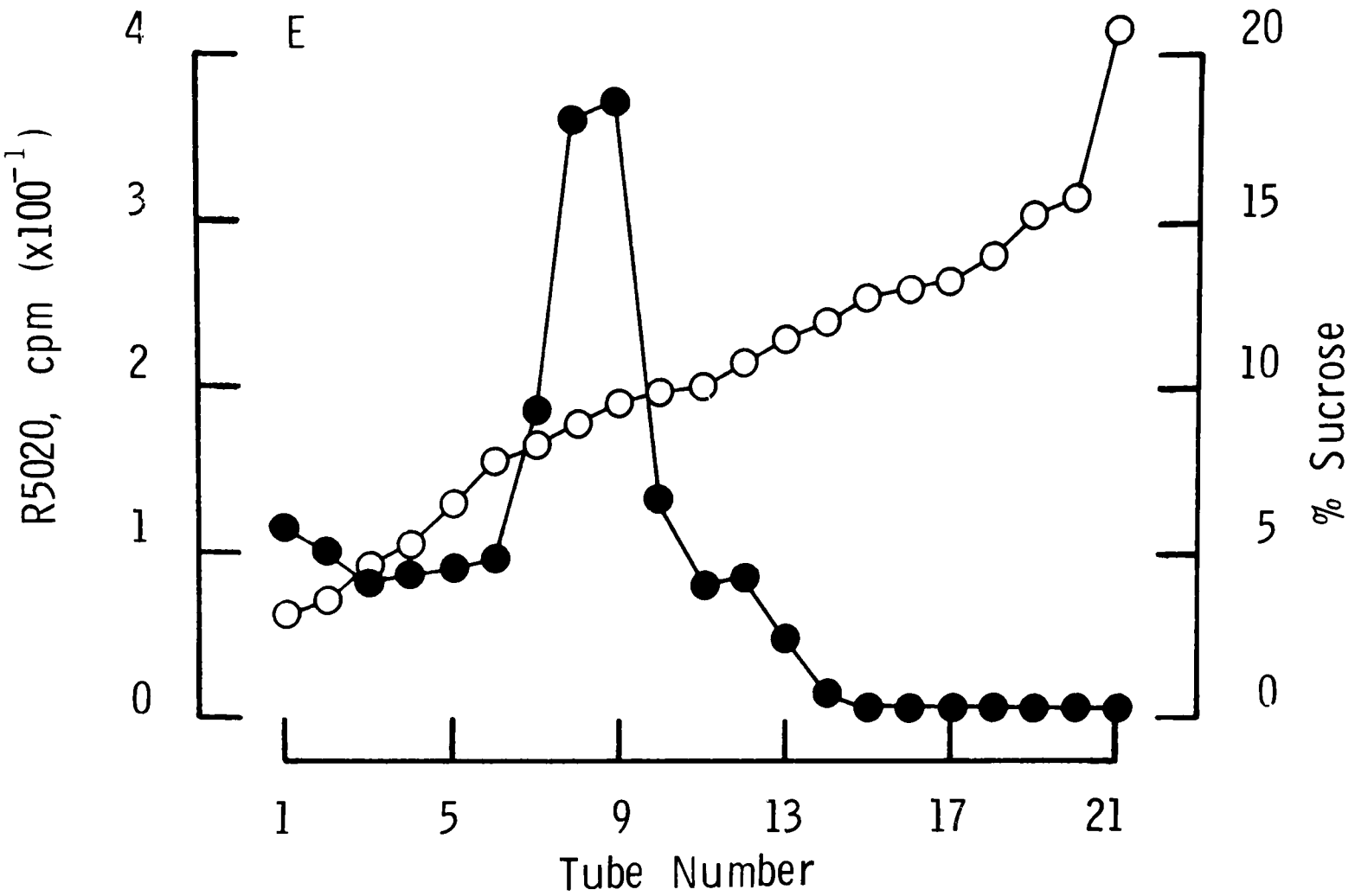
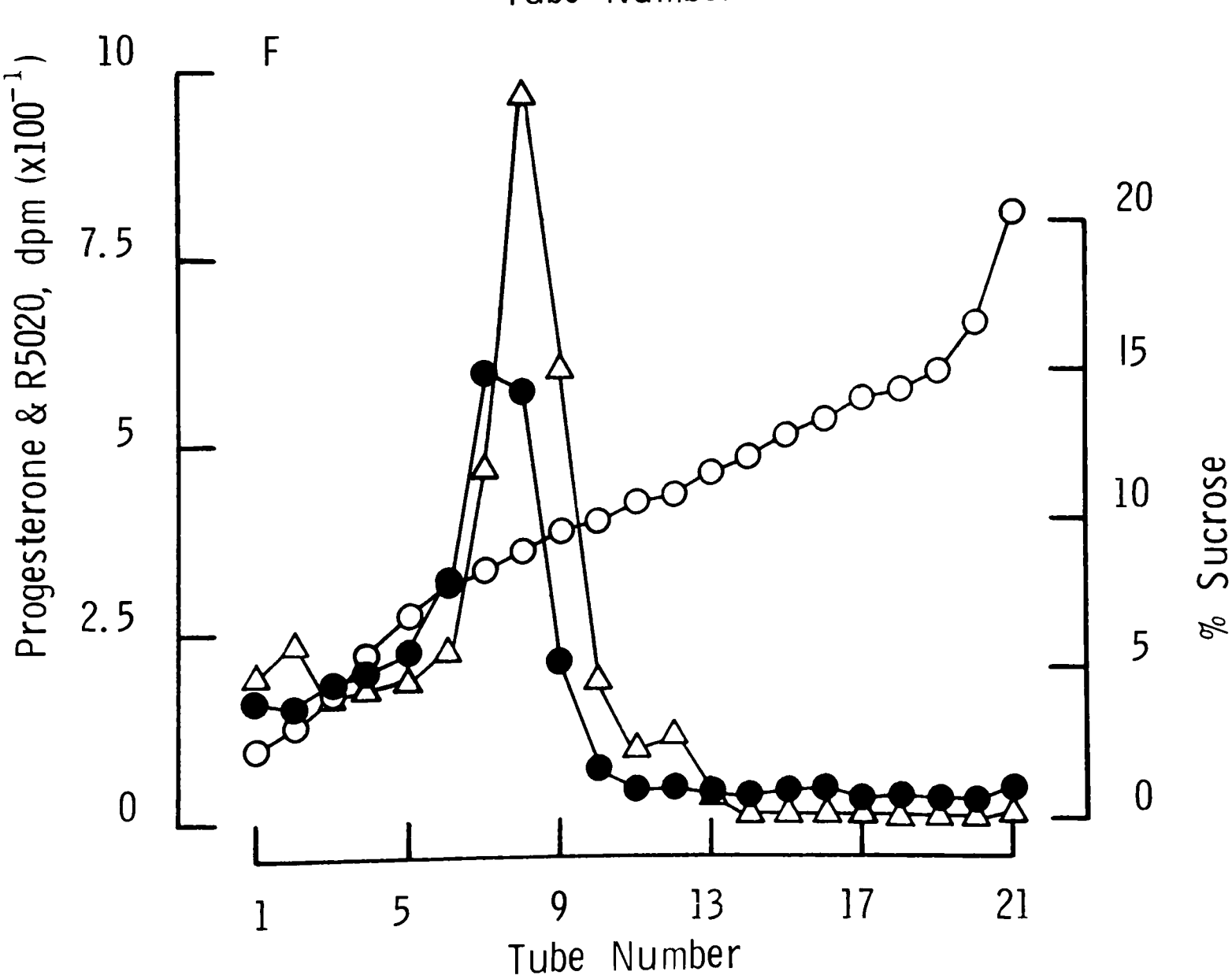

D
4
3
2
1
0
Danazol, cpm (x100⁻¹)
20
15
10
5
0
% Sucrose
1
5
9
13
17
21
Tube Number

Fig. 4-1E,F: Sucrose density gradient centrifugation of the cytosolic fraction of bovine corpus luteum

E) ● = R5020, cpm.0.5 ml^{-1}
○ = % Sucrose, w/w

F) ● = Progesterone, dpm.0.5 ml^{-1}
△ = R5020, dpm.0.5 ml^{-1}
○ = % Sucrose, w/w


E
R5020, cpm (x100^{-1})
4
3
2
1
0
20
15
10
5
0
% Sucrose
1
5
9
13
17
21
Tube Number


F
Progesterone & R5020, dpm (x100^{-1})
10
7.5
5
2.5
0
20
15
10
5
0
% Sucrose
1
5
9
13
17
21
Tube Number

bound. Thus, the progesterone-binding activity of the cytosol was not due to CBG.

Removal of most of the endogenous steroid in the cytosol with DCC, prior to incubation with ^{3}H-progesterone, did not alter the shape or position of the latter on sucrose gradients. Also, the presence of 0.4 M KCl in both untreated and DCC-treated cytosols and in the gradient gave profiles of ^{3}H-progesterone analogous to those obtained in the absence of KCl. Thus, the progesterone-binding peak sedimented as a 4S entity at both low and high concentrations of salt (Chamness and McGuire, 1975b), in contrast to the uterine receptor proteins for progesterone or oestrogen (Janne et al., 1978).

Calculation of the sedimentation coefficient and apparent molecular weight of the progesterone-binding activity was undertaken according to Martin and Ames (1961).

Table 4-1 shows that both the sedimentation coefficient and molecular weight of the cytosolic steroid-binding component were slightly less than those of BSA. The calculation compared the average concentration of sucrose at which the different radioactive steroids banded (8.93 ± 0.11 (SEM) % w/w (n=5)) with the sucrose concentration (9.50 ± 0.06 (SEM) % w/w (n=3)) at which the peak of BSA occurred. Three assumptions were made (Martin and Ames, 1961):

(i) sedimentation equilibrium was reached,

(ii) the partial specific volumes of the two proteins were approximately equal (viz., 0.725), and

(iii) both proteins were approximately spherical in shape.

Protein	$S_{20,w}$	Molecular weight
BSA (Marker)	4.3[a]	67,000
Binding protein (Expt 1)	4.04	61,061
Binding Protein (Expt 2)	4.19	64,735
	Mean 4.12	Mean 62,900

Table 4-1: Estimation of the sedimentation coefficient and molecular weight of a cytosolic steroid-binding protein from bovine corpus luteum by sucrose gradient centrifugation.

Aliquots of cytosol were prepared from bovine corpora lutea, equilibrated with radioactive steroids and centrifuged in 5-20% w/w sucrose gradients as described under Methods.

The $S_{20,w}$ values and molecular weights were calculated as described by Martin and Ames (1961).

a; According to Miller and Golder (1952).

4.3.1.2 Multiple Equilibrium Dialysis

The cytosolic fraction of bovine corpus luteum was subjected to MED to provide a quantitative estimate of the progesterone-binding interaction and an indication of its stability to various manipulative procedures (Table 4-2). The equilibrium association constant (K_A) for the progesterone-binding reaction of cytosol was of the order of 10^8 M^{-1}. This was in excess of the K_A for progesterone determined for BSA (10^4-10^5 M^{-1}) and similar to that determined for CBG (ca.10^8 M^{-1}; Westphal, 1971). Therefore, the progesterone-binding activity of cytosol from bovine corpora lutea was a high-affinity binding, in agreement with Leymarie and Gueriguian (1970). Although there was considerable variability in the numerical value of the K_A for progesterone in these initial experiments (Table 4-2), the pattern of variability did not indicate that the binding activity required the presence of sulphydryl groups in the buffer for stability, or that the removal of endogenous steroid by gel filtration through Sephadex G-25, rather than by treatment with DCC, provided greater preservation of the binding activity. However, one cycle of freezing and thawing of the cytosol, or its passage through Sephadex G-25, appeared to lower the number of binding sites. Scatchard analysis of frozen and thawed cytosol showed that a significant shift of the high-affinity binding points to lower total progesterone concentrations occurred when cytosol was so treated.

4.3.1.3 Hydroxylapatite Column Chromatography

Leymarie and Gueriguian (1969, 1970) reported that the progesterone-binding activity of the cytosolic fraction of bovine corpora lutea was resolvable into more than one component

	K_A, X 10^8 M^{-1}	K_D, X 10^{-9} M	n(M), pmol.mg^{-1}
Freshly-prepared cytosol	2.40	4.16	16.95
DTE-buffered cytosol	3.57	2.80	18.4
Sephadex G-25-treated cytosol	7.55	1.33	5.4
Frozen and thawed cytosol	10.31	0.97	8.4

Table 4-2: Multiple equilibrium dialysis and Scatchard analysis of the progesterone-binding activity of the cytosolic fraction of bovine corpus luteum.

The cytosol was freshly-prepared and aliquoted immediately into dialysis bags, with one exception (see below). Dialysis was carried out at 4°C for 44.5 h over a range of total progesterone extending from 0 to 3000 pmol per dialysis vial. The buffer was 10 mM Trizma 7.0 (pH 7.5 at 5°C) containing 0.25 M sucrose in the inside compartment. The buffer was dispensed using a zipette.

The conditions of MED were varied as follows:
(i) A portion of the freshly prepared cytosol was frozen (-40°C for 5 min, followed by 60 min at -20°C) and then thawed on ice and aliquoted into dialysis bags.
(ii) The endogenous steroid of another portion of cytosol was removed by passage of the cytosol through two large columns of Sephadex G-25 (4.4(i.d) x 15 cm) operating at room temperature (22°C).
(iii)A portion of DCC-treated cytosol was made 5 mM in dithioerythritol (DTE). The same concentration of this reagent was also included in the buffer added to the dialysis vials.

on columns of hydroxylapatite. Pilot studies established that the ^{3}H-progesterone-binding component(s) of cytosol were not bound by DEAE- and CM-cellulose, or by DEAE-, QAE- and SP-Sephadex. Therefore, the fractionation of the cytosolic proteins by selective de-adsorption from HAP was investigated (Fig. 4-2). Protein was eluted from the column at each concentration of KPB, but only those proteins which were eluted by 150 mM KPB appeared to bind added progesterone specifically. The trailing edge of the peak of unadsorbed material (eluting at 5 mM KPB) and the fractions between this and the 150 mM peak contained unbound progesterone. Cortisol was not retained by any of the adsorbed peaks of protein but eluted with the unadsorbed material. In another experiment, the cytosol was dialyzed against base buffer before application to a duplicate column (data not shown). Again cortisol was recovered totally in the unadsorbed peak, but the amount of unbound radioactive progesterone that eluted between the 5 mM and 150 mM peaks was increased relative to the latter. When radioactive progesterone and cortisol only were applied to the same HAP column, cortisol was completely unadsorbed and eluted exactly as previously. However, progesterone was eluted maximally some 21-24 ml behind cortisol, suggesting that the latter, more polar, steroid interacted weakly with the beads of Sephadex G-25 in the HAP column. Taken together, these findings suggested that in the case of the cytosol experiments, the progesterone recovered in the fractions between the 5 mM and 150 mM peaks was derived from two sources: firstly, from unbound progesterone which eluted behind cortisol and, secondly, by dissociation of some of the progesterone bound to the protein eluting at 150 mM KPB.

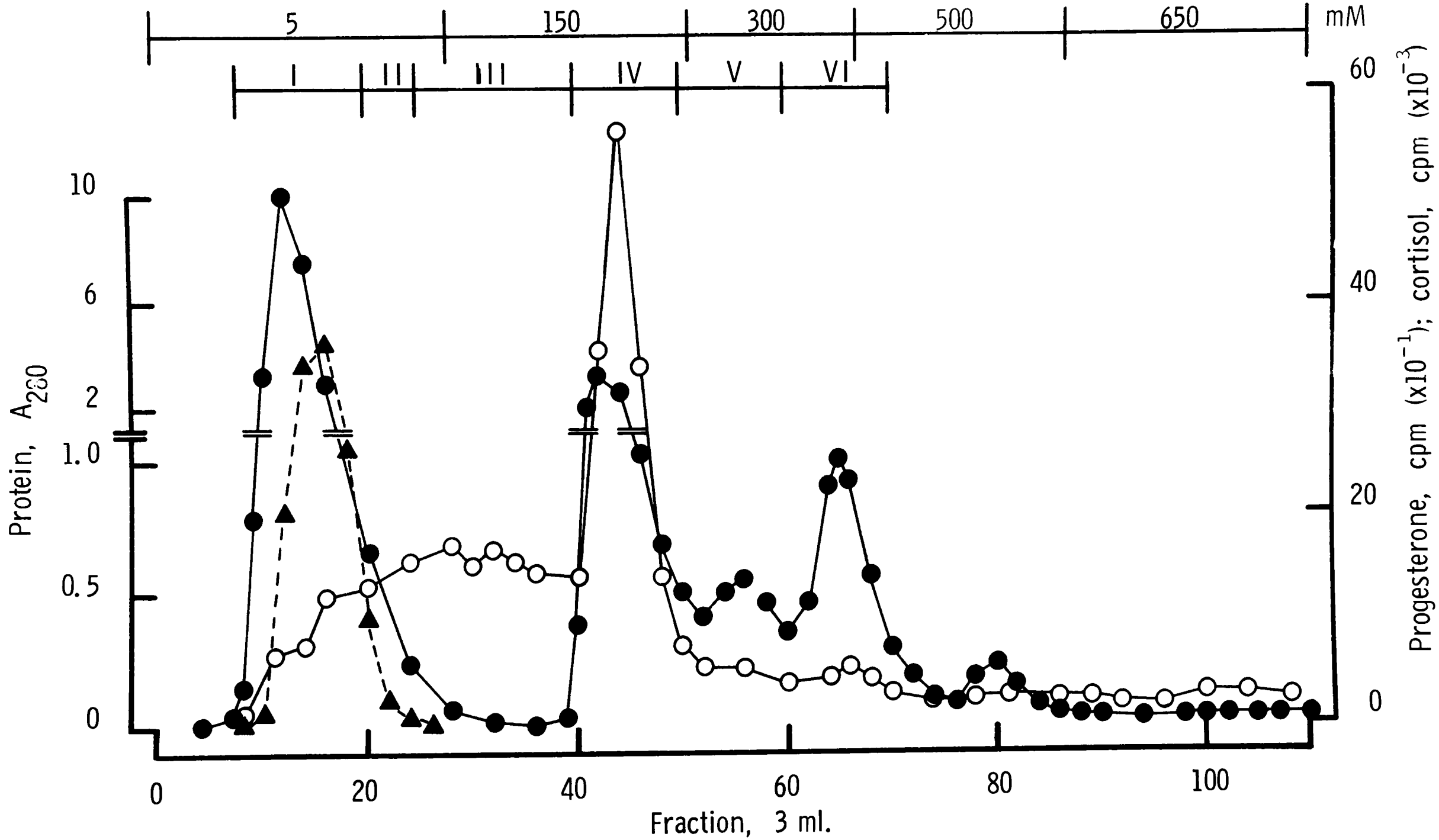
Fig. 4-2: Hydroxylapatite column chromatography of the cytosolic fraction of bovine corpus luteum

Cytosol was prepared from a 1:5 w/v homogenate of bovine corpora lutea, buffered by 5 mM KPB, pH 6.8. An aliquot of the cytosol (10 ml) was incubated with 0.1 ml of radioactive steroids in ethanol for 30 min at 22°C and 3.75 h at 0-4°C. The incubated cytosol (9.5 ml) was applied to an HAP column (1.7 (i.d.) x 17 cm) and developed at 4°C with the following sequence of potassium phosphate buffers, pH 6.8: 81 ml x 5 mM; 60 ml x 150 mM; 60 ml x 300 mM; 60 ml x 500 mM; and 60 ml x 650 mM. Fractions of 3 ml were collected at a flow rate of 26.5-18 $ml.h^{-1}$ and pooled into 6 aliquots (I-VI).

The absorbances at 280 nm of the column fractions were measured, and the radioactivities of 0.5 ml aliquots were determined in 8 ml of Cocktail N scintillant on double label settings. The spillover of ^{14}C into the ^{3}H channel was not corrected.

The radioactive steroids applied to the column were:
^{3}H-Cortisol, 7.57×10^{5} cpm, 1.25 pmol (counting efficiency = 30%)
^{14}C-Progesterone, 5.11×10^{4} cpm, 216 pmol (counting efficiency = 49.5%)

● = Protein, A_{280} per ml
○ = ^{14}C-Progesterone, cpm per 0.5 ml
▲ = ^{3}H-Cortisol, cpm per 0.5 ml


5
150
300
500
650
mM
I
II
III
IV
V
VI
10
6
2
1.0
0.5
0
A_{280}
Protein,
60
40
20
0
Progesterone, cpm ($x10^{-1}$); cortisol, cpm ($x10^{-3}$)
0
20
40
60
80
100
Fraction, 3 ml.

Cytosol (equilibrated with ^{3}H-cortisol and ^{14}C-progesterone) was applied to another HAP column and developed with a 400 ml gradient of 5 mM to 550 mM KPB (data not shown). However, gradient elution under these conditions gave poorer resolution of both proteins and steroids compared with step elution.

MED of two sets of pooled fractions (numbers III and IV of the HAP column shown in Fig. 4-2) revealed that only the protein contained in pool IV, (eluted by 150 mM KPB) bound progesterone specifically with K_A of 0.97×10^8 M^{-1} and n(M) of 10.68 pmol.A_{280}^{-1} of cytosolic protein. The K_A was approximately 3-fold less than that obtained from MED of freshly-prepared cytosol. It was not known at this stage if this was a real difference or if the binding activity was partially destroyed during the course of the experiment.

4.3.2 Other Methods of Measuring Progesterone-Binding

The progesterone-binding activity was measureable by MED but this technique was very tedious and used large amounts of both steroid and protein. Therefore, attempts were made to develop alternative equilibrium and non-equilibrium methods of measuring the steroid-binding activity of cytosol. The objective was to develop a more rapid assay for the progesterone-binding activity.

4.3.2.1 Batch Sephadex Technique

The effect of removing endogenous steroid on the progesterone-binding activity of cytosol is shown in Table 4-3. Removal of ≥99% of the endogenous progesterone (as determined by RIA) increased the levels of total and "specific" binding. However, at this concentration of cytosol protein (approximately

	% Total bound	% Non-specific bound	% Specific bound
Untreated cytosol	42	29	13
	51.5	32	19.5
	48.5	24	24.5
	44	33	11
	51	36	15
	50	32	18
	48 ± 3.9(SD)	31.0 ± 4.1(SD)	16.8 ± 4.9(SD)
DCC-treated cytosol	70	39	31
	65	36	29
	65	43	22
	64	37	27
	62	43	19
	60	40	20
	64.3 ± 3.4(SD)	39.7 ± 2.9(SD)	24.7 ± 5.0(SD)

Table 4-3: Progesterone-binding of bovine corpus luteum cytosol, determined by the batch Sephadex technique, in the presence and absence of endogenous steroid.

The cytosolic fraction from a 1:10 w/v homogenate of bovine corpora lutea was thawed and used directly. Alternatively, 1 ml aliquots of it were incubated for 20 min on ice with the pellets from 1 ml of 1/10 DCC suspension, and centrifuged at 2000 x g_{max} for 5 min. The combined supernatants were taken for the assay. The assay tubes consisted of 0.8 ml of cytosol and 0.2 ml of 40 mM KPB, pH 7.4 in which ^{3}H-progesterone (6,240 cpm, ca. 0.1 pmol; total bound) or ^{3}H-progesterone plus radioinert progesterone (71.5 pmol; non-specific bound) had been equilibrated. The tubes were left at 4^{o}C for 90 min, after which 1 ml of Sephadex G-25 suspension (200 mg) was added. Four tubes did not contain Sephadex ("total count" tubes) and four tubes did not contain cytosol ("blank" tubes). After shaking for 1 h, the gel was allowed to settle for 1 h, and 0.2 ml aliquots of the supernatant were counted in 8 ml of scintillant. All steps were carried out at 0-4^{o}C.
Specific bound = Total bound - Non-specific bound.

10 mg.ml^{-1}) total binding of progesterone of about 60% gave specific binding (total minus non-specific binding) of only 25%. Also, a wide scatter of values was obtained for replicate determinations.

The first requirement of this method of assay (and most other methods) was to determine the protein concentration which simultaneously provided the greatest specific binding and the least non-specific binding of progesterone. Table 4-4 summarizes the result of such an experiment at 4^{o}C. Thus, total binding and non-specific binding of a constant amount of progesterone both declined as the cytosol was diluted. The difference between the upper and lower limits of binding ("specific" binding) did not form a discernible pattern with the variation in concentration of cytosolic protein.

There was close agreement (±5%) between replicates of the radioactivity (cpm) of the supernatants of the assay tubes. However, the manner in which the specifically-bound progesterone (giving rise to B/U ratios and masses of bound progesterone) were calculated in the batch Sephadex technique resulted in unacceptably large variations of multiple determinations of the same value (see Table 4-3). The original method was carried out at 25^{o}C (Pearlman and Crepy, 1967). Application of the method to the assay of progesterone-binding of corpus luteum cytosol was attempted at room temperature (22^{o}C) and, later, at 4^{o}C. Reproducibility between multiple determinations in the same assay were not improved at the lower temperature, when the binding components would be expected to be more stable because the rate of dissociation of bound progesterone would be lowered (Westphal, 1978). Furthermore, a dilution curve of the

Dilution	1:0	1:2	1:5	1:10	1:25	1:50
DCC-treated Cytosol						
% Total bound	57	58.4	17.3	0	0	0
	77	56.7	4.0	0	0	0
% Non-specific bound	70	53.3	7.7	0	0	0
	62.3	58.4	10.9	0	0	0
% "Specific" bound	-13	5.1	9.6	0	0	0
	14.7	- 1.7	- 6.9	0	0	0
Sephadex G-25-treated Cytosol						
% Total bound	47.5	31.7	19.6	11.8	9.7	11.9
	51.4	29.3	27.5	16.4	14.6	16.7
% Non-specific bound	48.3	28.8	17.0	22.9	19.7	16.8
	45.2	35.5	20.5	14.2	23.9	14.5
% "Specific" bound	- 0.8	2.9	2.6	-11.1	-10.0	4.9
	6.2	- 6.2	5.0	2.2	- 9.3	2.2

Table 4-4: Dilution curve of progesterone-binding activity of the cytosolic fraction of bovine corpus luteum determined by the batch Sephadex technique.

The cytosolic fraction was freshly prepared from a 1:5 w/v homogenate of bovine corpora lutea. Aliquots (1 ml) of the cytosol were treated with the pellets from 1 ml of 1/10 DCC suspension for 20 min on ice, centrifuged at 2000 g for 5 min, and passed through a Metrical filter. Another aliquot (10 ml) of cytosol was passed through a large column of Sephadex G-25 (4.4 (i.d.) x 15 cm) at 22°C in order to remove endogenous steroid. The batch Sephadex assay was carried out at 4°C as described under Methods and in the legend to Table 4-3. The "total binding" tubes contained 13,200 cpm of ^{3}H-progesterone (ca. 0.23 pmol) and the "non-specific binding" tubes contained 800 pmol of radioinert progesterone (ca. 3500 x excess).
Specific bound = Total bound - Non-specific bound.

progesterone-binding peak from the HAP column could not be obtained using this technique. R5020 is a synthetic progesterone which has been shown to dissociate less rapidly than progesterone from progesterone receptor proteins (Raynaud, 1977). However, its inclusion in the batch Sephadex technique for the assay of R5020-binding by the progesterone-binding components of corpus luteum cytosol was unsuccessful.

In summary, the inability to obtain suitable dilution curves with acceptable reproducibility among multiple determinations precluded the use of the batch Sephadex technique as a means of measuring the progesterone-binding activity of corpus luteum cytosol.

4.3.2.2 Fluorescent Progesterone-Binding Assay

Stroupe et al. (1975) developed an assay for progesterone-binding based on measurement of the quenching of protein fluorescence which accompanied the binding of progesterone to PBG (guinea pig) or AAG (human). The fluorescent method was applied to the measurement of the progesterone-binding activity of a preparation of binding protein (BPI), partially-purified from the cytosolic fraction of bovine corpus luteum (see section 4.3.5.1 for details of the purification of the binding protein).

Firstly, the difference spectrum of absorbance of progesterone plus BPI versus BPI was obtained. It revealed a broad peak of absorbance between 235 and 270 nm, with a maximum at 250 nm; absorbance above 270 nm was constant (data not shown). The fluorescence spectrum of BPI in the absence and presence of an excess of progesterone revealed that binding of the steroid to the protein caused a 23.5% drop in the relative

fluorescence. (Fig. 4-3A). The fluorescence quenching titration of the binding protein with progesterone is shown in Fig. 4-3B. The affinity constant and concentration of binding sites were determined from the curve as $2.5 \times 10^{8}\ M^{-1}$ and 11.2×10^{-6}M, respectively, which was within the range of values determined by MED. The calculation assumed that there was only one steroid binding site per protein molecule since the concentration of the binding protein was calculated from the concentration of progesterone at the equivalence point (Stroupe et al. 1975).

Ethanol reduced the fluorescence of the binding protein by ⩽1% up to a concentration of 4% v/v, and even at a concentration of 9% v/v the decrease in fluorescence was only 4%. The titration experiments did not exceed an ethanol concentration of 4.8% v/v.

However, the use of fluorescence as a routine means of measuring progesterone-binding activity was precluded because of three factors:

(i) The magnitude of K_A determined by fluorescence was very susceptible to small differences in the slope of the line drawn through the data points obtained at low, sub-saturating concentrations of progesterone. Multiple determinations of the K_A of the same solution of BPI for progesterone varied from $1.1\text{-}3.8 \times 10^{8}\ M^{-1}$ (n=5); Furthermore, fluorescence quenching may provide lower values of K_A and n(M) than MED (Stroupe et al., 1975).

(ii) The method was of unproven efficacy for cytosols, due to the possible presence of substances which could enhance or reduce the fluorescence changes in a non-systematic manner.

Fig. 4-3A: Fluorescence excitation and emission spectra of partially-purified binding protein I in the presence and absence of progesterone

The binding protein was purified from ovine UOV plasma as described in section 4.3.5.1. The solution of binding protein I was diluted to an A_{280} of 0.6, incubated with 0.2 volume of DCC-suspension for 20 min on ice, and centrifuged at 2000 g for 10 min. The supernatant was diluted to an A_{280} of 0.03 with 50 mM KPB, pH 7.4. The fluorescence of 2.5 ml of the binding protein solution was determined in the presence of 50 µl of ethanol with and without added progesterone (50 µmol). Emission of the excitation spectra was measured at 340 nm; excitation of the emission spectra was measured at 280 nm.

