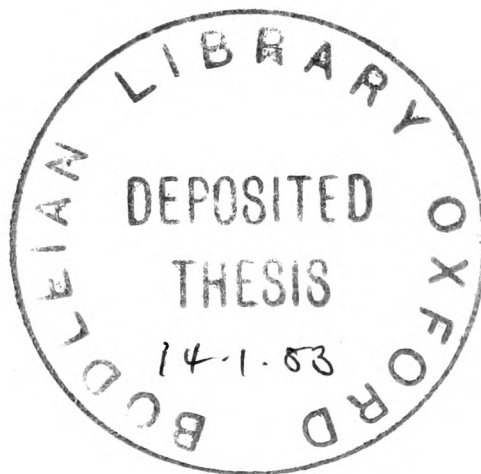


THE NEUROENDOCRINE CONTROL OF GONADOTROPHIN
SECRETION IN THE FEMALE RAT WITH SPECIAL EMPHASIS
ON THE EFFECTS OF CONTINUOUS EXPOSURE TO LIGHT

by

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For my Father,

who would liked to have seen it.

ABSTRACT

The neuroendocrine control of gonadotrophin secretion in the female rat with special emphasis on the effects of continuous exposure to light.

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The effects of constant light (LL) exposure on different patterns of gonadotrophin release have been studied in the female rat. Exposure of animals with regular oestrous cycles to LL during the 3 days preceding pro-oestrous, results in a 3 h delay and a 40% reduction in the height of the pro-oestrous LH surge. This delay and reduction may involve a reduction in the sensitivity of the hypothalamo-hypophyseal complex to oestradiol and progesterone. Diurnal and pulsatile patterns of LH release (seen in ovariectomized rats in the presence and absence of oestradiol) are driven by different neural mechanisms; LL inhibits diurnal but not pulsatile LH release. Analysis of tissue using HPLC suggests that in the suprachiasmatic nucleus alterations in the release and reuptake mechanisms of 5-HT may contribute to the loss of diurnal LH release. The pulsatile LH release found in rats ovariectomized after 60 days in LL, exhibits a lower mean plasma LH concentration, pulse amplitude, and pituitary responsiveness to LHRH, than rats ovariectomized in LD. Injection of oestradiol into gonadectomized animals shows that the centres upon which oestradiol acts to inhibit pulsatile LH release, are neither sexually differentiated nor affected by neonatal inhibitory action of oestradiol. LH release after progesterone treatment in LL exposed female rats, is accompanied by increases in the pituitary responsiveness to LHRH and the magnitude of the priming effect of LHRH. Analysis of plasma steroid concentrations after mating in females exposed to LL, suggests that progesterone may potentiate mating-induced ovulation. Studies using female rats with spontaneous degeneration of the retina, show that the effects of LL on gonadotrophin secretion are due to effects upon the brain, and are not a consequence of retinal degeneration.

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CHAPTER I

Introduction

The development of the ovarian follicle in the female rat is dependent upon the pituitary gonadotrophins luteinizing hormone (LH) and follicle stimulating hormone (FSH). The latter stages of follicular development and ovulation are stimulated by surges of plasma LH and FSH. The surges of gonadotrophin release are the result of complex interactions between gonadal steroids, environmental factors, neural elements and gonadotrophs of the anterior pituitary gland.

Different patterns of gonadotrophin release occur in various physiological and experimental situations. Ovulation is stimulated by surges of LH and FSH both spontaneously, every 4 days in female rats kept on a summer lighting schedule (14 h light : 10 h dark), and, as a reflex response to mating in female rats kept on constant light. LH and, to a lesser extent FSH, are released in a pulsatile pattern (one pulse every 20-120 min) in a number of physiological and environmental situations, but is most easily seen in ovariectomized rats. LH, and possibly FSH, are released in a diurnal pattern (one peak every day) in ovariectomized rats with continually elevated plasma oestradiol. Gonadotrophin release from the anterior pituitary gland is controlled by neural elements in the brain, which trigger the release of luteinizing hormone releasing hormone (LHRH) from neuronal terminals in the median eminence into hypophysial portal blood.

The object of this thesis is to investigate some aspects of how neural elements, gonadal steroids and environmental lighting interact to control gonadotrophin release. Attention has been focused on how constant light modifies the release of LH. Constant light has been used as a non-invasive tool to investigate the neural mechanisms which control the different patterns of LH release.

1.1 Neurohumoral Control of Gonadotrophin Secretion

1.1.1

Observations from the field, experimental investigations (Cushing, 1932; Marshall, 1936, 1937) and clinical studies (Cushing, 1932, Theobald, 1936) led to the concept of central nervous control over gonadal activity. Investigations were extended by Harris (1937) and Haterius and Derbyshire (1937) who showed that electrical stimulation of the hypothalamus led to ovulation in the rabbit. More detailed studies (Markee, Sawyer and Hollinshead, 1946; Harris, 1948) later showed that ovulation in the rabbit only occurred if the electrical stimulus was applied to the tuber cinereum and not in the anterior pituitary gland.

1.1.2 Humoral or nervous control?

Bougery (1845) described a nervous input to the anterior pituitary gland, and later studies described the innervation of the pituitary stalk and associated blood vessels supplying the pituitary gland with sympathetic nerve fibres (Dandy, 1913; Rasmussen, 1938; Truscott, 1944), parasympathetic nerve fibres (Chorobski and Renfield, 1932; Cobb and Finesinger, 1932; Zacharias, 1941) and nerve fibres from the hypothalamus (Cajal, 1894). The role of this nerve supply in mediating secretion from the pars distalis of the adenohypophysis was much debated, but the relative lack of nerve fibres in the pars distalis of many species (Rasmussen, 1938; Green, 1951) seemed to exclude any role in controlling the secretion from the pars distalis.

The first descriptions of prominent blood vessels in the pituitary stalk (Popa and Fielding, 1930; Wislocki and King, 1936) allowed for the possibility of a vascular link between the hypothalamus and the pituitary gland. The original accounts by Popa and Fielding (1930, 1933)

suggested that blood flowed from the pituitary gland to the hypothalamus, but, based on histological evidence, Wislocki (1937, 1938) suggested a flow from the hypothalamus to the pituitary gland. This was confirmed by direct microscopic observation in anaesthetized amphibians (Houssay, Biassotti and Sammartino, 1935; Green, 1947) and rats (Green and Harris, 1949). More recent evidence has suggested that some vessels may allow blood to flow from the anterior pituitary to the hypothalamus (Torek, 1962; Ambach, Palkovits and Szentagothai, 1976; Oliver, Mical and Porter, 1977; Bergland and Page, 1978) perhaps through the neurohypophysis (Bergland and Page, 1979).

The importance of the hypophysial portal vessels as a functional connection between the hypothalamus and the pituitary gland was demonstrated in experiments using pituitary stalk section and transplantation of anterior pituitary tissues from the sella turcica to other sites within the body. When anterior pituitary tissue was placed in the anterior chamber of the eye of hypophysectomized animals (Westman and Jacobsohn, 1940; Cutuly, 1941) there was no evidence of regenerated gonadal function even though the tissue was well vascularized. Only when the gland was transplanted under median eminence and a vascular connection allowed to develop was there any regeneration of gonadal function (Harris and Jacobsohn, 1952). The use of transplanted pituitary was extended by Nikitovitch-Winer and Everett (1958) who showed that when pituitary tissue was transplanted under the kidney capsule, reproductive function ceased. When the same pituitary tissue was replaced under the median eminence, normal reproductive activity resumed.

Early studies using pituitary stalk section gave conflicting results, with some workers claiming little disturbance to reproductive

function while others demonstrated gonadal atrophy. A careful study by Harris (1950) showed that if stalk section was not accompanied by the placement of a barrier into the cut, the hypophysial portal vessels would regenerate and normal reproductive function would return. However, because of the severe pituitary necrosis caused by pituitary stalk section, results from this type of preparation have to be interpreted with caution. The lack of regenerating nerve fibres after stalk section (Fisher, Ingram and Ranson, 1938; Magoun, Fisher and Ranson, 1939) and the evidence outlined above, led to the theory that neurones in the hypothalamus secreted substances which were transmitted to their target cells in the pars distalis by the hypophysial portal vessels (Green and Harris, 1947; Harris, 1955; Harris, 1972).

1.1.3 Isolation of luteinizing hormone releasing hormone

Studies in the laboratories of McCann (McCann, Taleisnik and Friedman, 1960), Harris (Campbell, Fever and Harris, 1964) and Schally (Schally and Bowers, 1964) suggested that the hypothalamus contained a substance capable of releasing LH and FSH from the pituitary. Many workers were involved in the attempted isolation of the LH-releasing factor (LRF) (reviewed by Harris, 1972; Schally, Kastin and Arimura, 1973), but eventually Schally and co-workers isolated and characterized it as a decapeptide (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂, from porcine hypothalamic fragments (Matsuo, Baba, Nair, Arimura and Schally, 1971; Schally, Arimura, Baba, Nair, Matsuo, Redding, Debeljuk and White, 1971), findings which were confirmed by Guillemin and co-workers (Burgos, Butcher, Amoss, Ling, Monahan, Rivier, Fellows, Blackwell, Vale and Guillemin, 1972) using ovine hypothalamic fragments. The LRF decapeptide (now more commonly called luteinizing hormone releasing hormone (LHRH)),

also releases FSH and, although the existence of a separate FSH-RF cannot be ruled out, no biochemical evidence of a separate FSH-RF has so far been reported. Most patterns of gonadotrophin release can be explained by the existence of one LHRH, whose effects on the pituitary are modulated by gonadal steroids.

All of Harris' criteria for a releasing factor (Harris, 1972) have been fulfilled for LHRH. That is: "a) that the substance is extractable from hypothalamic or stalk : median eminence tissue; b) that it is present in hypophysial portal blood in greater amounts than in systemic blood; and c) that varying concentrations of the substance in portal blood are related to varying secretion rates of one of the anterior pituitary hormones under a number of different experimental and environmental conditions".

1.1.4 Luteinizing hormone releasing hormone in hypophysial portal blood

By exposing the portal vessels in a manner similar to Green and Harris (1949), Worthington (1966) developed a technique for collecting the blood from the cut end of the pituitary stalk. Using this technique, LH releasing activity was found to be higher in hypophysial portal blood than peripheral blood (Fink, Nallar and Worthington, 1967; Fink and Harris, 1970). With the isolation and characterization of LHRH (Matsuo *et al.*, 1971; Burgos *et al.*, 1972) and the development of a specific radioimmunoassay for LHRH (Nett, Akbar, Niswender, Hedlund and White, 1973), Fink and Jamieson (1976) found greater concentrations of LHRH in hypophysial portal blood than peripheral blood. Eskay, Mical and Porter (1977), using a different surgical approach to expose and cut the portal vessels, confirmed the findings of Fink and Jamieson (1976). Neither Fink and Jamieson (1976) using Urethane anaesthesia,

nor Eskay *et al.* (1977) using sodium pentobarbitone anaesthesia, were able to show an increase in the LHRH secretion into the hypophysial portal blood during the LH surge on pro-oestrus. However, Sarkar, Chiappa, Fink and Sherwood (1976), using an anaesthetic (Althesin) which did not completely block ovulation if administered during the critical period of pro-oestrus, demonstrated a significant increase in the release of LHRH at the time of the pre-ovulatory LH surge; findings which have recently been confirmed by Ching (1982).

The pulsatile release of LH release most evident in long-term ovariectomized females (Gay and Sheth, 1972; Gallo, 1980a), is dependent upon increased LHRH release from the median eminence. Snabes and Kelch (1979) showed that antiserum to LHRH injected intravenously into ovariectomized rats would inhibit pulsatile LH release. Direct measurement of LHRH in portal plasma have shown (Sarkar and Fink, 1980) that ovariectomized rats have fluctuating LHRH concentrations compatible with pulsatile LH release; although the surgical preparation used in these studies, would not allow direct correlation between peripheral plasma LH and portal plasma LHRH concentrations.

The diurnal surge of LH seen in oestrogen treated long-term ovariectomized rats (Caligaris, Astrada and Taleisnik, 1971a; Legan, Coon and Karsch, 1975; Henderson, Baker and Fink, 1977a) is stimulated by corresponding surge of LHRH (Sarkar and Fink, 1980).

Fink and Jamieson showed that electrical stimulation of the preoptic area (POA) resulted in increased release of LHRH into hypophysial portal blood. The findings of Fink and Jamieson (1976) were extended by Chiappa, Fink and Sherwood (1977), who showed that electrical stimulation of the median eminence resulted in greater release of LHRH into the hypophysial portal vessels than stimulation of the POA.

Chiappa *et al.* (1977) also showed that electrical stimulation of the suprachiasmatic nucleus (SCN) resulted in less LHRH release into the hypophysial portal vessels than stimulation of the POA; and that stimulation of the amygdala or the dorsal or ventral hippocampus had no effect on LHRH release. Modulation of the LHRH response to POA stimulation by steroid hormones was demonstrated by Sherwood, Chiappa and Fink (1976); oestradiol increased and progesterone decreased the LHRH response to POA stimulation. Studies into the role of steroid hormones in LHRH release were extended by Sarkar and Fink (1979), who investigated the modulatory effects of steroid hormones on the LHRH surge during the afternoon of pro-oestrus, and found that oestradiol stimulated LHRH release, while progesterone either had no effect or inhibited LHRH release.