● = Binding protein

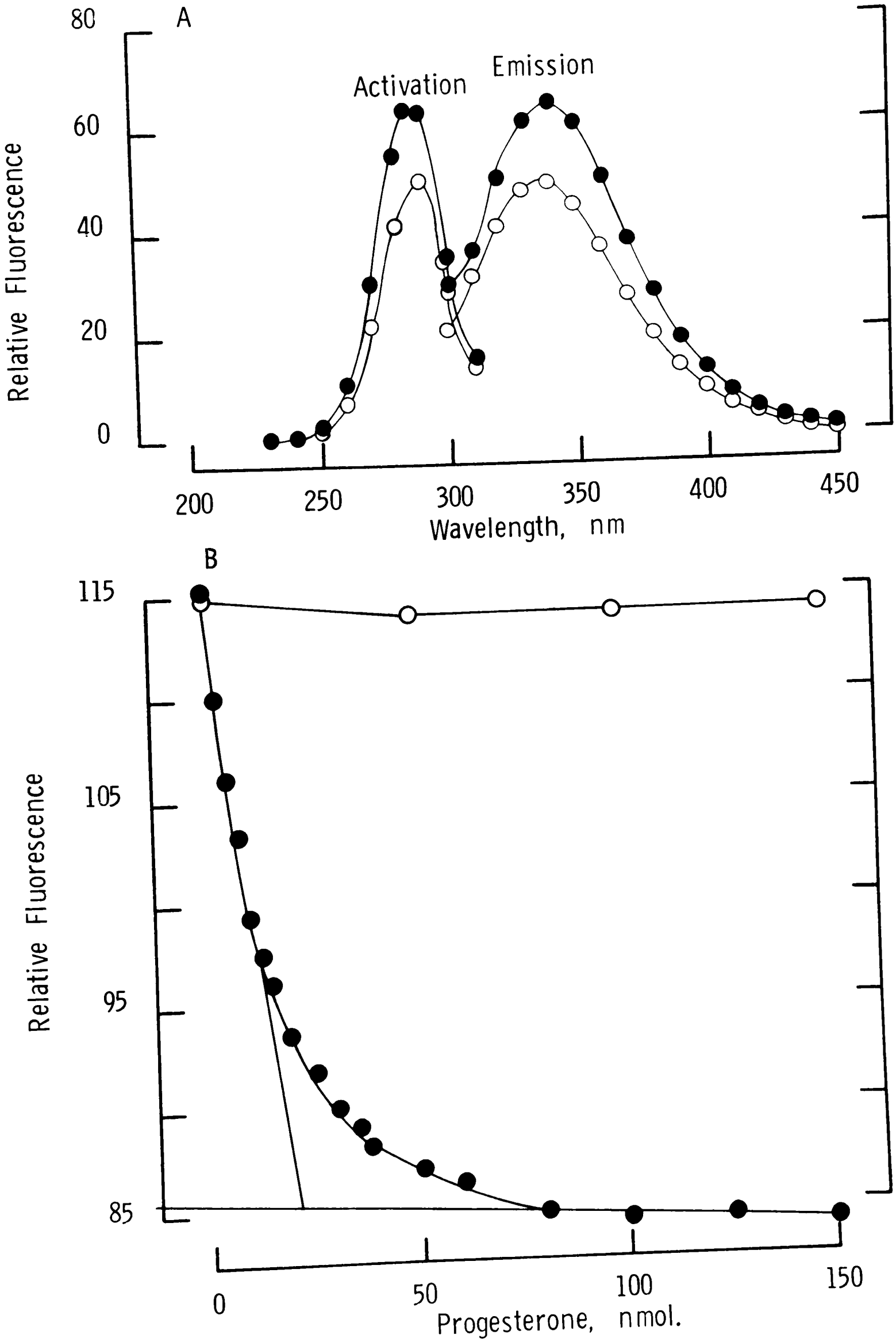
○ = Binding protein + progesterone

Fig. 4-3B: Fluorescence quenching titration of binding protein I with progesterone

The solution of partially-purified binding protein I was diluted to an A_{280} of 0.02 and 2.5 ml aliquots were titrated with 0 to 0.15 ml of 1 mM progesterone dissolved in ethanol. The changes in relative fluorescence were corrected for increasing dilution of the protein solution. At the equivalence point, 21 nmol of progesterone had been added to 2.5 ml of binding protein I solution.

● = Titration curve of BPI with progesterone

○ = Effect of ethanol on fluorescence of BPI


A
80
60
40
20
0
Relative Fluorescence
Activation
Emission
200
250
300
350
400
450
Wavelength, nm
B
115
105
95
85
Relative Fluorescence
0
50
100
150
Progesterone, nmol.

(iii) Fluorescence-quenching required that the steroid undergoing binding should contain the Δ^4-3-keto structure. Although progesterone and R5020 contain this structure the relative binding affinity of the cytosolic fraction for steroids lacking this grouping was also required. The ability of steroids lacking the Δ^4-3-keto structure to prevent the quenching of protein fluorescence by progesterone (or R5020) was not measureable with sufficient precision. This applied particularly to the determination of the relative binding affinities of those progestogenic steroids with structures similar to that of progesterone.

4.3.2.3 Non-Equilibrium Methods

The applicability of non-equilibrium methods of separating protein-bound progesterone from unbound progesterone (such as adsorption to charcoal, molecular sieving through filters of defined porosity, and gel filtration) were evaluated.

1. Charcoal. The assays were carried out as described under Methods. The cytosolic fraction of bovine corpora lutea was used with and without prior removal of endogenous steroid by concentrated DCC suspension, and the assays were set up using either tritiated and radioinert progesterone or tritiated and radioinert R5020, and variously diluted (1/10, 1/20, 1/50 and 1/100) suspensions of DCC.

In all cases the maximum levels of total binding (23-27%) and "specific" binding (3.5%) were obtained for undiluted cytosol. With decreasing protein concentration "specific" binding decreased to zero at a cytosolic dilution of 1:7.5. Thus, the use of DCC to separate protein-bound progesterone or R5020 from unbound steroid resulted in the complete loss of the

high-affinity binding.

2. Filters. The evaluation of various filters for separating protein-bound from soluble progesterone (summarized in Table 4-5) indicated that none of the filters tested were suitable for the assay of progesterone-binding activity of corpus luteum cytosol.

3. Minicolumns. The partitioning of soluble progesterone or R5020 from protein-bound progesterone or R5020 was investigated using small columns of Sephadex G-25, Sephadex LH-20, Biogel P-10, and amorphous and spheroidal HAP.

The greatest effort was expended on the evaluation of minicolumns of Sephadex G-25 (fine and coarse). The following is a chronological account of the attempt to develop an assay for the progesterone-binding activity of corpus luteum cytosol using such minicolumns.

(i) The elution of blue dextran and aqueous ^{3}H-progesterone through minicolumns of Sephadex G-25 (fine) and LH-20 indicated that the void volumes of columns (packed to a height of 8 cm) were 1.3 ml and 1.5 ml for sample volumes of 0.5 ml and 1.0 ml, respectively; ^{3}H-progesterone was first recovered at 3.6 ml and 3.6 ml, respectively. Thus, it was concluded that for minicolumns of Sephadex G-25 and sample volumes of 0.5 ml and 1.0 ml, the collection of the first 2.0 ml and 2.5 ml, respectively, of effluent would provide complete recoveries of protein-bound steroid free from soluble steroid.

(ii) Determination of the progesterone-binding activity of human plasma from the second trimester of pregnancy (HPP) was undertaken to provide a test of the methodology. The dilution curve and binding assay were performed on DCC-treated plasma.

Filter	Comments
HAWP (Millipore)	Retained soluble progesterone but rate of flow of cytosol or human plasma was very slow, causing breakdown of the binding interaction between protein and progesterone.
GF/C (Whatman) GF/F (Whatman)	Did not retain either soluble progesterone or cytosolic proteins.
PSED (Millipore) PTGC (Millipore)	Required positive pressures >10 atmospheres in order to obtain even slow rates of flow. Impractical (and expensive) for the simultaneous determination of multiple samples.
DE81 (Whatman)	Did not retain either soluble progesterone or cytosolic proteins.

Table 4-5: Evaluation of various filters for the separation of soluble progesterone from protein-bound progesterone in the cytosolic fraction of bovine corpus luteum.

The reaction volume was 1.0 ml and consisted of 0.8 ml of diluted HPP and 0.2 ml of 40 mM KPB, pH 7.4 in which a range of concentrations of radioactive and radioinert progesterone were equilibrated (see section on charcoal assay under Methods). After incubation for 4 h at 4^{o}C, the reaction mixtures (1 ml) were applied to minicolumns of Sephadex G-25 and the first 2.5 ml of effluent were collected and counted for radioactivity (Table 4-6).

(iii) Dilution curves of cytosolic fractions of bovine corpora lutea were obtained by the same methods as above (Fig. 4-4A). Several binding assays were carried out using both frozen-thawed and freshly-prepared cytosols at a dilution of 1:4.5 (approximately 1 mg.ml^{-1} of protein) over a range of progesterone concentration of 5-2500 pmol.ml^{-1}. As Fig. 4-4B shows, the variability among multiple determinations of the cytosolic progesterone-binding activity was unacceptably high using minicolumns compared with MED over similar concentration ranges of progesterone and protein.

(iv) A test of the reproducibility of the minicolumn assay technique was undertaken at two concentrations of progesterone:

0.2 pmol progesterone : % bound = 23.60 ± 5.43 SD, (n=6)

100.2 pmol progesterone : % bound = 20.90 ± 2.11 SD, (n=6)

At this stage it was also determined that treatment of 1 part cytosol with 1 part DCC gave 7% greater binding of progesterone compared with treatment of 1 part cytosol with 0.25 part DCC.

(v) The elution characteristics of cytosol and human plasma in the presence of excess progesterone through minicolumns of Sephadex G-25 (fine and coarse) were investigated. Whilst progesterone bound to HPP was completely eluted in a total

Dilution	=	1:100
K_A	=	4.12×10^{8} M^{-1}
K_D	=	2.39×10^{-9} M
n(M)	=	9.16×10^{-9} $mol.g^{-1}$
n(M)	=	7.50×10^{-10} mol. ml^{-1} of undiluted plasma

Table 4-6: Determination of the progesterone-binding activity of human plasma of pregnancy by Sephadex G-25 mini-column assay.

Endogenous progesterone remaining after DCC-treatment was measured by RIA and included in the calculations. The cpm data has been converted to Scatchard form for determination of binding parameters.

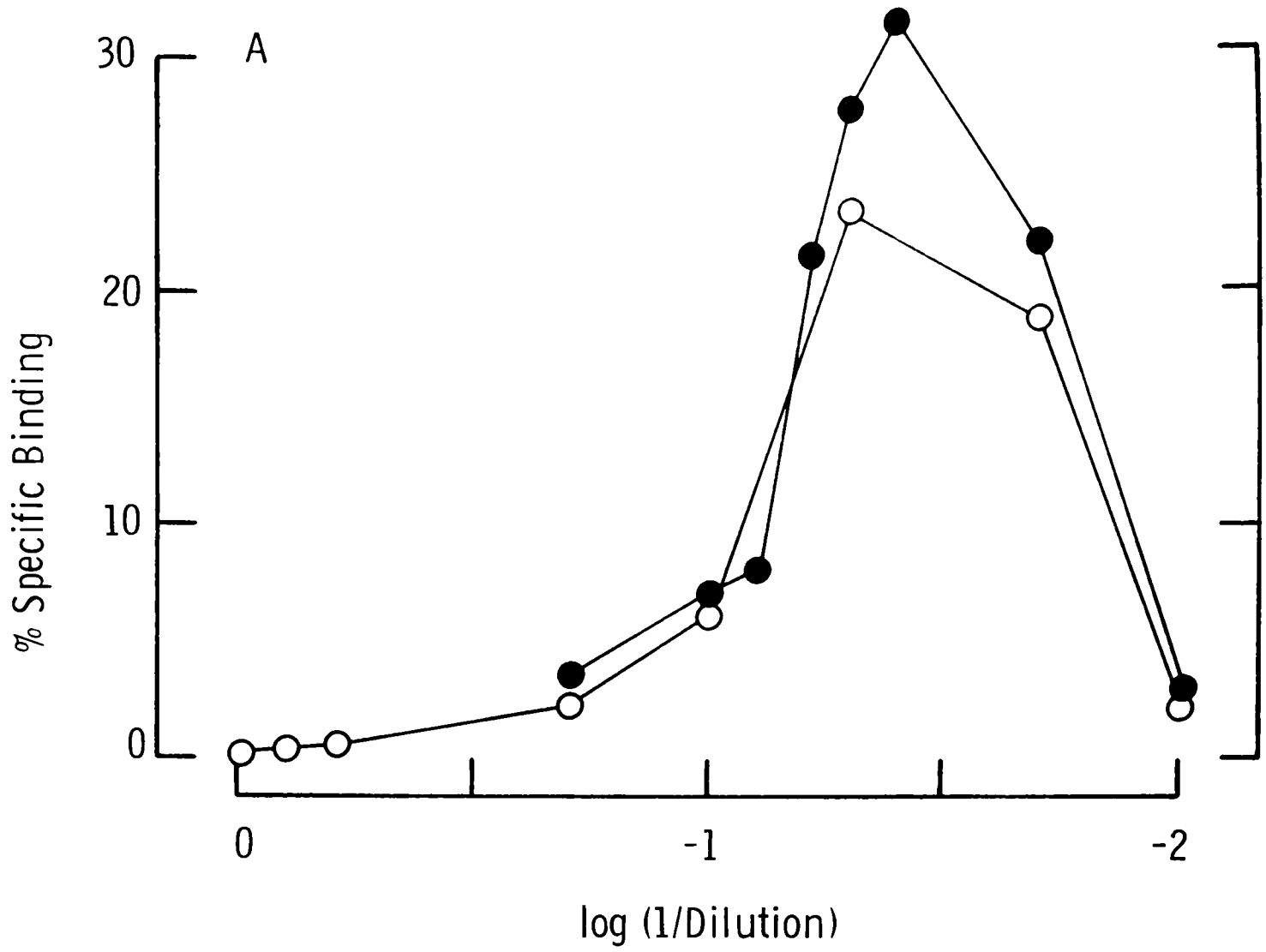
Fig. 4-4A: Dilution curves of the cytosolic fraction of bovine corpora lutea obtained using minicolumns of Sephadex G-25

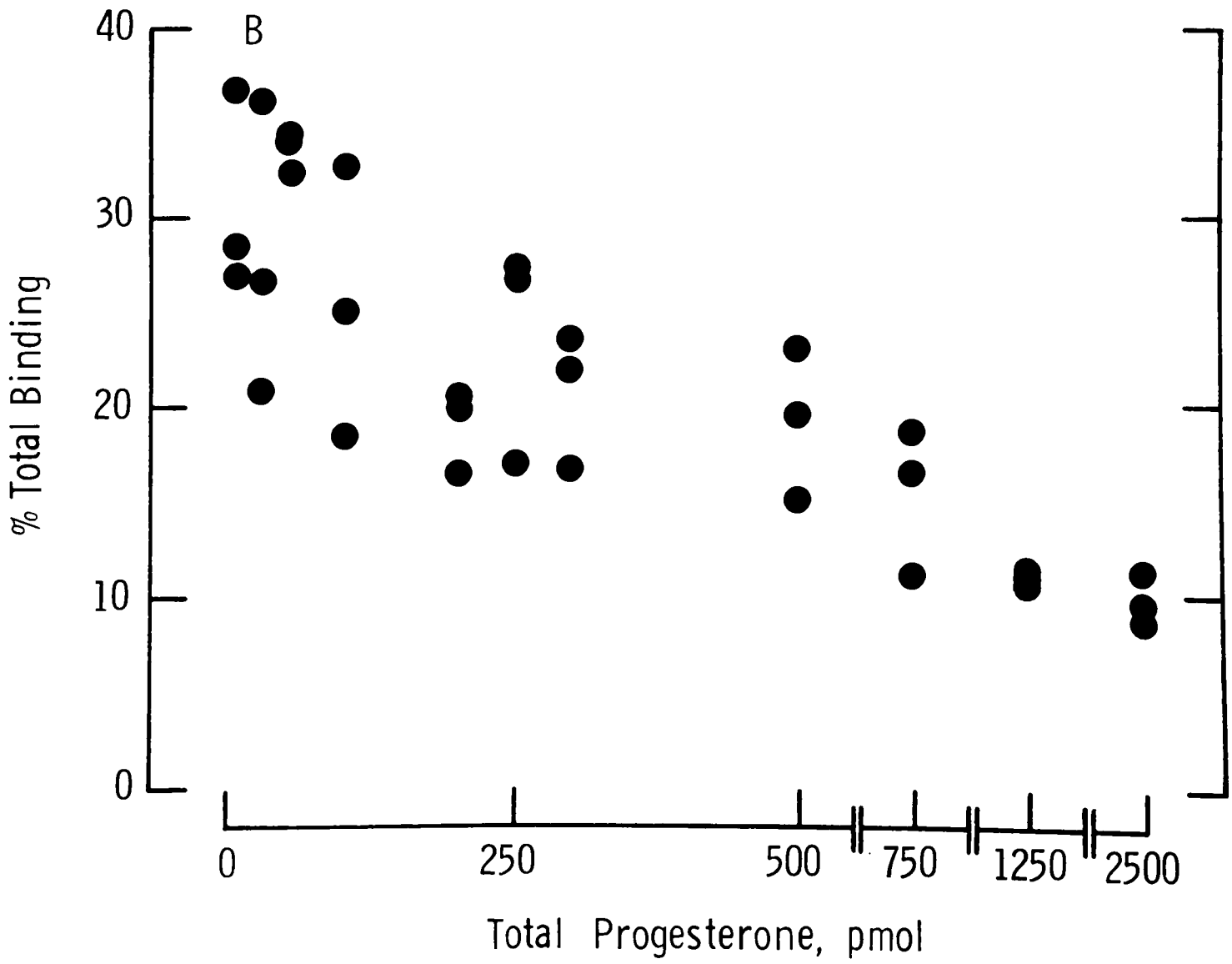
Freshly-prepared and frozen-thawed cytosol were prepared from different 1:5 w/v homogenates of bovine corpora lutea. The assay was carried out as described in the text.
% Specific binding = Total binding - Non-specific binding (determined at 2500 pmol).

● = Freshly-prepared cytosol
○ = Frozen-thawed cytosol

Fig. 4-4B: Progesterone-binding assay of the cytosolic fraction of bovine corpora lutea using minicolumns of Sephadex G-25 to separate protein-bound from unbound steroid

The cytosolic fraction was freshly-prepared from a 1:5 w/v homogenate of bovine corpora lutea and the assay was performed in triplicate as described in the text.


A
30
20
10
0
% Specific Binding
0
-1
-2
log (1/Dilution)


B
40
30
20
10
0
% Total Binding
0
250
500
750
1250
2500
Total Progesterone, pmol

volume of 2.5 ml (fraction 25; Fig. 4-5A), progesterone bound to cytosolic protein was not (Fig. 4-5B). In the latter case, there were two, interconnected peaks of A_{280}-absorbing material; the material in the second peak was retarded upon passage through Sephadex G-25[3]. The fact that the peak of bound progesterone did not coincide with the peak of high-molecular weight protein so that it "tailed" at its trailing edge suggested that the binding interaction between cytosolic protein(s) and progesterone was perturbed upon passage of the complex through Sephadex G-25. This conclusion was supported by the finding that, in the absence of protein, ^{3}H-progesterone eluted between fractions 38 and 55 (i.e., 3.8 ml and 5.5 ml).

Although the overall protein and progesterone profiles were similar at low (0.2 pmol) and high (100.2 pmol) concentrations of steroid, the height of the trailing edge of ^{3}H-progesterone varied in an apparently random manner. This explained the variability between replicates in the earlier determination of progesterone-binding by the cytosolic fraction using this method. The "tailing" of bound progesterone upon passage through Sephadex G-25 was not due to the presence of the second high-molecular weight peak of cytosolic protein (Fig. 4-6A), since removal of the latter did not alter the radioactivity profile. Also it was not due to inadequate times of incubation of cytosol with progesterone, since the characteristic progesterone profile of the high-molecular weight cytosolic proteins was established by 30 min and was stable for 24 h.

3: The 2 peaks of protein obtained upon passage of cytosol through Sephadex G-25 will be designated "high-molecular weight" and "low-molecular weight" respectively, even though it is recognized that their separation may be due partly to differences other than molecular size.

Fig. 4-5A: Elution profile through a minicolumn of Sephadex G-25 of progesterone bound to human plasma of pregnancy

Plasma from a woman 22 weeks pregnant was treated with DCC and 0.8 ml was mixed with 0.2 ml of 40 mM KPB, pH 7.4 containing ^{3}H-progesterone (ca.0.2 pmol) and incubated for 2 h at 4^{o}C. The solution was applied quantitatively to a minicolumn of Sephadex G-25 (fine). The column was packed to a height of 8 cm. One ml of 40 mM KPB, pH 7.4 containing ^{3}H-progesterone (ca.0.2 pmol) was applied to a duplicate column. In both cases, 2-drop fractions were collected and their radioactivity was determined in 8 ml of Cocktail N scintillant.

● = Progesterone equilibrated with human plasma of pregnancy, cpm
○ = Progesterone in buffer, cpm

Fig. 4-5B: Elution profile through a minicolumn of Sephadex G-25 of progesterone equilibrated with the cytosolic proteins of bovine corpus luteum

Cytosol (0.8 ml) was incubated with 0.2 ml of 40 mM KPB, pH 7.4 containing ^{3}H-progesterone (ca.0.2 pmol) and incubated for 2.5 h at 4^{o}C. The solution was applied quantitatively to a minicolumn of Sephadex G-25 (fine), and 2-drop fractions were collected. The fractions were diluted with 1 ml of KPB, the absorbance at 280 nm was determined and the radioactivity of 0.75 ml of each fraction was measured in 8 ml of Cocktail N scintillant.

● = Progesterone, cpm
○ = Protein, A_{280}

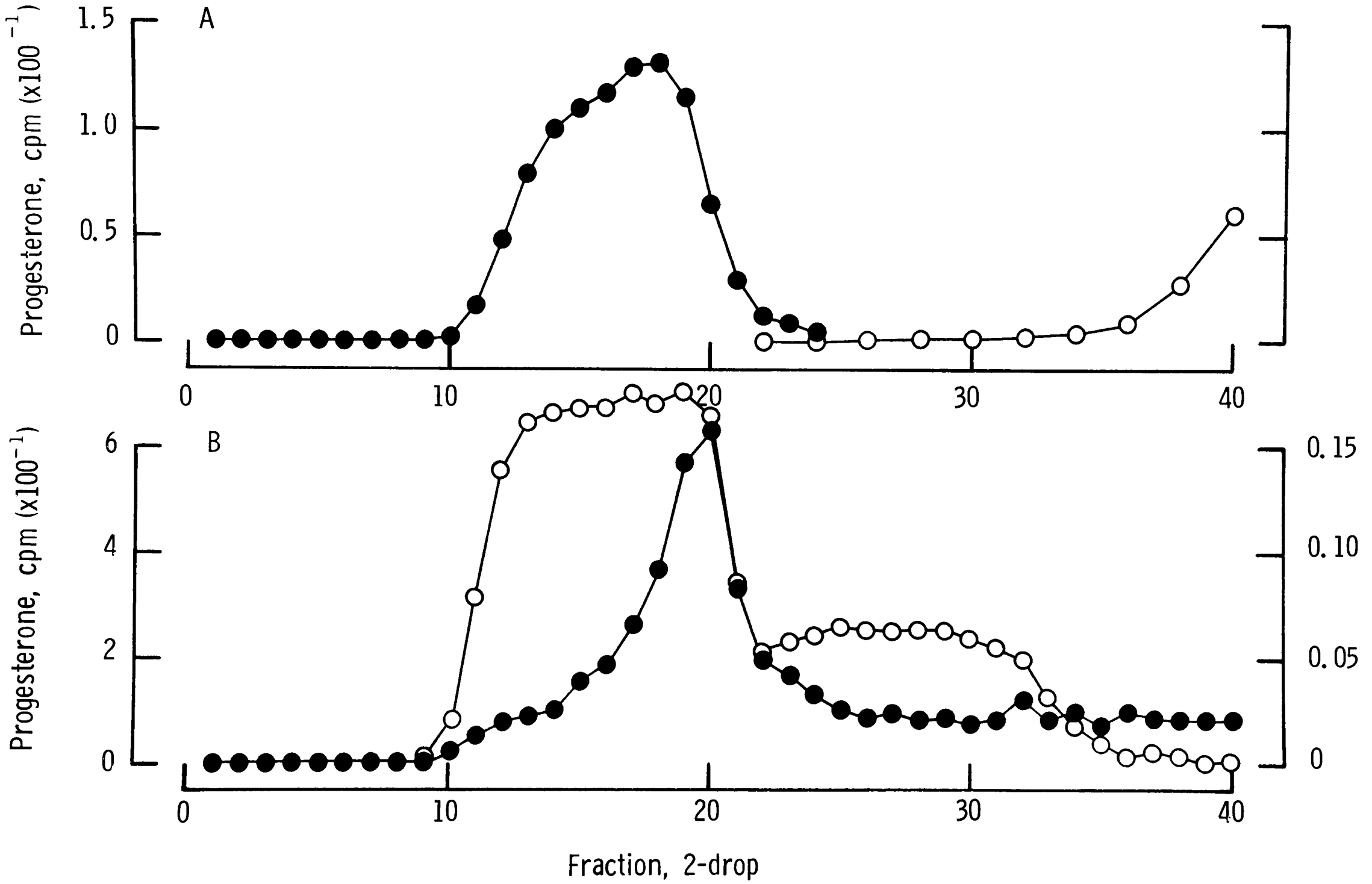

A
B
Progesterone, cpm (x100^{-1})
Protein, A_{280}
1.5
1.0
0.5
0
6
4
2
0.15
0.10
0.05
0
0
10
20
30
40
Fraction, 2-drop

Fig. 4-6A: Application of the high-molecular weight cytosolic proteins of bovine corpus luteum to a minicolumn of Sephadex G-25

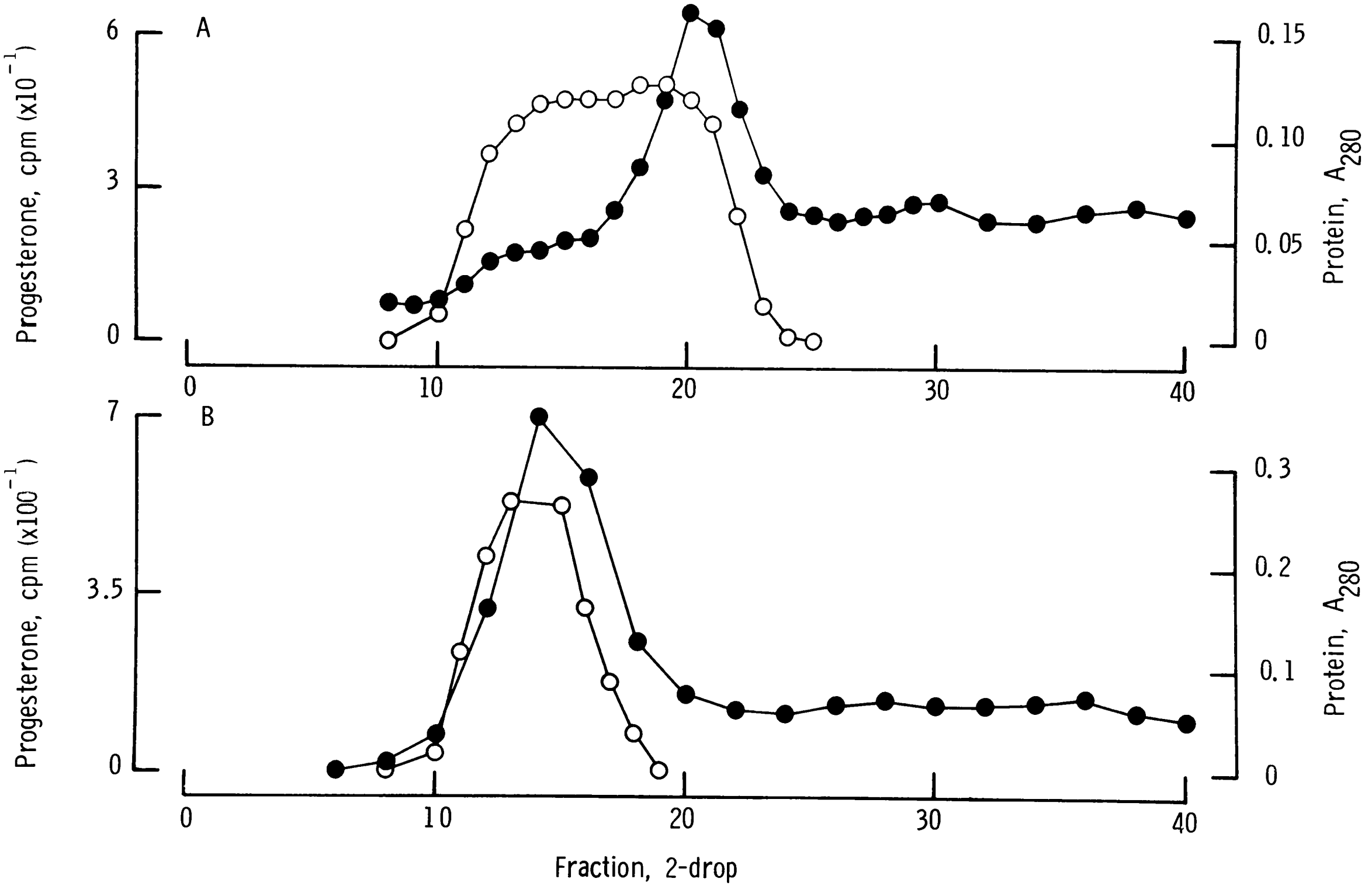
The endogenous steroid contained in thawed corpus luteum cytosol was removed with DCC and a 3 ml aliquot was incubated with ^{3}H-progesterone (ca.6 pmol) for 2 h on ice. It was applied to a Sephadex G-25 column (2 (i.d.) x 26 cm) and developed with 40 mM KPB, pH 7.4. Fractions of 3 ml were collected and their absorbance at 280 nm and radioactivity determined. The fractions containing the excluded material were pooled and 1 ml aliquot was applied to a minicolumn of Sephadex G-25 as described in Fig. 4-5B.

● = Progesterone, cpm
○ = Protein, A_{280}

Fig. 4-6B: Application of partially-purified binding protein I incubated with ^{3}H-progesterone to a minicolumn of Sephadex G-25

BPI was partially-purified from ovine UOV plasma as described in section 4.3.5.1. An aliquot (A_{280} = 1.2) was thawed, concentrated 4-fold with an Amicon pressure cell (Model 202) and incubated with ^{3}H-progesterone (ca.0.2 pmol) for 2.5 h on ice. An aliquot (0.5 ml) of the incubated mixture was applied to a minicolumn of Sephadex G-25 as described in Fig. 4-5B.

● = Progesterone, cpm
○ = Protein, A_{280}


A
6
3
0
0
10
20
30
40
0.15
0.10
0.05
0
Progesterone, cpm (x10^{-1})
Protein, A_{280}
B
7
3.5
0
0
10
20
30
40
0.3
0.2
0.1
0
Progesterone, cpm (x100^{-1})
Protein, A_{280}
Fraction, 2-drop

(vi) Application of partially-purified BPI to Sephadex G-25 minicolumns after incubation with ^{3}H-progesterone yielded a steroid profile analogous to that obtained with corpus luteum cytosol. BPI was eluted in a smaller volume than the cytosolic proteins (Fig. 4-6B).

(vii) The use of R5020 in place of progesterone reduced the magnitude of "tailing", but did not abolish it (Fig. 4-7A).

(viii) Minicolumns were prepared with known amounts of Sephadex G-25 (fine and coarse) in place of being packed to a constant height (8 cm). The elution characteristics of cytosol (0.5 ml) incubated with ^{3}H-R5020 were determined for minicolumns containing either 0.5 g or 0.7 g of coarse or fine Sephadex G-25. Combinations of 0.7 g of Biogel in perspex minicolumns and of 0.5 g and 0.7 g of both grades of Sephadex G-25 in 20 cc glass syringes did not reduce the "tailing" of ^{3}H-R5020. From these calibration studies, it was decided to apply 0.5 ml of sample to columns containing 0.7 g of coarse Sephadex G-25, wash with 0.5 ml of KPB and collect the next 2.25 ml in a plastic vial for counting in 10 ml of two-phase scintillant (4 g PPO + 0.1 g POPOP per litre of toluene).

Fifty minicolumns were standardized with aliquots of the same solution of cytosol. The cytosol had been DCC-treated, diluted 4.5-fold and equilibrated with ^{3}H-R5020. Only those columns which gave amounts of total binding to within ±5% of the mean value for all the columns were retained. The standardization procedure was repeated to provide a total of 47 calibrated minicolumns of coarse Sephadex G-25.

The optimal dilution of cytosol derived from a 1:5 w/v homogenate of bovine corpora lutea was re-determined using

Fig. 4-7: Application of the cytosolic fraction of bovine corpus luteum incubated with R5020 to minicolumns of Sephadex G-25

Cytosol (2 ml) was thawed and incubated with ^{3}H-R5020 (ca.0.2 pmol) for 2.5 h at 4°C. It was applied to 8 cm minicolumns of Sephadex G-25. Fractions of 0.5 ml were collected and analyzed as described in Fig. 4-5B.

A) R5020 equilibrated with cytosolic proteins, cpm

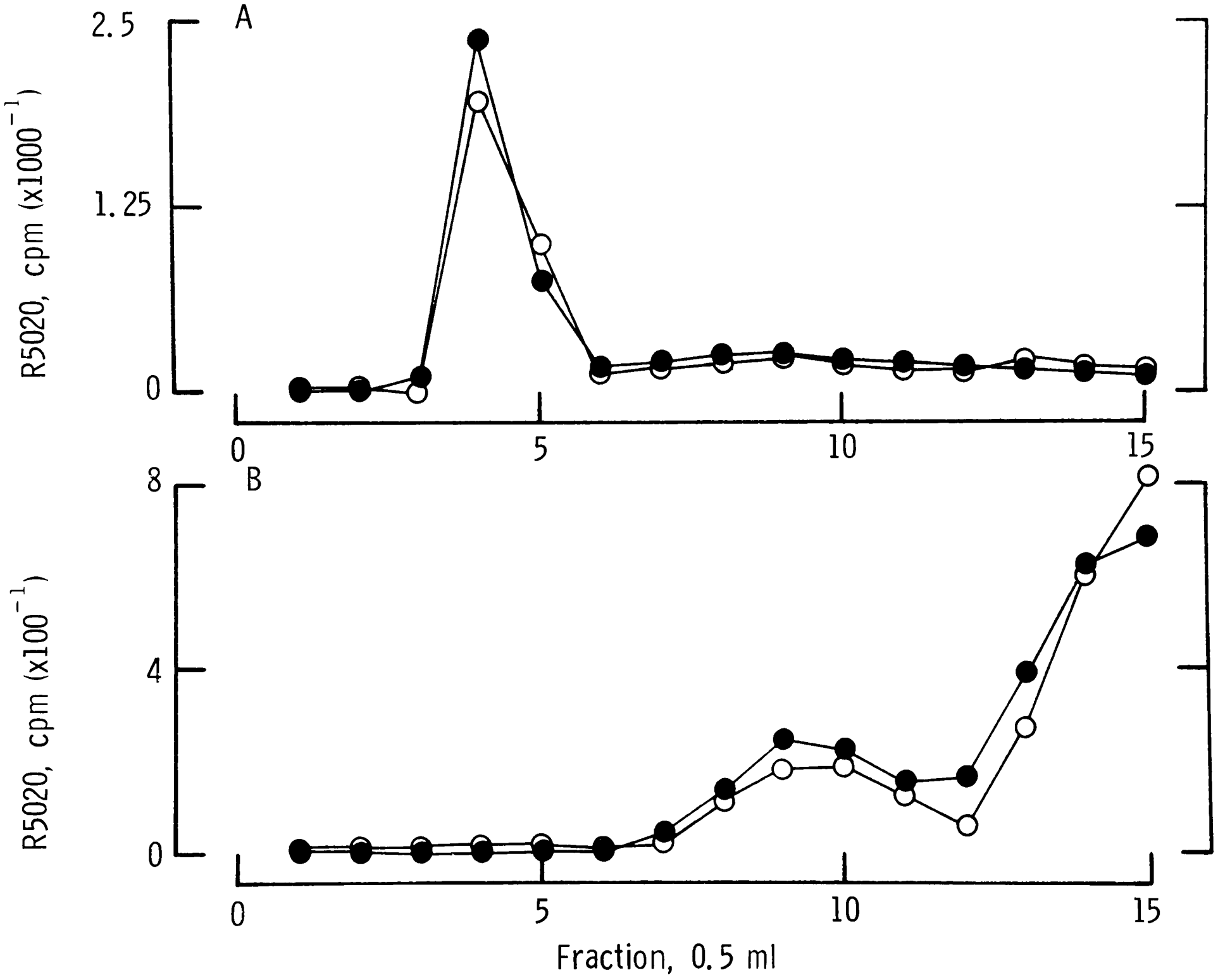
● = Column 1

○ = Column 2

B) R5020 in buffer, cpm

● = Column 3

○ = Column 4


A
2.5
1.25
0
R5020, cpm (x1000^{-1})
0
5
10
15
B
8
4
0
R5020, cpm (x100^{-1})
0
5
10
15
Fraction, 0.5 ml

these columns to be 1:5, equivalent to a protein concentration of approximately 1 $mg.ml^{-1}$. Binding assays at this dilution of cytosol were set up over a concentration range of R5020 (and progesterone) of 0-2500 $pmol.ml^{-1}$. In all cases the variation between multiple determinations of the total binding was reduced to 5-10%. However, the increase in the level of specific binding in going from the low to the high mass points was only 13-14% in the case of R5020 and 5-7% in the case of progesterone (Table 4-7). These differences were too small to form the basis of a reliable binding assay for either steroid by the cytosolic proteins.

The separation of cytosol-bound progesterone (or R5020) from unbound steroid by minicolumns consisting of media other than Sephadex were also tested. Samples flowed through Biogel P-10 less readily than through Sephadex without achieving a compensating improvement in the levels of protein-bound steroid. Minicolumns of amorphous HAP (consisting of a mixture of HAP and Sephadex G-25 in the ratio 1:2) retained BPI and a peak of protein at 5 mM KPB, pH 6.8, but these proteins did not retain as much steroid as they did in passing through Sephadex G-25. This may have been due, at least in part, to the fact that it took approximately 4 h to elute a minicolumn of amorphous HAP. Spheroidal HAP eluted as rapidly as coarse Sephadex G-25 (5-10 min) but did not retain the specific progesterone-binding proteins. (This was subsequently confirmed by applying cytosol to a large column of spheroidal HAP and developing it with a gradient of increasing ionic strength).

In summary, the use of minicolumns of Sephadex and other chromatographic media to separate protein-bound progesterone

Mass of R5020, pmol	% Total bound	% Bound - % $Bound_o$	% Specific bound
2500[a]	24.6	13.2	0
100	30.7	19.3	6.1
50	32.3	20.9	7.7
20.1	34.2	22.8	9.6
10.1	36.6	25.2	12.0
5.1	36.0	24.6	11.4
2.6	35.0	23.6	10.4
1.1	35.3	23.9	10.7
0.6	34.8	23.4	10.2
0.4	39.2	27.8	14.6
0.1	37.9	26.5	13.3
No P[b]	11.4		

Table 4-7: Determination of the R5020-binding activity of the cytosolic fraction of bovine corpus luteum using minicolumns of Sephadex G-25.

The cytosolic fraction of bovine corpus luteum was thawed, treated with DCC, and diluted 5-fold to a protein concentration of approximately 1 $mg.ml^{-1}$. Aliquots (0.4 ml) of diluted cytosol were added to 0.1 ml of 40 mM KPB, pH 7.4 containing radioactive R5020 (0.1 pmol) or radioactive plus radioinert R5020 (0.3-2500 pmol), and incubated at 4°C for 1.5 h. The reaction mixtures were quantitatively transferred to standardized minicolumns of 0.7 g of coarse Sephadex G-25. The columns were washed with 0.5 ml KPB, and the next 2.25 ml of effluent was collected and counted in 10 ml of two-phase scintillant. The reaction was carried out in triplicate.

a = Non-specific binding point (NSB)

b = Sample contained no protein; these minicolumns determined the amount of spillover of soluble R5020 into the region of the columns where protein-bound R5020 eluted = $Bound_o$.

% Specific bound = % Bound - % $Bound_o$ - % NSB.

(or R5020) from unbound steroid for the assay of binding activity of cytosol (and BPI) was unsuccessful. In all cases extensive dissociation of protein-bound steroid occurred as the cytosol or BPI samples passed through the columns.

4.3.3 The Distribution of Progesterone-Binding Proteins

4.3.3.1 The Bovine Corpus Luteum

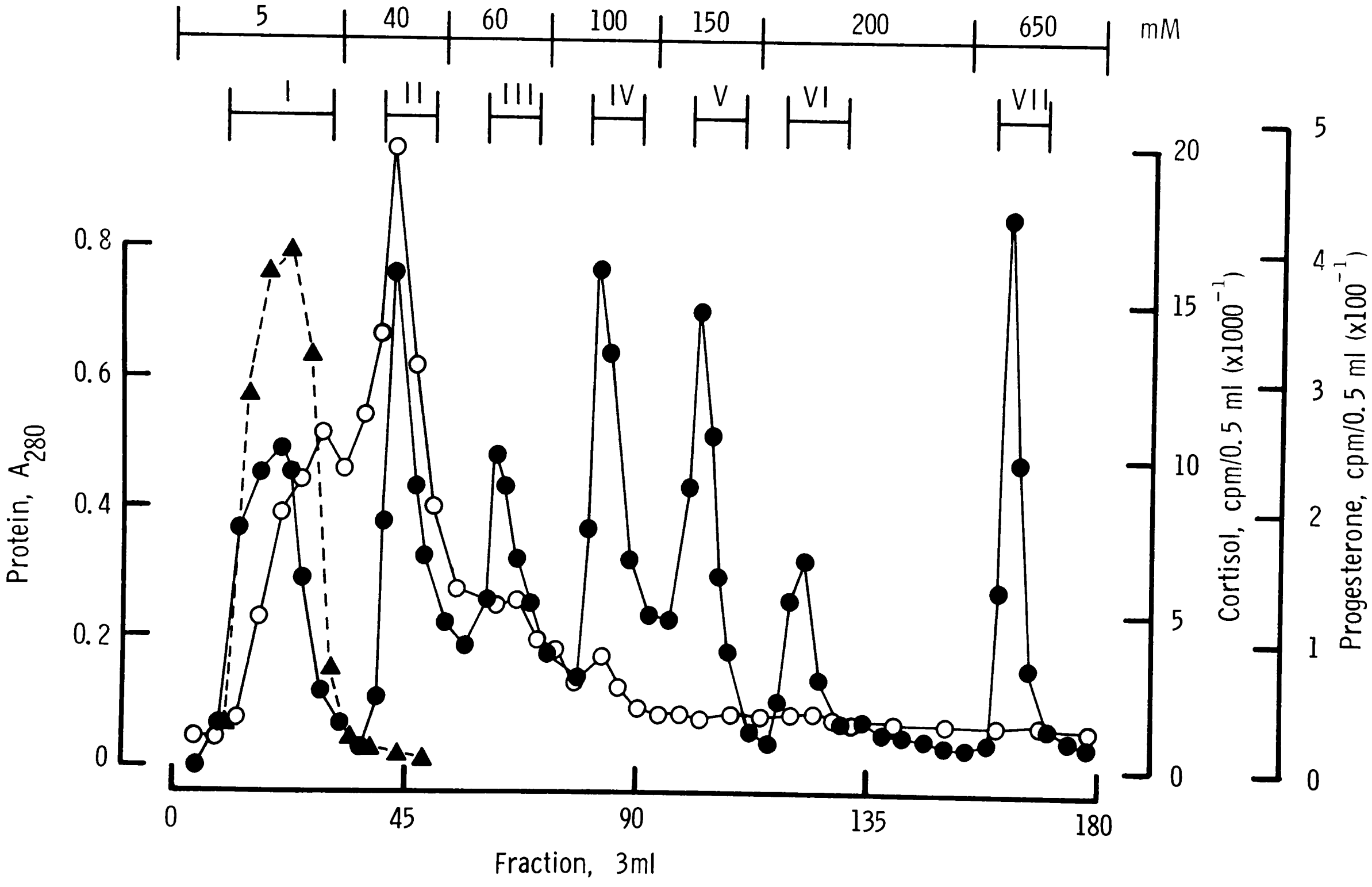
The fractionation of cytosol on HAP was repeated using more buffer changes than in the preliminary study (4.3.1.3), according to Leymarie and Gueriguian (1970) and Leymarie (personal communication). Added progesterone was specifically associated with the protein which eluted at 40 mM KPB (40 mM BP or BPI; Fig. 4-8). This was the material that was recovered at 150 mM KPB in the preliminary experiments (see Fig. 4-2). Added progesterone was not specifically associated with the protein eluted at ionic strengths above 40 mM, except for small amounts which were recovered with the protein eluted at 60 mM and 100 mM KPB. Fractions 15-18 of the column contained ^{14}C-progesterone (Fig. 4-8). This was probably soluble steroid because it was these fractions which contained the bulk of progesterone when the latter was applied without cytosol to a duplicate column (see 4.3.1.3). As in the preliminary HAP fractionation of cytosol, added cortisol was recovered entirely with the peak of unadsorbed material which eluted at 5 mM KPB. Most of the protein was eluted from the column at ⩽650 mM KPB; 1.25 M KPB eluted negligible additional protein.

Subsequent experiments showed that the profile of HAP fractionation of cytosol was unchanged when it was dialyzed against base buffer prior to its application to the column. However, dialysis reduced the size of the unadsorbed peak of

Fig. 4-8: Hydroxylapatite column chromatography of the cytosolic fraction of bovine corpus luteum

Cytosol was prepared from a 1:10 w/v homogenate of mid-cycle bovine corpora lutea. The buffer was 5 mM KPB, pH 6.8. The cytosol (55 ml) was dialyzed for 18 h against 3 x 2 L of 5 mM KPB, pH 6.8, containing progesterone (1 $mg.L^{-1}$) and 0.1% w/v sodium azide. The volume of dialyzed cytosol was 96.5 ml. An aliquot (40 ml) was incubated with ^{14}C-progesterone (1.33×10^5 cpm; ca.3.8 nmol) and ^{3}H-cortisol (1.76×10^5 cpm; ca.3.2 pmol) for 30 min at 22°C and 60 min at 0-4°C, and 39 ml (A_{280} = 4.78) was applied to a column of HAP (2.1 (i.d.) x 7.9 cm). The column was developed with the following sequence of potassium phosphate buffers, pH 6.8: 42 ml x 5 mM; 60 ml x 40 mM; 60 ml x 60 mM; 60 ml x 100 mM; 60 ml x 150 mM; 128 ml x 200 mM; 84 ml x 650 mM; and 60 ml x 1.25 M. Fractions of 3 ml were collected at a flow rate of 26 - 12 $ml.h^{-1}$. The fractions were analyzed as described in the legend to Fig. 4-2.

● = Protein, $A_{280}.ml^{-1}$
○ = Progesterone, $cpm.0.5\ ml^{-1}$
▲ = Cortisol, $cpm.0.5\ ml^{-1}$


5
40
60
100
150
200
650
mM
I
II
III
IV
V
VI
VII
0.8
0.6
0.4
0.2
0
Protein, A_{280}
20
15
10
5
0
Cortisol, cpm/0.5 ml (x1000^{-1})
5
4
3
2
1
0
Progesterone, cpm/0.5 ml (x100^{-1})
0
45
90
135
180
Fraction, 3ml

A_{280}-absorbing material. Therefore, cytosols and plasmas were not routinely dialyzed before application to columns of HAP.

The fractionation profile on HAP of cytosol derived from corpora lutea of pregnant cows (6-12 weeks) was identical to the fractionation profile of cytosol derived from corpora lutea of non-pregnant animals. Gradient elution of cytosol on columns of either amorphous HAP mixed with Sephadex G-25 (1:2) or of spheroidal HAP gave poorer resolution of the 40 mM BP from other proteins than step elution. Thus, the 7-step elution procedure or an abbreviated form of it, consisting of just 5 mM and 40 mM buffer steps, was used in the HAP fractionation of plasma, lymph and cytosol from other sources.

4.3.3.2 The Ovine Mid-Cycle Corpus Luteum

The protein profile obtained by HAP fractionation of the cytosolic fraction of ovine corpus luteum was similar to that of bovine corpus luteum, except that a smaller peak was eluted at 150 mM KPB (Fig. 4-9). The proteins of the sheep corpus luteum which were eluted at 40 mM KPB, like those of the cow, bound added progesterone. Only small amounts of added progesterone were recovered in the unadsorbed peak of protein. Less dissociation of protein-bound progesterone occurred in this experiment, compared to the bovine experiment, because it was run on a smaller scale and was completed in a shorter time.

4.3.3.3 Ovine Utero-Ovarian Venous Plasma

The fractionation profile of ovine UOV plasma is shown in Fig. 4-10. The protein profile was slightly different from that of the cytosol of bovine corpus luteum in that smaller and larger peaks were eluted at 60 mM and 150 mM, respectively. However, the 40 mM BP was shown to be present by steroid-binding

Fig. 4-9: Hydroxylapatite column chromatography of the cytosolic fraction of ovine corpus luteum

Cytosol was prepared from a 1:5 w/v homogenate of ovine corpus luteum as described under Methods (sections 4.2.1 and 4.2.2). An aliquot (31 ml; A_{280} = 14) was incubated with 0.1 ml ethanol containing ^{3}H-progesterone (3.2×10^{6} cpm; ca.48 nmol) for 55 min on ice. The incubated cytosol (30.5 ml) was applied to an HAP column (1.7 (i.d.) x 15 cm). The column was developed with the following sequence of potassium phosphate buffers, pH 6.8: 90 ml x 5 mM; 69 ml x 40 mM; 135 ml x 60 mM; 117 ml x 100 mM; 140 ml x 150 mM; 129 ml x 200 mM; and 69 ml x 650 mM. Fractions of 3 ml were collected at a flow rate 29-24 $ml.h^{-1}$. The absorbance at 280 nm of the column fractions was measured, and the radioactivity of 0.5 ml aliquots were counted in 8 ml of Cocktail N scintillant.

● = Protein, A_{280}
○ = Progesterone, $cpm.0.5\ ml^{-1}$

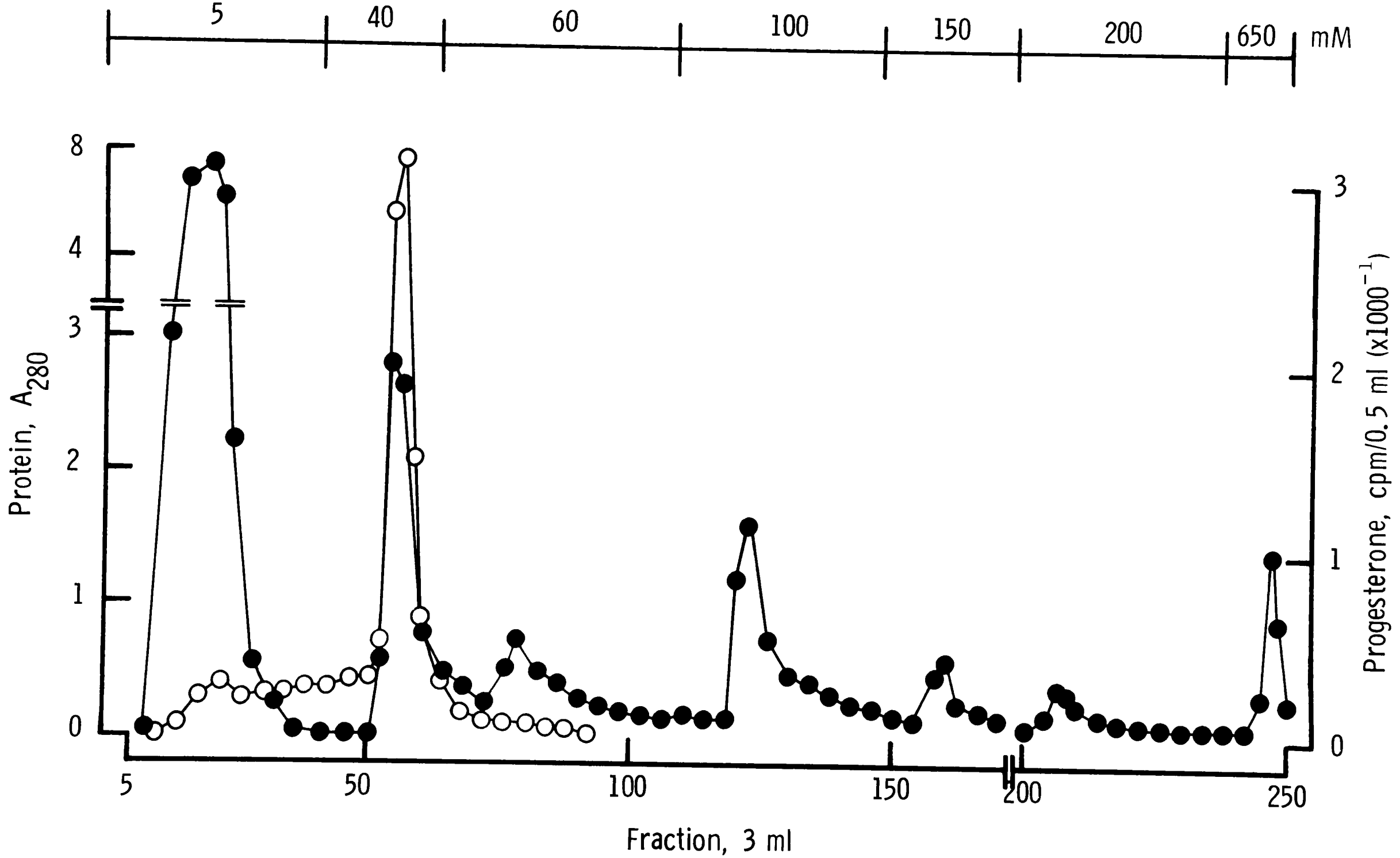

5
40
60
100
150
200
650
mM
Protein, A_{280}
Progesterone, cpm/0.5 ml (x1000^{-1})
8
4
3
2
1
0
3
2
1
0
5
50
100
150
200
250
Fraction, 3 ml

Fig. 4-10: Hydroxylapatite column chromatography of ovine utero-ovarian venous plasma

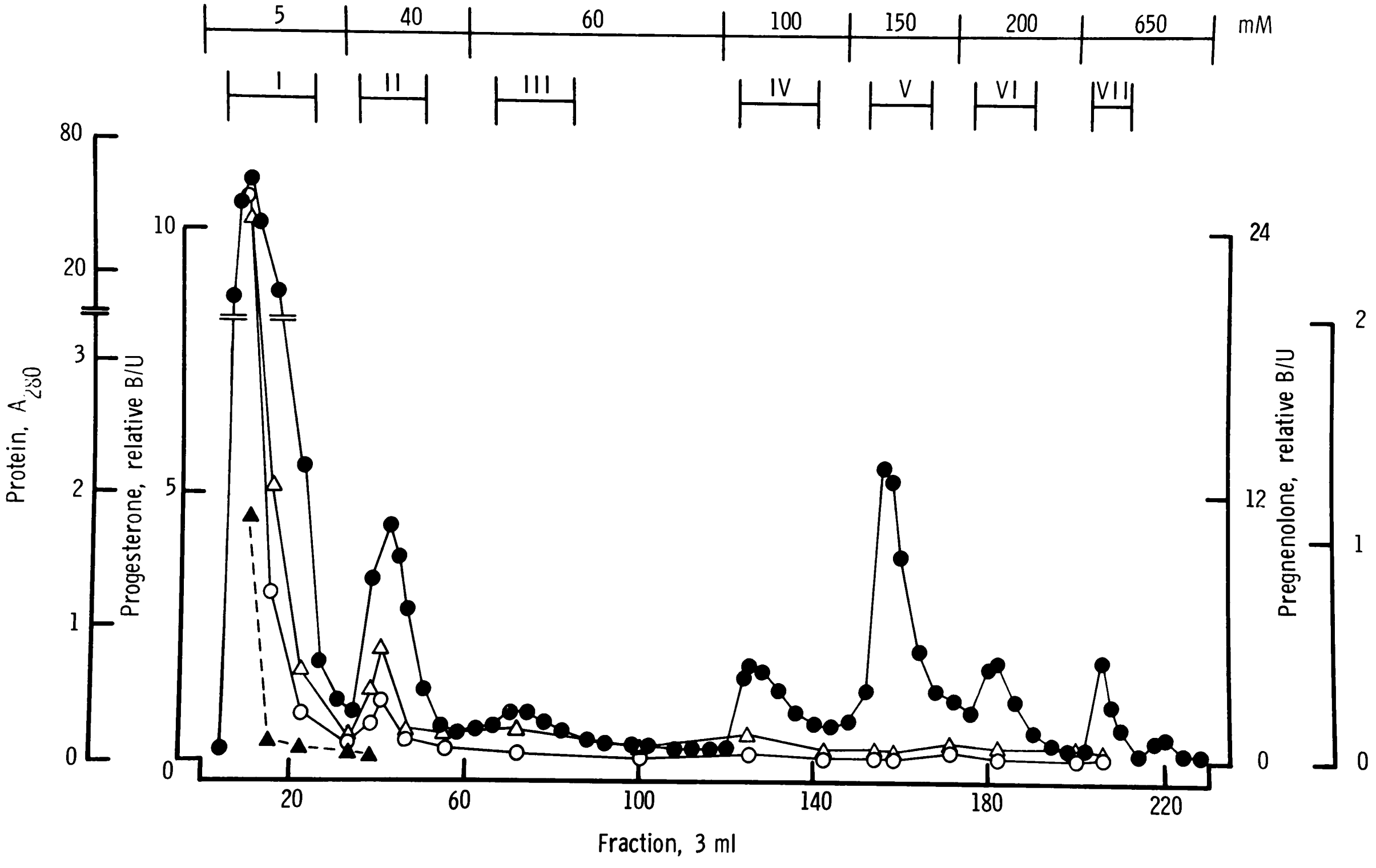
UOV blood was collected twice-daily for 2 days from a ewe with an indwelling catheter. The plasma was obtained within 15 min of collection as described under Methods (section 4.2.1) and stored at -20°C. The thawed plasma was centrifuged at 70,000 x g_{av} for 90 min at 4°C; no pellet was obtained. The centrifuged plasma (22.5 ml, A_{280} = 78.5) was applied to an HAP column of 2.05 (i.d.) x 8 cm and eluted with the following sequence of potassium phosphate buffers, pH 6.8: 66 ml x 5 mM; 95 ml x 40 mM; 190 ml x 60 mM; 95 ml x 100 mM; 75 ml x 150 mM; 88 ml x 200 mM; and 90 ml x 650 mM. Fractions of 3 ml were collected at a flow rate of 24.5-23.5 $ml.h^{-1}$. The absorbance at 280 nm of the column fractions was measured, and 0.9 ml of selected fractions were subjected to batch Sephadex-scanning assays as described under Methods (section 4.2.7.1). The amounts of radioactive steroid used in the reaction tubes of the scanning assays were 6,300 cpm (ca.0.1 pmol) of ^{3}H-pregnenolone, 8,300 cpm (ca.0.1 pmol) of ^{3}H-progesterone, and 6,100 cpm (ca.0.1 pmol) of ^{3}H.cortisol.

● = Protein, A_{280}

○ = Progesterone, relative B/U

△ = Pregnenolone, relative B/U

▲ = Cortisol, relative B/U


5
40
60
100
150
200
650
mM
I
II
III
IV
V
VI
VII
Protein, A_{280}
Progesterone, relative B/U
Pregnenolone, relative B/U
Cortisol, relative B/U
Fraction, 3 ml

scanning assays performed on selected column fractions using the batch Sephadex technique (Gueriguian and Pearlman, 1968). This method of assessing the relative progesterone-binding activities of the column fractions was introduced in place of monitoring radioactive progesterone added prior to fractionation because of the dissociation of bound steroid that occurred during elution with the latter procedure. It also showed that cortisol, pregnenolone and progesterone were bound by the unadsorbed material which suggested that CBG was present in this material. The latter has been shown to bind these three steroids (Westphal, 1970).

The proteins which eluted at 40 mM KPB bound progesterone and pregnenolone, but not cortisol. If this peak contained a progesterone-binding protein, then it would be expected to show binding affinity for structurally-related steroids such as pregnenolone. The proteins eluted at greater phosphate ionic strengths did not bind any of these steroids according to this method of determination.