1.2 Different Patterns of Gonadotrophin Secretion

1.2.1 Spontaneous gonadotrophin secretion during the oestrous cycle

The anterior pituitary gland of female rats maintained in a light regime of 14 h light : 10 h dark, stimulates ovulation once every 4 days with an increase in the release of LH and FSH during the afternoon of pro-oestrus. The following section describes the patterns of steroid hormone and gonadotrophin secretion, and the status of steroid and LHRH receptors, during the oestrous cycle of the female rat.

a) Oestrogens:

The major oestrogen secreted by the ovary is oestradiol-17 β . During the 4-day oestrous cycle the plasma concentration of oestradiol-17 β begins to rise in the ovarian vein and peripheral vessels during the afternoon of dioestrus, and reach a peak during the morning and early afternoon of pro-oestrus (Yoshinga, Hawkins and Stocker, 1969;

Brown-Grant, Exley and Naftolin, 1970; Shaikh, 1971; Dupon and Kim, 1973; Shaikh and Shaikh, 1975; Tapper and Brown-Grant, 1975; Henderson, Baker and Fink, 1977b). Although oestrone concentrations in ovarian vein plasma parallel the surge of oestradiol-17 β seen during the morning of pro-oestrus, peripheral plasma concentrations remain very low and do not fluctuate throughout the oestrous cycle (Shaikh and Shaikh, 1975).

b) Progestins:

Progesterone is the major progestin secreted during the oestrous cycle. The initial increase in progesterone which begins during the early afternoon of pro-oestrus before the LH surge, is probably due to adrenal secretion (Feder, Brown-Grant and Corker, 1971; Barraclough, Collu, Massa and Martini, 1971; Mann and Barraclough, 1973; Shaikh and Shaikh, 1975). Later stages of the progesterone surge are the result of ovarian secretion and concentrations reach a maximum in plasma during the afternoon of pro-oestrus, but at a later time than the peak of the LH surge (Barraclough *et al.*, 1971, Feder *et al.*, 1971; Piacsek, Schneider and Gay, 1971; Shaikh and Shaikh, 1975). Although plasma concentrations of 17 α - and 20 α -hydroxyprogesterone do not change in the peripheral plasma throughout the oestrous cycle (Barraclough *et al.*, 1971; Shaikh and Shaikh, 1975), the ovarian secretion of these metabolites increases during the afternoon of pro-oestrus (Goldman, Kamberi, Siiteri and Porter, 1969; Barraclough *et al.*, 1971; Piacsek *et al.*, 1971; Shaikh and Shaikh, 1975).

c) Steroid receptors:

Steroid hormones interact with the target cell by binding to an intracellular receptor in the cytoplasm. The resulting complex moves into the nucleus to modify nuclear function (Jensen and De Sombre, 1972; O'Malley and Means, 1974; Edelman, 1975; Taylor, Swaneck and Smith, 1980). Cytosolic and nuclear receptors for progesterone (Kato and Onouchi, 1977; Kato, Onouchi and Okinaga, 1978; Lee, Davis and Ryan, 1979) and oestradiol (Kato, 1973; Ginsburg, MacLusky, Morris and Thomas, 1975; Menon and Gunaga, 1976; White, Lim and Thrower, 1977; Sen and Menon, 1978; White, Thrower and Lim, 1978; Thrower, Neething, White and Lim, 1981) have been measured in the hypothalamus and pituitary. The number of high affinity cytosolic binding sites for oestradiol in the pituitary and hypothalamus decreases during pro-oestrus, but then increases at oestrus and metoestrus (Ginsburg *et al.*, 1975; White *et al.*, 1977). However, the ratio of nuclear to cytosolic receptors in the pituitary increases dramatically from dioestrus to pro-oestrus, where peak numbers occur at the time of maximum pituitary responsiveness to LHRH (Sen and Menon, 1978). Progesterone binding sites in the hypothalamus and pituitary seem to be dependent upon previous exposure to oestradiol (MacLusky and McEwen, 1978; Moguileusky and Raynaud, 1979; Parsons, MacLusky, Krey, Pfaff and McEwan, 1980).

d) Luteinizing hormone:

The concentration of LH in peripheral plasma remains low during the oestrous cycle, except during the afternoon of pro-oestrus when a surge of LH occurs about 17.00–19.00 h, with plasma concentrations up to 50 times those found during dioestrus (Monroe, Rebar, Gay and Midgley, 1969; Goldman, *et al.*, 1969; Gay, Midgley and Niswender,

1970; Brown-Grant *et al.*, 1970; Naftolin, Brown-Grant and Corker, 1972; Aiyer, Fink and Greig, 1974b; Blake, 1976a). Gallo (1981b) has suggested that the pro-oestrus LH surge is caused by an increase in amplitude and frequency of pulsatile LH release similar to that demonstrated at midcycle in humans (Midgley and Jaffe, 1971; Yen, Tsai, Naftolin, Vandenberg and Ajabar, 1972) and cows (Rahe, Owens, Fleeger, Newton and Harms, 1980).

e) Follicle stimulating hormone:

Like LH, concentrations of FSH remain low in peripheral plasma during the oestrous cycle until the afternoon of pro-oestrus. Concentrations in the plasma begin to rise at the same time as LH but peak values do not occur until the early hours of oestrus (Gay *et al.*, 1970; Daane and Parlow, 1971a; Fink and Aiyer, 1974).

f) Luteinizing hormone releasing hormone and its pituitary receptors:

The concentrations of LHRH in the hypophysial portal blood increase rapidly during the afternoon of pro-oestrus reaching a peak at the same time as the peripheral plasma concentrations of LH (Sarkar *et al.*, 1976; Ching, 1982). The surge of LHRH correlates with a decrease of immunohistochemical LHRH in the median eminence during the morning of pro-oestrus (Polkowska and Jutisz, 1979), and a decrease in radioimmunoassayable LHRH in the hypothalamus (Asai and Wakabayashi, 1975; Kalra and Kalra, 1977; Barr and Barraclough, 1978; Oshima, Morishita, Omura and Saito, 1978; Wise, Rance, Selmanoff and Barraclough, 1981). A second smaller surge of LHRH occurs in hypophysial portal blood between midnight of pro-oestrus and 02.00 h of oestrus (Sarkar *et al.*, 1976), and may be related to the pro-oestrus surge of FSH, which is longer in duration than the pro-oestrus

surge of LH (Daane and Parlow, 1971a; Fink and Aiyer, 1974), or to sexual receptivity, which is maximal around this time (Feder *et al.*, 1971).

Using a radioligand assay for high-affinity LHRH binding sites in the pituitary (Clayton, Shakespear, Duncan and Marshall, 1979) it has recently been possible to measure an increase in the pituitary content of LHRH receptors between the morning of metoestrus and the morning of pro-oestrus (Clayton, Solano, Garcia-Vela, Dufau and Catt, 1980). Clayton *et al.* (1980) suggest that this increase may contribute to the increase in pituitary responsiveness to LHRH found during pro-oestrus.

g) The critical period of pro-oestrus:

Everett, Sawyer and Markee (1949) demonstrated that dibenamine or atropine would block ovulation in the majority of rats with 4-day oestrous cycles if given before 14.00 h of pro-oestrus, but neither drug blocked ovulation if given later than 16.00 h. These findings led to the concept of a critical period on the afternoon of pro-oestrus, before and during which the administration of neuropharmacological drugs (e.g. barbiturates, opiates, etc.) would block the preovulatory gonadotrophin surge and ovulation (Everett, 1964). The onset of the critical period is linked to the time at which the lights are switched on (Everett, 1961, 1977). For example, animals maintained in a 14 h light : 10 h dark regime, the critical period is between 14.00 h and 16.00 h (Naftolin, *et al.*, 1972; Aiyer *et al.*, 1974a).

1.2.2 Role of steroid hormones in regulation of gonadotrophin release

- a):

The importance of the relationship between the anterior pituitary and the ovaries was first recognized and studied by Moore and Price (1932). They concluded that the oestrous cycle was the result of a reciprocal relationship between the ovary and the 'hypophysis'; gonadal secretion was stimulated by 'hypophysial activity', and 'hypophysial' secretion was reduced by increased gonadal activity. Hohlweg and Junkman (1932) and Hohlweg (1936) demonstrated that the brain was an essential component of the interaction between the ovary and the anterior pituitary when they found castration cells did not develop in the anterior pituitary of ovariectomized rats if the pituitary was transplanted away from the sella turcica.

The role of steroid hormones in the control of the oestrous cycle was further explored by Everett (1944, 1948, 1964) and other workers (McCann, 1962; Bogdanove, 1963; Rothschild, 1965) and, taken together they demonstrated the complex interrelationship between steroid hormones and centres controlling the oestrous cycle.

During the greater part of the oestrous cycle, gonadotrophin levels in the plasma are kept low, probably through inhibition by basal plasma levels of oestradiol in the presence of progesterone (McCann, 1962; Goodman, 1978; Fink, 1979). However, during the morning and early afternoon of pro-oestrus, oestradiol switches from an inhibitory to a facilitatory role in the control of gonadotrophin release.

The surge of gonadotrophins during the afternoon of pro-oestrus, is dependent upon the surge of oestradiol, which occurs during the evening of dioestrus and the morning of pro-oestrus (Ferin, Tempone, Zimmering and Vande Wiel, 1969; Schwartz, 1969; Aiyer and

Fink, 1974; Fink, 1977). The facilitatory effect of oestradiol is enhanced by plasma concentration of progesterone, secreted in response to LH during the afternoon of pro-oestrus (Everett, 1964; Rothschild, 1965; Brown-Grant and Naftolin, 1972; Lawton, 1972; Mann and Barraclough, 1973; Aiyer and Fink, 1974; McCann, 1974; Fink and Henderson, 1977a). Progesterone alone has little effect on the release of gonadotrophins (Everett, 1964; Brown-Grant and Naftolin, 1972; Aiyer and Fink, 1974) perhaps because the presence of progesterone receptors in the hypothalamus and pituitary seem to depend on exposure to oestradiol (see Section 1.2.1c).

- *b) The pituitary responsiveness to LHRH and the effect of oestrogen and progesterone:*

The pituitary responsiveness to LHRH increases gradually from 13.30 h of dioestrus to 15.00 h of pro-oestrus, and then abruptly between 15.00 h and 18.00 h pro-oestrus (Aiyer *et al.*, 1974a; Cooper, Fawcett and McCann, 1974; Fink and Aiyer, 1974; Gordon and Reichlin, 1974). The gradual increase in pituitary responsiveness seems to be dependent upon increasing levels of plasma oestradiol (Aiyer and Fink, 1974; Fink and Henderson, 1977a) which initially inhibit, then (12 h after oestradiol treatment has begun) facilitate pituitary responsiveness to LHRH (Henderson *et al.*, 1977b). Oestradiol probably acts both at the brain and the anterior pituitary gland to facilitate pituitary responsiveness (Fink and Henderson, 1977b; Henderson *et al.*, 1977b; Greeley, Volcan and Mahesh, 1978; Hsueh, Erickson and Yen, 1978).

Aiyer *et al.* (1974b) noted that between 15.00 h and 22.30 h of pro-oestrus there was a significant positive correlation between the plasma LH concentration before and the plasma LH response after an

LHRH injection, and that in animals given pentobarbitone at 13.30 h of pro-oestrus, the plasma LH response to LHRH at 18.30 h was considerably lower compared to animals anaesthetized at 18.00 h of pro-oestrus. These two observations suggested that the abrupt increase in pituitary responsiveness to LHRH occurring between 15.00 and 18.00 h of pro-oestrus was partly dependent upon a priming effect of LHRH (Aiyer *et al.*, 1974b). This priming effect of LHRH was evident after 2 i.v. injections of LHRH (50 ng/100 g b.w.), when the response to a second injection was significantly greater than the response to the first, and was maximal when the 2 injections were separated by 60 min (Aiyer *et al.*, 1974b). The primary effect was greatest in magnitude during the afternoon of pro-oestrus, and was absent during the afternoon of oestrus (Aiyer *et al.*, 1974b). Fink, Chiappa and Aiyer (1976) showed that the priming effect could be elicited by POA stimulation, intravenous infusion of LHRH or multiple injections of LHRH. The priming effect does not seem to be mediated by adrenal or ovarian hormones (Aiyer *et al.*, 1974b; Fink and Pickering, 1980), but ovariectomy on dioestrus reduces the magnitude of the priming effect during the following afternoon of pro-oestrus, an effect partly reversible by oestradiol benzoate injected shortly after ovariectomy. The priming effect of LHRH could be elicited in *in vitro* preparations of hemipituitaries (Pickering and Fink, 1976a,b, 1979; Fink and Pickering, 1980), and using this preparation, Pickering and Fink (1977) demonstrated that LHRH would also prime the anterior pituitary gland with respect to FSH secretion, a phenomenon difficult to demonstrate *in vivo* (Aiyer *et al.*, 1974b).

1. 2. 3 Models for investigating gonadal steroid involvement in the LH surge

Three models have been developed to investigate the mechanisms whereby steroid hormones modulate gonadotrophin secretion.

a) Female rats are ovariectomized during the morning of dioestrus and immediately given an injection of oestradiol benzoate. The following day (expected day of pro-oestrus) an injection of progesterone is given at the beginning of the afternoon, resulting in an LH surge and increases in pituitary responsiveness to LHRH similar to those found in sham-operated animals. This model has been used by various workers (Kalra, Fawcett, Krulich and McCann, 1973; Mann and Barraclough, 1973; Aiyer and Fink, 1974; Tapper, Brown-Grant and Greig, 1974; Fink and Henderson, 1977a) in an attempt to reproduce the changing plasma steroid hormone concentrations found during dioestrus and pro-oestrus, and therefore, to study the role of steroids in the development of the LH surge and pituitary responsiveness to LHRH.

b) Female rats are ovariectomized and at least 21 days later are given either 2 injections of oestradiol benzoate (24 h apart), or a subcutaneously implanted Silastic capsule containing oestradiol-17 β . Every afternoon, beginning 2 days after oestradiol exposure, a surge of LH occurs, the size of which is dependent upon the dose of oestradiol (Caligaris *et al.*, 1971a; Legan *et al.*, 1975; Chazal, Fraudon, Gogan, Héry, Kordon and Laplante, 1977; Henderson *et al.*, 1977a). The surge of LH in this preparation is accompanied by a surge of LHRH in pituitary portal blood (Sarkar and Fink, 1980), but not by an increase in pituitary responsiveness to LHRH measured *in vivo* (Chazal *et al.*, 1977; Henderson *et al.*, 1977a). However, Wilkinson,

and Moger (1981) claim that there is an increase in pituitary sensitivity to LHRH between morning and afternoon of oestradiol-treated female rats, if pituitary glands are maintained *in vitro* in the presence of LHRH for 3 h (Wilkinson and Moger, 1981). Daily surges of FSH can only be elicited using pentobarbitone injections on alternate days (Henderson *et al.*, 1977a).