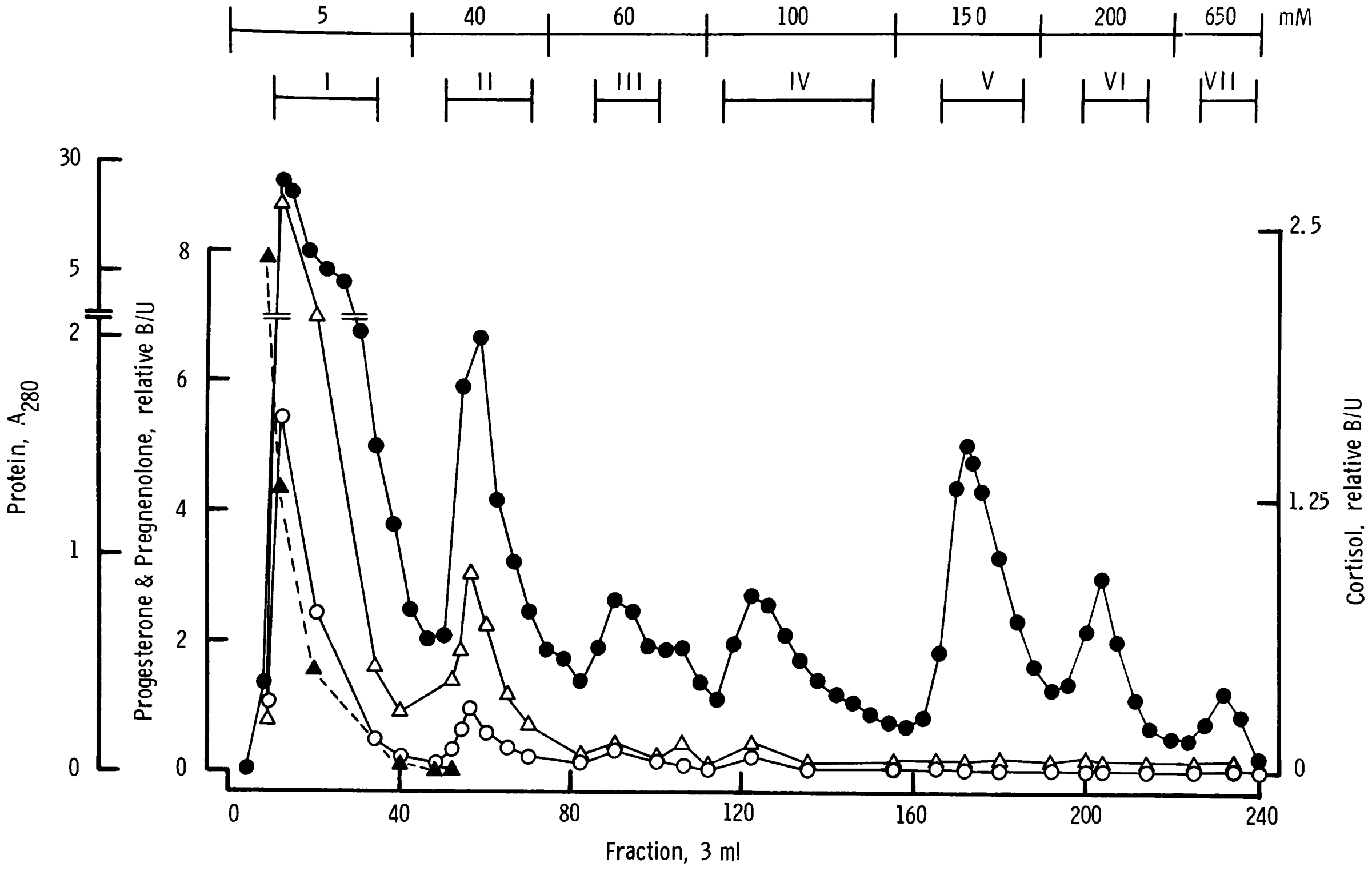
4.3.3.4 Ovine Ovarian Lymph

The fractionation of ovarian lymph from sheep is shown in Fig. 4-11. Protein was eluted at all seven buffer concentrations. The unadsorbed material (eluted at 5 mM KPB) showed binding activity for cortisol, progesterone and pregnenolone, whereas the protein peak which eluted at 40 mM KPB bound progesterone and pregnenolone, but negligible amounts of cortisol. Small amounts of progesterone and pregnenolone were also bound by the protein contained in the peaks which eluted at 60 mM and 100 mM KPB.

Fig. 4-11: Hydroxylapatite column chromatography of ovine ovarian lymph

Ovarian lymph was collected from an indwelling catheter continuously over 36 h on ice, and aliquots were removed every 4-8 h and frozen at -20°C. The thawed lymph was centrifuged at 2,000 x g_{max} for 5 min to remove dust and a small amount of flocculent material. The supernatant (18.8 ml; A_{280} = 64.4) was applied to a column of HAP (2.6 (i.d.) x 8.5 cm) and developed with the following potassium phosphate buffers, pH 6.8: 96 ml x 5 mM; 100 ml x 40 mM; 100 ml x 60 mM; 100 ml x 100 mM; 100 ml x 150 mM; 100 ml x 200 mM; and 100 ml x 650 mM. Fractions of 3 ml were collected at a flow rate which varied between 29 and 15 $ml.h^{-1}$. The absorbance at 280 nm of the column fractions was determined, and 0.9 ml of selected fractions were subjected to batch Sephadex-scanning assays as described under Methods (section 4.2.7.1). The amounts of radioactive steroid used in the scanning assays were 6,100 cpm (ca.0.1 pmol) of ^{3}H-progesterone; 5,700 cpm (ca.0.1 pmol) of ^{3}H-pregnenolone; and 6,450 cpm (ca.0.1 pmol) of ^{3}H-cortisol.

● = Protein, A_{280}
○ = Progesterone, relative B/U
△ = Pregnenolone, relative B/U
▲ = Cortisol, relative B/U


5
40
60
100
150
200
650
mM
I
II
III
IV
V
VI
VII
30
5
2
1
0
Protein, A_{280}
8
6
4
2
0
Progesterone & Pregnenolone, relative B/U
2.5
1.25
0
Cortisol, relative B/U
0
40
80
120
160
200
240
Fraction, 3 ml

4.3.3.5 Bovine and Ovine Jugular Venous Plasmas

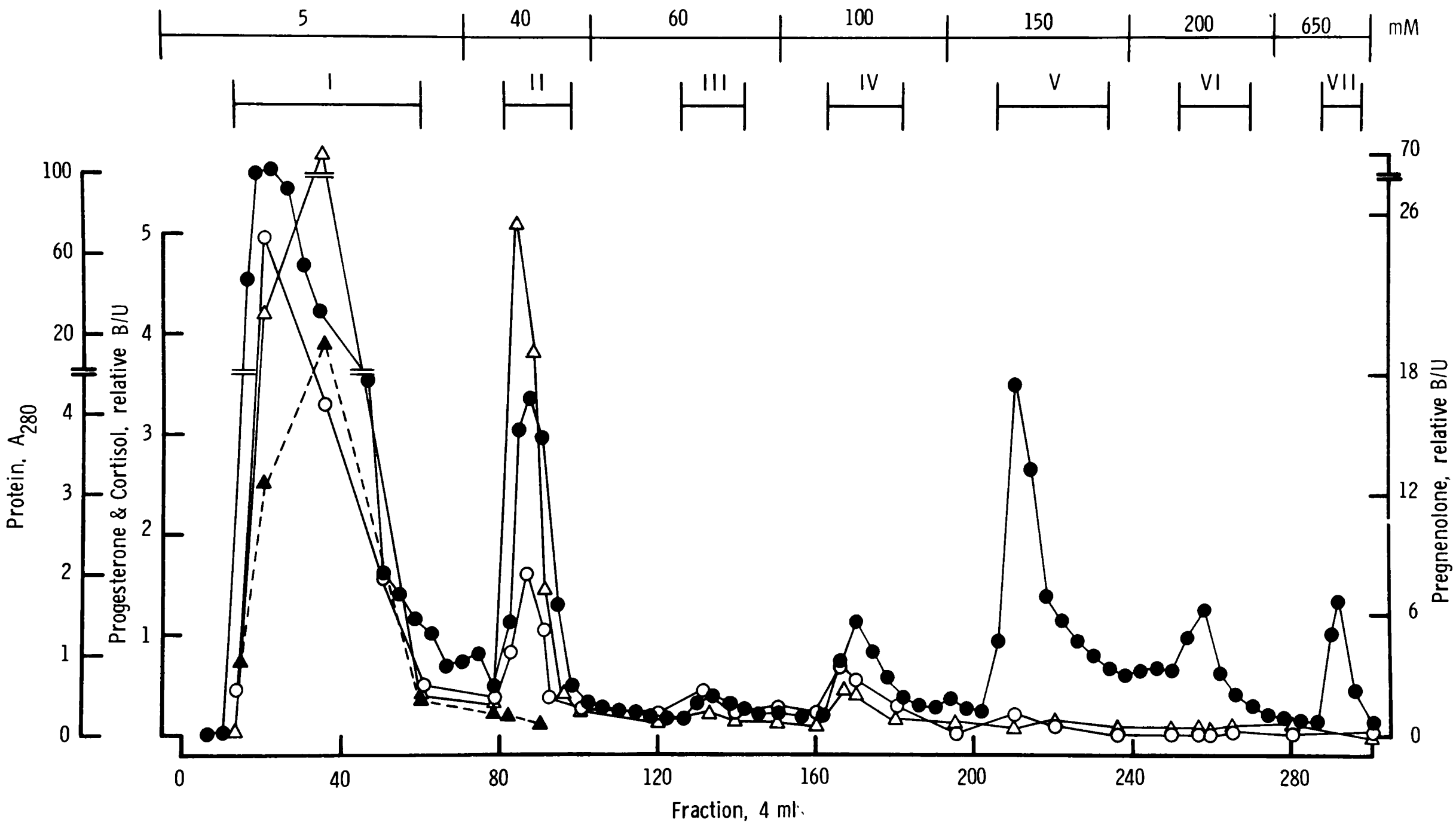
The finding of a 40 mM binding protein activity in UOV plasma and lymph analogous to that found in the cytosol of bovine and ovine corpora lutea raised three possibilities. Either it was a secretory product of the corpus luteum, and/or it was a circulating plasma protein. To examine the latter possibilities fractionation of JV plasma on HAP was undertaken in order to determine whether BPI was present in the peripheral circulation. Fig. 4-12 shows the result of such a fractionation Protein was eluted at all seven buffer concentrations; however, in the sheep, the peaks eluted at 200 mM and 650 mM KPB were larger for JV plasma than for UOV plasma. Scanning assays of column fractions showed that JV plasma, like UOV plasma, contained two steroid-binding peaks. The peak of unadsorbed material bound progesterone, pregnenolone and cortisol, whereas the peak eluted at 40 mM KPB bound only progesterone and pregnenolone. Small amounts of three steroids were also bound by the proteins which eluted at 60 mM, 100 mM, 200 mM and 650 mM KPB, but the level of binding in all was sufficiently low to be possibly artifactual. Analogous results were obtained when bovine JV plasma was applied to a column of HAP and developed with 5 mM and 40 mM KPB.

In summary, a 40 mM binding activity for progesterone and pregnenolone was obtained upon HAP fractionation of the cytosolic fractions of bovine and ovine corpora lutea, UOV plasma and ovarian lymph of sheep and JV plasma of both species. A binding activity for progesterone, pregnenolone and cortisol, possibly due to the presence of CBG or some related protein, was found in the material unadsorbed to HAP derived from the

Fig. 4-12: Hydroxylapatite column chromatography of ovine jugular venous plasma

Jugular venous blood was collected and stored from a ewe at the mid-luteal phase of the oestrus cycle as described under Methods (section 4.2.1). The thawed plasma was centrifuged at 2,000 x g_{max} for 10 min; no pellet was obtained. The supernatant (76.5 ml; A_{280} = 86) was applied to an HAP column (2.75 (i.d.) x 16 cm) and developed with the following sequence of potassium phosphate buffers, pH 6.8: 188 ml x 5 mM; 172 ml x 40 mM; 160 ml x 60 mM; 180 ml x 100 mM; 184 ml x 150 mM; 150 ml x 200 mM; and 150 ml of 650 mM. Fractions of 4 ml were collected at flow rates which varied between 33 and 15 $ml.h^{-1}$. The absorbance at 280 nm of the column fractions was determined, and 0.9 ml of selected fractions were subjected to batch Sephadex-scanning assays as described under Methods (section 4.2.7.1). The amounts of radioactive steroid used in the reaction tubes of the scanning assays were 6,350 cpm (ca.0.1 pmol) of ^{3}H-progesterone; 6,700 cpm (ca.0.1 pmol) of ^{3}H-pregnenolone; and 6,250 cpm (ca.0.1 pmol) of ^{3}H-cortisol.

● = Protein, A_{280}
○ = Progesterone, relative B/U
△ = Pregnenolone, relative B/U
▲ = Cortisol, relative B/U


5
40
60
100
150
200
650
mM
I
II
III
IV
V
VI
VII
Protein, A_{280}
Progesterone & Cortisol, relative B/U
Pregnenolone, relative B/U
Fraction, 4 ml

plasma and lymph samples.

4.3.4 Multiple Equilibrium Dialysis and Steroid Cross Reaction Studies of Cytosol from Bovine Corpora Lutea

4.3.4.1 Multiple Equilibrium Diaylsis

The progesterone-binding activity of the cytosolic fraction of corpora lutea obtained from mid-cycle cows was determined by MED either separately or as part of the steroid cross reaction experiments. A typical Scatchard plot of the MED data is shown in Fig. 4-13. The bovine corpus luteum was shown to contain a soluble, high-affinity, progesterone-binding activity of the order of 10^8 M^{-1} (Table 4-8). In all of these later MED experiments the protein concentrations were 0.75-1.5 $mg.ml^{-1}$. Dialysis was initially carried out for 36-46 h but since equilibration was achieved by 16 h, dialysis times were reduced to 18-24 h. However, as the progesterone-BP(s) complex was stable for 50-55 h, the experiments using the longer periods of dialysis were also valid (Fig. 4-14). Endogenous progesterone was shown to exchange with exogenous progesterone with a half-time of approximately 9.8 h (Fig. 4-15).

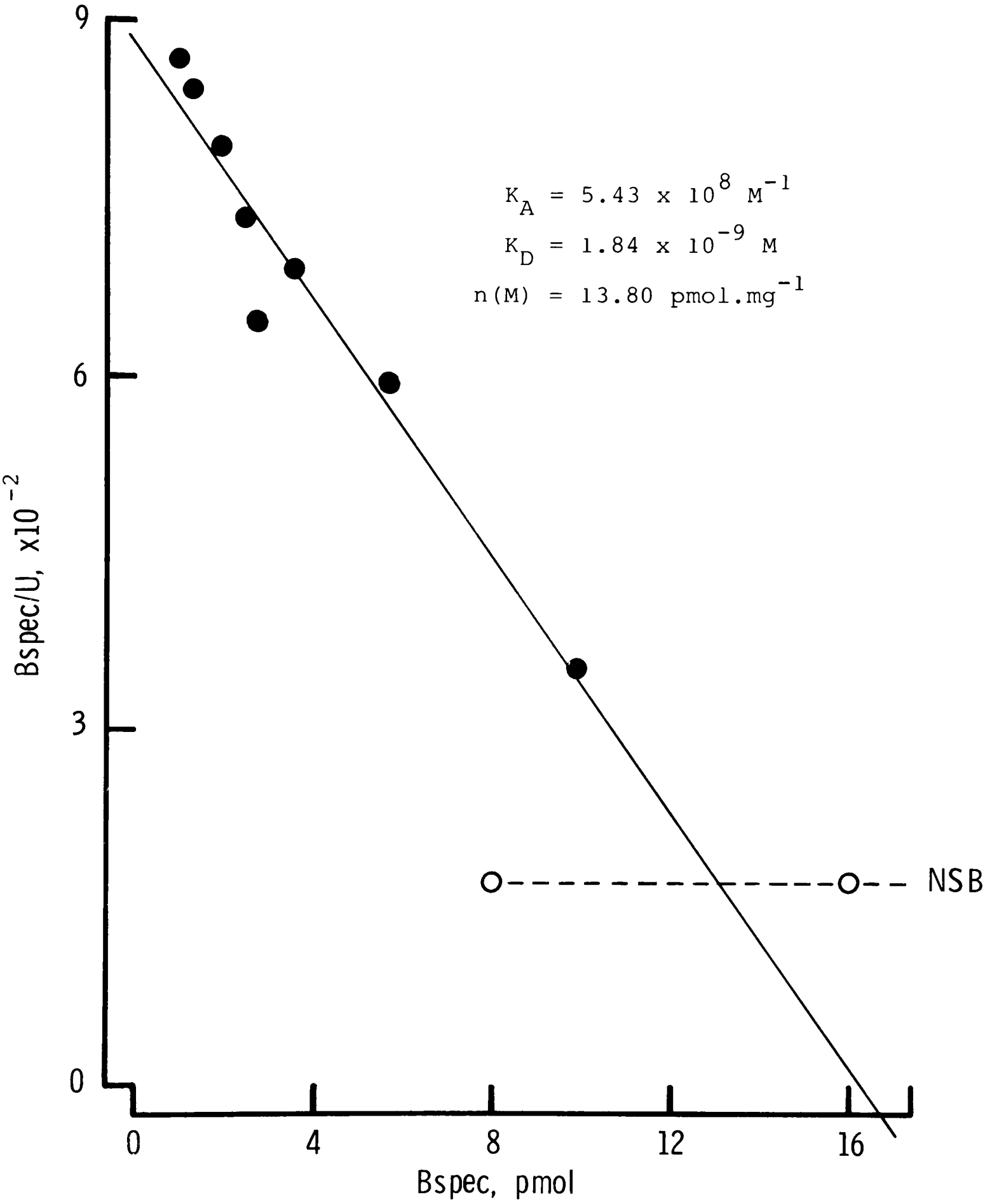
It was necessary to convert all measurements of radioactivity to dpm because radioactivity of the protein-containing (inside) compartment was detected less efficiently than the radioactivity of the buffer (outside) compartment. The concentration of residual endogenous steroid in the cytosol (determined by RIA) remaining after DCC-treatment was included in the computation of the masses of progesterone in each dialysis vial. Table 4-9 shows that the estimation of K_A was decreased by 22%, and the magnitude of n(M) was increased by 11%, when radioactivities were not converted to dpm. If the

Fig. 4-13: Scatchard plot of the progesterone-binding activity of the cytosolic fraction of bovine corpus luteum determined by multiple equilibrium dialysis

The cytosol was prepared and subjected to multiple equilibrium dialysis as described under Methods (sections 4.2.2 and 4.2.5). The concentration of progesterone in the dialysis vials ranged from 2.5-9,000 pmol. The DCC-treated cytosol was dialyzed at a protein concentration of 1.174 $mg.ml^{-1}$ for 42 h at $4^{o}C$. The concentration of endogenous progesterone was 7.50 $pmol.ml^{-1}$, and this was added to the masses of exogenous progesterone for computation of the total amount of steroid available for binding. The radioactivities were used in the form of dpm in the calculations. Additional data points at high concentrations of progesterone are off-scale to the right. The line was fitted by least mean-squares regression analysis.

● = High-affinity binding

○ = Low-affinity (non-specific) binding


9
6
3
0
Bspec/U, x10^{-2}
K_A = 5.43 x 10^{8} M^{-1}
K_D = 1.84 x 10^{-9} M
n(M) = 13.80 pmol.mg^{-1}
NSB
0
4
8
12
16
Bspec, pmol

Sample	K_A, x 10^8 M^{-1}	K_D, x 10^{-9} M	n(M), pmol.mg^{-1}
Freshly-prepared cytosol	2.40	4.16	16.95
	3.57	2.80	18.40
	7.55	1.33	5.42
	5.43	1.84	13.80
	8.94	1.12	10.63
	1.70	9.32	46.52
	2.45	4.07	23.14
	4.13	2.42	19.67
	2.01	4.98	48.30
	4.235	2.36	9.05
Frozen and thawed cytosol	10.31	0.97	8.40
	2.06	4.85	17.46
	2.94	3.40	13.63
Freshly-prepared cytosol from pregnant animal	1.64	6.11	37.99
Mean	4.19	3.55	20.67
SEM	0.77	0.61	3.69

Table 4-8: Multiple equilibrium dialysis and Scatchard analysis of the progesterone-binding activity of the cytosolic fraction of bovine corpus luteum, determined in separate experiments.

Fig. 4-14: Equilibration and stability of the cytosolic proteins of bovine corpus luteum upon dialysis

A series of identical dialyses were initiated simultaneously and triplicates were terminated sequentially at times ranging from 4 h to 72.75 h. The dialysis bag (inside) contained 1 ml of DCC-treated cytosol (protein = 0.941 $mg.ml^{-1}$) and the dialyzing solution (outside) consisted of 15 ml of 40 mM KPB, pH 7.4 containing radioactive and radioinert progesterone (29.15 pmol). The buffer solution was dispensed as 2 x 7.5 ml using a zipette. Dialysis was at $4^{o}C$. Bound = inside - outside (at equilibrium).

○ = Inside
▲ = Outside
● = Bound

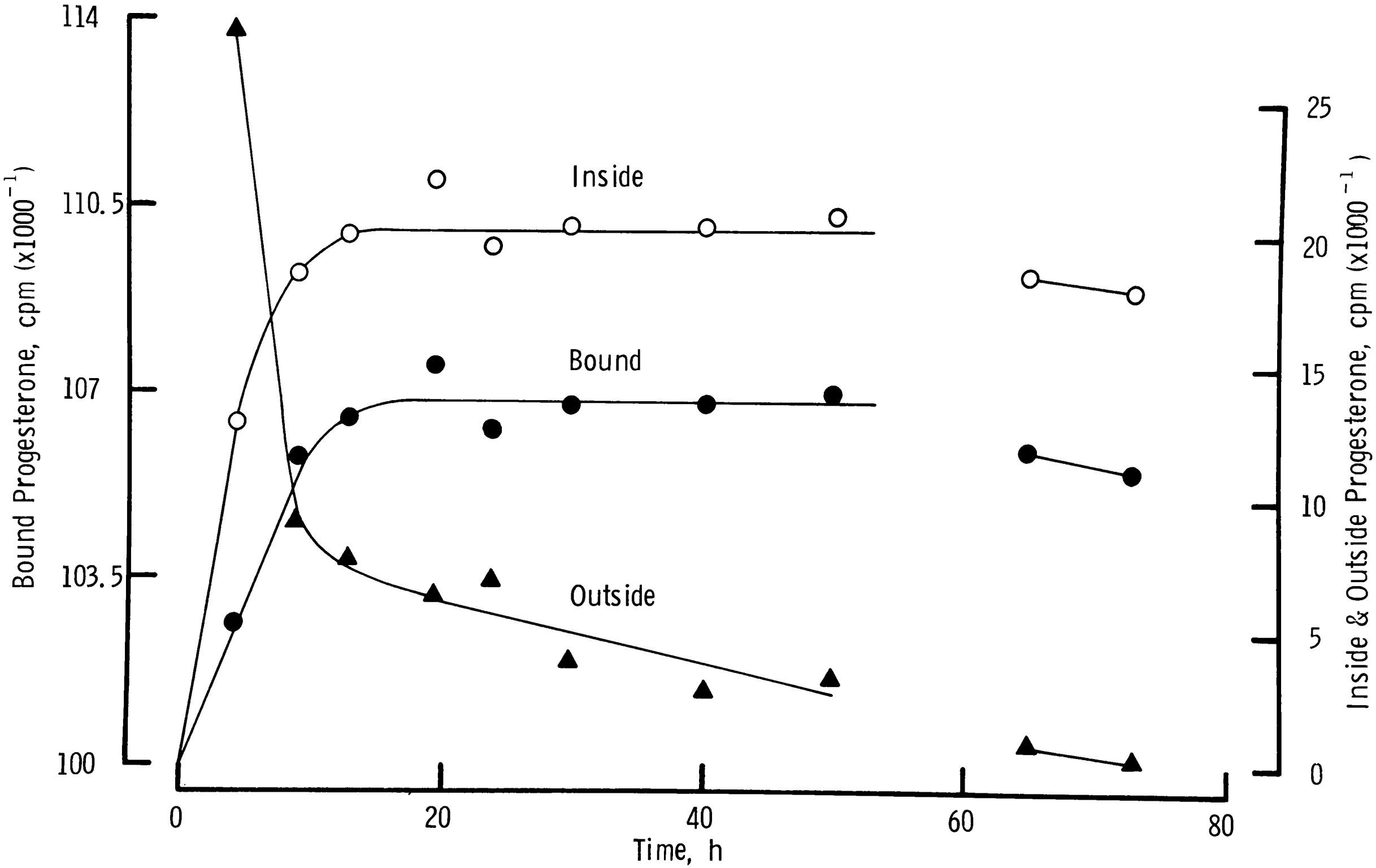
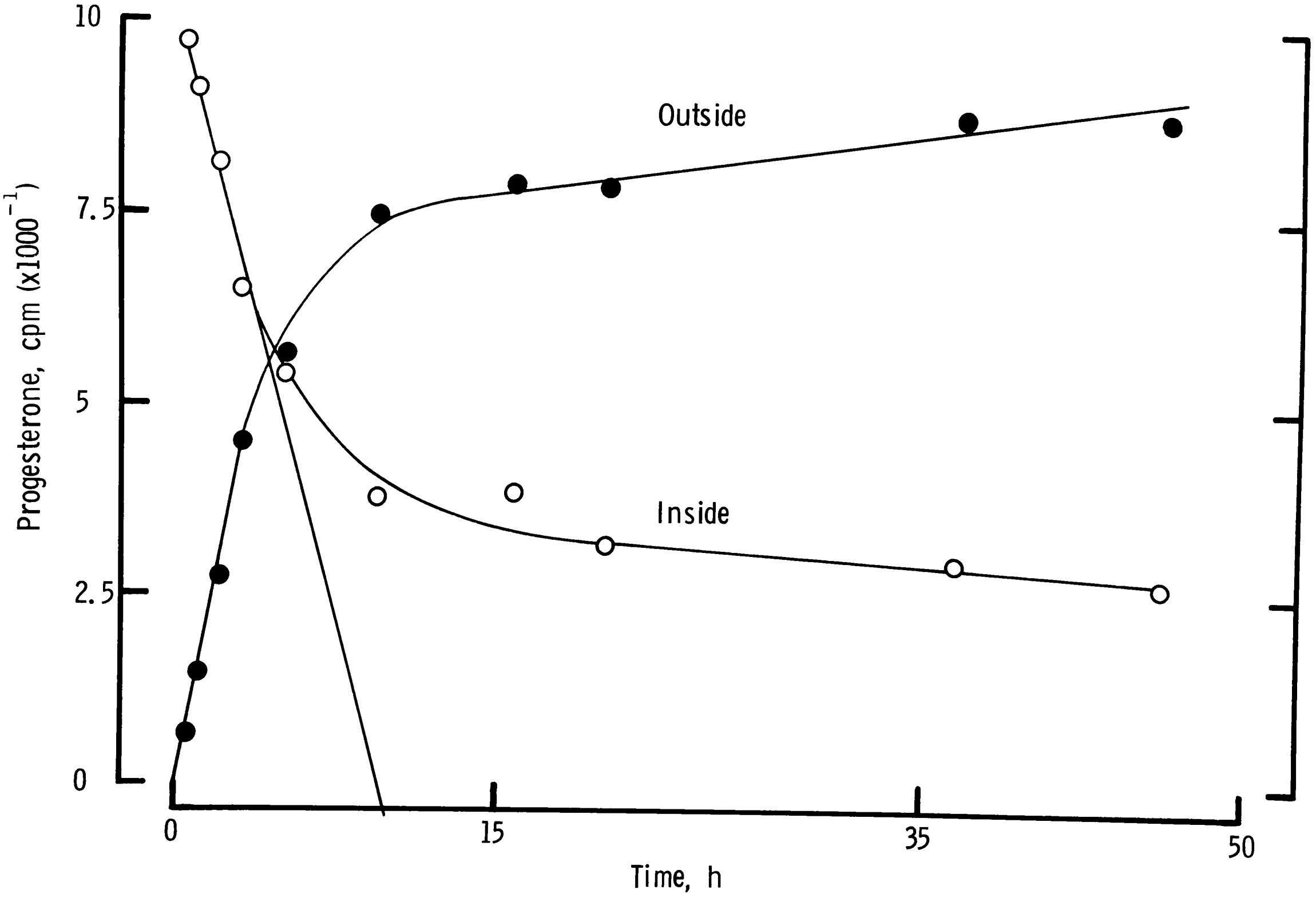

114
110.5
107
103.5
100
Bound Progesterone, cpm (x1000^{-1})
Inside
Bound
Outside
25
20
15
10
5
0
Inside & Outside Progesterone, cpm (x1000^{-1})
0
20
40
60
80
Time, h

Fig. 4-15: Dissociation of bound progesterone from the cytosolic proteins of bovine corpus luteum

Forty ml of DCC-treated cytosol was incubated for 3.33 h with ^{3}H-progesterone on ice. Unbound steroid was removed by incubating with 8 ml of DCC suspension for 20 min, and removal of the charcoal by centrifugation. The supernatant was dispensed in aliquots of 1 ml (protein = 0.941 mg.ml^{-1}; inside) and dialyzed at 4^{o}C against 15 ml of buffer containing 100 pmol of radioinert progesterone (outside). The dialyzes were terminated sequentially, in triplicate, at times ranging between 0.5 h and 46.17 h. The straight line is an extrapolation of the initial dissociation rate of high-affinity complexes plus diffusion time.

○ = Inside
● = Outside


10
7.5
5
2.5
0
Progesterone, cpm (x1000^{-1})
Outside
Inside
0
15
35
50
Time, h

	K_A x 10^8 M^{-1}	K_D, x 10^{-9} M	n(M), pmol.mg^{-1}
A. Radioactivity as cpm	6.97	1.44	11.81
Radioactivity as dpm	8.94	1.12	10.63
B. Endogenous progesterone excluded	5.26	1.90	11.20
Endogenous progesterone included	5.43	1.84	13.80

Table 4-9: The effect of converting radioactivities to dpm and of including the concentration of endogenous progesterone in the calculation of the progesterone-binding parameters of the cytosolic fraction of bovine corpus luteum.

Cytosol was prepared from mid-cycle bovine corpora lutea and subjected to MED. B: Scatchard analysis including or excluding the contribution of endogenous progesterone to the total mass of steroid in each dialysis vial. The radioactivities were converted to dpm. A and B were different experiments.

contribution of endogenous progesterone to the total masses of progesterone was not allowed for, then the estimation of K_A was reduced by only 3%, but the estimation of n(M) was decreased by 19%. Thus, failure to correct for the quenching of radioactivity by protein led to about a 20% underestimation of K_A, whereas failure to allow for the contribution of endogenous progesterone to the total mass of steroid available for dialysis caused about a 20% decrease in the estimation of n(M) which was not fully compensated by using radioactivity data in the form of cpm.

The 40 mM peak of protein obtained by HAP fractionation of cytosol and plasma samples bound both progesterone and pregnenolone in the batch Sephadex-scanning assays of column fractions. Therefore, MED using pregnenolone was carried out on the cytosolic fraction of bovine corpus luteum and on the 40 mM BP peak obtained by fractionation of cytosol on HAP. However, in three separate experiments a high-affinity binding activity for pregnenolone could not be demonstrated by MED.

Measurement of the progesterone-binding activity by MED was carried out on the peaks of protein obtained by HAP fractionation of the cytosol of bovine corpus luteum and on the 40 mM peak of protein derived from ovine UOV plasma (Table 4-10). The cytosol of bovine corpus luteum contained two progesterone binding activities, one of which eluted at 40 mM KPB (40 mM BP or BPI), and another which eluted at 100 mM KPB (100 mM BP or BPII). They both displayed equilibrium association constants for progesterone of the order of 10^8 M^{-1}, although the magnitude of the K_A of BPI was greater than that of unfractionated cytosol (compared with the preliminary

Sample			K_A, x 10^8 M^{-1}	K_D, x 10^{-9} M	n(M), pmol.mg^{-1}
Cytosol of bovine corpus luteum					
Peak I	(5 mM)	Expts 1,2	No high-affinity binding		
Peak II	(40 mM)	Expt 1	8.61	1.16	6.58
		Expt 2	[a]6.87	1.46	11.95
			[a]6.58	1.52	10.80
Peak III	(60 mM)		No high-affinity binding		
Peak IV	(100 mM)	Expt 1	[a]1.51	6.61	87.15
			[a]1.78	5.63	84.36
		Expt 2	2.77	3.61	122.80
Peak V	(150 mM)	Expts 1,2	No high-affinity binding		
Peak VI	(200 mM)	Expts 1,2	No high-affinity binding		
Peak VII	(650 mM)	Expts 1,2	No high-affinity binding		
Ovine UOV Plasma					
Peak II	(40 mM)	Expt 3	3.42	2.92	16.02

Table 4-10: Multiple equilibrium diaylsis and Scatchard analysis of the progesterone-binding activity of the peaks of protein obtained by HAP fractionation of the cytosol of bovine corpus luteum and of ovine utero-ovarian venous plasma.

a = Separate determinations (each performed in duplicate in the same experiment).

The binding capacity, n(M), of Peak II was significantly different from that of cytosol ($p<0.02$, two-tailed Mann-Whitney test; Siegal, 1972).

experiment described under 4.3.1.3) and was some 3.5-fold greater than the magnitude of the K_A of BPII. Generally, the latter had a 12-fold greater concentration of binding sites than BPI, assuming that in both cases one molecule of protein bound one molecule of steroid. UOV plasma contained a progesterone-binding activity similar to BPI of the bovine corpus luteum. The peak of protein derived from UOV plasma which eluted at 100 mM KPB was not subjected to MED.

4.3.4.2 Steroid Cross Reaction Studies

The specificity of the cytosolic binding activity for progesterone and other steroids was investigated using MED. Fig. 4-16 shows typical cross reaction plots for progesterone and some other steroids, and Table 4-11 summarizes the data obtained from three experiments. In some cases the amount of competing radioinert steroid in the dialysis vials exceeded their aqueous solubilities. However, the protein inside the dialysis bags would have increased the solubility of the steroids in this compartment, but the extent to which this occurred was unknown.

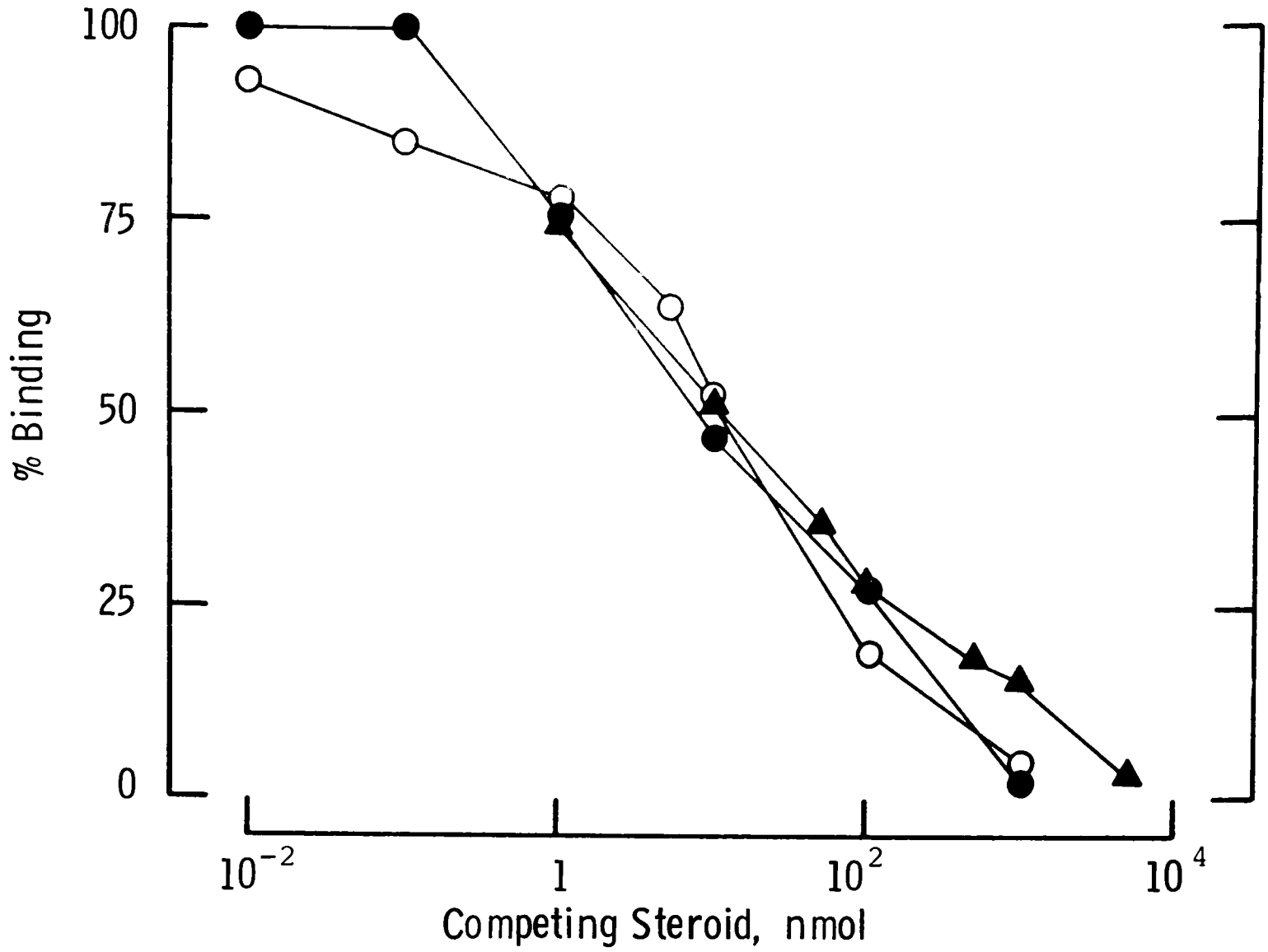
Those steroids whose structures most closely resembled that of progesterone, such as pregnenolone, 5α-pregnanedione and deoxycorticosterone, cross-reacted most effectively with progesterone. R5020 also competed for progesterone-binding, but another synthetic progestogen, norgestrel, was a less effective competitor than both R5020 and danazol. The oestrogens and cortisol were weak competitors, whilst cholesterol did not cross-react at all.

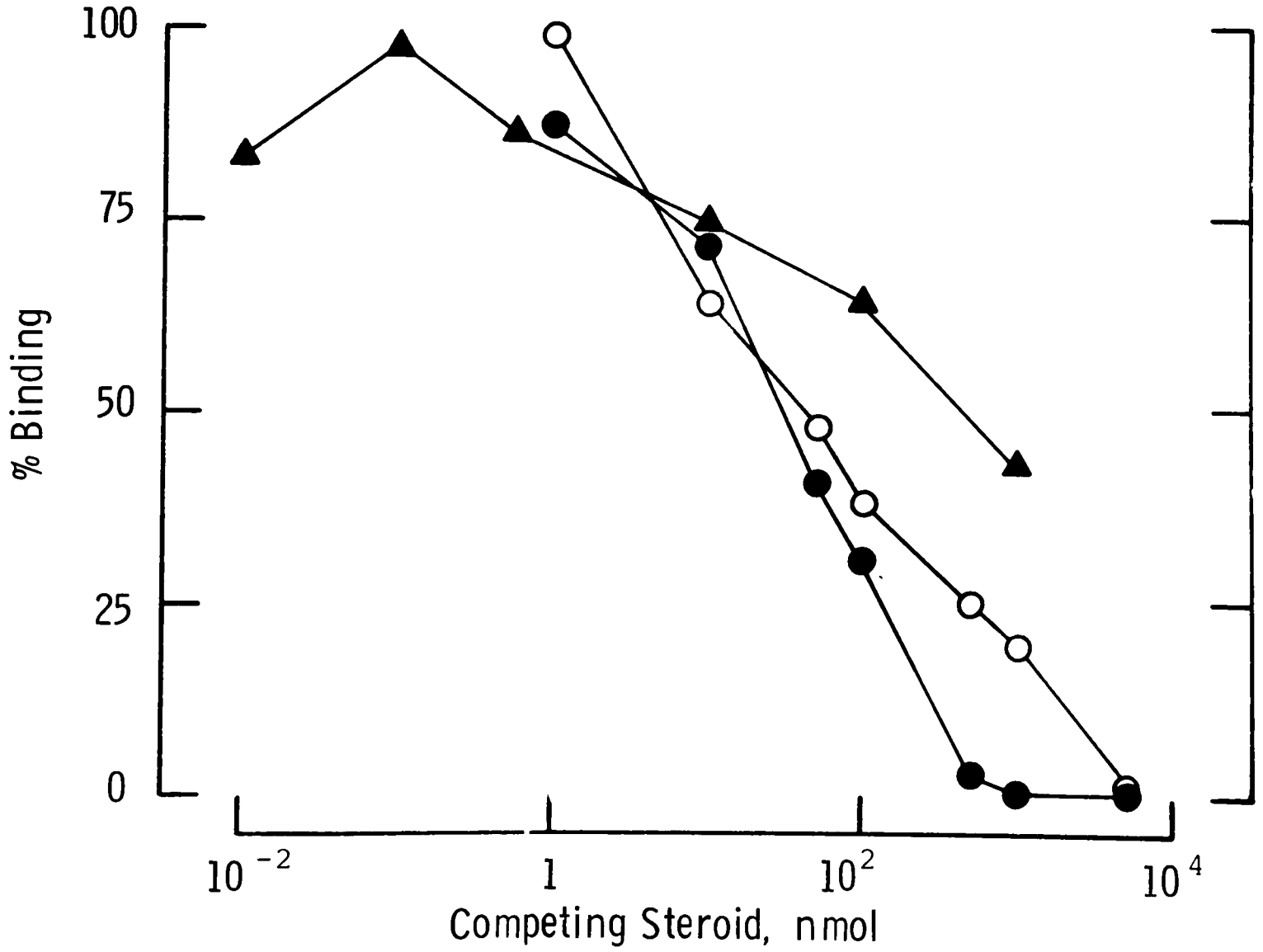
Fig. 4-16: Profiles of cross reaction of different steroids for the progesterone-binding activity of the cytosolic proteins of bovine corpus luteum

The cross reaction of different steroids for the progesterone-binding activity of cytosol was measured using MED as described under Methods (section 4.2.6). Results are expressed relative to the amount of progesterone bound by the high-affinity components when only 10 pmol of progesterone was present in the dialysis vials. This was set at 100%.

A) ● = Progesterone (control curve)
○ = Pregnenolone
▲ = Deoxycorticosterone

B) ● = Danazol
○ = Androstenedione
▲ = Cortisol


100
75
50
25
0
% Binding
10^{-2}
1
10^{2}
10^{4}
Competing Steroid, nmol


100
75
50
25
0
% Binding
10^{-2}
1
10^{2}
10^{4}
Competing Steroid, nmol

Steroid	% Cross Reaction			
	Expt 1	Expt 2	Expt 3	Average
Progesterone	100	100	100	100
Pregnenolone		70.8/63.1		67.0
5α-Pregnanedione			62.37	62.4
Deoxycorticosterone		63.1	47.31	55.2
17α-Hydroxyprogesterone			42.17	42.2
Testosterone	12.58	79.0	19.95	37.2
R5020	17.76	44.7	45.18	35.9
Androstenedione	3.16	44.7	10.59	19.5
17α-Hydroxypregnenolone			18.84	18.8
Danazol	12.60	28.2	14.29	18.4
Dihydrotestosterone			16.79	16.8
Norgestrel			10.12	10.1
Oestradiol-17β	3.16	14.20	3.58	7.0
Dihydroepiandrosterone	4.46	4.50		4.5
Cortisol	1.78	4.7		3.2
Oestrone	1.12	2.5		1.8
Oestradiol-17α	0.79	0.79		0.8
Cholesterol	0			0

Table 4-11: Relative affinities of the progesterone-binding components of the cytosolic fraction of bovine corpora lutea for other steroids.

4.3.5 Partial Purification and Characterization of Progesterone Binding Protein I

4.3.5.1 Ovine Utero-Ovarian Venous Plasma

Two hundred and thirty-two ml of pooled ovine UOV plasma was applied to a large HAP column, and the peak of protein that eluted at 40 mM KPB was recovered. These proteins showed high-affinity binding for progesterone (see Table 4-10). They were concentrated and resolved by gel filtration on Sephadex G-200 into three proteins of differing molecular weights (Fig. 4-17). The protein of lowest molecular weight (peak III) displayed the greatest binding affinity for progesterone and pregnenolone, although small amounts of these steroids were also bound by the protein of intermediate molecular weight. Multiple equilibrium dialysis using progesterone and pregnenolone was carried out on the material of Peak III but high affinity binding was not obtained in either case. The steroid-binding activity was probably destroyed as the purification progressed, since during the course of both HAP and Sephadex G-200 column chromatography endogenous steroid was removed from the protein. The progesterone-binding activities of bovine corpus luteum were also unstable, especially when the concentration of endogenous progesterone was reduced to low levels. BPI comprised approximately 2% of the protein of ovine UOV plasma and approximately 40% of the protein of the 40 mM peak from the HAP column (Table 4-12).

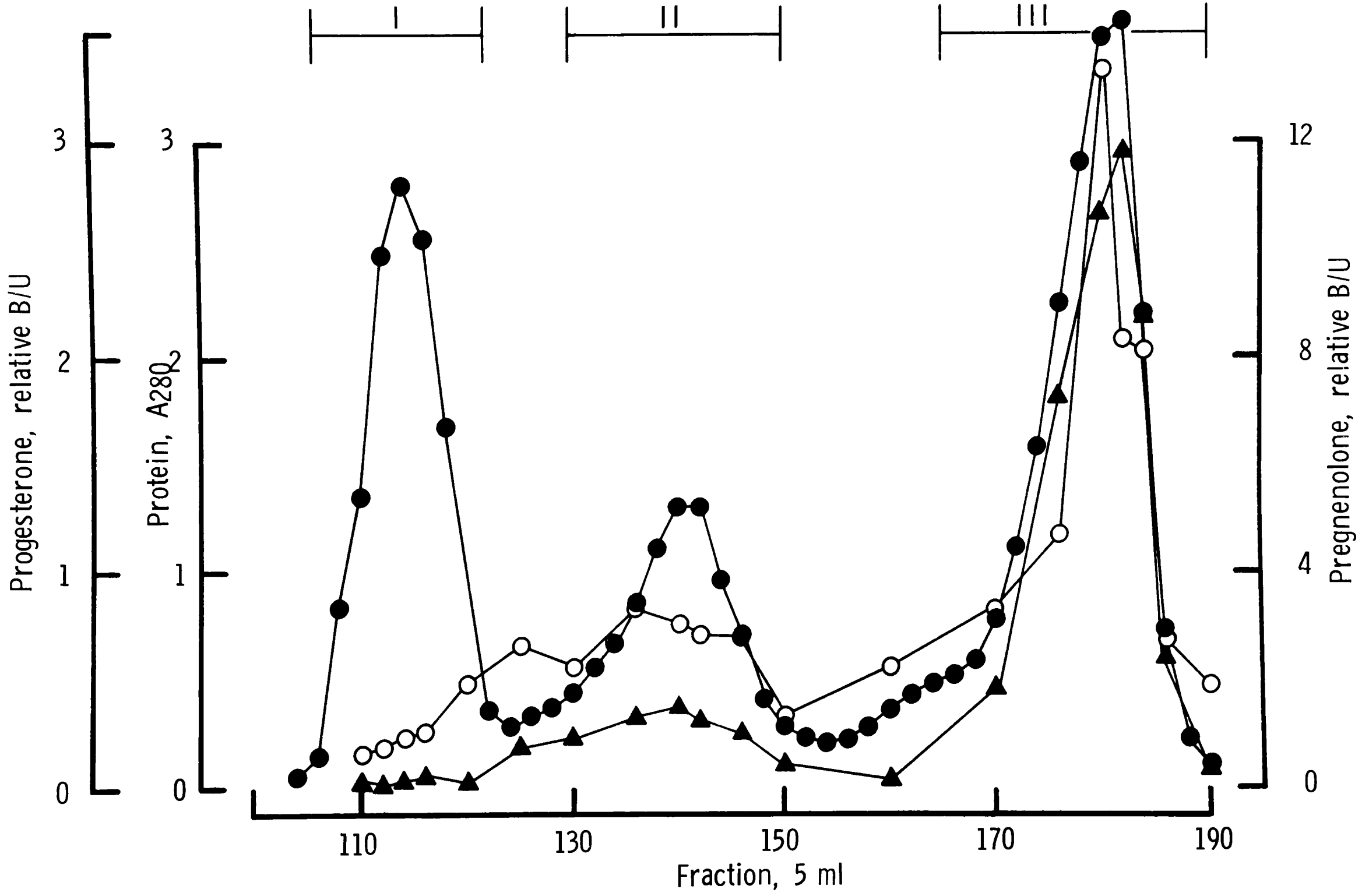
4.3.5.2 Bovine Mid-Cycle Corpus Luteum

BPI was also partially-purified from the cytosolic fraction of bovine corpus luteum using the same procedure that was employed for sheep UOV plasma. The proteins of bovine corpus

Fig. 4-17: Sephadex G-200 column chromatography of the 40 mM progesterone-binding protein peak obtained from HAP fractionation of ovine UOV plasma

Ovine UOV plasma (232 ml, 19.1 g protein) was fractionated on a column of HAP (4.5 (i.d.) x 15.5 cm), and 275 ml (63%) of the protein peak eluted at 40 mM KPB, pH 6.8 was concentrated (with 90% recovery) through a PM-10 membrane (Amicon, model 202). The concentrated protein solution (1.9 ml) was applied to a column of Sephadex G-200 (4.5 i.d. x 90 cm), which was developed with 40 mM KPB, pH 7.4. Fractions of 5 ml were collected at a flow rate of 17-23 $ml.h^{-1}$. The absorbance at 280 nm of the column fractions was determined and 0.9 ml aliquots of selected fractions were subjected to batch Sephadex-scanning assays using ^{3}H-progesterone (6,000 cpm; ca.0.1 pmol) or ^{3}H-pregnenolone (6,000 cpm; ca.0.1 pmol) in each assay tube.

● = Protein, A_{280}
○ = Progesterone, relative B/U
▲ = Pregnenolone, relative B/U


I
II
III
Progesterone, relative B/U
Protein, A280
Pregnenolone, relative B/U
3
2
1
0
3
1
0
12
8
4
0
110
130
150
170
190
Fraction, 5 ml

Purification Step	Volume, ml	Protein, $mg.ml^{-1}$	Total Protein mg	Yield, %
UOV plasma	232	82.43	19,124	100
HAP peak II	437	2.04	819.5	4.7
Sephadex peak III	92	2.13	346	1.8

Table 4-12: Partial purification of BPI from ovine UOV plasma.

Protein concentrations were determined according to Lowry et al. (1951). Further details of the purification are given in the legend to Fig. 4-17.

Purification Step	Volume, ml	Protein, A_{280}	Total Protein A_{280}	Yield, %
Cytosol	162	20.5	3321	100
HAP peak I	448	0.57	255.4	7.7
Sephadex peak III	80	1.33	106.7	3.2

Table 4-13: Partial purification of BPI from the cytosolic fraction of bovine corpus luteum.

Further details of the purification are given in the legend to Fig. 4-18.

luteum which eluted at 40 mM KPB on HAP were resolved into four peaks on Sephadex G-200 (Fig. 4-18), compared to three peaks for the HAP proteins derived from UOV plasma. In both cases the major peak from the Sephadex G-200 step bound added progesterone and pregnenolone. BPI comprised approximately 3% of the protein of the cytosolic fraction of bovine corpus luteum and approximately 40% of the protein of the 40 mM peak from the HAP column (Table 4-13).

4.3.5.3 Polyacrylamide Gel Electrophoresis

1. Estimation of Purity. The peaks of protein obtained from fractionation of bovine corpus luteum together with HAP peak II (40 mM peak) and Sephadex G-200 peaks I-III from the purification of BPI from UOV plasma were concentrated and applied to cylindrical polyacrylamide gels run in the presence of detergent. Fig. 4-19 shows that the cytosolic fraction of bovine corpus luteum consisted of a complex mixture of proteins, with a wide range of monomer molecular weights. The 40 mM peak of protein obtained from fractionation of corpus luteum cytosol or UOV plasma on HAP (gels 3 and 9) contained considerably less protein than the cytosolic fraction of bovine corpus luteum. In both cases, approximately 50% of the 40 mM peak consisted of one, relatively low monomer molecular weight, protein which formed a wide band at the gel-loadings used in this experiment. A protein monomer of similar size was also present in HAP peak III (gel 4), but HAP peak IV (gel 5), did not contain a protein of similar size. This substantiated the demonstration by MED that the progesterone-binding activities of HAP peaks II and IV were due to different proteins.

The partially-purified BPI fraction derived from ovine UOV

Fig. 4-18: Sephadex G-200 column chromatography of the 40 mM progesterone-binding protein peak obtained from HAP fractionation of the cytosolic fraction of bovine corpus luteum

Minced corpora lutea (29.8 g) were homogenized (215 ml) in 5 mM KPB, pH 6.8, and centrifuged to yield a cytosolic fraction (167 ml) as described under Methods (section 4.2.2). An aliquot of the cytosolic fraction (162 ml) was fractionated on an HAP column (4.5 (i.d.) x 13.5 cm). Eighty-four % of the protein eluted at 40 mM KPB, pH 6.8 was concentrated with 90% efficiency through a PM-10 membrane (Amicon, model 202). The concentrated solution (10.5 ml) was applied to a Sephadex G-200 column (4.5 (i.d.) x 90 cm) and developed with 40 mM KPB, pH 7.4. Fractions of 5 ml were collected at a flow rate of 17 $ml.h^{-1}$. The absorbance at 280 nm of the column fractions was determined and 0.9 ml aliquots of selected fractions were subjected to batch Sephadex-scanning assays using ^{3}H-progesterone (5,700 cpm; ca.0.1 pmol) or ^{3}H-pregnenolone (6,200 cpm; ca.0.1 pmol) in each assay tube.

● = Protein, A_{280}
○ = Progesterone, relative B/U
▲ = Pregnenolone, relative B/U

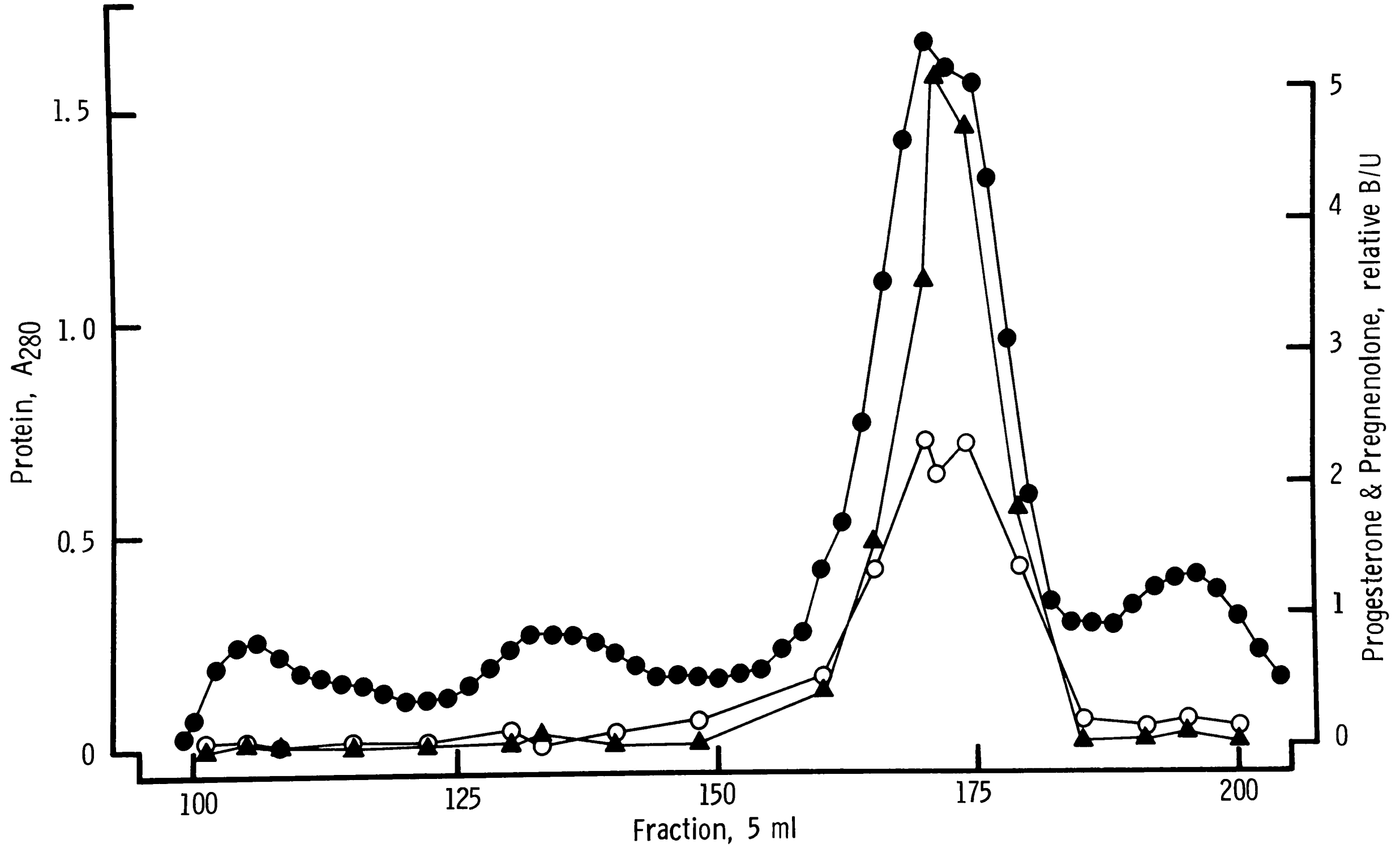
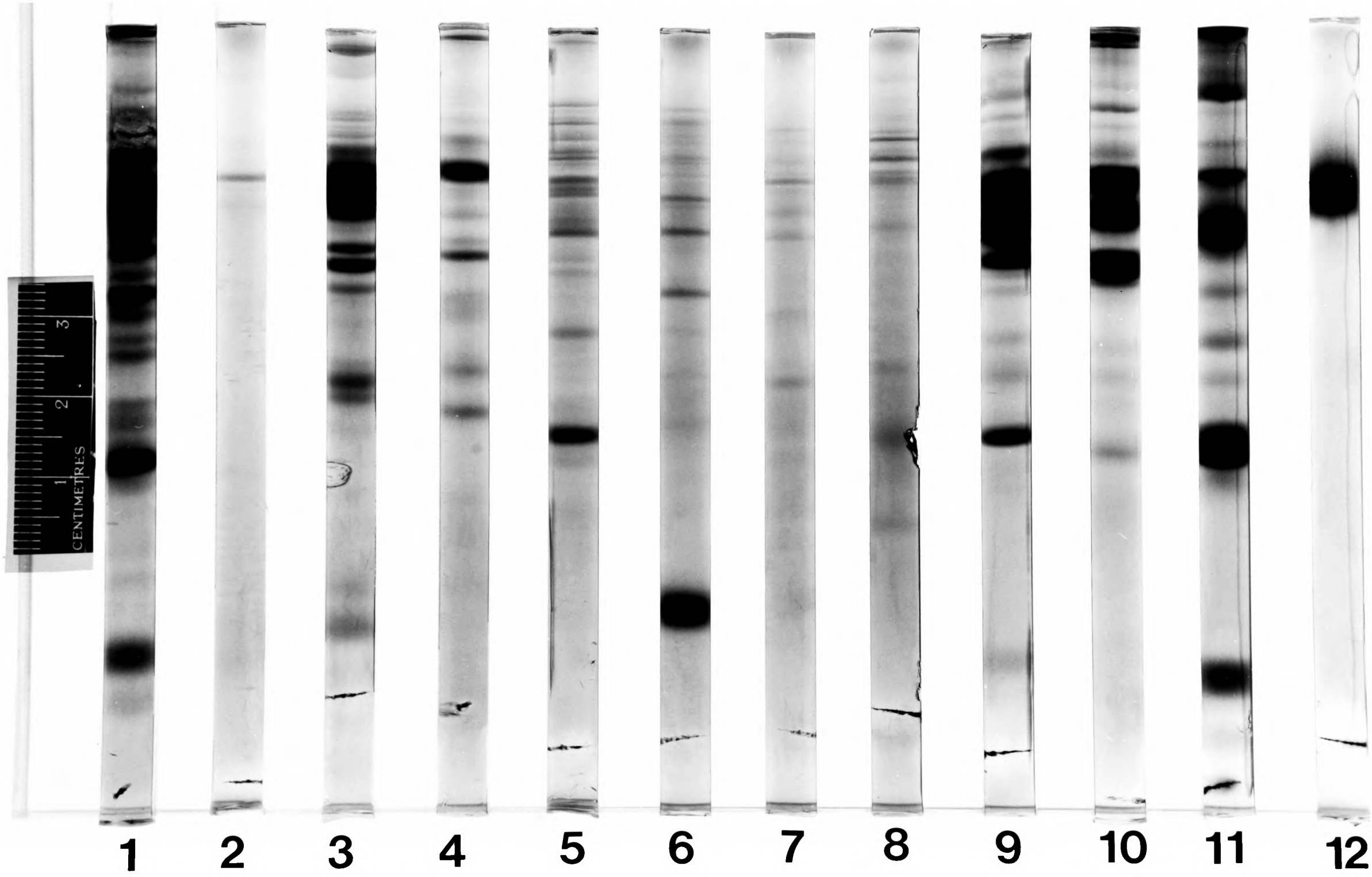

Protein, A_{280}
1.5
1.0
0.5
0
100
125
150
175
200
Fraction, 5 ml
5
4
3
2
1
0
Progesterone & Pregnenolone, relative B/U

Fig. 4-19: Polyacrylamide gel electrophoresis of the peaks of protein obtained by hydroxylapatite and Sephadex G-200 column chromatography

These samples were treated and subjected to electrophoresis as described under Methods (section 4.2.8.1). The peaks of protein obtained from fractionation of the cytosolic fraction of bovine corpus luteum and from the partial purification of BPI from ovine UOV plasma were subjected to electrophoresis for 48 mA and 38V for 15 min, 72 mA and 30V for 3.75 h, and 48 mA and 38V for 6 h. The gels were loaded as follows:

Gel	Sample	Protein, µg
	Bovine corpus luteum	
1.	Cytosol	96
2.	HAP peak I	ca. 5
3.	HAP peak II	ca.40
4.	HAP peak III	ca.10
5.	HAP peak IV	ca.10
6.	HAP peak V	ca.10
7.	HAP peak VI	ca. 5
8.	HAP peak VII	ca. 7.5
	Ovine UOV plasma	
9.	HAP peak II	40
10.	Sephadex peak I	38
11.	Sephadex peak II	10
12.	Sephadex peak III	26


CENTIMETRES
1
2
3
1
2
3
4
5
6
7
8
9
10
11
12

plasma (Sephadex G-200 peak III, gel 12), consisted of principally one protein of comparable monomer molecular weight to the major protein of HAP peak II (gels 3 and 9). Therefore, the MED and polyacrylamide gel data showed that the progesterone-binding activities associated with HAP peak II were due to the same (or similar) proteins in both corpus luteum cytosol and UOV plasma.