A daily neural signal for gonadotrophin release was first disclosed by Everett and Sawyer (1950) and the diurnal LH surge in ovariectomized-oestrogen treated rats has been used to study this daily signal. Oestrogen treatment resulted in a diurnal LH surge in female but not in male rats (Henderson *et al.*, 1977a), or female rats treated neonatally with androgen (Gogan, Beattie, Héry, Laplante and Kordon, 1980). Other workers have shown the diurnal LH surge to be dependent upon the SCN (Coen and MacKinnon, 1980; Kawakami, Arita and Yoshioka, 1980; Wiegand and Terasawa, 1982) and serotonin (5-HT) neural systems (Héry, Laplante and Kordon, 1976, 1978; Coen and MacKinnon, 1979; Héry, Faudon, Dusticier and Héry, 1982). Colombo, Baldwin and Sawyer (1974) showed that light was an entraining stimulus for the diurnal LH surge, that is, the timing of the LH surge altered in parallel with a change in the time at which the lights were switched on.

c) Female rats are ovariectomized, and at least 21 days later are

given an injection of oestradiol, followed 72 h later by an injection of progesterone. This regime results in a surge of plasma LH with peak concentrations occurring 5 h after the progesterone injection (Caligaris, Astrada and Taleisnik, 1968; Brown-Grant, 1974).

However, unlike model b) subsequent surges of plasma LH are abolished (Caligaris *et al.*, 1968; Freeman, Dupke and Croteau, 1976). The surge of LH can be abolished by sodium pentobarbitone injection

(Brown-Grant, 1974), is accompanied by a massive increase in pituitary responsiveness to LHRH (Aiyer, Sood and Brown-Grant, 1976), but not by a significant increase in portal plasma LHRH concentration (Sarkar and Fink, 1980). The ability of progesterone to induce an LH surge in oestrogen primed ovariectomized rats, is subject to diurnal variation (Caligaris *et al.*, 1968, 1971b; Brown-Grant and Raisman, 1977) and there is evidence for the involvement of 5-HT neurons (Franks, McElhone, Young, Kraubs and Ruf, 1980). However, lesions of the medial raphe nucleus (MRN) (Barofsky, 1975) or the SCN (Wiegand, Terasawa, Bridson and Gay, 1980; Wiegand and Terasawa, 1982) do not abolish the progesterone-induced LH surge; whereas lesions of the medial preoptic nucleus (rostral and in the same coronal plane as the SCN) do abolish the surge (Wiegand *et al.*, 1980; Wiegand and Terasawa, 1982).

The height of the LH surge induced in model c) appears to be higher than in either a) or b), although the doses of steroid used in model c) are non-physiological (up to 20 µg of oestradiol and 4 mg of progesterone per rat). Model c) has been used to investigate the effects of steroids on catecholamine turnover rates in areas of the hypothalamus important in controlling gonadotrophin secretion (Crowley, O'Donohue, Wachslicht and Jacobowitz, 1978; Honma and Wuttke, 1980; Wise, Rance and Barraclough, 1981).

1.2.4 The reflex surge of gonadotrophins

Female rats exposed to long-term constant light (LL) respond to mating with surges of LH and FSH (Brown-Grant, Davidson and Greig, 1973; Davidson, Smith and Bowers, 1973), peak concentrations of which occur 60 min after mating. Smith and Davidson (1974) showed that a significant decrease in the hypothalamic content of bioassayable

LH releasing activity occurred within 20 min of mating and suggested that mating-induced gonadotrophin release was accompanied by the release of LHRH. The pituitary sensitivity to LHRH of LL rats was similar to female rats on control lighting (LD) on the morning of pro-oestrus (Smith and Davidson, 1974; Fink, 1975). Fink (1975) showed that in LL rats the LH response to 250 ng/100 g body weight (b.w.) LHRH was similar to the LH response to 1000 ng/100 g b.w. LHRH, and, since the LH response to 1000 ng/100 g b.w. LHRH was similar to that found after mating (Brown-Grant *et al.*, 1974), Fink (1975) suggested that mating led to the release of most of a readily releasable pool of LH.

1.2.5 Pulsatile release of gonadotrophins

Gonadotrophin release in a number of physiological situations, seems to be in a pulsatile or episodic pattern (Midgley and Jaffe, 1971; Yen *et al.*, 1972; Kapen, Boyar, Hellman and Weitzman, 1973; Rahe *et al.*, 1980). Pulsatile gonadotrophin secretion in the rat is most easily seen in gonadectomized males and females, and is more pronounced for LH than for FSH (Gay and Sheth, 1972; Gallo, 1980a), probably as a consequence of the longer half life of FSH when compared to LH (Gay and Sheth, 1972). Pulsatile LH release has recently been demonstrated in intact female rats during each day of the oestrous cycle, with a decrease in the frequency of the LH pulses occurring between the morning of oestrus and the morning of pro-oestrus (Gallo, 1981a,b). Pulsatile LH release is characterized by rapid increases in plasma LH concentration (over a period of 5-10 min) with a slower decline over the following 15-20 min (Gay and Sheth, 1972; Gallo, 1980a; Weick, 1981). The functional significance of pulsatile gonadotrophin release may be related to the refractoriness of the LH response of the anterior

pituitary, seen in response to constant infusions of LHRH (Schuiling, de Koning, Zurcher, Gnodde and van Rees, 1976; Belchetz, Plant, Nakai, Keogh and Knobil, 1978).

Studies (see Section 1.1.4) involving the use of anti-LHRH serum (Snabes and Kelch, 1979), measuring portal plasma LHRH concentrations in ovariectomized females (Sarkar and Fink, 1980), as well as studies with pulsatile or constant infusion of LHRH into pento-barbitone treated and conscious ovariectomized rats (Schuiling and Gnodde, 1976; Blake, Sridaran, Elias, Ashiru and Rush, 1980) suggest that pulsatile LH release is a centrally controlled phenomenon reflecting pulsatile LHRH release from the median eminence. Anterior pituitary tissue from ovariectomized rats in *in vitro* preparations, does not appear to possess an endogenous mechanism for pulsatile LH release (Osland, Gallo and Williams, 1975).

Oestradiol, administered as an i.v. injection, has a rapid inhibitory action on pulsatile LH release, reducing both the mean plasma LH concentration and the amplitude of the pulses (Blake, Norman and Sawyer, 1974; Sarkar and Fink, 1980; Higuchi and Kawakami, 1982). However, progesterone given alone does not seem to affect pulsatile LH release, nor, if given in conjunction with oestradiol, does progesterone modulate the inhibitory action of oestradiol (Weick, 1977). Oestradiol probably acts both at the brain to reduce LHRH release (Sarkar and Fink, 1980), and at the pituitary to reduce pituitary sensitivity to LHRH (Higuchi and Kawakami, 1982).

The interactions between central areas controlling pulsatile LH release is complex. Both intra- and extra-hypothalamic areas seem to be involved (Blake and Sawyer, 1974; Soper and Weick, 1980), with the arcuate nucleus (ARC) perhaps contributing to the intrahypothalamic

neural drive. Electrical stimulation of the ARC rapidly inhibits pulsatile LH release (Gallo and Osland, 1976), an effect which is reversed by oestradiol priming (Gallo and Osland, 1976) or depletion of brain 5-HT concentrations (Gallo and Moberg, 1977). Electrolytic lesions of the SCN do not inhibit pulsatile LH release (Arendash and Gallo, 1979a; Soper and Weick, 1980), although electrical stimulation of the peri-SCN region (Arendash and Gallo, 1979b) or the dorsal raphe nucleus (DRN) (Arendash and Gallo, 1978) do inhibit pulsatile LH release.

Investigations into the neurotransmitters involved in controlling pulsatile LH release have revealed a complex situation. Depletion of brain 5-HT concentrations does not inhibit pulsatile LH release (Gallo, 1980b; Rasmussen, Jacobs, Kissinger and Malven, 1981) but studies using intraventricular infusions of catecholamines (Gallo and Drouva, 1979; Leung, Arendash, Whitmoyer, Gorski and Sawyer, 1982), administration of catecholamine agonists or antagonists (Drouva and Gallo, 1976, 1977; Gallo, 1981c; Kinoshito, Nakai, Katakami, Kato and Imura, 1981; van Dielen, van Valkenburg, de Koning and van Rees, 1981; Leung *et al.*, 1982) and electrical stimulation (Leung, Arendash, Whitmoyer, Gorski and Sawyer, 1981) strongly suggest the involvement of noradrenaline (NA) and dopamine (DA) in the control of pulsatile LH release.

1.2.6 Diurnal release of gonadotrophins

The daily signal for ovulation, first demonstrated by Everett and Sawyer (1950), can be observed as a daily surge of plasma LH in the female rat only when the hypothalamo-hypophysial system is exposed to continually elevated plasma oestradiol concentrations. Since the experimental protocol used to study the diurnal surge of gonadotrophin

secretion, and the findings of other workers have already been described (see Section 1.2.3b) no further details are given here.

1.3. The Effects of Light on Reproductive Function

1.3.1 The first investigations into the effect of light on reproductive function were carried out in birds. Using the junco finch, Rowan (1926) found that if the duration of the light period was increased during the winter, the testes would be enlarged and sperm production initiated. Rowan (1930) described similar results in crows, while Bissonette (1933) found that wavelength and intensity of light were important parameters in gonadal stimulation in starlings. These studies were extended to mammals by Baker and Ranson (1932) who found that in the ^{field mouse,} a day consisting of 9 h light reduced the breeding capacity when compared to a day of 15 h light. Bissonette (1932) found that during winter, the ferret would come into oestrus more quickly if given extra illumination, an observation confirmed by Hill and Parkes (1933). Marshall and Bowden (1934) showed that within a range from red to near ultraviolet (650 nm to 365 nm), the intensity of extra illumination was more important than the wavelength in accelerating the onset of oestrus in female ferrets during the winter. The converse effect was found by Bissonette (1935a) when he found sexual regression in the ferret resulted from a decrease in either the intensity or duration of the light period. Hart (1950, 1951) found that in sheep and ferrets the amount of light within the light/dark cycle were important in accelerating oestrus; that is sheep responded to short light and longer dark periods, while the ferret responded to long light and shorter dark periods.

1.3.2 The pathway mediating the effects of light on reproductive function

- a):

Various workers demonstrated that the pituitary was an essential part of the pathway through which light exerts its effects. Hill and Parkes (1933) showed that hypophysectomized ferrets did not respond to extra illumination with an accelerated onset of oestrus. Bissonette (1935b) confirmed the results of Hill and Parkes (1933), and also showed that light led to histological changes in the anterior pituitary gland similar to those found in castrated animals (Bissonette, 1935b, 1936). Marshall reviewed (1936, 1937) experimental studies and observations from the field and concluded "that the oestrous cycle and its successive phases are not regulated solely by the endocrine mechanisms of the gonads and the pituitary, but are controlled also by exteroceptive or other external factors which probably act upon the hypothalamo-hypophysial mechanism through the nervous system" (Marshall, 1937).

The effects of extra illumination seem to be mediated through the retina and optic nerves. Ceni (1928) found degenerated testes in blinded cocks, while Benoit (1934) found that the stimulating effect of light on the testis of the drake, did not occur if the eyes were covered by a hood. Ferrets with severed optic nerves showed either an absence of oestrous cycles or a lack of photic influence on oestrous cycles (Bissonette, 1938; Clark, McKeown and Zuckerman, 1939). Using a combination of sectioning or crushing the optic nerve, and sectioning the central artery and ciliary vessels in the eye, Thompson (1951) concluded that in ferret, the message was transmitted to the pituitary through a nervous pathway and that the integrity of the ganglion cell layer of the retina was essential.

- b) *The retinohypothalamic pathway (RHP):*

The demonstration of a retinohypothalamic pathway (RHP) eluded neuroanatomists for many years. The presence of the RHP could be inferred indirectly from the results of Clark *et al.* (1939). They found that in ferrets, lesions of the superior colliculi, or interruption of the visual pathway to the dorsal nucleus of the lateral geniculate body and visual cortex, did not interfere with the gonadal response to extra illumination. However, Jefferson (1940) failed to find any connection between the main optic pathway and the hypothalamus in the ferrets used by Clark *et al.* (1939). Such a pathway was described by Frey (1951) in the dog, but no confirmation occurred in other species.

Attempts to demonstrate the RHP in mammals using classical neuroanatomical techniques met with considerable difficulties (see Nauta and Haymaker, 1969; Moore and Lenn, 1972). However, by injecting tritiated amino acids into the vitreous of the eye and observing their retrograde accumulation by autoradiography (Taylor and Weiss, 1965), a pathway terminating in the suprachiasmatic nucleus (SCN) of the hypothalamus was identified (Moore, Karapas and Lenn, 1971; Hendrickson, Wagoner and Cowan, 1972; Moore and Lenn, 1972; Moore, 1973). The presence of a retino-suprachiasmatic pathway has now been demonstrated in many species (Hendrickson *et al.*, 1972; Moore, 1973; Thorpe, 1975; Mason and Lincoln, 1976; Tigges, Bos and Tigges, 1977). In the rat the SCN is elongated along the rostro-caudal plane with a length of 700-950 μm . Each nucleus is about 300 μm wide at the broadest point of the ventrodorsal plane (Van den Pol, 1980; Lydic, Albers, Tapper and Moore-Ede, 1982). No retinal axons enter the most rostral 400 μm , but the remaining caudal portion of the nucleus

is innervated by fine unmyelinated fibres from the optic chiasm, starting at the ventral surface of the SCN and spreading caudally to supply the ventrolateral part of the nucleus (Moore, 1973; Millhouse, 1977; Moore, 1978). Neurons of the caudal SCN of the rat and cat have recently been shown to respond electrophysiologically to a light stimulus to the retina (Groos and Mason, 1980). The SCN therefore, is the part of the hypothalamus that receives information directly from the retina and probably acts as an integrator for neuroendocrine and behavioural rhythms which require light as an entraining stimulus (Moore, 1978; Rusak and Zucker, 1979). Recent studies in the mouse (Silver and Brand, 1979) have shown that neurones of the preoptic area of the hypothalamus have dendritic connections in the optic chiasm with synapses on retinal axonal boutons. Itaya, van Hoesen and Jenq (1981) have also demonstrated a direct retinal input into the limbic system in the rat brain. The possibility therefore exists, of connections between the retina and areas of the forebrain other than the hypothalamic SCN.