The gel of BPI derived from ovine UOV plasma (gel 12) showed that this material was highly purified compared with the 40 mM protein peak from the HAP column (gel 9). Although the photographed gel shows only one major band, other experiments using even higher loadings revealed the presence of 2-3 contaminants of low molecular weight. The preparation of BPI derived from the cytosolic fraction of bovine corpuS luteum also appeared to contain the low molecular weight contaminants. In both cases, purification of the protein was visually estimated to be ≥95%.

2. Determination of Molecular Weights. The monomer molecular weights of both preparations of BPI were estimated on a slab gel of SDS-polyacrylamide (Table 4-14). The molecular weight of BPI from corpus luteum was very similar to the molecular weight of BPI from UOV plasma. The mean molecular weight of BPI was 65,450, which compared favourably with the value obtained for the corpus luteum protein of 62,900, determined from sucrose density gradients (this chapter, section 4.3.1.1). The close agreement between the two methods implied that the protein was not broken down into subunits when exposed to SDS.

Marker protein		Molecular weight	Distance on gel, cm	Mobility, %	Estimated molecular weight
Cytochrome C[a]		12,500	8.0	99.4	
Chymotrypsinogen A		25,000	7.85;7.55	97.5;96.8	
Albumin from hen egg		45,000	6.1;6.0	78.2;76.9	
Albumin from bovine serum		67,000	4.15	53.2	
Aldolase		158,000	6.43	82.4	
Albumin polymers	1[a]	14,300	7.9	101.3	
	2	28,600	6.6	84.6	
	3	42,900	5.25	67.3	
	4	71,500	3.9	50.0	
BPI (bovine corpus luteum)			4.1	52.6	67,143
			4.1	52.6	67,143
			4.15	53.2	66,069
					66,785
BPI (ovine UOV plasma)			4.25	54.5	64,121
			4.23	54.2	64,565
			4.28	54.8	64,122
					64,122

Table 4-14: Determination of the molecular weight of BPI purified from the cytosolic fraction of bovine corpus luteum and from ovine utero-ovarian venous plasma.

All samples were run together on the same gel. The individual marker proteins were run on each side of the gel and the polymers of albumin and experimental samples were run at three spaced positions across the gel.

a = not included in the analysis.

4.3.6 Sucrose Density Gradients of the Two Progesterone Binding Proteins of the Bovine Corpus Luteum

Since MED showed the presence of two discrete progesterone-binding activities in the bovine corpus luteum, the relative affinities of the separated proteins for different steroids on sucrose density gradients were investigated. The purified preparation of BPI was used. BPII was obtained in a peak eluted from a column of HAP at 650 mM KPB, after BPI had been removed by elution at 40 mM KPB. MED experiments had shown that only one high-affinity progesterone-binding component was present in this fraction.

Fig. 4-20 shows that BPI bound added progesterone, danazol, and R5020, but not cortisol. However, unlike unfractionated cytosol, BPI did not bind completely all of the added progesterone such that some of the bound hormone appeared to dissociate from the protein as centrifugation progressed (Fig. 4-20, A and B). The concentration of sucrose in these gradients rose steeply so that BPI did not penetrate far into them. This adversely affected the accuracy with which the molecular weight of the protein could be determined by the method of Martin and Ames (1961). Nonetheless, comparison of the sucrose concentrations at the protein maxima of BSA and BPI yielded a molecular weight for the latter of 65,360. This value was in close agreement with the values obtained previously on polyacrylamide gel and sucrose gradients of unfractionated cytosol (66,800 and 62,900, respectively).

The relative steroid-binding affinities of BPII were different from those of BPI (Fig. 4-21). Thus, added progesterone and danazol were only weakly bound to form a small shoulder

Fig. 4-20A,B: Sucrose density gradient centrifugation of BPI purified from the cytosolic fraction of bovine corpus luteum

BPI, partially purified from the cytosolic fraction of mid-cycle corpora lutea, was incubated in aliquots of 1 ml with radioactive steroids, and 0.5 ml of each incubated mixture was resolved on 5-20% w/w sucrose gradients, and analyzed as described under Methods (section 4.2.3). Details of the gradients were as follows:

Gradient	Exogenous Steroid	Radio-activity applied	Mass applied,	% Recovery of radio-activity[a] from gradient
A	^{3}H-Progesterone	11,295 cpm	0.17 pmol	70
B	^{14}C-Progesterone	59,210 dpm	0.50 nmol	67
	^{3}H-R5020	136,460 dpm	0.71 pmol	71
D	^{3}H-Cortisol	14,080 cpm	0.26 pmol	99
E	^{3}H-Danazol	3,485 cpm	0.20 nmol	75
F	^{3}H-R5020	56,450 cpm	0.99 pmol	72
	Marker			
C	BSA		2 mg	

a: Assumed a loss of 7.5% upon reading of refractive indices.

A) ● = Progesterone, cpm.0.5 ml^{-1}
△ = Protein, A_{280}
○ = % Sucrose, w/w

B) ● = Progesterone, dpm.0.5 ml^{-1}
△ = R5020, dpm.0.5 ml^{-1}
○ = % Sucrose w/w

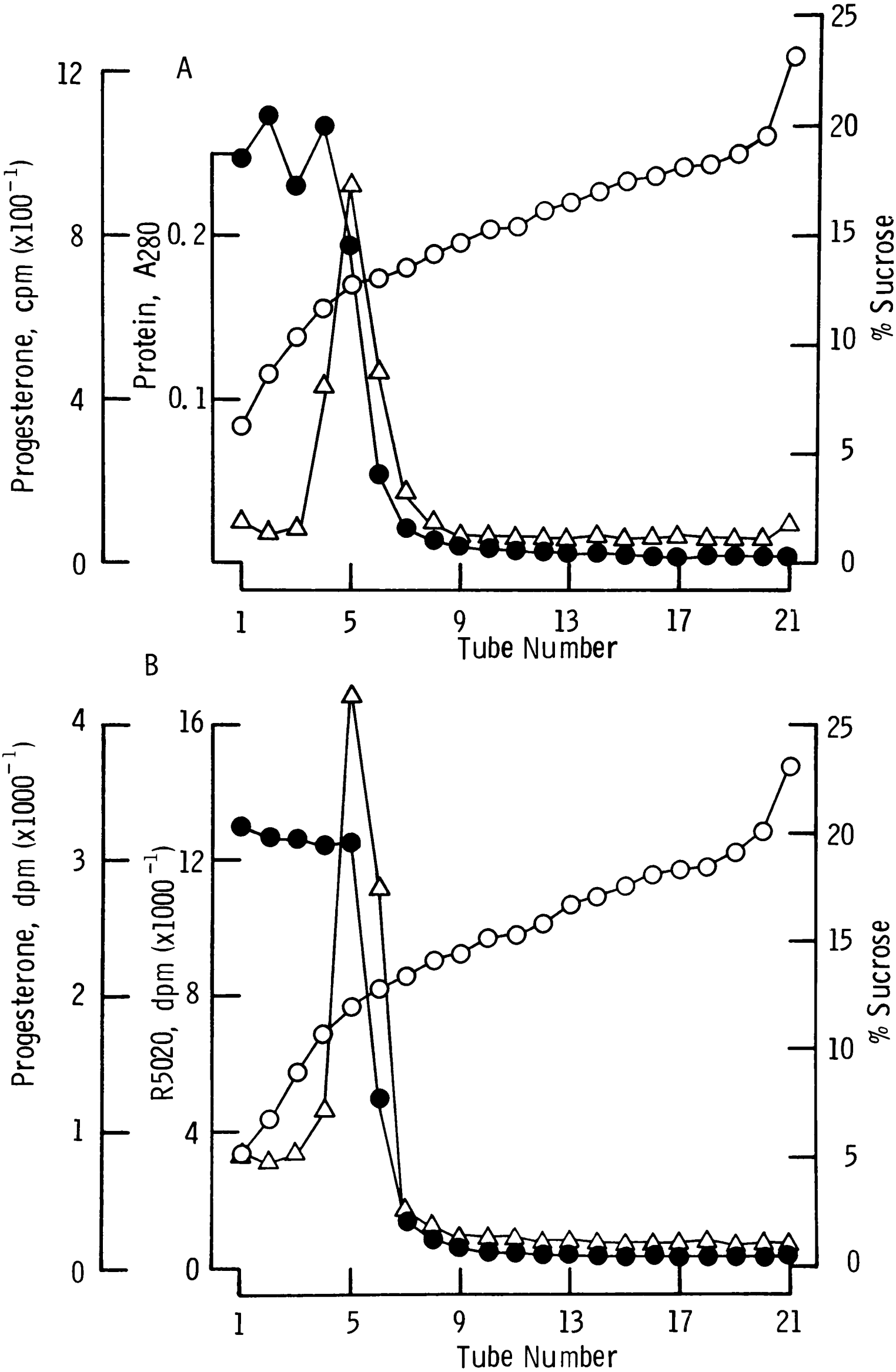

A
Progesterone, cpm (x100^{-1})
Protein, A280
% Sucrose
Tube Number
B
Progesterone, dpm (x1000^{-1})
R5020, dpm (x1000^{-1})
% Sucrose
Tube Number

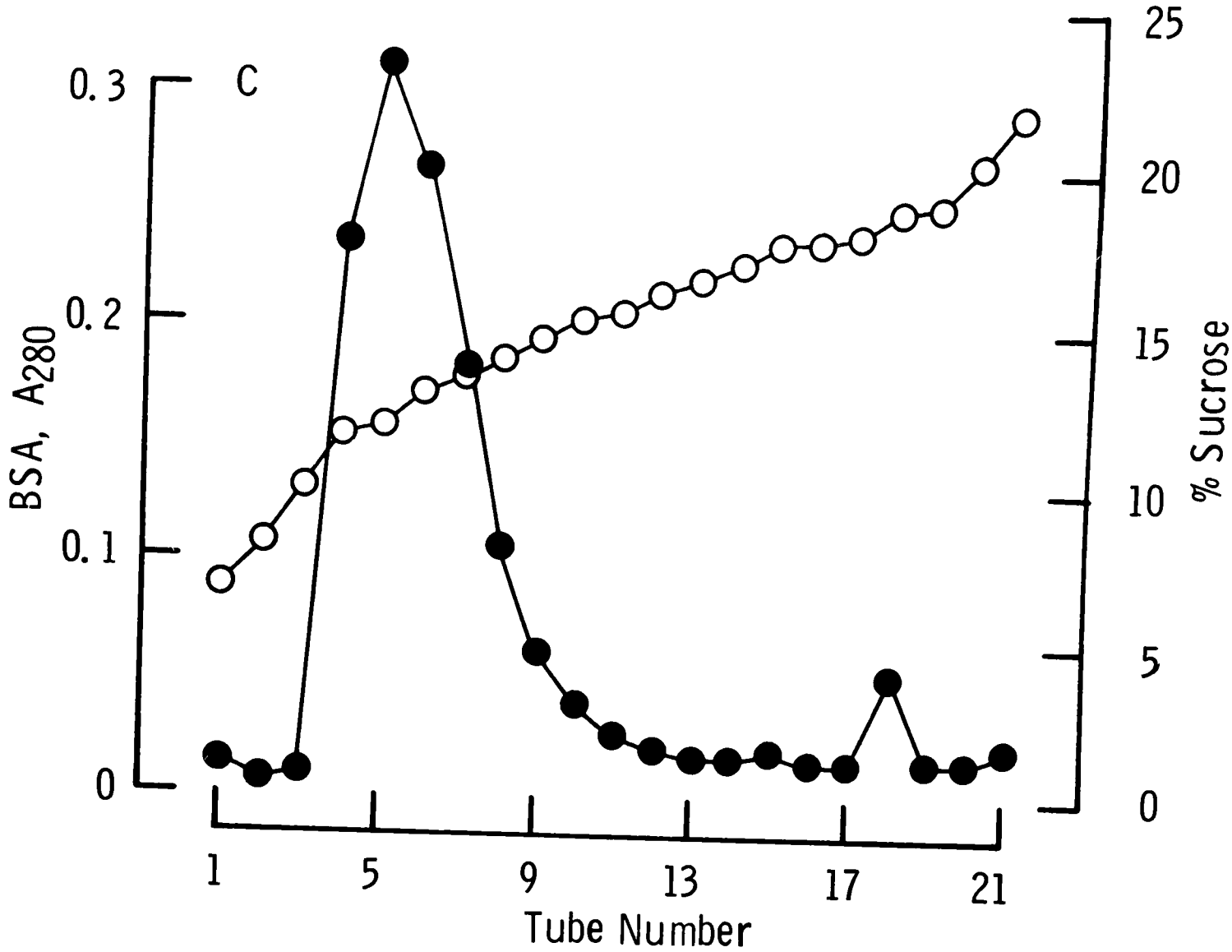
Fig. 4-20C,D: Sucrose density gradient centrifugation of BPI purified from the cytosolic fraction of bovine corpus luteum

C) ● = BSA, A_{280}

○ = % Sucrose, w/w

D) ● = Cortisol, cpm.0.5 ml^{-1}

○ = % Sucrose, w/w


C
0.3
0.2
0.1
0
BSA, A280
25
20
15
10
5
0
% Sucrose
1
5
9
13
17
21
Tube Number

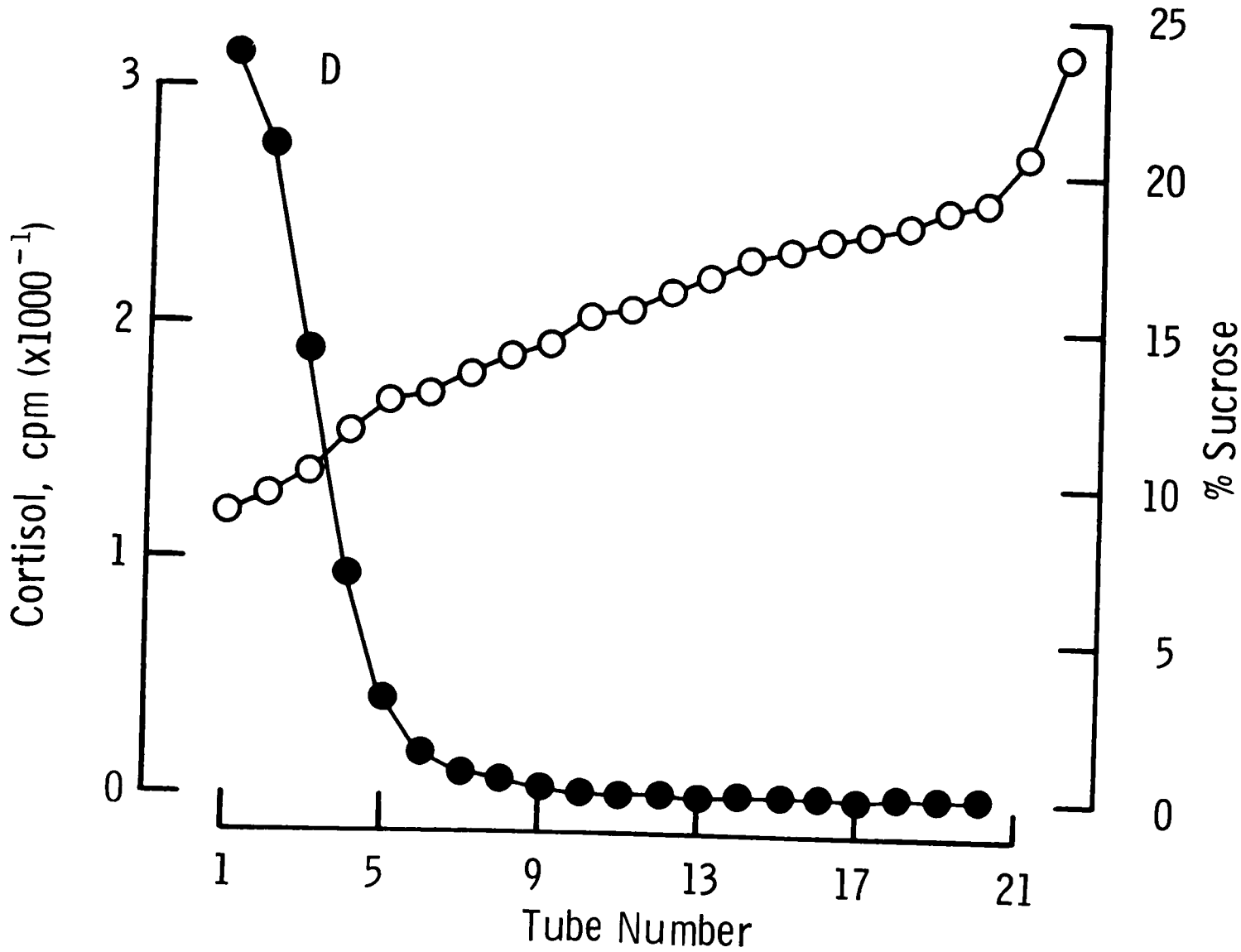
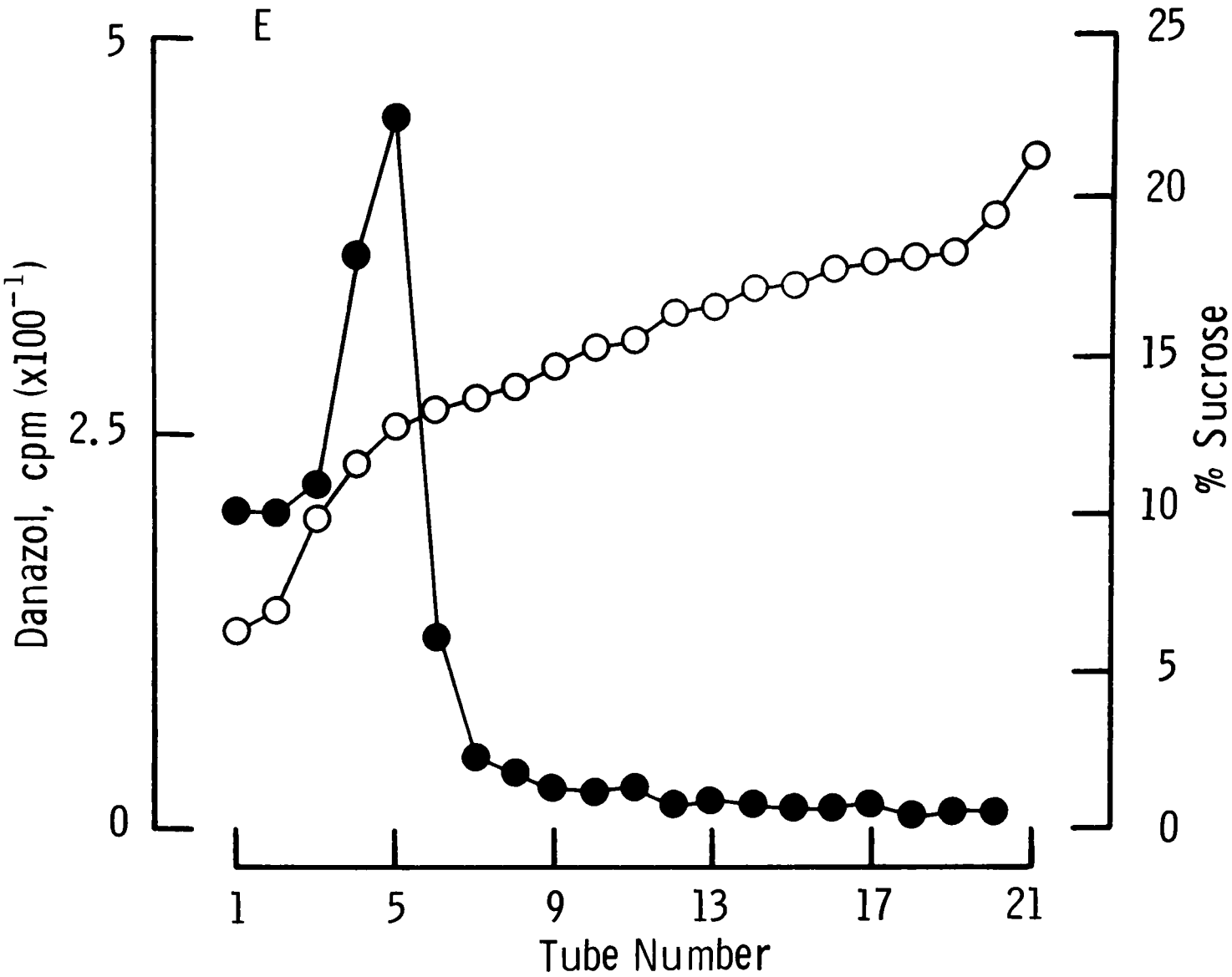

D
3
2
1
0
Cortisol, cpm (x1000⁻¹)
25
20
15
10
5
0
% Sucrose
1
5
9
13
17
21
Tube Number

Fig. 4-20E,F: Sucrose density gradient centrifugation of BPI purified from the cytosolic fraction of bovine corpus luteum

E) ● = Danazol, cpm.0.5 ml^{-1}
○ = % Sucrose, w/w

F) ● = R5020, cpm.0.5 ml^{-1}
○ = % Sucrose, w/w


E
5
2.5
0
Danazol, cpm (x100^{-1})
25
20
15
10
5
0
% Sucrose
1
5
9
13
17
21
Tube Number

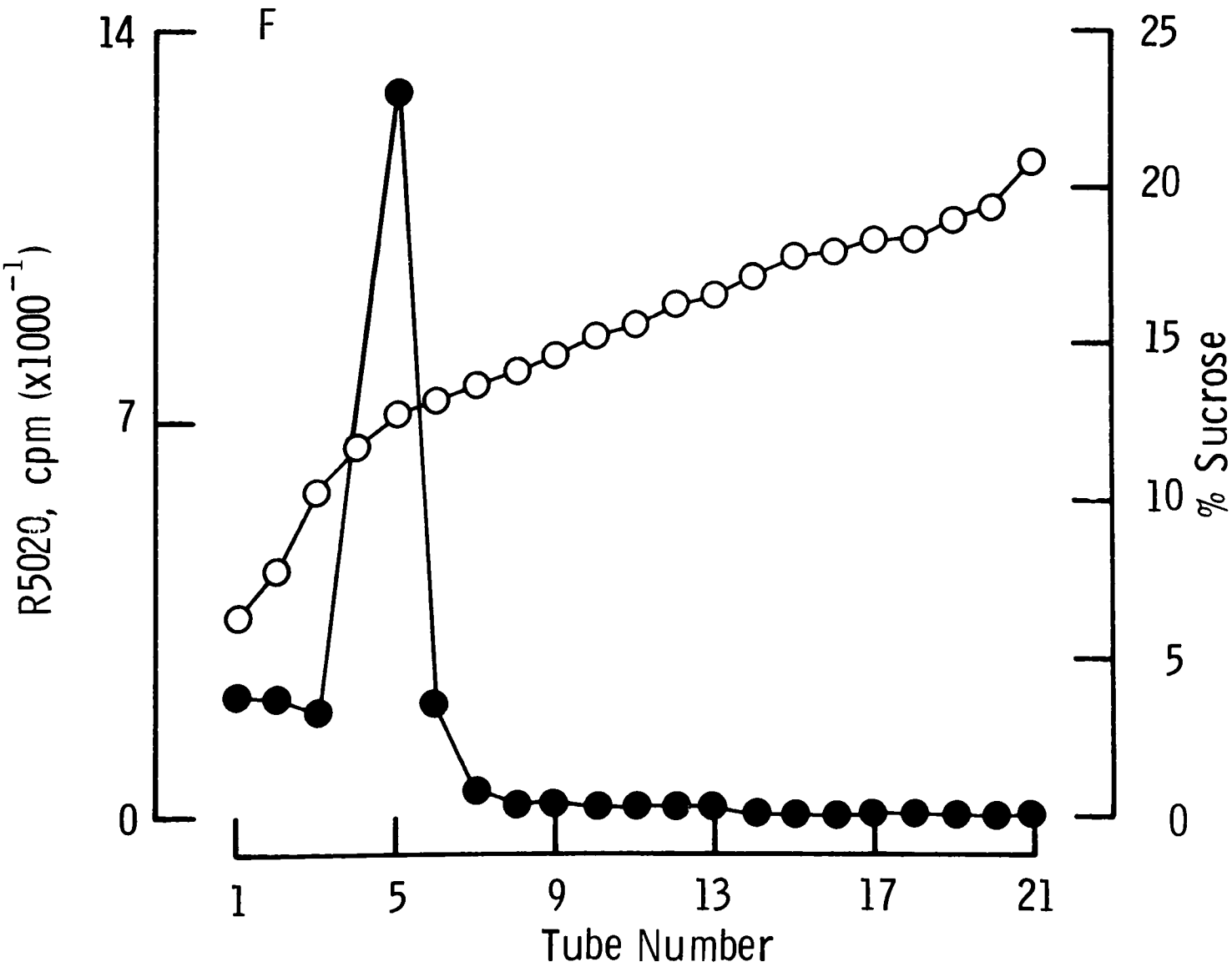

F
14
7
0
R5020, cpm (x1000^{-1})
25
20
15
10
5
0
% Sucrose
1
5
9
13
17
21
Tube Number

Fig. 4-21A,B: Sucrose density gradient centrifugation of BPII obtained from fractionation of the cytosolic fraction of bovine corpus luteum on hydroxylapatite

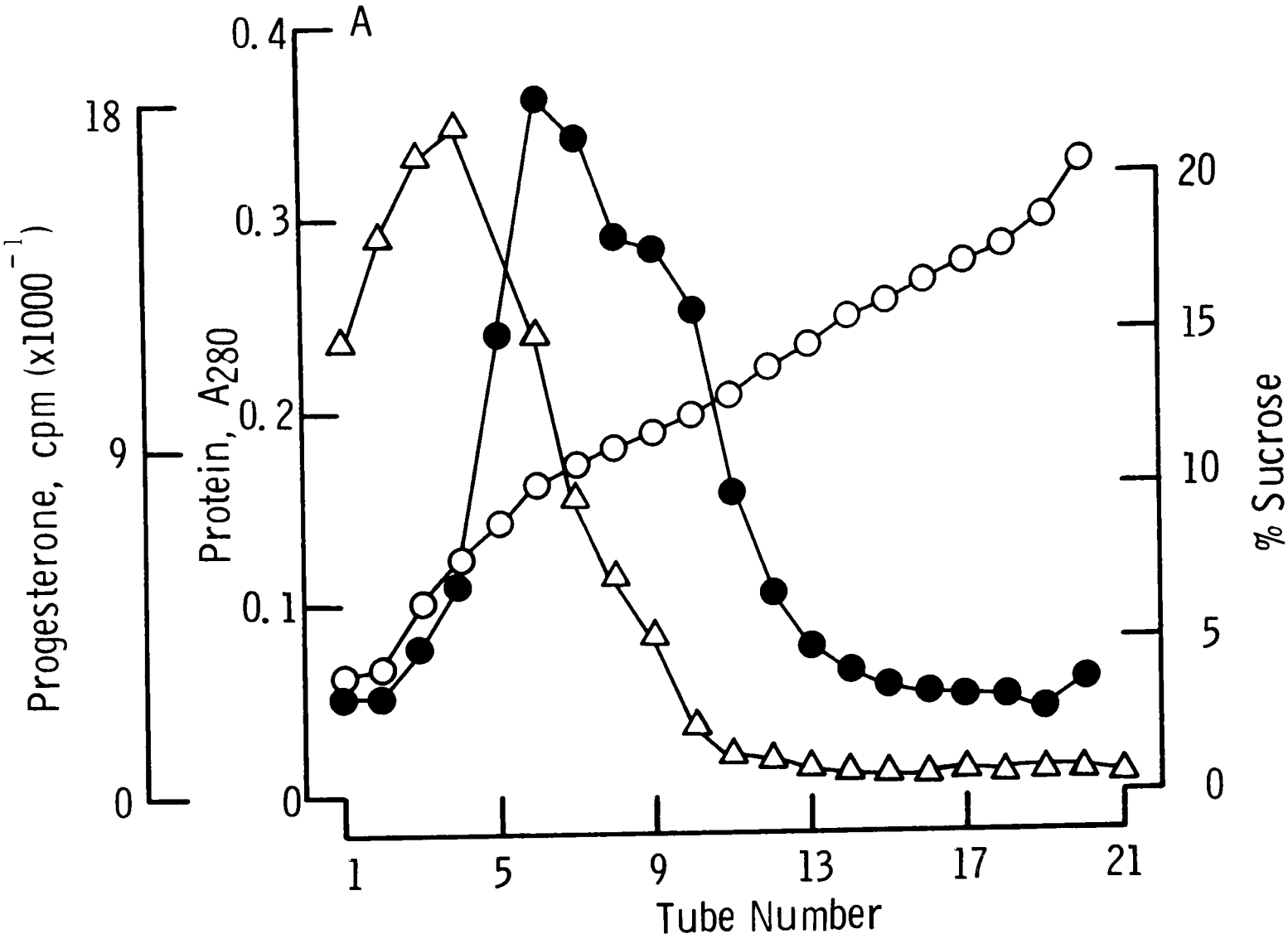
BPII was obtained in a peak eluted from a column of HAP at 650 mM KPB, after BPI had been removed from the column by elution with 40 mM KPB. The BPII preparation was incubated in 0.75 ml aliquots with radioactive steroids, and 0.5 ml (A_{280} = 3.5) of the incubated mixtures were centrifuged in polyallomer tubes containing gradients of 5-20% w/w sucrose as described under Methods (section 4.2.3). Details of the gradients were as follows:

Gradient	Exogenous Steroid	Radio-activity applied	Mass applied	% Recovery of radio-activity[a] from gradient
A	^{14}C-Progesterone	29,740 cpm	0.84 nmol	82
C	^{3}H-Cortisol	37,030 cpm	0.68 pmol	96
D	^{3}H-R5020	25,210 cpm	0.44 pmol	72
E	^{3}H-Danazol	6,950 cpm	0.39 nmol	72
F	^{14}C-Progesterone	35,260 dpm	0.30 nmol	82
	^{3}H-R5020	59,018 dpm	0.31 pmol	71
	Marker			
B	BSA		2 mg	

a: Assumed a loss at 7.5% upon reading of refractive indices.

A) △ = Progesterone, cpm.0.5 ml^{-1}
● = Protein, A_{280}
○ = % Sucrose, w/w

B) ● = BSA, A_{280}
○ = % Sucrose, w/w


A
0.4
0.3
0.2
0.1
0
18
9
0
Progesterone, cpm (x1000^{-1})
Protein, A_{280}
20
15
10
5
0
% Sucrose
1
5
9
13
17
21
Tube Number

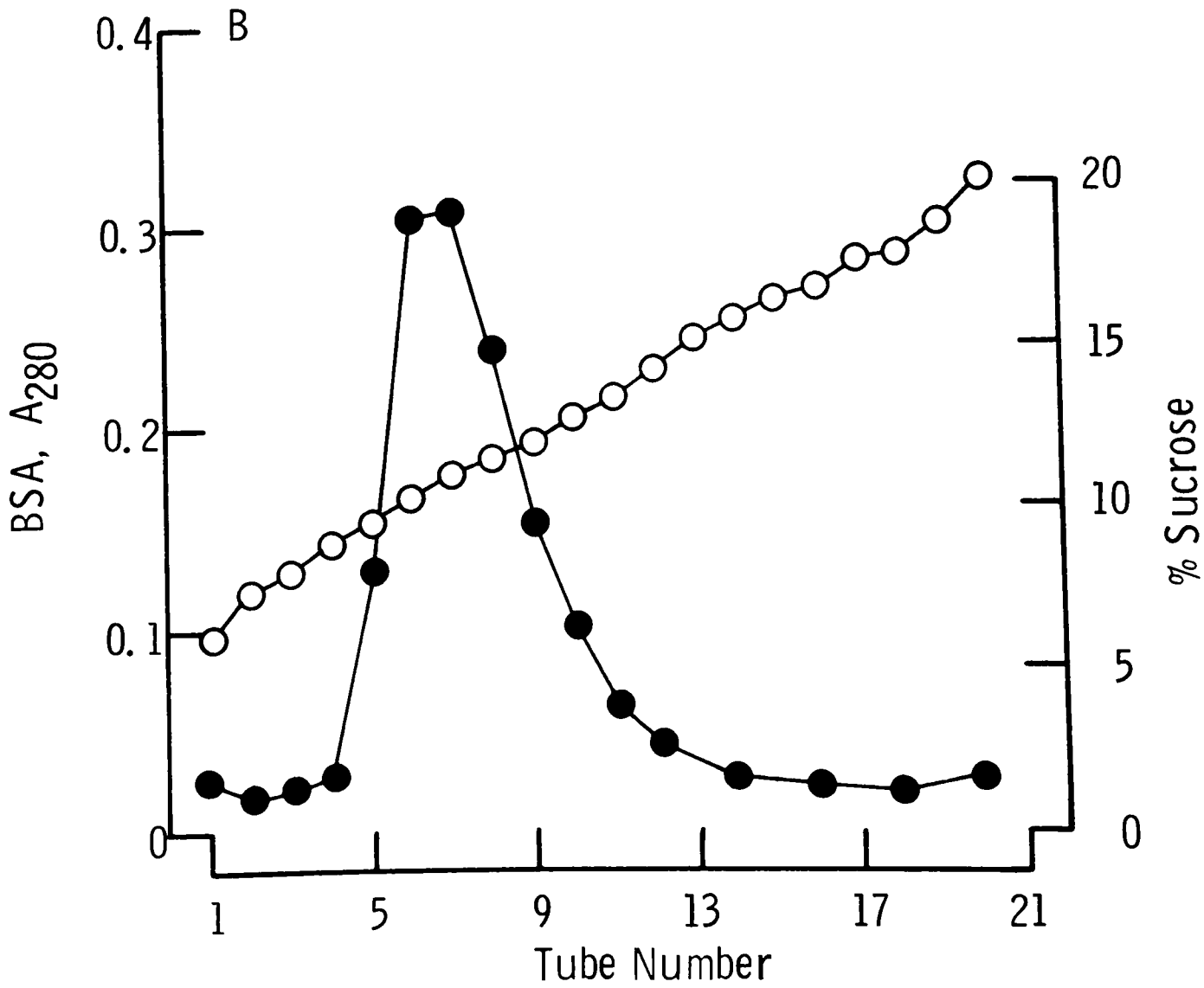

B
0.4
0.3
0.2
0.1
0
BSA, A_{280}
20
15
10
5
0
% Sucrose
1
5
9
13
17
21
Tube Number

Fig. 4-21C,D: Sucrose density gradient centrifugation of BPII obtained from fractionation of the cytosolic fraction of bovine corpus luteum on hydroxylapatite

C) ● = Cortisol, cpm.0.5 ml^{-1}
△ = Protein, A_{280}
○ = % Sucrose, w/w

D) ● = R5020, cpm.0.5 ml^{-1}
△ = Protein, A_{280}
○ = % Sucrose, w/w

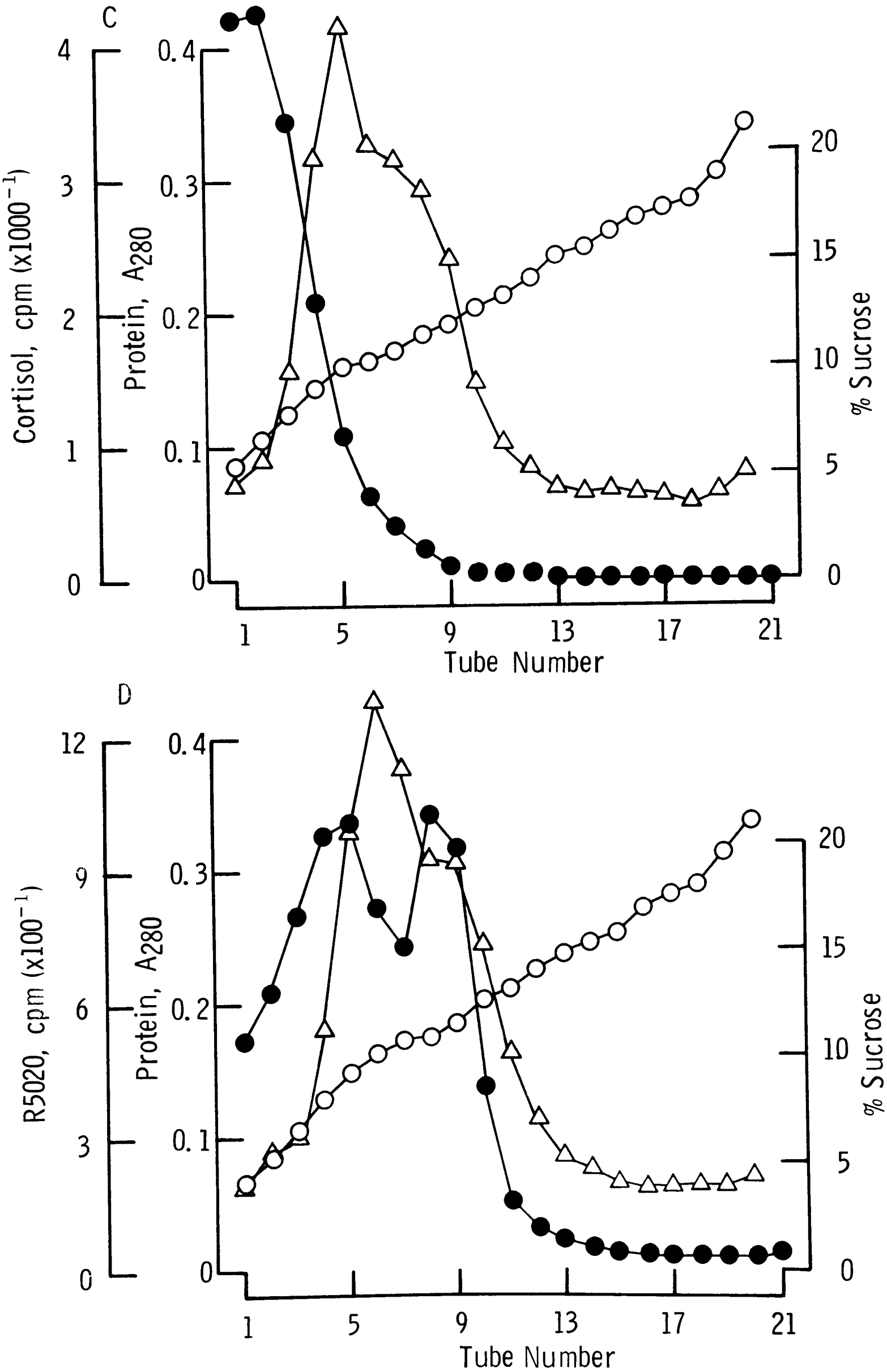
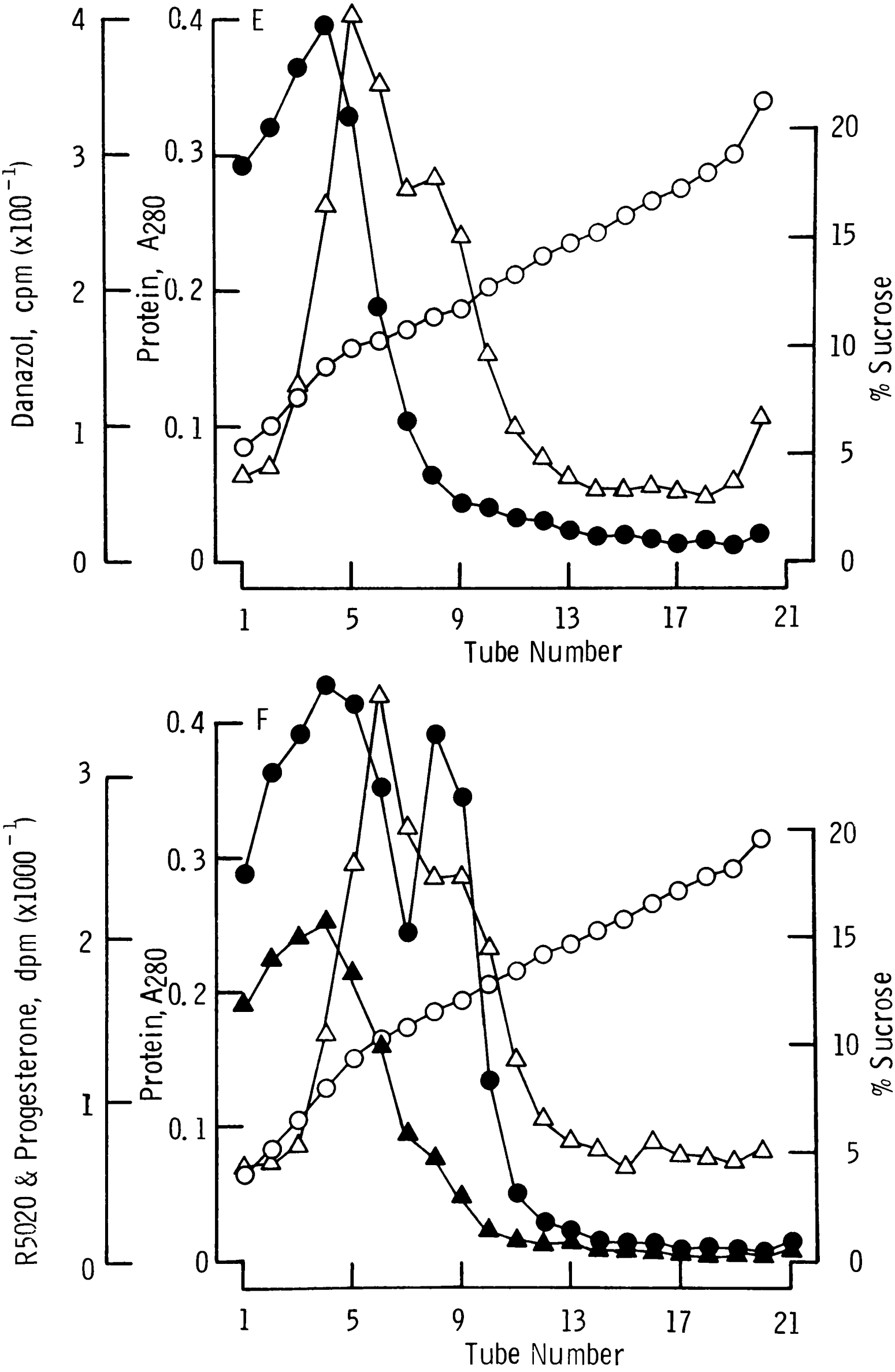

C
Cortisol, cpm (x1000^{-1})
Protein, A_{280}
% Sucrose
Tube Number
D
R5020, cpm (x100^{-1})
Protein, A_{280}
% Sucrose
Tube Number

Fig. 4-21E,F: Sucrose density gradient centrifugation of BPII obtained from fractionation of the cytosolic fraction of bovine corpus luteum on hydroxylapatite

E) ● = Danazol, cpm.0.5 ml^{-1}
△ = Protein, A_{280}
○ = % Sucrose, w/w

F) ▲ = Progesterone, dpm.0.5 ml^{-1}
● = R5020, dpm.0.5 ml^{-1}
△ = Protein, A_{280}
○ = % Sucrose, w/w


E
Danazol, cpm (x100^{-1})
Protein, A_{280}
% Sucrose
Tube Number
F
R5020 & Progesterone, dpm (x1000^{-1})
Protein, A_{280}
% Sucrose
Tube Number

in the radioactivity profiles concomitant with the shoulder in the profile for protein. Some binding of added progesterone and danazol was indicated from the finding that these steroids were maximal 2 fractions further into the gradient than added cortisol. The latter, like BPI, was not bound by BPII. Possibly, progesterone and danazol dissociated from BPII during the course of centrifugation and migrated to lower densities. Added R5020 (Fig. 4-21, D and F) formed two peaks, one at 11.3%-11.5% and another (like added progesterone and danazol) at lower density. The denser peak of R5020 was taken into the gradient specifically and appeared to be bound by the component which occurred as a shoulder to the protein profile at these densities.

Calculation of the sedimentation coefficient and molecular weight according to Martin and Ames (1961) yielded values for BPII of 4.6S and 74,726 respectively. The banding position of BPII was estimated from the maxima of the R5020 peak which occurred at greatest density. Therefore, a comparison of the sedimentation characteristics and relative steroid-binding affinities of BPI and BPII on sucrose gradients, and of their binding parameters as measured by MED, showed that they were different proteins.

4.4 DISCUSSION

The existence of a soluble progesterone-binding activity in bovine corpus luteum was first reported by Leymarie and Gueriguian (1969, 1970). The present study has confirmed and extended their observations. A peak of progesterone-binding was obtained when cytosol was applied to sucrose gradients, which suggested that only one binding component was present, but another binding component, displaying slightly lower affinity for progesterone, was revealed when different aliquots of fractionated cytosol were applied to sucrose gradients.

The progesterone-binding activities detected in the cytosolic fraction of bovine corpus luteum were not due to CBG or a CBG-like protein since added cortisol was not taken into the gradients and the sedimentation coefficient of CBG (3.6S, Bernutz et al., 1978) was less than either of the cytosolic components (4.1S and 4.6S, respectively). The molecular weight of CBG is 52,000 daltons (Muldoon and Westphal, 1967), whereas the molecular weights of BPI and BPII from the corpus luteum were estimated from their sedimentation characteristics on density gradients as 63,000 and 74,700 daltons, respectively. These values for the molecular weights of the binding proteins of the corpus luteum also distinguished them from BSA (67,000 daltons, Martin and Ames, 1961) and SHBG (98,000-115,000 daltons, Gueriguian and Pearlman (1968) or 52,000 daltons, Mercier-Bodard et al. (1970)). Additional evidence that the progesterone-binding proteins of the corpus luteum were distinct from CBG was their retention on HAP at phosphate ionic strengths of 5 mM and above. Under these conditions, CBG is not adsorbed to HAP (Westphal, 1970; Uniyal and Laumas, 1976).

R5020, which does not bind to CBG (Philibert and Raynaud, 1973) and danazol were taken into the gradient to band at the same densities as progesterone. Like R5020, danazol is a synthetic steroid, but its structure is not easily correlated with its properties. It is androgenic in structure but displays progestogenic properties in addition to acting as a weak androgen (Andrews and Wentz, 1975; Potts, 1977). Danazol lowers SHBG concentrations (Wynn, 1977), inhibits ovarian steroidogenesis *in vitro* (Tsang et al., 1979), and competes effectively with progesterone for occupancy of the progesterone receptor in human myometrial and endometrial cytosols (Jenkin and Cookson, unpublished observations). Therefore, its binding to the cytosol components of the corpus luteum might have been anticipated.

Equilibration of exogenous radioactive steroids with cytosol was readily accomplished even in the presence of large amounts of endogenous progesterone (approximately 4.5 $nmol.ml^{-1}$), making it unnecessary to remove the latter prior to the addition of other steroids. This suggests that the rates of dissociation of endogenous progesterone from the binding components were rapid.

Steroid receptor proteins characteristically undergo dis-aggregation from 8-9S forms in the absence of salt to 4S forms in the presence of salt (Erdos, 1968; Shulster et al., 1976). The progesterone-binding activities of the cytosol of corpus luteum remained as 4S entities in the presence or absence of salt, suggesting that they were due to binding proteins rather than receptors. The corpus luteum is not generally considered to be a target tissue for progesterone and therefore would not

be expected to contain a progesterone receptor. However, the corpus luteum is responsive to oestrogen and does have an oestrogen receptor (Kimball and Hansel, 1974). The sedimentation pattern of the glucocorticoid binding protein found in the bovine adrenal cortex was also unchanged when exposed to different salt conditions (Cochet et al., 1977). Its very rapid binding process contrasted with the slower binding kinetics associated with the glucocorticoid receptors in liver cytosol (Cochet and Chambaz, 1974).

A more rapid method than MED for assaying the steroid-binding components of cytosol from corpus luteum, and one which required less cytosol and reagents, was sought. A number of equilibrium and non-equilibrium methods of separating cytosol-bound progesterone (or R5020) from unbound steroid were evaluated. All the methods that were tested were found to be unsuitable for this purpose. Charcoal (usually coated with dextran) has been used to adsorb and precipitate unbound steroid in the assay of oestrogen receptors (Milgrom and Baulieu, 1969; Korenman, 1969; Kimball and Hansel, 1974), progesterone receptors (Haukkamaa and Luukainen, 1974; Kontula et al., 1975), and in steroid radioimmunoassays (Challis et al., 1973; Odell et al., 1975). The interaction between the specific binding components of cytosol and progesterone was destroyed by DCC. The separation of bound from unbound progesterone (or R5020) was attempted using various filters but in all cases the steroid that was bound at equilibrium in solution partitioned with the unbound steroid when the equilibrated mixtures were passed through the different filters.

The demonstration that the binding of progesterone by BPI

was accompanied by a decrease in the protein fluorescence showed that BPI bound the steroid. The association constant of BPI determined by fluorescence titration was comparable to that determined by MED. However, fluorescence titration was useful only for the assay of steroids such as progesterone (containing the Δ^4-3-keto structure) to purified proteins (Stroupe et al., 1975), and so was unsuitable for the determination of the binding activity of cytosols.

Sephadex equilibration was introduced by Pearlman and Crepy (1967) to study the testosterone-binding properties of human serum. When the method was applied to the determination of progesterone-binding by corpus luteum cytosol, progesterone did not partition ideally between the aqueous and gel compartments so that as much as 30% of it was adsorbed to the Sephadex. This appears to be a general phenomenon for steroids; for example, Edwards et al. (1975) reported that 42% of cortisol (2 pmol) was adsorbed to Sephadex in this system. Progesterone appears to be strongly adsorbed to Sephadex, since it eluted behind cortisol from columns of HAP containing Sephadex.

The batch Sephadex technique was unsuitable for the assay of the progesterone-binding activity of cytosol from corpus luteum because it was not possible to obtain ratios of bound to unbound progesterone of $\geqslant 1$, which is an absolute requirement of the method (Pearlman and Crepy, 1967; Puig-Duran, 1977; Ryan et al., 1977). Reduction of the temperature at which the batch Sephadex technique was performed, from 22°C to 4°C, did not improve the bound to unbound steroid ratio or reduce the variability between replicates. The alteration of the ratios of gel to aqueous compartments did not improve the stoichiometry

of binding, and is thought to be a relatively unimportant factor in this technique (Edwards et al., 1975).

The batch Sephadex technique is convenient and straightforward to use, but a better understanding of the factors responsible for the adsorption of steroids to Sephadex is necessary before it can be used routinely in the quantitative measurement of steroid-protein interactions. Pearlman and Crepy (1967) recognized the problem and attempted to allow for it by measuring the unequal distribution of radioactive steroid in tubes containing only buffer and gel. Ryan et al. (1977) showed that the adsorption effect of steroid onto Sephadex was not independent of protein. It caused an overestimate of the binding capacity but other, unknown factors are also operating since the binding capacity determined in parallel by MED was 20-30% higher, which was opposite to what would be predicted by adsorption effects alone (Ryan et al., 1977). The technique was used to determine the relative steroid-binding activities of constant aliquots of column fractions since added radioactive progesterone was not retained by the cytosolic proteins eluted at 100 mM KPB on HAP. In this mode, constant amounts of radioactive progesterone were used so that its adsorption to Sephadex was a systematic effect. BPII did not bind either progesterone or pregnenolone as extensively as BPI when tested by the batch Sephadex technique. This may have been due to the lower association constant of BPII for progesterone and/or to a greater loss of its binding activity upon chromatography.

The use of small columns of Sephadex and other chromatographic media for the separation of cytosol-bound steroid from unbound steroid was also unsuccessful. The progesterone-binding

interactions of corpus luteum cytosol did not remain intact under conditions which were suitable for the measurement of the high-affinity binding of progesterone by human plasma obtained at 22 weeks of pregnancy. The classical methods used to separate unbound from bound steroids, such as DCC, ammonium sulphate precipitation, or MED also did not give reproducible results when used in the assay of cholesterol binding by specific adrenal proteins (Lefevre et al., 1978).

The binding reactions between cytosol and progesterone (or R5020) were established by 30 min and were stable for 50-55 h in solution. However, comparable dissociation of bound progesterone occurred whether cytosol was equilibrated with hormone for 30 min or for longer periods. Dissociation was least when the passage of cytosol through the minicolumns was rapid, as in the case of minicolumns of coarse Sephadex G-25, and when R5020, rather than progesterone, was the binding ligand. R5020 has been used to identify and characterize progesterone receptors in a number of tissues (Wittliff et al., 1977; Horwitz and McGuire 1977; Vu Hai and Milgrom, 1978). It does not bind to plasma proteins such as CBG and PBG, and it dissociates from receptors more slowly than progesterone, although it binds onto them at rates similar to that of the natural steroid (Philibert and Raynaud, 1974, 1977).

The high-affinity interactions between progesterone and the binding proteins of the corpus luteum were formed readily and remained stable in solution for long periods, but were easily destroyed when the conditions of aqueous equilibrium were perturbed. This suggests that the cytosolic progesterone-binding reactions were characterized by similar rates of

association and dissociation (k_1 just greater than k_2). Since the rates of association were not substantially greater than those of dissociation, significant amounts of dissociated steroid were not quickly re-bound to the binding proteins when cytosol was applied to minicolumns of Sephadex. The steroid released by dissociation was weakly adsorbed to the gel particles so that it became progressively less able to reassociate with the faster moving protein compartment. This contrasted with that reported for other binding proteins and receptors. Progesterone was bound to PBG in the pregnant guinea pig some 24-times more rapidly than it was released (Westphal et al., 1977), whilst human CBG bound progesterone and cortisol 4.4-times and 3.6-times, respectively, more rapidly than it released either steroid (Stroupe et al., 1978). The same relationship holds for the progesterone receptor of the chick oviduct, whose affinity for progesterone was 10-fold greater than either the corpus luteum proteins or CBG (Westphal, 1971) for the hormone. Thus, its A and B subunits bound progesterone 150-times and 260-times, respectively, more quickly than they released it at 4°C (Schrader and O'Malley, 1972). The liver receptor of the rat bound dexamethasone 7.3-times more rapidly than it released it (Koblinsky et al., 1972).

The equilibrium association constant of cytosol from bovine corpus luteum determined by MED was somewhat variable. This variation may have been partly due to the use of abattoir material, but sensitivity of the binding proteins to disruption of the tissue and prolonged dialysis in diluted form are also likely to have been important factors. In addition, BPII may be less stable than BPI, since high values for the association

constant of cytosol were generally associated with lower binding capacities (see Table 4-8). The close agreement between duplicate determinations of the binding parameters of fractionated BPI and BPII suggests that methodological variations were not as significant as the biological variations (see Table 4-10).

The extent to which a variety of steroids could compete for the progesterone-binding activity of cytosol was determined by MED. A wide concentration range of competing steroid was used in these studies because the binding proteins of the corpus luteum had large capacities compared with classical steroid receptors. Those steroids whose structures most closely resembled that of progesterone competed most effectively with it for binding (see Table 4-11). Deoxycorticosterone and testosterone were also effective competitors of progesterone binding to PBG (Westphal et al., 1977) and to the uterine progesterone receptor of the rat (Booth and Colas, 1977). In the rabbit, uteroglobin bound 5α-pregnanedione some 3-times more avidly than progesterone but it did not bind R5020 (Milgrom, 1978). This contrasts with the uterine receptors of the rabbit and rat, which bound R5020 1.5-times and 5-times, respectively,better than progesterone (Milgrom, 1978; Vu Hai and Milgrom, 1978). Both 5α- and 5β-pregnanedione were better competitors for progesterone-binding than 20α-dihydroprogesterone to the receptors of human endometrial and myometrial cytosols (Verma and Laumas, 1973; Young and Cleary, 1974). BPI of the corpus luteum was different from the uterine receptors in that it did not bind synthetic progestogens such as R5020 and danazol more avidly than natural progestogens but the overall spectrum of binding was similar to that reported for

other progesterone binding proteins and receptors. This implies that the unusual feature of the high-affinity binding proteins of the corpus luteum, namely the similarity in their rates of steroid uptake and release at equilibrium, was not reflected in the pattern of cross reaction by steroids for progesterone-binding.

Cholesterol did not cross-react with progesterone for binding to cytosolic components, so that the latter were different from the cholesterol carrier proteins of the bovine adrenal cortex (Teicher et al., 1978) and corpus luteum (Kan et al., 1974). The binding proteins of the corpus luteum are also distinct from the pregnenolone and pregnenolone sulphate binding proteins of the adrenal cortex since the latter have lower affinities for their homologous steroids than the corpus luteum proteins and do not bind progesterone significantly (Strott, 1977; Kream and Sauer, 1977; Strott and Lyons, 1978). Progesterone does not occur in plasma in conjugated form (Gower, 1975) so that possible cross-reaction of conjugated steroids to the binding proteins of the bovine corpus luteum was not determined. Pregnenolone sulphate and other conjugated steroids may bind to these proteins or the corpus luteum may contain discrete binding proteins for them. In the blood conjugated steroids are generally considered to be as biologically inactive as protein-bound steroids, although they participate directly in some steroid pathways (Heap et al., 1973), such as in the synthesis of dehydroepiandrosterone sulphate (Roberts and Lieberman, 1970).

It may be significant that danazol was bound by BPI and, to a lesser extent, by BPII. Tsang et al. (1979) have shown

that this steroid can inhibit directly both basal and LH-stimulated progesterone secretion by granulosa or luteal cells isolated from pig ovaries. It inhibits microsomal 3β-hydroxy-steroid dehydrogenase in hamster ovary and rat testis and adrenal *in vitro* (Barbieri et al., 1977). Since danazol is taken in large quantities (up to 800 mg daily; Ward, 1977) for the management of menorrhagia, endometriosis and chronic cystic mastitis (Chimbira, 1977; Ward, 1977; Buckle, 1977), it may also partly manifest its effects through competing for progesterone-binding in the corpus luteum in women.

BPI was identified in the soluble fraction of sheep corpus luteum from its analogous behaviour to the bovine protein on columns of HAP. The presence of BPII in the sheep corpus luteum was not shown directly since radioactive progesterone was not retained by the proteins eluted at 100 mM KPB when either sheep or cow cytosol was fractionated on HAP. MED of the peaks of protein obtained from the fractionation of sheep cytosol was not carried out. Further experiments are required to determine if the sheep corpus luteum contains a binding protein analogous to BPII of the cow corpus luteum.

BPI purified from bovine cytosol was comparable to BPI purified from ovine UOV plasma. Both proteins displayed similar adsorption and elution characteristics on HAP and Sephadex G-200, respectively. Batch Sephadex scanning assays of fractions from the preparative Sephadex G-200 columns showed that both proteins bound progesterone and pregnenolone. The proteins banded maximally at the same density and showed the same relative steroid affinities on sucrose density gradients. The 40 mM binding proteins comprised similar proportions of the

total protein of UOV plasma and luteal tissue (5 and 8%, respectively), and they migrated similarly on polyacrylamide gels to give molecular weights of 64,100 and 66,800, respectively.

The association constant for progesterone of the UOV plasma proteins recovered at 40 mM KPB from HAP was determined in one experiment to be about half that of the same proteins obtained from freshly-prepared cytosol. The plasma had been stored frozen prior to its fractionation on HAP. When BPI from both sources was subjected to MED after further purification on Sephadex G-200, very little high affinity binding for progesterone was obtained. This suggests that BPI lost biological activity as endogenous progesterone was progressively removed during the course of its purification. A similar loss of biological activity during purification has been reported for PBG (Lea, 1973), CBG (Seal and Doe, 1962; Muldoon and Westphal, 1967), SHBG (Gueriguian and Pearlman, 1968; Mercier-Bodard et al., 1970) and various steroid receptors (Tu and Moudrianakis, 1973; Schrader et al., 1977; Kuhn et al., 1977). However, rapid, one-step purifications utilizing affinity chromatographic techniques have enabled retention of the steroid-binding activities of PBG (Cheng et al., 1976), SHBG (Rosner and Smith, 1975; Iqbal et al., 1978), CBG (Rosner and Bradlow, 1975), and the progesterone receptor of the chick oviduct (Kuhn et al., 1975). This approach offers the prospect of purifying both BPI and BPII rapidly so that their progesterone-binding activities are retained.

The progesterone-binding proteins of the corpus luteum were different from each other in ways other than their binding

characteristics. The proteins migrated differently on polyacrylamide gels and BPII banded at greater density on sucrose gradients than BPI to provide an estimated molecular weight some 10,000 daltons greater than BPI (64,500 and 74,700 daltons, respectively). BPII displayed relative steroid affinities on sucrose density gradients that were different from those of bovine cytosol or purified BPI; it bound R5020 better than either progesterone or danazol. When BPII was equilibrated with different steroids and applied to sucrose gradients, progesterone and danazol largely dissociated from the protein during centrifugation to band at positions intermediate between the banding proteins of cortisol and protein. On the other hand, R5020 was recovered as two large peaks, one consisting of dissociated steroid at the same density as dissociated progesterone and danazol, and another which was bound to BPII at greater density (see Fig. 4-21). Small amounts of bound progesterone and danazol were also recovered at the same position as the second peak of R5020. Therefore, during the course of centrifugation on sucrose gradients BPII lost most of its bound progesterone and danazol and 50-60% of its bound R5020. This behaviour reflects its high rate of steroid dissociation under non-equilibrium conditions. The exogenous steroids were not present in excess since very little progesterone, danazol or R5020 was recovered in the sample zone at the top of the gradients.