1.3.3 The effects of constant illumination on reproductive function in female rats

Browman (1937) and Hemmingsen and Krarup (1937) first observed that LL led to a state of prolonged oestrus, findings which were confirmed by Fiske (1941) and Everett (1942). The breakdown of regular oestrous cycles and the development of longer periods of vaginal cornification usually begins a few days after the start of LL exposure (Everett, 1942; Lawton and Schwartz, 1967; Campbell and Schwartz, 1980). The synchronization of the LH surge with uterine distention and the consequent oestrous vaginal smear gradually disappears with each successive cycle in LL (Lawton and Schwartz, 1967) until the

animals eventually become anovulatory with cystic follicular ovaries and long periods of vaginal cornification (Fiske, 1941; Everett, 1961; Critchlow, 1963; Everett, 1964; Lawton and Schwartz, 1967; Reiter and Klein, 1971; Brown-Grant, Davidson and Greig, 1973). These changes are accompanied by a switch from regular spontaneous ovulation to reflex ovulation in response to mating (Dempsey and Searles, 1943; Brown-Grant *et al.*, 1973). The rapid reflex response to mating is shown by an LH surge (described in Section 1.2.4) with increased plasma LH concentrations detectable within 10 min of mating (Davidson *et al.*, 1973). Ovulation will also occur in a proportionate number of female rats exposed to long-term LL in response to progesterone (Critchlow, 1963; Singh and Greenwald, 1967; Brown-Grant *et al.*, 1973), human chorionic gonadotrophin (Singh and Greenwald, 1967), LHRH (Fink, 1975), laparotomy (Brown-Grant *et al.*, 1973), and exposure to a period of darkness (Takahashi, Ford, Yoshinaga and Greep, 1977).

Reiter and Klein (1971) showed the presence of stratified squamous metaplasia in the uterus in a proportion of female rats exposed to long-term LL, a type of uterine morphology that has been associated with persistent oestrogen secretion (Reiter, 1969). The persistent vaginal cornification in LL-exposed rats suggests the plasma concentrations of oestrogen are elevated in such animals. This was shown to be the case by Naftolin *et al.* (1972) who measured oestradiol concentrations in peripheral plasma, and by Takahashi *et al.* (1977) who measured oestradiol concentrations in plasma from the ovarian vein. Both Naftolin *et al.* (1972) and Takahashi *et al.* (1977) found the oestradiol concentration in the plasma of LL rats was intermediate between those found in LD females during dioestrus evening and pro-oestrus morning.

Long-term LL exposure results in irreversible degeneration of photoreceptor cells in the retina (Noell, Walker, Kang and Berman, 1966; Kuwabara and Gorn, 1968; Grignolo, Orzalesi, Castellazzo and Vittone, 1969; O'Steen, 1970; Anderson and O'Steen, 1971; Reiter and Klein, 1971; Bennett, Dyer and Dunn, 1972, 1973; O'Steen and Anderson, 1972; Fifkova, 1972). These degenerative changes are dependent upon the age at which animals are exposed to LL (O'Steen, Anderson and Shear, 1974), the intensity of the illumination (Noell and Albrecht, 1971; Weisse, Stotzer and Seitz, 1974) and the wavelength of the light. Shorter wavelengths are the most damaging (Anderson, Coyle and O'Steen, 1972; Ham, Mueller and Sliney, 1976; Moon, Clarke, Ruffolo, Mueller and Ham, 1978). Despite the almost total absence of photoreceptors, LL-exposed animals are still capable of discriminating between light and dark in behavioural tests (Anderson and O'Steen, 1972; Bennett, Dyer and Dunn, 1972, 1973; Lemmon and Anderson, 1979a; Anderson and Lemmon, 1980). Some workers (Cicerone, 1976; LaVail, 1976) claim that this may be due to residual cone cells, but this is disputed by Lemmon and Anderson (1979b).

Pineal melatonin secretion is thought to have antigonadal activity and may act to inhibit gonadal function in seasonal breeders (Reiter, 1973). Rats exposed to LL lose the diurnal rhythms of the melatonin synthetic enzymes n-acetyltransferase and hydroxy^{indole}o-methyltransferase (Wurtman, Axelrod and Phillips, 1963; Klein and Weller, 1970; Reiter and Klein, 1971). However, denervation of the pineal (Brown-Grant and Ostberg, 1974) or pinealectomy (Hoffman and Cullin, 1975) do not alter the effect of LL on oestrous cycles of female rats or the ovulatory response of LL rats to mating, and these results suggest that the pineal does not play an important role in LL-induced ovulation.

1.4 Neuroanatomy of monoamine and LHRH neural pathways

1.4.1:

Interactions between neurotransmitter and LHRH neural pathways determine the patterns of gonadotrophin secretion from the anterior pituitary gland (see Sections 1.1.4, 1.2). The following section describes the anatomical arrangement of the neural pathways relevant to this thesis.

1.4.2 LHRH pathways

Studies using bioassay, radioimmunoassay and immunohistochemistry have shown that majority of LHRH terminals are located in the median eminence (ME) around the capillary plexus of the portal vessels; a second group is located in the organum vasculosum of the lamina terminals (OVLT). Using colchicine to block axonal transport, several workers were able to localize cell bodies within the medial preoptic area (MPoA), SCN and ARC nuclei of the guinea pig (Barry, Dubois and Poulain, 1973; Silverman and Krey, 1978). In the rat, however, difficulty has occurred in locating perikarya within the hypothalamus (Leonardelli, Barry and Dubois, 1973; Baker, Dermody and Reel, 1974; King, Parsons, Enlandsen and Williams, 1974; Kordon, Kerdelhue, Pattou and Jutisz, 1974). The presence of perikarya in the preoptic-suprachiasmatic area of the rat is well-documented (e.g. Barry, Dubois and Poulain, 1973; Setalo, Vigh, Schally, Arimura and Flerko, 1976; Kawano and Daikoku, 1981; Rethelyi, Vigh, Setalo, Merchenthaler, Flerko and Petrusz, 1981) but the presence of perikarya in the mediobasal hypothalamus has been much debated (Naik, 1975a,b; Setalo *et al.*, 1976). Recent work (Kawano and Daikoku, 1981; Rethelyi *et al.*, 1981) suggests that the majority of LHRH perikarya in the rat hypothalamus are located in the septal-preoptic region with

no evidence for LHRH synthesizing perikarya within the ARC region. The work of Kawano and Daikoku (1981) and Rethelyi *et al.* (1981) suggests 2 pathways (preoptico-infundibular tracts) from the perikarya in the anterior hypothalamus to the ME region: i) a mediobasal tract running from the preoptic area ventral and caudally through the SCN region and ARC to the medial part of the ME; ii) a laterobasal tract from the septal area and lateral preoptic area running laterally around and in close proximity to the optic chiasma and converging caudally on the lateral margin of the ME.

Rethelyi *et al.* (1981) also describe a caudal LHRH tract with fibres running from an unknown origin to the medial mammillary nucleus and the preoptico-infundibular tracts joining those at the retrochiasmatic area. They also describe fibres from both tracts (i.e. the preoptic infundibular tract and the caudal tract) to the OVLT.

1.4.3 Serotonin innervation

The early histochemical work of Fuxe and co-workers (Dahlstrom and Fuxe, 1964; Fuxe, 1965) showed that most of the 5-HT innervation of the brain originated from cell bodies in the raphe nuclei in the mid-brain. This was confirmed by, histofluorescence studies of the brain after lesioning 5-HT pathways either surgically (Ungerstedt, 1971; Kuhar, Aghajanian and Roth, 1972) or cytotoxically (Bjorklund, Nobin and Stenevi, 1973; Fuxe and Jonsson, 1974), autoradiographic tracing of tritiated 5-HT (Chan-Palay, 1977; Parent, Descarries and Beaudet, 1981) and immunohistochemical studies (Steinbusch, 1981; Consolazione and Cuello, 1982).

Within the hypothalamus 5-HT innervation is dense (Descarries and Beaudet, 1978; Kent and Sladek, 1978; Parent *et al.*, 1981) particularly in the SCN and ARC, which have the highest concentrations

of 5-HT (Saavedra *et al.*, 1974), although most nuclei have some detectable 5-HT. Electrolytic lesions of the DRN leads to significant reductions of 5-HT in several hypothalamic nuclei (Palkovits, Saavedra, Jacobowitz, Kizer, Zaborsky and Brownstein, 1977) as does deafferentation of the mediobasal hypothalamus (Brownstein, Palkovits, Tappaz, Saavedra and Kizer, 1976). Recent work by Van de Kar and Lorens (1979) has suggested that the hypothalamus is differentially innervated from the raphe nuclei, with the MRN supplying the SCN, anterior hypothalamus and MPoA and both the MRN and DRN supplying the antero-lateral hypothalamus and ARC. An intrinsic hypothalamic 5-HT system is suggested by autoradiographic studies which have shown that various nuclei have ^3H -5-HT labelled cell bodies (Chan-Palay, 1977; Descarries and Beaudet, 1978; Beaudet and Descarries, 1979; Parent *et al.*, 1981). However, the presence of immunoreactive cell bodies within the hypothalamus remains unconfirmed (Steinbusch, 1981; Consolazione and Cuello, 1982).

1.4.4 Noradrenaline innervation

Noradrenaline (NA)-containing neurons, like 5-HT neurons, have their cell bodies in the brain stem (Dahlstrom and Fuxe, 1964; Ungerstedt, 1971; Palkovits and Jacobowitz, 1974; Swanson and Hartman, 1975; Lindvall and Bjorklund, 1978). Two groups of cell bodies supply the brain; firstly, a system which originates in the locus coeruleus and contains 43% of the cell bodies of NA fibres in the brain (Swanson and Hartman, 1975) and secondly, neurons whose cell bodies originate in the lateral tegmentum. The hypothalamus receives the majority of its NA innervation from the lateral tegmental group with only the supraoptic, dorsomedial, paraventricular and periventricular nuclei receiving fibres from the locus coeruleus (Segal, Pickel and

Bloom, 1974; Jones and Moore, 1977). Biochemical (Palkovits, Brownstein and Saavedra, 1974), histofluorescence (Fuxe, 1965; Hokfelt and Fuxe, 1972; Jacobowitz and Palkovits, 1974) and immuno-histochemical studies have shown that the hypothalamus contains a rich, diffuse NA-innervation with greatest concentrations in the retrochiasmatic area, paraventricular and dorsomedial nuclei. The median eminence also contains some NA-terminals as shown by the existence of dopamine β -hydroxylase (Fuxe *et al.*, 1978). The principal innervation to the hypothalamus comes by way of the principal noradrenergic bundle which projects rostrally through the dorsal mesencephalic tegmentum and zona incerta (Swanson and Hartman, 1975).

1.4.5 Dopamine innervation

Unlike NA and 5HT, dopaminergic neuronal systems appear to be localized with cell bodies located in various parts of the brain. A detailed description of all these systems is outside the scope of this Chapter, but is described in the reviews of Moore and Bloom (1978) and Palkovits (1981). However, three of these systems are of relevance here. First, the mesocortical system with cell bodies located in the ventral tegmentum and substantia nigra. Some of their projections run to the amygdala (Fallon and Moore, 1976a,b) and possibly to the SCN, ventromedial nucleus and ME (Kizer, Palkovits and Brownstein, 1976). Second, the incerto-hypothalamic system, cell bodies of which are found in the posterior hypothalamus and zona incerta, and the periventricular nucleus (Lindvall, Bjorklund, Moore and Stenevi, 1974; Bjorklund, Lindvall and Nobin, 1975). These two cell systems supply their surrounding areas with short diffuse fibres (Bjorklund *et al.*, 1975). Finally, the tuberoinfundibular dopaminergic system. Cell bodies are located in the ARC and periventricular nuclei (Dahlstrom and Fuxe,

1964; Hokfelt and Fuxe, 1972; Bjorklund, Falck, Nobin and Stenevi, 1973a) with projections to 3 areas; the neural lobe of the pituitary (Smith and Fink, 1972), the intermediate lobe of the pituitary (Smith and Fink, 1972) and the ME (Bjorklund, Falck, Hromek, Owman and West, 1970; Smith and Fink, 1972; Jonsson, Fuxe and Hokfelt, 1972; Bjorklund *et al.*, 1973a,b; Ajika and Hokfelt, 1975).

1.5 Aims of this thesis

The aims of this thesis are to use constant light exposure to investigate the neural mechanisms which control spontaneous, pulsatile and diurnal LH release from the anterior pituitary gland of female rats. In addition, studies are also described which investigate the effect of steroid hormones on the neural apparatus which control gonadotrophin secretion in rats maintained in control or constant lighting.

CHAPTER II

Materials and Methods

2.1 Animals

Animals used for the majority of experiments were Wistar Cobs (caesarian originated barrier sustained) rats supplied by Charles Rivers Ltd. (Margate, Kent) or by the colonies maintained by the departments in Oxford or Edinburgh. For the experiments described in Chapter IX, 3 further strains were used: Hunter, RCS (Royal College of Surgeons), and PVG. These 3 strains were all derived from breeding colonies maintained in the Department of Pharmacology, Edinburgh.

Animals were maintained under either controlled (LD: lights on 05.00 h - lights off, 19.00 h) or constant illumination (LL) supplied by 30W fluorescent tubes 12 cm above the wire grill roof of each cage. Animals in both regimes were maintained at a temperature of 22°C and were allowed free access to food (Diet 41B, E. Dixon and Sons Ltd., Ware, or diet 41B, Oxoid, Basingstoke) and tap water. For animals supplied by Charles Rivers Ltd. about 10 days were allowed for acclimatization to the animal house before any experimental manipulation was performed.

Oestrous cycles were determined by daily inspection of vaginal smears usually obtained between 08.30 h and 10.30 h. Only LD animals showing 4-day oestrous cycles were used. Each day was termed metoestrus, dioestrus, pro-oestrus or oestrus, depending upon the predominant cell type present in the smear. That is,

- metoestrus - mixture of predominantly leucocytes, epithelial and cornified cells.
- dioestrus - leucocytes.
- pro-oestrus - pre-nucleated epithelial cells.
- oestrus - cornified epithelial cells.

Only females that had showed at least 2 consecutive cycles in LD were used for experiments.

2.2 Surgical and Experimental Manipulations

2.2.1 Anaesthetics

Five anaesthetics were used:

- i) Avertin- tribromoethanol in amylene hydrate 1 gm/ml, diluted (2 ml + 8 ml) in absolute alcohol and then finally in 0.9% saline solution (1 ml + 9 ml). The dose used (unless indicated) was 0.25 gm/kg body weight (b.w.) injected intraperitoneally (i.p.).
- ii) Sodium pentobarbitone - purchased as Nembutal (Abbott laboratories) or Sagatal (May and Baker Ltd., Dagenham) and consisted of 60 mg/ml of sodium pentobarbitone in alcohol: propylene glycol vehicle (10 : 20 v/v). The dose used was 36 mg/kg b.w. injected i.p. either undiluted or in some cases, freshly diluted 1 : 10 in 0.9% saline solution.
- iii) Althesin (Glaxo, Greenford, Middlesex) - 9 mg of alphaxalone 3 α -hydroxy-5 α -pregnane-11,20-dione) and 3 mg alphadalone acetate (21-acetoxy-3 α -hydroxy-5 α -pregnane-11,20-dione) per ml of isotonic aqueous vehicle, administered i.p. at a dose of 0.4 - 0.6 ml/100 g b.w.
- iv) Anaesthetic ether - purchased from Macfarlane Smith Ltd., Edinburgh.
- v) Halothane - purchased as Fluothane (I.C.I. Pharmaceuticals, Cheshire). Anaesthesia was induced by placing the rat in a

plastic box, through which a 5% Halothane/air mixture was circulated by a 'Vapor-Halothane' vaporiser. After induction, anaesthesia was maintained by circulating a 1.5% Halothane/air mixture to the animal through a face mask.

2.2.2 Ovariectomy

Ovariectomy was carried out bilaterally by way of two flank incisions under Avertin or halothane anaesthesia. The animal was shaved in the posterior flank region and after making a small (1.5 cm) incision, the ovary and distal end of the uterus exteriorised. The ovary and oviduct only were excised and the incision sutured.

2.2.3 Orchidectomy

Orchidectomy was carried out bilaterally under Avertin anaesthesia by way of a single midline incision in the ventral surface of the scrotal sac. After making an incision in the tunica vaginalis each testis was exteriorised and a ligature made around the spermatic cord. Each testis was excised and the incisions sutured.

2.2.4 Treatment of blood samples

Blood samples were always collected into syringes (usually 1 ml) heparinised with 1000 IU/ml heparinised saline. After collection, blood was expelled into plastic tubes (LP2 - Luckhams Ltd., Burgess Hill, Sussex) that had been cooled on ice. The samples were centrifuged at 4°C and the plasma taken off by pipette into plastic tubes (PT0944 - Luckhams Ltd.) for storage at -25°C.

2.2.5 Collection of peripheral blood - conscious animals

Peripheral blood was collected from conscious animals by way of a Silastic cannula that had been previously inserted under Avertin

anaesthesia down the external jugular vein and into the right atrium, according to the method of Greig (1971). Each cannula consisted of an intravenous portion (34-40 mm in length depending on the body weight of the animal) made up of Silastic tubing (0.5 mm I.D., 0.93 mm O.D., cat. no. 602-135: Dow Corning Corp. Michigan, U.S.A.) connected to extravenous Tygon tubing (0.25 mm I.D., 2.25 mm O.D., Technicon Instrument Corp., Michigan, U.S.A.) by a 9 mm stainless steel connector made from 23 gauge hypodermic needle tubing. Before insertion, each cannula was soaked in 1 : 250 chlorohexidine (Hibitane: I.C.I. Pharmaceuticals Ltd., Macclesfield, Cheshire) in distilled water for at least 1 h, and then washed in saline. The cannula was filled with a solution of heparin in saline (250 IU/ml) by way of a 1 ml syringe which was left connected to the Tygon tubing.

After induction of anaesthesia, the animal was secured in a supine position and the neck area washed with absolute alcohol. An incision (1.5 - 2.0 cm) was made longitudinally over the right external jugular vein (about 1 cm anterior to the clavicle). The tissue overlying the right external jugular vein was gently retracted to expose the vein. Four silk sutures (3/0) were passed underneath the vein, the two anterior ones being tied immediately. Gentle traction was applied to the most anterior suture and a small incision made on the ventral surface of the vein with a small pair of iridectomy scissors, anterior to the two untied sutures. The Silastic portion of the cannula was then introduced into the vein and inserted into the atrium. After patency of the cannula had been checked, the two posterior sutures were tied around the connector so holding it in place. Finally, the most anterior suture was tied around the Tygon tubing external to the vein so holding it in place. The heparinised saline syringe was removed from the Tygon tubing and

a stainless steel dental plug (1 cm x 0.5 mm) inserted into the open end. The cannula was brought out through the skin between the scapulae with the aid of a stainless steel trochar. After suturing the ventral incision, the cannula was again checked for patency and filled with heparinised saline. The Tygon tubing was trimmed to allow about 4 cm to protrude. The syringe was finally removed and the stainless steel dental plug replaced. Animals were placed in individual cages for the duration of the experiment.

Blood samples were obtained by removing the plug and inserting a sample cannula. This consisted of a length (15 cm) of Tygon tubing filled with heparinised saline (250 IU/ml) one end of which contained a 23 gauge needle connector which was inserted into the open end of the jugular cannula. Into the other end of the sample cannula a 1 ml syringe and a 23 gauge needle filled with heparinised saline (250 IU/ml) was inserted. Heparinised saline from the jugular cannula (along with any air) was withdrawn until blood reached the syringe, which was then replaced with the sample syringe and the appropriate volume of blood withdrawn. The sample cannula was clamped with the thumb and first finger proximal to the open end (to prevent air embolism) and the syringe containing heparin/saline replaced. Blood in the jugular cannula was replaced with heparinised saline.

In some experiments (Chapters IV, V and IX), the sample cannula was inserted at every sample time, while in others (Chapter VI) the sample cannula was positioned at the beginning of each experiment and the free end brought outside the wire grill roof of the cage remaining there for the duration of the sampling period, the animals therefore being undisturbed by the sampling manipulation.

2.2.6 Collection of peripheral blood -anaesthetized animals

This was achieved by exposing the external jugular vein as described in the previous section (2.2.5). A 25 gauge hypodermic needle was introduced into the vein through the hyoid muscle, and the appropriate blood volume was withdrawn into a heparinised syringe. This method of blood collection allowed repeated blood sampling at frequent intervals with minimal trauma to the vessel.

2.2.7 Collection of pituitary stalk blood

Blood from the hypothalamo-hypophysial portal vessels was collected essentially according to the method first described by Worthington (1966) and used in this laboratory by Fink and Jamieson (1976) with minor modifications. The method was based on the trans-pharyngeal approach used by Green and Harris (1949) to expose and visualize the hypophysial portal vessels.

The animal was anaesthetized and placed in a supine position on an operating board. A longitudinal midline incision about 10 cm long was made in the lower lip and extended caudally. The skin and subcutaneous tissue were retracted to expose the muscles overlying the trachea. A 1 cm longitudinal midline incision was made in these muscles and a small suture passed under the exposed trachea. A small transverse incision was made in the trachea rostral to the suture and a 1.5 cm polythene tube introduced into the incision for a distance of about 1 cm (rostral to the junction of the bronchi). This was tied in place with the suture. A long silk suture (3/0) was placed through the tip of the tongue to allow gentle traction to be applied to it. To stop bleeding from branches of the lingual arteries, a tight ligature was placed around each mandibular ramus, with the aid of a pair of forceps to avoid damage to the tongue. A suture was inserted through

each of the flaps of the divided lower lip and one of these was secured to the operating board. With manual traction applied to the other lip suture, the lower jaw was split with a midline longitudinal incision starting between the incisor teeth. The incision was continued caudally and eventually divided to go parallel each side of the mandible so resulting in mobilization of the tongue. Care was exercised in extending these incisions caudally, since over extension resulted in damage to the lingual arteries, while insufficient extension resulted in insufficient mobilization of the tongue. A suture was placed around the base of the tongue, and with the aid of the suture through its tip, the tongue was withdrawn and secured in position over the chest, resulting in visualization of the soft palate and the epiglottis. The preparation was placed under a Zeiss binocular operating microscope. The soft palate was incised with an iris electrocautery longitudinally along its midline from about 1 mm anterior to the epiglottis to the posterior edge of the hard palate, and laterally to the pterygoid processes of the sphenoid. This incision exposed the mucosa overlying the basophenoid which was removed using cotton wool. A midline hole was drilled in the basosphenoid bone about 1 mm posterior to the now visible transverse venous sinus, with the hole having the sphenoid-basosphenoid suture in its posterior portion. The hole was drilled deep enough to visualize the inner table of bone, and was packed (along with the trough over the basosphenoid bone) with bone wax (Ethicon Ltd., Edinburgh). The drill hole was extended rostrally as far as the basosphenoid - presphenoid suture taking care to avoid rupture of the transverse venous sinus. Any osseous bleeding was controlled with further application of bone wax and/or cotton wool. When the inner palate was exposed along the full length of the trough

it was 'polished' carefully around its edge with the drill, taking care to avoid excessive pressure. When sufficiently thin, the inner table was removed carefully with watchmakers forceps. Removal of the inner table of bone exposed the dura mater overlying the vessels, anterior portion of the adenohypophysis and the median eminence. The dura mater overlying the median eminence and pituitary stalk was cut in a V-shape using a small piece of razor blade held in a pin cluck. The cuts were started over the pituitary gland and extended rostrally, lateral to the portal vessels to meet just rostral to the median eminence. Cerebrospinal fluid was removed with small pieces of cotton wool. The flap of dura mater and arachnoid mater so formed was reflected caudally to expose the portal vessels. The animal was given 2,500 IU of heparin (heparin sodium - mucous, injection solution 5,000 IU/ml; Weddel Pharmaceuticals, London) into the external jugular vein. Using small iridectomy scissors (Weiss Ltd., London), the portal vessels were cut close to the pituitary gland to include as many portal vessels as possible. Blood was collected from the trough using a Pasteur pipette; fluid collected for the first 5 min after cutting was discarded to allow any tissue debris to be removed. The actual collection period commenced after this initial 5 min period and blood was collected using a Pasteur pipette and was placed in a small pre-cooled tube (LP2, Luckham Ltd.) kept on ice. At the end of the collection period the volume of blood was recorded, as was the volume of blood cells and plasma obtained after centrifugation. The plasma was stored at -25°C .

2.2.8 Preparation and implantation of oestradiol capsules

Silastic capsules containing oestradiol- 17β were prepared according to the method of Karsch, Dierschke, Weick, Yamaji, Hotchkiss and Knobil

(1973) and used in this laboratory by Henderson, Baker and Fink (1977b). Medical grade Silastic tubing (2.6 mm I.D. x 4.9 mm O.D., cat. no. 601-331, Dow Corning Corp. Michigan, U.S.A.) was cut into lengths determined by the size of the desired capsule. One end of the capsule was sealed with Silastic elastomer which was made by mixing catalyst and elastomer (approximately 1 : 1000 v/v; cat. no. 382, Dow Corning Corp.). The inside of the capsule was then packed with crystalline oestradiol-17 β (Sigma Ltd., London) and the open end then sealed with the elastomer. Capsules were implanted subcutaneously into the back of animals through a small incision using a stainless steel trochar (7.5 cm length, 6.5 mm O.D. x 5.5 mm I.D.). The capsules were incubated in donor animals for at least 24 hours and were taken out of the donors immediately before implantation into experimental animals.

2.2.9 Steroids for subcutaneous injection

Steroids used for subcutaneous injection were oestradiol benzoate, progesterone and testosterone propionate (all obtained from Paines and Byrne Ltd., Greenford, Middlesex). All were supplied in ethyl oleate and were diluted to the working concentration with arachis oil (Hopkins and Williams, Romford, Essex). The diluted steroids were injected subcutaneously (under light ether anaesthesia) or intramuscularly into the hind leg.

2.2.10 Determination of ovulation

Ovulation was determined in animals with regular oestrous cycles on the morning of oestrus (09.00 - 10.30 h) or 24 h after experimental manipulation in animals exposed to long-term LL. The timing in LL exposed animals was based on the observations of Singh and Greenwald

(1967) and Brown-Grant, Davidson and Greig (1973). Animals were killed by cervical dislocation and the ovaries and oviducts excised. The oviducts were dissected from the ovaries placed on a glass microscope slide in a drop of 0.9% saline solution and examined under a Zeiss transilluminating microscope. Any ova present (usually in a dilated segment) were extruded and counted.

2.2.11 Determination of uterine and uterine fluid weights

Animals were killed by cervical dislocation after experimentation and the uterus carefully dissected out and trimmed free of fat, taking care not to lose any fluid present. The uterus was weighed on a balance (Mettler) after which any fluid was extruded. The dry uterus was reweighed and the difference between the two weights taken as the fluid weight.

2.2.12 Determination of pituitary responsiveness to luteinizing hormone releasing hormone (LHRH)

The responsiveness of the anterior pituitary gland was determined according to the method of Aiyer, Fink and Greig (1974a). That is, the maximum increment in plasma LH concentration was determined in each animal after an i.v. injection of (50 ng/100 g b.w.) synthetic LHRH (I.C.I. Pharmaceuticals). The dose of LHRH was the minimum necessary to result in ovulation in rats that had been blocked with sodium pentobarbitone during the critical period on pro-oestrus (Aiyer *et al.*, 1974a).

Animals were anaesthetized with sodium pentobarbitone (36 mg/kg b.w.) and 30-45 min later given an i.v. (external jugular vein) injection of 50 ng/100 g body weight synthetic LHRH. Blood samples (0.45 ml) were withdrawn into heparinised syringes 2 min before and

10, 15, 20, 30 and 60 min after the injection of LHRH. The plasma was removed and stored as previously described (2.2.4). Plasma LH concentrations were determined by radioimmunoassay as described below (2.3.3). The maximum increment in plasma LH concentration was determined for each animal by subtracting the plasma LH concentration before LHRH injection from the maximum post-injection value. In the vast majority of cases the value at 60 min was lower than the maximum but in only a few cases had it returned to the value before the LHRH injection.