The heterogeneity of the progesterone- and/or cortisol-binding components in the bovine corpus luteum reported by Leymarie and Gueriguian (1969, 1970) was not confirmed in this study. In their initial fractionation of cytosol on HAP, these

workers obtained three proteins, two of which bound progesterone and cortisol and one of which bound only progesterone (Leymarie and Gueriguian, 1969). They identified one of these proteins as CBG, even though this protein is not adsorbed to HAP (Chader and Westphal, 1968; Uniyal and Laumas, 1976) and they determined that blood contamination of the cytosolic fraction was negligible (Leymarie and Gueriguian, 1969). The protein that bound only progesterone had a high capacity so they concluded that this was probably serum albumin (Leymarie and Gueriguian, 1970). However, serum albimin would be expected to bind cortisol as well as progesterone (Westphal, 1971), so this component may not have been albumin but rather the protein identified as BPII in the present study. The third protein peak was resolvable on HAP using different elution conditions into two steroid-binding proteins, one of which bound both progesterone and cortisol and another which was specific for progesterone (Leymarie and Gueriguian, 1969,1970). In contrast to the results of these workers, two discrete progesterone-binding activities were identified in the present study in the soluble fraction of bovine corpus luteum, neither of which bound cortisol. No evidence was obtained for either CBG or a CBG-like binding component in the corpus luteum. These discrepancies may have been due to the different methods used to measure steroid-binding. Thus, Leymarie and Gueriguian (1969, 1970) used the batch Sephadex technique as developed by Pearlman and Crepy (1967) and Gueriguian and Pearlman (1968) to estimate the K_A for progesterone of the specific binding component as $2.8 \times 10^6\ M^{-1}$. This was 100-fold less than the association constants of the binding proteins determined here

by MED. For the reasons discussed above, the batch Sephadex technique may not be suitable for estimating the progesterone-binding parameters of these proteins.

The synthesis and secretion of steroid hormones is accompanied by the synthesis and secretion of specific proteins in the adrenal cortex (Laychock and Rubin, 1974; Rubin et al., 1974), Leydig cells of the testis (Janszen et al., 1977) and luteal cells (Sawyer et al., 1977; Abel et al., 1977). Electron micrographs of mid-cycle luteal cells of the sheep and cow reveal large numbers of osmiophilic granules in the cytoplasm. These granules are secreted into the extracellular space, where their contents appear to disintegrate. If the granules are vehicles for the secretion of progesterone as has been proposed (Gemmell et al., 1974), they may contain a binding protein for progesterone in order to maintain the hormone in high concentrations. The extracellular spaces into which the granules are secreted are closely juxtaposed to capillaries. Therefore, the finding of BPI in (ovine) UOV plasma was consistent with a granular mode of progesterone secretion. BPI may also have been one of the proteins recovered in the medium after *in vitro* incubation of slices of ovine luteal tissue (Abel et al., 1977).

There are large numbers of lymphatic vessels in the ovary and many of them are organized around the corpus luteum at mid-cycle (Morris and Sass, 1966). The rate of lymph formation and its rate of flow are increased by the presence of a functional corpus luteum (Lindner et al., 1964b) so that there is close contact between lymph vessels and capillaries (Morris and Sass, 1966). BPI (and progesterone) were found in lymph

obtained from vessels draining the ovary at mid-cycle. It has been suggested that lymph may play a role in the transport of steroid hormones (Anderson, 1926; in Morris and Sass, 1966), although Lindner et al. (1964b) have estimated that the amount of progesterone carried in ovarian lymph does not exceed 10% of the total output of the corpus luteum. BPI (and progesterone) presumably drain into ovarian lymph from the extracellular spaces surrounding the luteal cells as well as leaking from adjacent capillaries. Extensive exchange of material between capillaries and lymph has been shown to occur, so that the latter contains 73% of the protein content of plasma (Morris and Sass, 1966).

Since BPI was detected in bovine and ovine JV plasmas the latter circulates generally. If the protein is synthesized by, and released from, the corpus luteum then its concentration in UOV plasma would be expected to be greater than its concentration in peripheral plasma.

The relative steroid specificities of BPI and its physicochemical behaviour on sucrose density gradients, polyacrylamide gels, and HAP columns distinguished it from both CBG and SHBG, the two other major circulating steroid binding proteins in sheep and cows (Seal and Doe, 1965; Murphy, 1968; Martin et al., 1976; Cook et al., 1977). The present study is the first description of this circulating progesterone-binding protein. Previous workers have usually used non-equilibrium techniques for separating bound from unbound steroid in the measurement of binding proteins in blood. Such techniques include the use of DCC (Mercier-Bodard et al., 1970; Suzuki et al., 1977), polyacrylamide electrophoresis (Cook et al., 1977), agar

electrophoresis (Wagner, 1978), filters of DEAE-cellulose (Mickelson and Petra, 1975), and gel filtration (Mercier-Bodard et al., 1970), which are unsuitable for the detection of this protein. When equilibrium techniques have been used, as in the measurement of steroid-binding to CBG (Westphal, 1970, 1971) or SHBG (Pearlman and Crepy, 1967), progesterone has either not been used as the binding ligand or its binding to high-affinity blood proteins has been carried out in the presence of CBG (Davies and Ryan, 1973; Davies et al., 1974), PBG (Westphal, 1978), AAG (Westphal and Ganguly, 1968) or uteroglobin (Fridlansky and Milgrom, 1976). The latter proteins have rates of steroid association considerably in excess of dissociation so that binding of progesterone to BPI would have been masked.

It may be significant that during human pregnancy, when the total circulating concentrations of cortisol and progesterone both rise (Heap et al., 1973), the absolute amount of progesterone bound to CBG and to unidentified high-affinity proteins rises (Rosenthal et al., 1966). Nonetheless, the relative distribution of the hormone among free, albumin-bound, and high-affinity-bound compartments remains constant (Rosenthal et al., 1966).

In conclusion, the corpora lutea of cows and sheep have been shown to contain a high-affinity progesterone-binding protein (BPI), which is distinct from CBG. This protein was also detected in UOV and JV plasmas, suggesting that it also circulates. The bovine corpus luteum (and possibly the ovine corpus luteum) contains another progesterone-binding protein (BPII) which has a greater capacity, together with a slightly lower affinity, for the hormone. The functional significance

of these differences in binding characteristics of the two proteins with respect to intracellular transport and secretion of progesterone from luteal cells remains to be elucidated.

CHAPTER 5

GENERAL DISCUSSION

GENERAL DISCUSSION

The proposal that steroid hormones are secreted from endocrine cells by exocytosis of hormone-containing granules (Rubin et al., 1974; Gemmell et al., 1974) has been investigated with respect to the secretion of progesterone from the bovine corpus luteum. Three conditions are required for granular secretion of progesterone to occur. Firstly, the concentration of secretory granules in the cytoplasm of luteal cells should vary directly with the rate of progesterone secretion, since steroid hormones are secreted as soon as they are formed (Short, 1965; Jaanus et al., 1970; Short, 1972; Heap et al., 1973). Secondly, some of the progesterone in luteal cells should be localized within granules and therefore be separable from the soluble, newly-synthesized steroid. Thirdly, specific proteins are required to constrain progesterone within membrane-bound granules at concentrations above those of the surrounding milieu. These proteins may also participate in the biosynthesis of the hormone, either as intracellular carrier proteins or as components of multi-constituent enzyme complexes. Three different, but complementary, experimental approaches were followed in this thesis, each designed to provide information on these different aspects of granular steroid secretion.

Previous ultrastructural observations have shown that the first condition, namely, correlation of large numbers of granules and evidence of their secretion with high rates of progesterone biosynthesis, is met in the corpora lutea of sheep and goats (Gemmell et al., 1974, 1977b; Corteel, 1975). The number of small osmiophilic granules (0.2-0.4 μ in diameter)

in cat adrenocortical cells rose 4-fold when stimulated by ACTH to secrete glucocorticoid (Gemmell et al., 1977a), and secretion of granules is a feature of avian interrenal cells (Belt et al., 1965). Secretory granules were also prevalent in the cytoplasm of bovine luteal cells and they were commonly observed in the extracellular space immediately adjacent to the margin of the luteal cells during mid-cycle. Conversely, at the beginning and end of the oestrus cycle, granules were much less prevalent intracellularly and were rarely observed in the extracellular space. Therefore, the correlation of secretory granules with high secretory rates also applies to the corpus luteum of the cow.

Direct evidence of the secretory process was unattainable by electron microscopy. However, granules located outside of the luteal cell did not possess outer membranes, which is consistent with the view that they were extruded from luteal cells by exocytosis, analogously to exocytosis of chromaffin granules from adrenal medulla cells (Douglas, 1966, 1974b). The adrenal medulla serves as the prototype tissue on which this concept is based, since an overwhelming amount of biochemical and morphological evidence has accrued to support the idea that exocytosis is the underlying mechanism by which acetylcholine stimulates the chromaffin cells to secrete catecholamines (Douglas, 1966, 1974b; Smith and Winkler, 1972). The influx of calcium is thought to trigger the process, but it is not known if the participation of calcium is essential to the exocytosis process (Douglas, 1974b). However, steroidogenesis in isolated adrenal cells is stimulated by calcium (Neher and Milani, 1978). Another unresolved aspect of

granular secretion in the bovine corpus luteum was the lack of discernible membrane recovery structures such as membrane vesicles. Conversely, in tissues such as the adrenal medulla and neurohypophysis, endocytosis of membrane vesicles accompanies exocytosis of hormone-containing granules (Douglas, 1974b; Allison and Davies, 1974).

Movement of the secretory granules to the outer edge of the cell in a number of different endocrine glands is generally thought to be mediated by interaction with microtubules (Lacy et al., 1968; Poisner and Berstein, 1971; Stephens and Edds, 1976; Gemmell, et al., 1977c) although visual evidence of this is rarely obtained (Gemmell et al., 1977a). The densely-staining granules of bovine luteal cells did not appear to be associated with microtubules even though these cells were secreting large amounts of steroid. However, microtubules were not prominent in well-fixed luteal tissue, so their participation in granule secretion cannot be ruled out, especially as secretion of progesterone and granules was disrupted in the ovine corpus luteum by administration of colchicine *in vivo* (Gemmell et al., 1977c).

During the most active phases of progesterone secretion (days 7-17 of the oestrus cycle), the secretory granules were present in large numbers even though they comprised only 2-3% of the cytoplasm of large luteal cells. However, differential centrifugation of luteal tissue homogenates showed that up to 40% of the progesterone of the corpus luteum was particulate, and that this hormone co-migrated with densely-staining granules on density gradients. Therefore, sufficient progesterone may be in granules for them to represent the major mode of steroid

hormone secretion from the bovine corpus luteum. The coincidence of progesterone and electron-dense granules on sucrose gradients provides support for the concept that the hormone is sequestered in the granules.

Quantitative morphological analysis of bovine luteal cells revealed that ger and polysomes increased as the oestrus cycle progressed, suggesting that increased protein synthesis accompanies increased steroidogenesis. The concommitant production and secretion of protein with steroid may be a consistent feature of steroidogenic cells since it has also been demonstrated in the corpora lutea of sheep and dogs (Abel et al., 1977; Sawyer et al., 1977), in rat testicular cells (Janszen et al., 1977) and in the adrenocortical cells of the cat (Laychock and Rubin, 1974; Rubin et al., 1974). The elevated level of protein synthesis occurring during mid-cycle in the bovine corpus luteum may be directed toward production of the binding proteins that are present in this tissue.

One (or both) of these binding proteins may function as the steroid-binding component in the secretory granules. However, such a function for one of these proteins, and their possible role in progesterone biosynthesis, remains to be demonstrated. A number of intracellular binding proteins have been described recently in other endocrine tissues, each with steroid affinities comparable to those that the corpus luteum proteins display for progesterone. It is possible that BPI is just one of a number of circulating binding proteins which are each specific for different steroid hormones. Thus, adrenocortical cells may produce a protein which binds various glucocorticoids specifically and is secreted with them in granules,

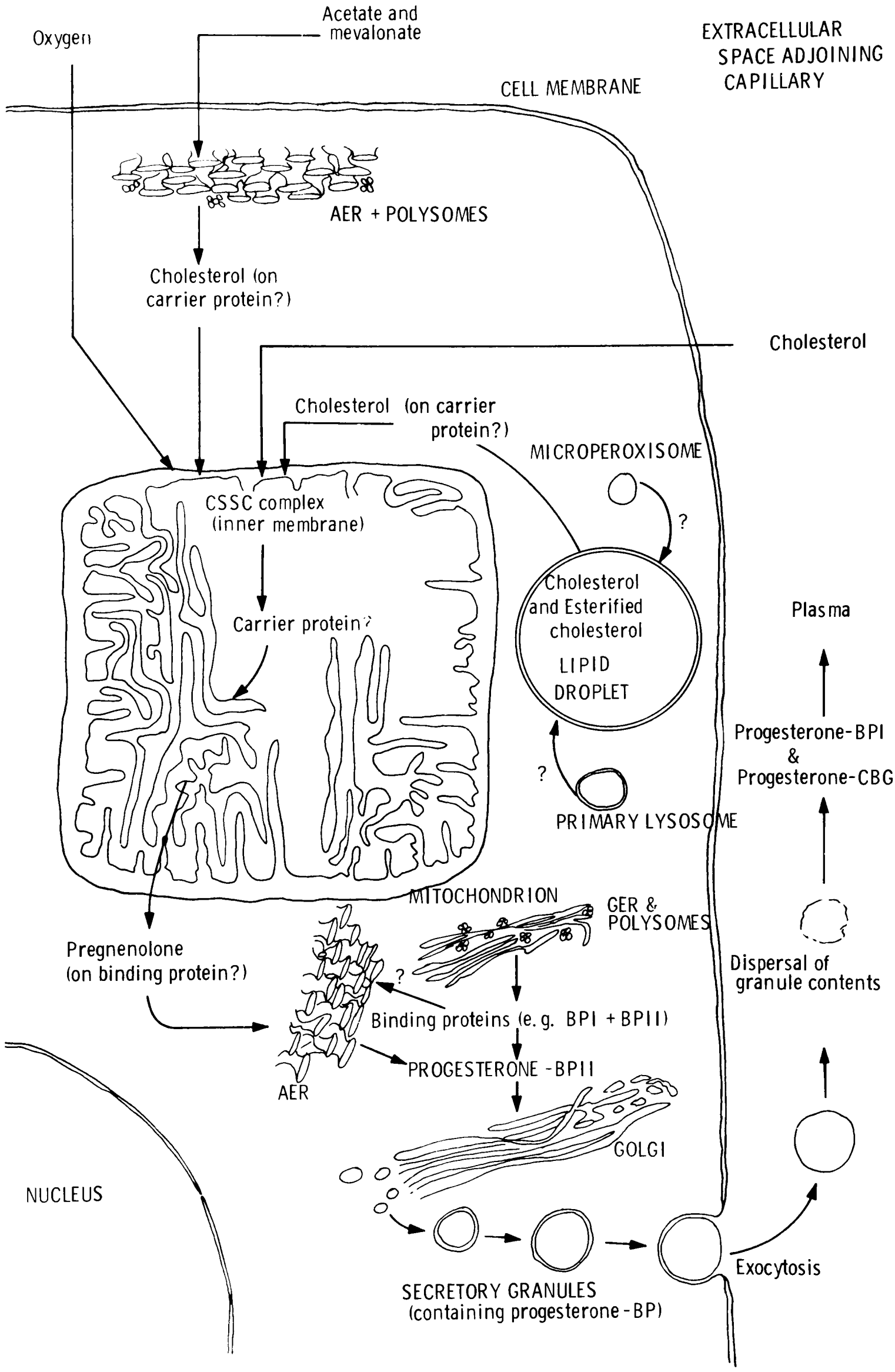
analogously to the secretion of progesterone in granules from the corpus luteum (Gemmell et al., 1974, 1976; this study). Secretory granules are found in the adrenal cortex of the cat (Gemmell et al., 1977a). A soluble glucocorticoid binding protein displaying rapid association and dissociation kinetics comparable to the binding kinetics of the progesterone binding proteins of the corpus luteum has been described in the cow adrenal cortex, but it is not known if the protein also occurs in blood (Cochet et al., 1977). Pregnenolone and its sulphate are secreted by the adrenal cortex, which contains discrete, high-affinity binding proteins for both substances. However, in the blood, these steroids are bound specifically to different proteins whose other properties are as yet undefined (Kream and Sauer, 1977; Strott, 1977; Strott and Lyons, 1978). The adrenal and luteal steroid binding proteins may fulfill comparable intracellular roles. If the proteins produced by the adrenal are only functional intracellularly, the corpus luteum may possess a pregnenolone-binding protein, since pregnenolone is an intermediate in the synthesis of progesterone. Specific binding proteins may transport steroids between different cell-types of an organ. For example, ABP is formed in the rat testis and transported with androgen to the epididymis in the efferent duct fluid (French and Ritzen, 1973) and, possibly, more slowly through lymph (Rommerts et al., 1976).

A role for specific cholesterol carrier proteins and steroid binding proteins in the biosynthesis and secretion of progesterone from the bovine corpus luteum is proposed in Fig. 5-1. Cholesterol is taken up directly from the blood, synthesized *de novo* in the aer, or mobilized from stores in

Fig. 5-1: Schematic representation of the biosynthesis and secretion of progesterone from a bovine luteal cell

Translocation of cholesterol and steroids between the different subcellular organelles is mediated by binding to specific, high-affinity proteins. Progesterone is packaged with protein into membrane-bound granules, which are secreted from the cell by exocytosis.

References: Bjersing, 1967b; Christensen and Gillim, 1969; Fawcett et al., 1969; Flint and Armstrong, 1971b; Gower, 1975a,b; Gemmell et al., 1974, 1977a; Strott, 1977.


Oxygen
Acetate and mevalonate
EXTRACELLULAR SPACE ADJOINING CAPILLARY
CELL MEMBRANE
AER + POLYSOMES
Cholesterol (on carrier protein?)
Cholesterol
Cholesterol (on carrier protein?)
MICROPEROXISOME
CSSC complex (inner membrane)
?
Carrier protein?
Cholesterol and Esterified cholesterol
LIPID DROPLET
Plasma
?
PRIMARY LYSOSOME
Progesterone-BPI & Progesterone-CBG
MITOCHONDRION
GER & POLYSOMES
Pregnenolone (on binding protein?)
Dispersal of granule contents
?
Binding proteins (e.g. BPI + BPII)
PROGESTERONE -BPII
AER
GOLGI
NUCLEUS
Exocytosis
SECRETORY GRANULES (containing progesterone-BP)

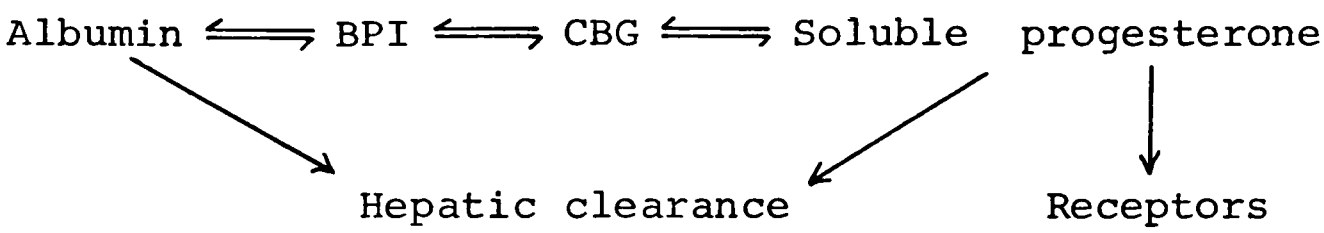
lipid droplets. Although cholesterol readily passes across the cell membrane, its movement through luteal cytoplasm to mitochondria may be mediated by a specific carrier protein such as sterol carrier protein (SCP; Ritter and Dempsey, 1971, 1973) or SCPI of Scallen et al. (1975). At least two other binding proteins for cholesterol have been described in steroidogenic tissues (Kan et al., 1972, 1974; Lefevre et al., 1978; Strott and Lyons, 1978), so that the cleavage of its side chain and movement within the mitochondrion may be protein-mediated also. Proteins occur with cholesterol within lipid droplets (Boyd et al., 1975), which may account partly for their osmiophility. It is possible that one of the cholesterol binding proteins described above sequesters the sterol in lipid droplets, or facilitates its translocation to mitochondria (Boyd et al., 1975). Cholesterol is compartmentalized functionally as well as structurally in luteal cells, so that not all of it exchanges readily with exogenous radioactive sterol or is available for promoting steroidogenesis in the short term (Major et al., 1967; Flint and Armstrong, 1971a,b; Dorrington, 1977). This compartmentalization may be enhanced by protein-binding effects. Microperoxisomes and lysosomes have both been implicated in the mobilization of the cholesterol stored in lipid droplets (Gulyas and Yuan, 1975; Szego, 1976; Elfont et al., 1977), and it has been suggested that they contain hydrolases and/or other enzymes for achieving this (Szego, 1976).

Once pregnenolone has been formed, it must be translocated to the aer, where 3β-HSD is situated (Savard and Telegdy, 1965; Savard, 1973; Gower, 1975a). It is likely that this step is also accomplished by movement of pregnenolone bound to a

specific protein since this would provide unidirectional transport of the steroid from the mitochondria to the aer. Diffusion of pregnenolone to aer would result in considerable amounts of the steroid becoming associated with organelles other than aer, even though this organelle is the major component of the cytoplasm. In steroidogenic cells such as the adrenocortical cells, binding of pregnenolone to protein may control the rate at which pregnenolone enters different steroid pathways.

Protein synthesis occurs concommitantly with steroidogenesis, as discussed above and indicated by the large numbers of polysomes scattered among the membranes of aer together with significant areas of ger in bovine luteal cells (see Chapter 2). This protein synthesis may be directed toward the provision of intracellular sterol and steroid binding proteins, especially those that leave the cell together with progesterone in granules. One of the progesterone binding proteins in the corpus luteum (BPI) binds the hormone with greater affinity but lower capacity than the other protein (BPII). Thus, progesterone may be bound to BPII after its synthesis in the aer and may be transferred by it to Golgi complexes. The latter form an increased proportion of luteal cell cytoplasm during mid-cycle. Progesterone may then be transferred in the Golgi membranes to BPI by virtue of its greater affinity for this protein and then packaged in high concentration with the protein into membrane-bound granules. These granules then move through the cytoplasm, via the microtubular system or by some other mechanism. At the cell border the granules undergo exocytosis, and their contents are released into the extracellular space.

Alternatively, the protein-bound hormone is secreted directly from the luteal cell. In either case, progesterone is extruded from the cell bound to BPI, from where it gets taken up by the blood and participates in the following interactions:



Although both corpus luteum binding proteins were recovered in the soluble fraction, their association with mitochondria and endoplasmic reticulum *in vivo* is not precluded since homogenization of the tissue may have disturbed their subcellular locations.

It is not known if BPII also circulates. It is unlikely that BPI recovered in cytosolic fractions of sheep and cow corpora lutea was derived from blood since it comprised approximately the same proportion of protein in both corpus luteum tissue and blood. Also, cortisol was not bound by the proteins of the cytosolic fraction. Leymarie and Gueriguian (1969) found that blood contamination of corpus luteum cytosol was minimal.

It is recoginized that many of the steps depicted in Fig. 5-1 are highly speculative. A direct demonstration of progesterone in granules is crucial to obtaining acceptance of the concept of secretion of steroids via granules. However, the granules comprise only 2-3% of the luteal cell cytoplasm, and less of the total volume of the tissue, so their isolation in quantities sufficient to enable a biochemical analysis of their composition is unlikely to be achieved with present technology. Therefore, *in situ* methods of demonstrating the localization of progesterone in granules must be sought, and

it is here that steroids present problems which do not occur with proteineous secretory products.

One way of testing the possibility of simultaneous diffusion and packaging into granules of steroids would be to determine their localization *in situ* at a particular instance and at timed intervals following exposure of the tissue to radioactively-labelled substrates. The latter, autoradiographic, approach has been successfully applied to the elucidation of the pathway and kinetics of zymogen granule secretion (Jamieson and Palade, 1967a,b; Palade, 1975). However, the intracellular localization of steroids, unlike proteins, is made difficult by the lack of suitable substrates and by the fact that the subcellular location of steroids is not maintained by the usual aldehyde fixation procedures of electron microscopy (Enders, 1973). This has long bedevilled attempts to determine the pathways of steroid synthesis and secretion at the subcellular level although it has been reported at the histochemical level (Sar and Stumpf, 1973,1974). It was not overcome by introducing frozen-section autoradiography (Christensen and Gillim, 1969) since this technique does not provide sufficient preservation of fine structure to enable the unequivocal location of subcellular components as small as the secretory granules of luteal cells.

Future work will be directed toward purifying BPI and BPII so that their biological activities are preserved. With anterisa against these proteins, it may be possible to demonstrate the presence of one or both of the proteins inside secretory granules by immunocytochemistry. The use of protein antigens will avoid the fixation problems associated with steroid antigens.

It will also be necessary to determine if BPII occurs in blood and to investigate the circulating concentration of BPI (and possibly BPII) throughout the oestrus cycle and during pregnancy in ruminants. More wide-ranging questions arising from this thesis include whether or not similar progesterone binding proteins occur in women and, if so, the clinical implications of the ability of synthetic steroids such as danazol and norgestrel to compete with them for progesterone-binding.

ADDENDUM: SOURCES OF MATERIALS

All chemicals used were analar grade and, unless specifically stated, were used without further purification.

Chemical	Source
Sucrose	Hopkins and Williams
N-2-hydroxyethylpiperazine-N'2-ethane-sulphonic acid (HEPES)	Sigma
Tris-(hydroxymethyl)-aminomethane, 7-9, 99-99.5%	
Trizma 7.0	
Cytochrome c, type III from horse heart	
Inosine diphosphate, sodium salt	
Dithioerytritol (DTE)	
3,3'-Diaminobenzidine-tetrahydrochloride (DAB)	
Dichlorophenol indophenol (DCPIP)	
Hydroxylapatite, type I	
Nicotinamide adenine dinucleotide, reduced form (NADH), grade II	
Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), type I	
Charcoal, activated	
Bovine serum albumin (BSA), fraction V	
Alcohol dehydrogenase (ADH), from yeast	
p-Nitrophenol	
p-Nitrophenyl phosphate, sodium salt	
p-Nitrophenyl-β-D-glucuronide, sodium salt	
2-Amino-2-methyl-1,3-propandiol (AMPD)	
Coomassie blue (R250)	
Sodium deoxycholate	

Chemical	Source
Δ^4-Pregnen-3,20-dione (progesterone)	Sigma
Δ^5-Pregnen-3β-ol-20-one (pregnenolone)	
Δ^4-Pregnen-11β,17α,21-triol,-3,20-dione (cortisol)	
$\Delta^{1,3,5\,(10)}$-oestratrien-3,17β-diol (oestradiol-17β)	
$\Delta^{1,3,5\,(10)}$-oestratrien-3,17α-diol (oestradiol-17α)	
$\Delta^{1,3,5\,(10)}$-oestratrien-3-ol-17-one (oestrone)	
Δ^4-Pregnen-18-al-11β,21-diol-3,20-dione (aldosterone)	
Δ^4-Androsten-3,17-dione (androstenedione)	
Δ^5-Androsten-3β-ol-17-one (dehydroepiandrosterone)	
Δ^4-Androsten-17β-ol-3-one(testosterone)	
Δ^5-Cholesten-3-ol (cholesterol)	
Δ^4-Pregen-21-ol-3,20-dione (deoxycorticosterone)	
5α-Androstan-17β-ol-3-one (DHT)	
5α-Pregnane-3,20-dione	
17α-Hydroxy-Δ^4-pregnen-3,20-dione	
3β,17α-Dihydroxy-Δ^5-pregnen-20-one	
dl-13β-Ethyl-17α-ethynyl-17βhydroxygon-4-ene-3-one (norgestrel)	
Petroleum ether, nanograde	Mallinkrodt
2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium (INT)	British Drug Houses
Triton X-100	
Biogel P-10	Bio-Rad Labs
2,5-Diphenyloxazole (PPO)	Koch-Light Labs
1,4-Di{2-(5-phenyloxazolyl)}benzene (POPOP)	

Chemical	Source
{1,2,6,7-^{3}H} Progesterone, 80-100 Ci/mmol	The Radiochemical Centre
{4-^{14}C} Progesterone, >50 mCi/mmol	
Δ^{5}{7(n)-^{3}H} Pregnenolone, 10-25 Ci/mmol	
{1,2,6,7-^{3}H} Cortisol, 80-105 Ci/mmol	
{4-^{14}C} Cortisol, >50 mCi/mmol	
{2,4,6,7(n)-^{3}H} Oestradiol-17β, 85-110 Ci/mmol	
{2,4,(n)-^{3}H} Oestradiol-17α, 40-60 Ci/mmol	
{2,4,6,7-^{3}H} Oestrone, 80-110 Ci/mmol	
{1,2,6,7-^{3}H} Androst-4-ene-3,17-dione, 80-110 Ci/mmol	
{1,2,6,7-^{3}H} Dehydroepiandrosterone, 60-90 Ci/mmol	
{1α,2α(n)-^{3}H} Testosterone, 45-60 Ci/mmol	
{1,2-^{3}H} Aldosterone, 40-60 Ci/mmol	
{1α,2α(n)-^{3}H} Cholesterol, 40-60 Ci/mmol	
17α,21-Dimethyl-19-norpregn-4,9-diene-3, 20-dione (R5020)	New England Nuclear
{17α-methyl-^{3}H}-R5020, 80-110 Ci/mmol	
17α-Pregn-4-en-20-yno{2,3-d}isoxazol-17-ol (danazol)	Winthrop
^{3}H-Danazol, ca. 10 Ci/mmol	
Sephadex G-25, G-200	Pharmacia
Dextran T-70	
Ficoll	
Spheroidal hydroxylapatite	Whatman
Glutaraldehyde, 25% v/v solution	EM-Scope
Araldite	

Chemical	Source
Cocktail N scintillant	Fisons
Ethanol, absolute	
Toluene	
Potassium dihydrogen phosphate	
Dipotassium hydrogen phosphate	
Potassium hydroxide	
Hydrogen peroxide, 100 volumes	
Sodium cacodylate	
Acrylamide	
N,N'-methylenebisacrylamide	
N,N,N',N'-tetramethylethylene diamine (TEMED)	

Material	Source
Visking tubing	Scientific and Medical Supplies
Metricel filters, 0.45 μ pore	Bio-Rad Labs
Glass fibre filters (GF/C, GF/F)	Whatman
HAWP, PSED, PTGC filters	Millipore

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