The LHRH used for these studies was stored at a concentration of 100 µg/ml in distilled water in 200 µl aliquots. The injection solution was made up fresh in 0.9% saline solution just before injection and the LHRH concentration checked after each experiment by radioimmunoassay (see below, 2.3.2). If the LHRH concentration was found to be significantly different from the expected concentration, the results from that experiment were discarded. The stock solution was stored at -25°C and used on several occasions (4-6 times).

2.2.13 Dissection of hypothalamus and preoptic area

Animals were decapitated and the brains quickly dissected onto ice. The optic nerve was carefully removed and, using fine scissors, a cut about 1-1.5 mm deep was made around the border (see Figure 2-S) of the hypothalamus and preoptic area. The cut was extended under the hypothalamus and the tissue removed for weighing and homogenization.

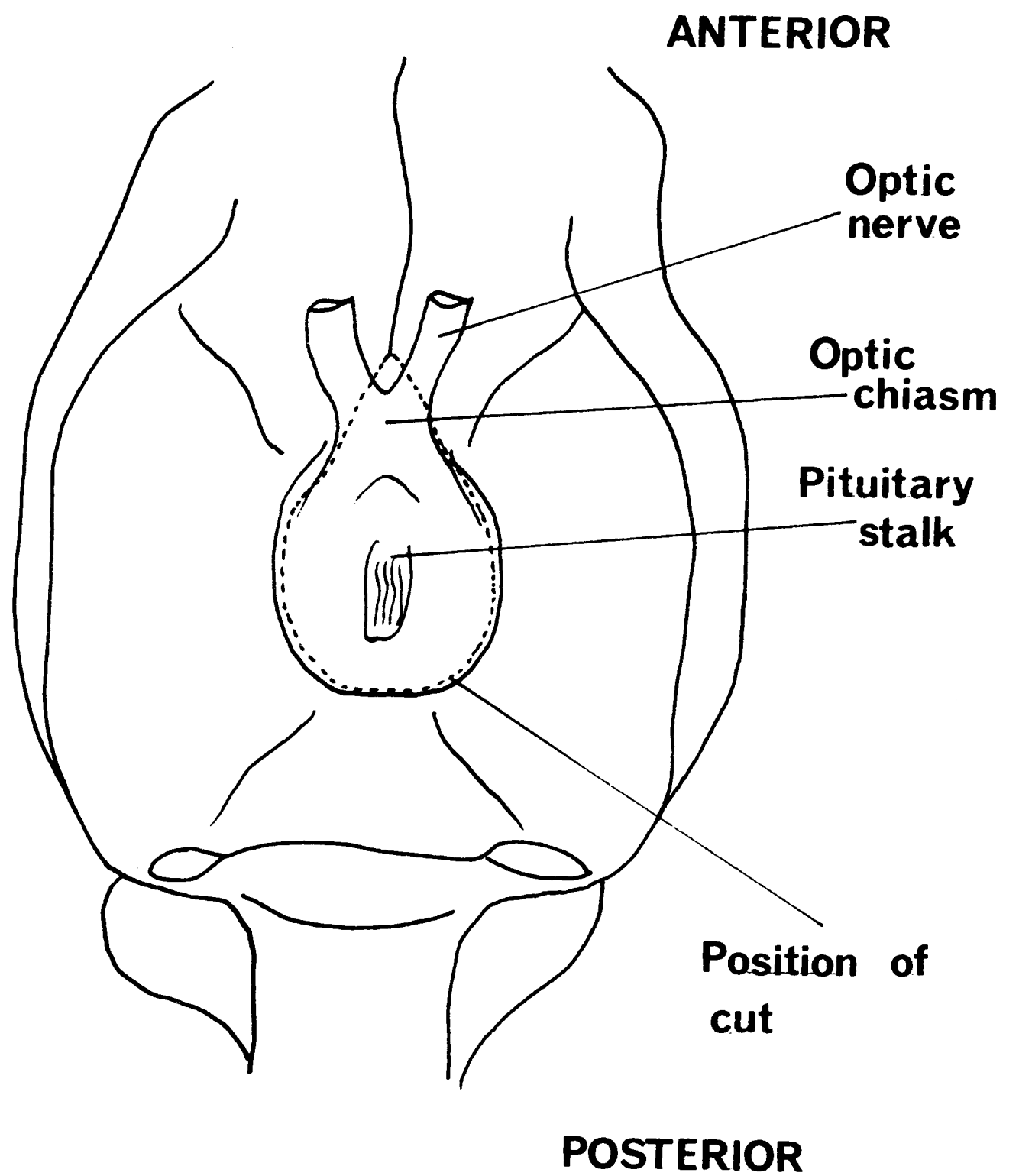


FIGURE 2-S: Ventral surface of rat brain showing position of the cut for optic-hypothalamic dissection.

2.3 Analytical Methods

2.3.1 Radioimmunoassay: Introduction

Radioimmunoassay (RIA) techniques were used to measure the concentrations of LHRH, LH, oestradiol-17 β and progesterone in rat plasma. Numerous reviews have described the basic principles and theory of RIA of peptide and steroid hormones (Midgley, Niswender and Rebar, 1969; Kirkham and Hunter, 1971; Rodbard, 1971; Abraham, 1974; Ekins, 1974; Yalow, 1980) and therefore only the relevant methodology is described here.

Synthetic LHRH and ovine LH were labelled with ^{125}I (Na ^{125}I ; Amersham International, Amersham, Bucks.) using a chloramine-T method modified from Greenwood, Hunter and Glover (1963) (Appendix 1). Labelled oestradiol-17 β (2,4,6,7- ^3H oestradiol-17 β) and progesterone (1,2,6,7- ^3H progesterone) were purchased from Amersham International. LH and LHRH were measured using double-antibody techniques (Utiger, Parker and Daughday, 1962), while oestradiol-17 β and progesterone were measured using dextran-charcoal separation RIA. In all assays, the bound hormone was retained for counting. The lower limit of assay sensitivity was determined as being the mean counts/min (cpm) minus 2 x standard deviation of the total bound tubes (Rodbard, 1971). All RIA were calculated using linear regression from standard curves (logit B/B_0 * on the ordinate, log concentrations on the abscissa). LH and LHRH RIA's used in Chapters III, IV and V were calculated using a Hewlett-Packard 9318 programmable desk-top

* B/B_0 = cpm of standard of sample - background/cpm of total bound - background. $\text{logit } B/B_0 = \ln\left(\frac{B/B_0}{1-B/B_0}\right)$.

calculator and a programme described by Pickering (1978). LH RIA's used in Chapters VI and IX were calculated using an automatic Berthold Mag 310 Gamma Counter (Scotlab Instrument Sales Ltd., Carlisle, Lanarkshire). The LH RIA's used in Chapter VII were calculated using both methods. Oestradiol-17 β and progesterone RIA's were calculated with an on-line 'Apple 2' microcomputer.

The quality controls of LH and LHRH RIA's were monitored using two pools in each assay. The pools were plasma from ovariectomized rats (high and low pools, LH) or plasma from male rats with either acid extract of rat hypothalamic tissue (high pool, LHRH) or synthetic LHRH (low pool, LHRH) added. The assays for oestradiol-17 β and progesterone were only used once (Chapter VII), and consequently no pools as such were used. Instead in each assay, one sample was assayed three times in duplicate, and the values of these samples were used to calculate intra-assay variation. Further quality control for LH and LHRH RIA was determined by monitoring the variation of the total counts, blanks, the total bound as a percentage of the total counts (% bound) and the equivalent concentrations of the $+2, 0, -2 \log_{10} B/B_0$. An assay was considered in the control limits if it was within 95% confidence limits of these parameters.

Samples for all RIA's were aliquoted into assay tubes (LP3, Luckhams Ltd.) using Gilson Pippetman (Scotlab Instrument Sales Ltd.) or Eppendorf automatic pipettes. Reagents were aliquoted using 'Micromedic' automatic pipetting systems.

2.3.2 Radioimmunoassay of luteinizing hormone releasing hormone

LHRH was measured using the double-antibody RIA developed by Nett, Akbar, Niswender, Hedlund and White (1973). The LHRH

RIA protocol used in this thesis (Appendix 2) was described by Jamieson (1974), Fink and Jamieson (1976) and Chiappa (1976).

Synthetic LHRH (I.C.I. Pharmaceuticals, Macclesfield) was used for standards (range 7.8 pg/ml - 500 pg/ml) and for iodination. The anti-LHRH antibody (R-42-anti-GnRH) was raised in rabbits and was provided by Dr. G.D. Niswender (NIAMDD, Bethesda, MD, U.S.A.). The antibody recognized the whole LHRH molecule and did not cross-react with LHRH analogues and fragments (Nett *et al.*, 1973). No cross-reactivity (<0.01%) was found with various peptides and pituitary hormones (Nett *et al.*, 1973; Jamieson and Fink, 1976; Chiappa, 1976). After incubation of the first antibody (working dilution 1 : 80,000), ^{125}I -LHRH and standard/sample, the bound hormone was separated from the free fraction by anti-rabbit gamma-globulin (ARGG) raised in donkeys (Wellcome Reagents Ltd., Beckenham, Kent). A working dilution of 1 : 30 was used for the second antibody. A typical standard curve is shown in Figure 2-A. Using a high pool (mean value 130 pg/ml, n=5) and a low pool (mean value 76 pg/ml, n=5), the intra- and inter-assay coefficients of variation were 11% and 8% respectively. The lower limits of sensitivity of the assays for 100 μl samples of hypophysial portal blood ranged from 10-12 pg/ml. The quality control of the parameters described in Section 2.3.1 are shown in Figure 2-B.

2.3.3 Radioimmunoassay of luteinizing hormone

LH was measured in rat plasma using the ovine-ovine RIA developed by Niswender, Midgley, Monroe and Reichert (1968). The LH RIA protocol used in this thesis (Appendix 2) was described by Aiyer (1974) and Aiyer and Fink (1974). Ovine LH (LER-1056-C2) provided by Dr. L.E. Reichert, Jr. (NIAMDD) was used for iodination

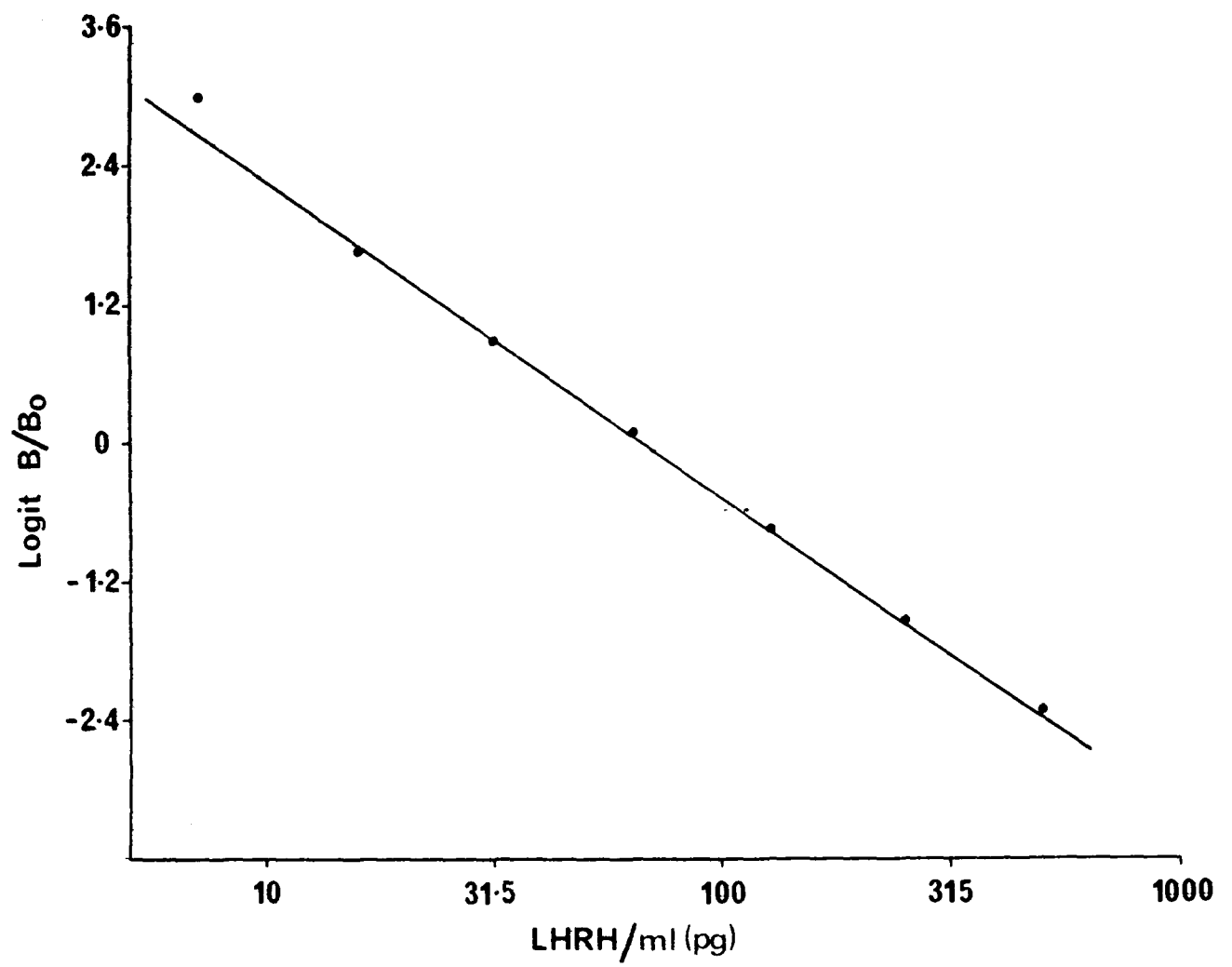


FIGURE 2-A: Standard curve of a representative assay for luteinizing hormone releasing hormone (LHRH). Each point represents the mean of 3 replicates.

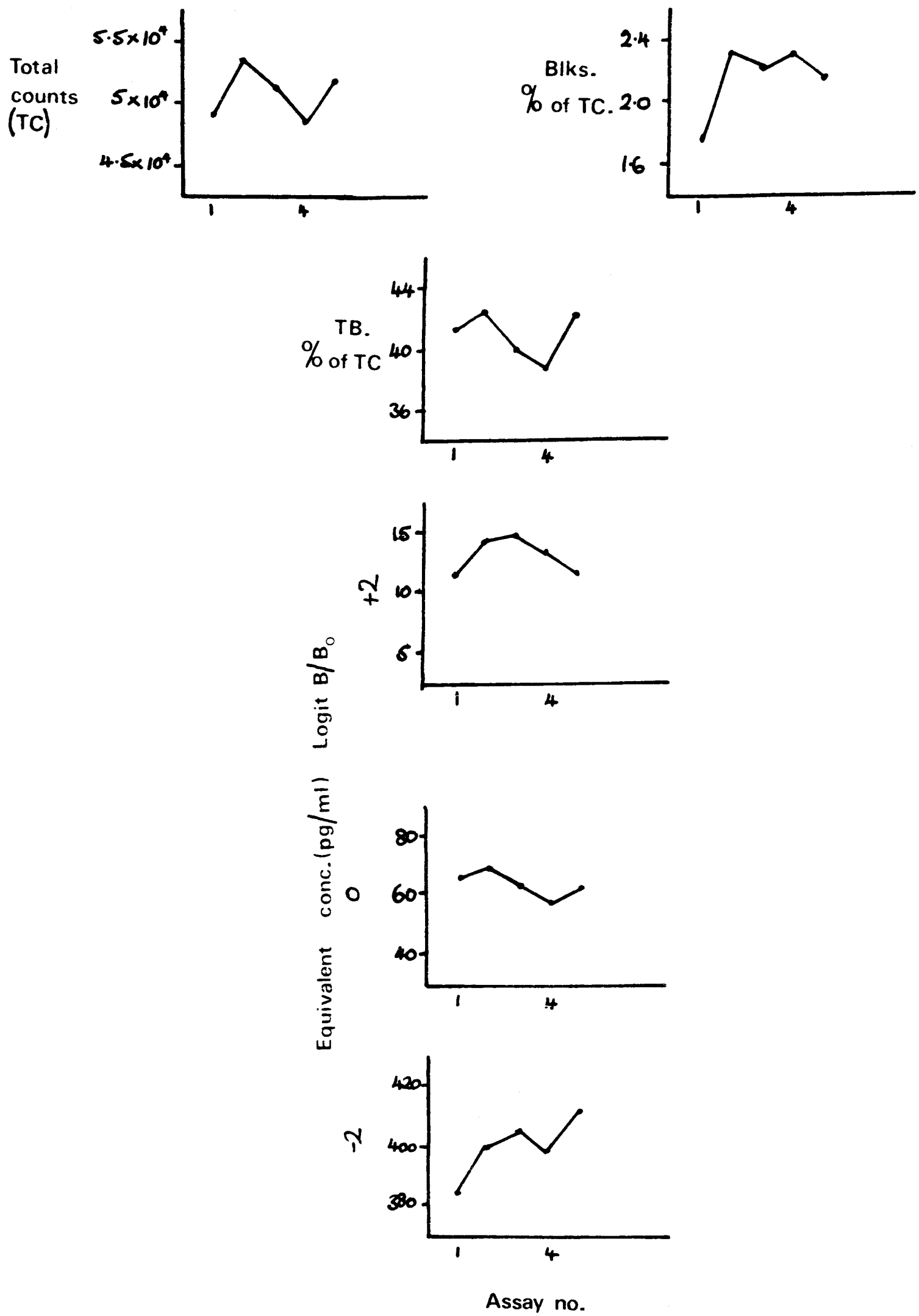


FIGURE 2-B: Quality control for luteinizing hormone releasing hormone (LHRH) RIA's.

(Appendix 1) and ovine LH (NIH-LH-S18) provided by Dr. A.F. Parlow (NIAMDD) was used for standards (range 0.25 - 16 ng/ml). The anti-ovine LH antiserum (GDN-15) was provided by Dr. G.D. Niswender (NIAMDD) and was used at a working dilution of 1 : 60,000. The characteristics of the antibody were described by Niswender *et al.* (1968). After incubation of ^{125}I -LH, anti-LH serum and the samples/standards, the bound hormone was separated from the free hormone using ARGG (Wellcome Reagents Ltd.) at a working dilution of 1 : 30. A typical standard curve is shown in Figure 2-C. The inter-assay coefficient of variation was 10% for a high pool of 42.8 ng/ml (mean, n=15) and 7% for a low pool of 13.2 ng/ml (mean, n=15). The intra-assay variation for the high and low pools was 7% and 9% respectively. The lower limits of sensitivity for 20 μl plasma samples were between 2.5 and 3.0 ng/ml and between 1.0 and 1.2 ng/ml for 50 μl plasma samples. The quality control of the LH RIA's used in this thesis is shown in Figure 2-D.

2.3.4 Radioimmunoassay of oestradiol-17 β

Oestradiol-17 β was measured in plasma samples using an RIA modified from that used by Dick, Culbert, Wells, Gilbert and Davidson (1978). The protocol used in this thesis is described in Appendix 3. The antiserum (73/349) was raised in rabbits against 6-keto-oestradiol-17 β -carboxy methyloxine and was provided by Dr. J.W. Wells (ARC Poultry Research Centre, Edinburgh). The cross reactivity with oestrone was <1%, and <0.01% with oestriol, progesterone, corticosterone, androsteredione, testosterone, 17 α -progesterone or 20 α -progesterone, up to concentrations of 1 ng/tube. Extraction of oestradiol-17 β from plasma was carried out using a modification of Culbert (1976) and resulted in 75-85% extraction of ^3H -oestradiol-17 β from plasma. Using

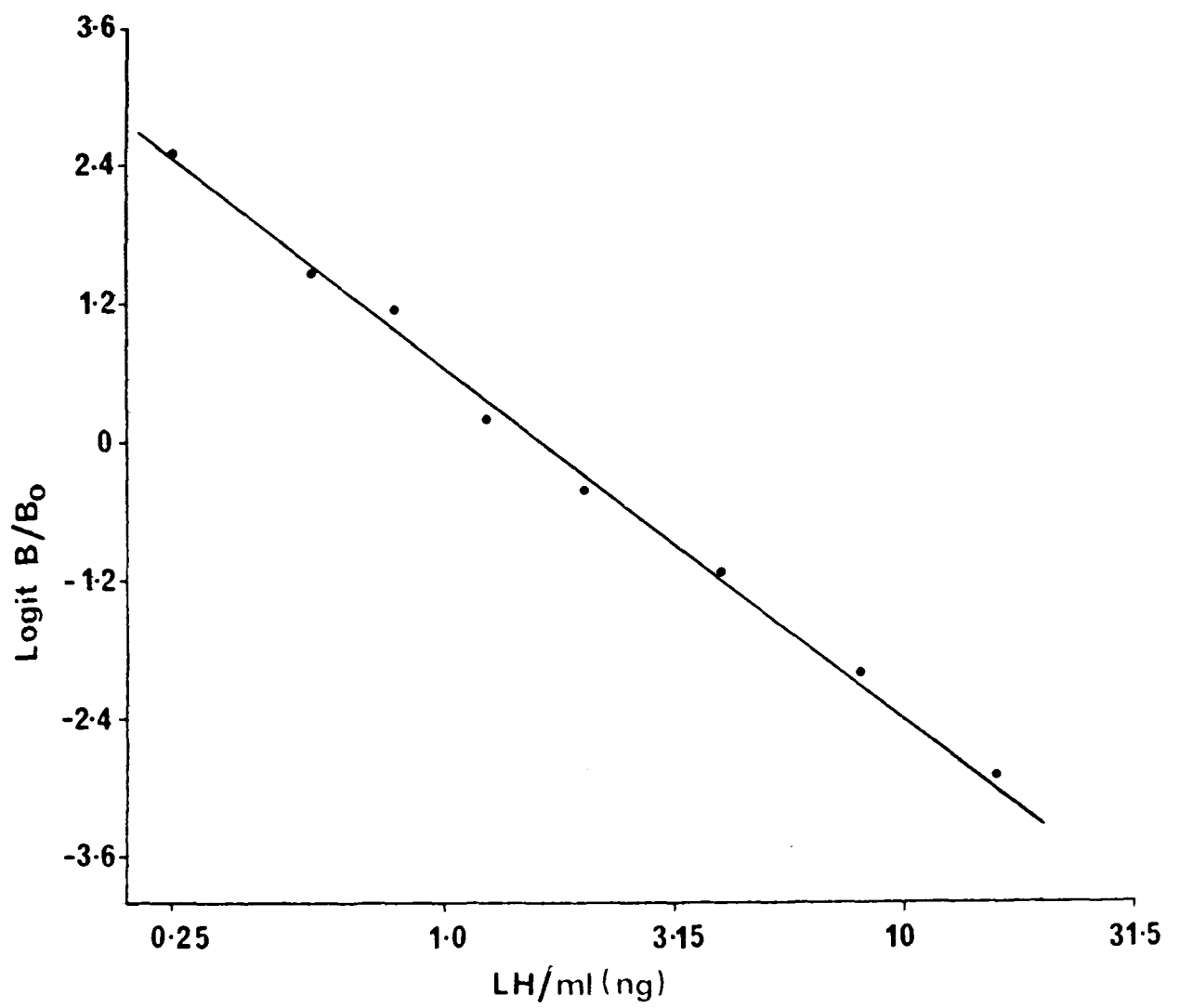


FIGURE 2-C: Standard curve of a representative assay for luteinizing hormone (LH). Each point represents the mean of 3 replicates.

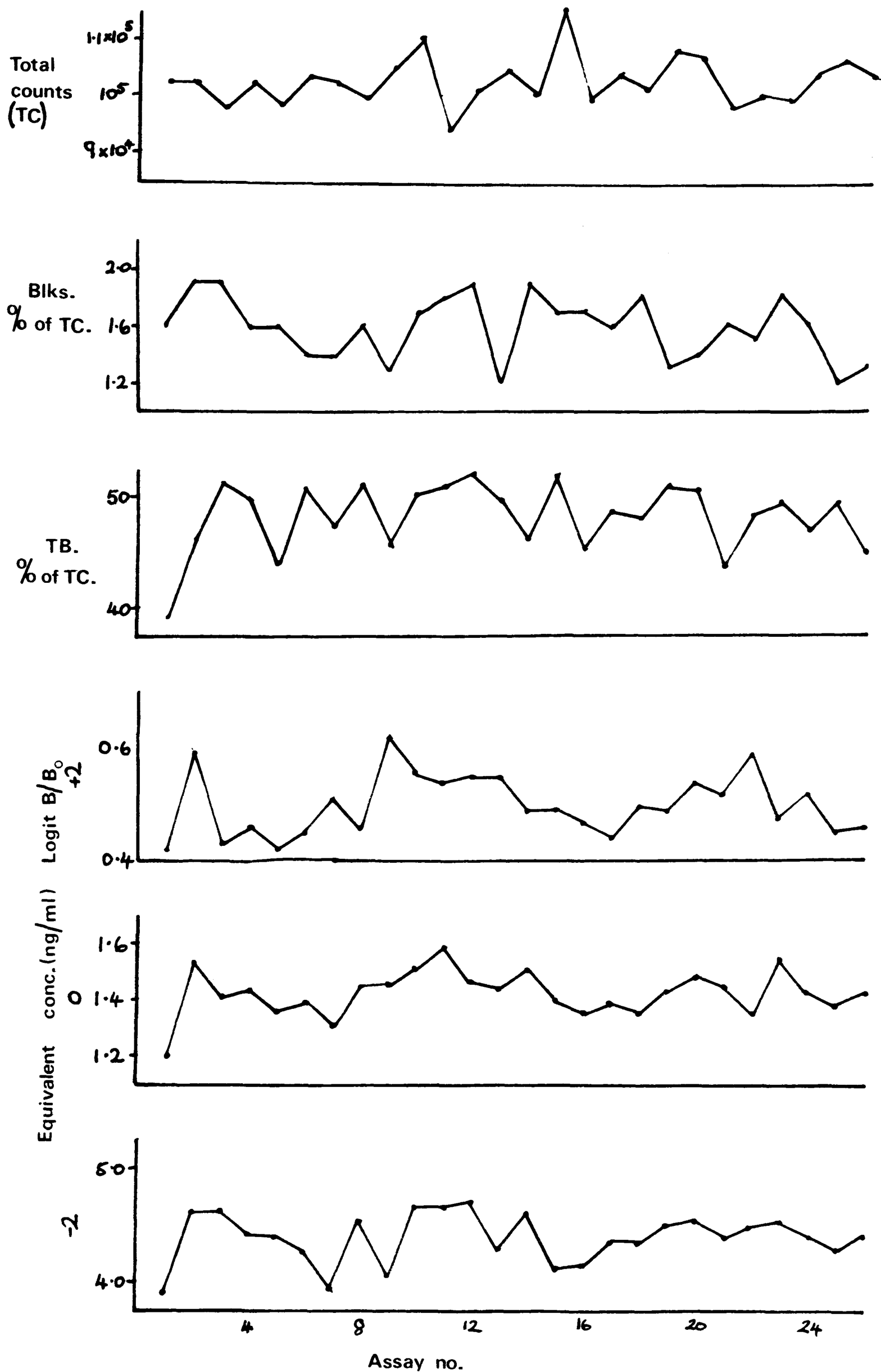


FIGURE 2-D: Quality control for luteinizing hormone (LH) RIA's.

oestradiol-17 β added to plasma, recoveries of 94% for 36 pg/ml and 98% for 144 pg/ml, 0.5 ml extracted plasma were found. The standard curve (Figure 2-E) was prepared from oestradiol-17 β (Sigma, London) and ranged from 4.5 - 144 pg/tube. After incubation of ^3H -oestradiol, antibody (working dilution, 1 : 300,000) and standards/samples, the bound hormone was separated from the free hormone using dextran-charcoal (Norit-A activated charcoal and dextran T-70: Sigma, London). 500 μl of the supernatant was taken for counting. The intra-assay coefficient of variation for a sample analysed three times in duplicate (mean 20.6 pg/ml) was 8%. The lower limit of sensitivity for 500 μl extracted plasma was 5 pg/ml.

2.3.5 Radioimmunoassay of progesterone

Progesterone was measured in plasma samples using a RIA modified from that described by Sarkar (1979). The antiserum was provided by the World Health Organization (W.H.O. RIA Reagent Program, batch 79/6), and using the assay conditions described in Appendix 3, did not significantly cross-react (<0.01%) with 17 α -progesterone, 20 α -progesterone, 20 β -progesterone, oestradiol-17 β and corticosterone. Plasma samples (50 μl or 200 μl) were extracted using petroleum ether at room temperature (Johansson, 1970; Sarkar, 1979), resulting in 50-77% extraction of ^3H -progesterone from plasma. After incubation of the ^3H -progesterone, antiserum (working dilution 8 : 30 of the W.H.O. 79/6 antibody of unspecified dilution) and standard/sample, the bound hormone was separated from the free hormone using dextran/charcoal (see Section 2.3.4). 500 μl of the supernatant was taken for counting. The standard curve (Figure 2-F) was prepared from progesterone (Sigma, London) and ranged from 6.6 to 660 pg/tube.

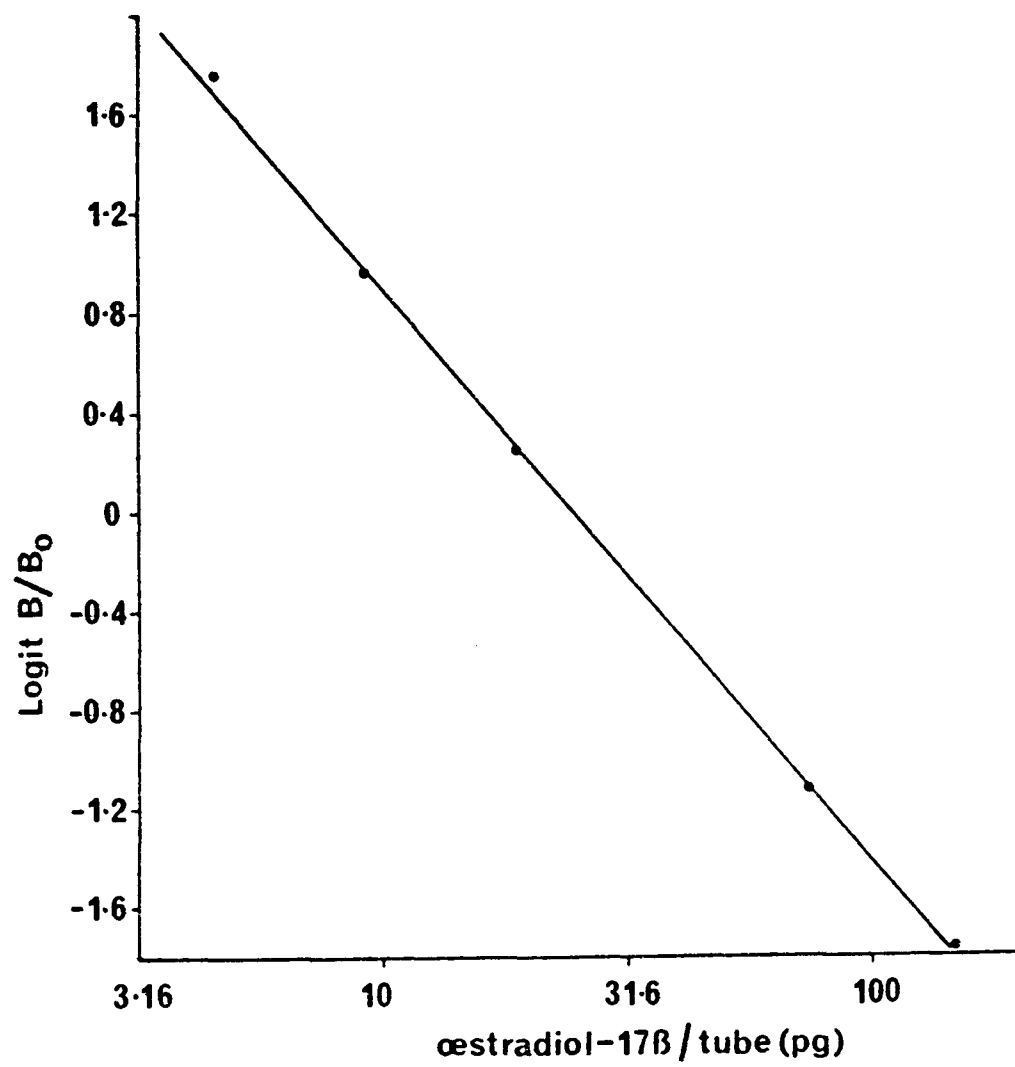


FIGURE 2-E: Standard curve of the assay for oestradiol-17 β . Each point represents the mean of duplicates.

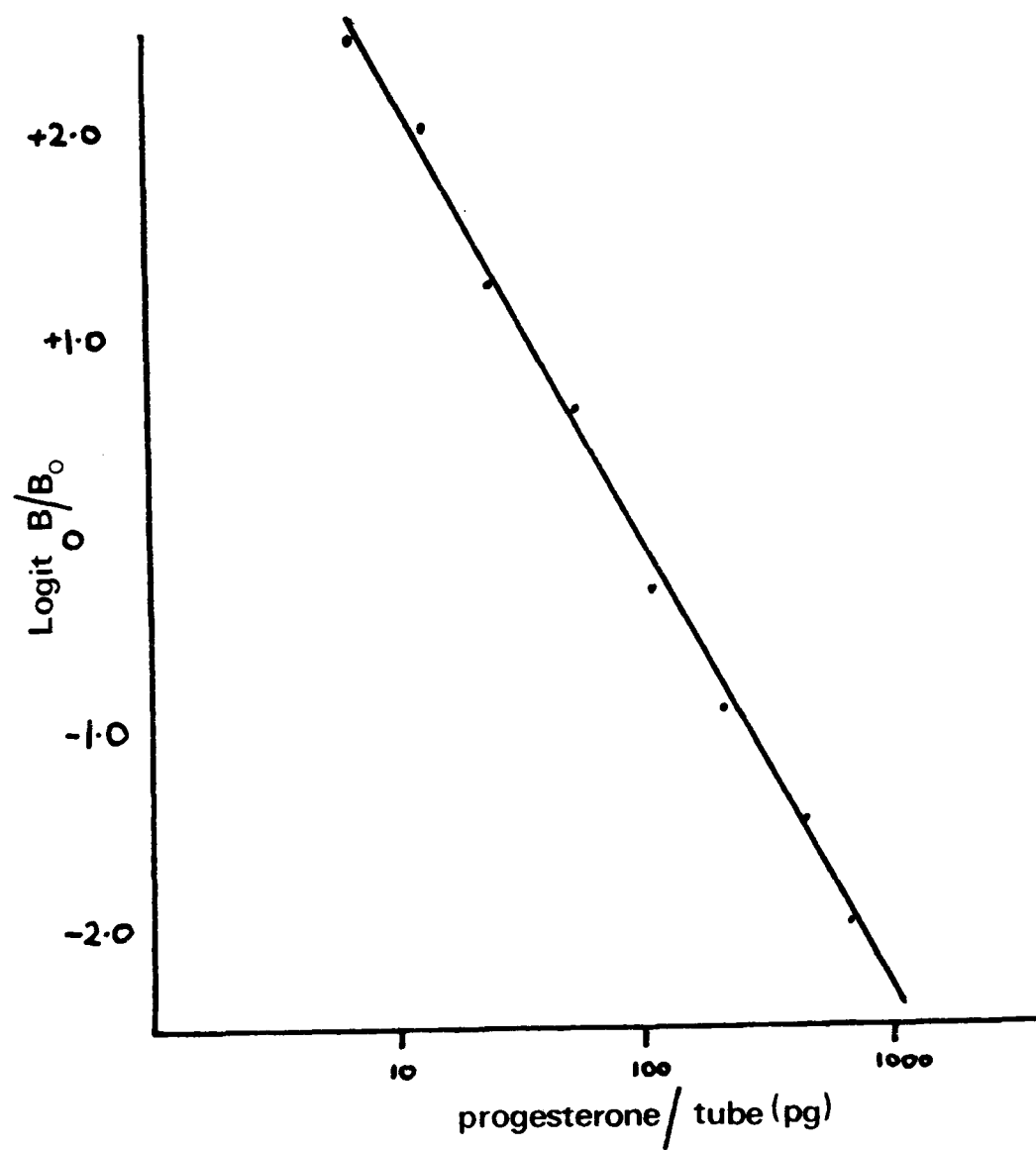


FIGURE 2-F: Standard curve of the assay for progesterone. Each point represents the mean of duplicates.

The lower limit of sensitivity was 1.2 ng/ml for 200 µl of extracted plasma. The intra-assay coefficient of variation for a sample assayed three times in duplicate (mean 8.8 ng/ml) was 12%.

2.3.6 High performance liquid chromatography

Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DoPAC), noradrenaline (NA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were measured by high performance liquid chromatography with electrochemical detection (LCED). The sensitivity of LCED was comparable to radioenzymatic assays available for catecholamines and indoleamines (Coyle and Henry, 1973; Saavedra, Brownstein and Axelrod, 1973; Cuello, 1978), however, LCED methods allowed simultaneous detection of DA, NA and 5-HT, and their metabolites with minimal sample preparation and assay procedure (Mefford and Barchas, 1980; Mefford, 1981).

2.3.7 Methods and materials

a) Instrumentation:

Figure 2-G shows schematically the system used in the present studies. Unless otherwise stated, all apparatus were purchased from Scotlab Instrument Sales Ltd., Carlisle.

The mobile phase was pumped through the system at a constant flow rate of 1.0 ml/min using an Altex 110A pump. The sample was introduced onto the columns using an SGE 25 µl injection syringe and a Rheodyne 7125 injection valve fitted with a 20 µl sample loop.

Figure 2-H shows the switching pattern of the injection valve. The valve allowed introduction of the sample onto the columns with minimal flow disturbance and loss of volume.

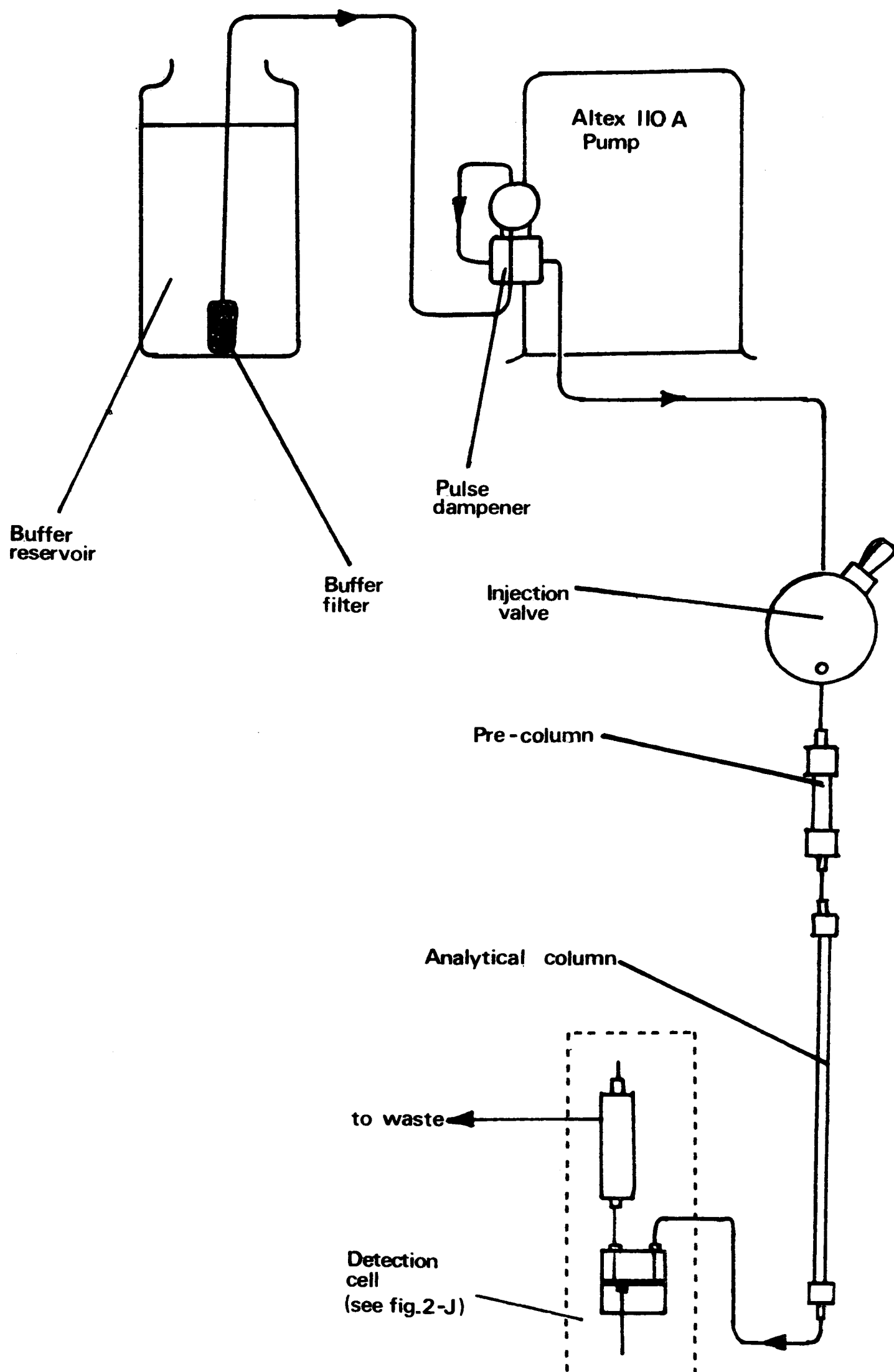


FIGURE 2-G: Schematic diagram of the LCED system.

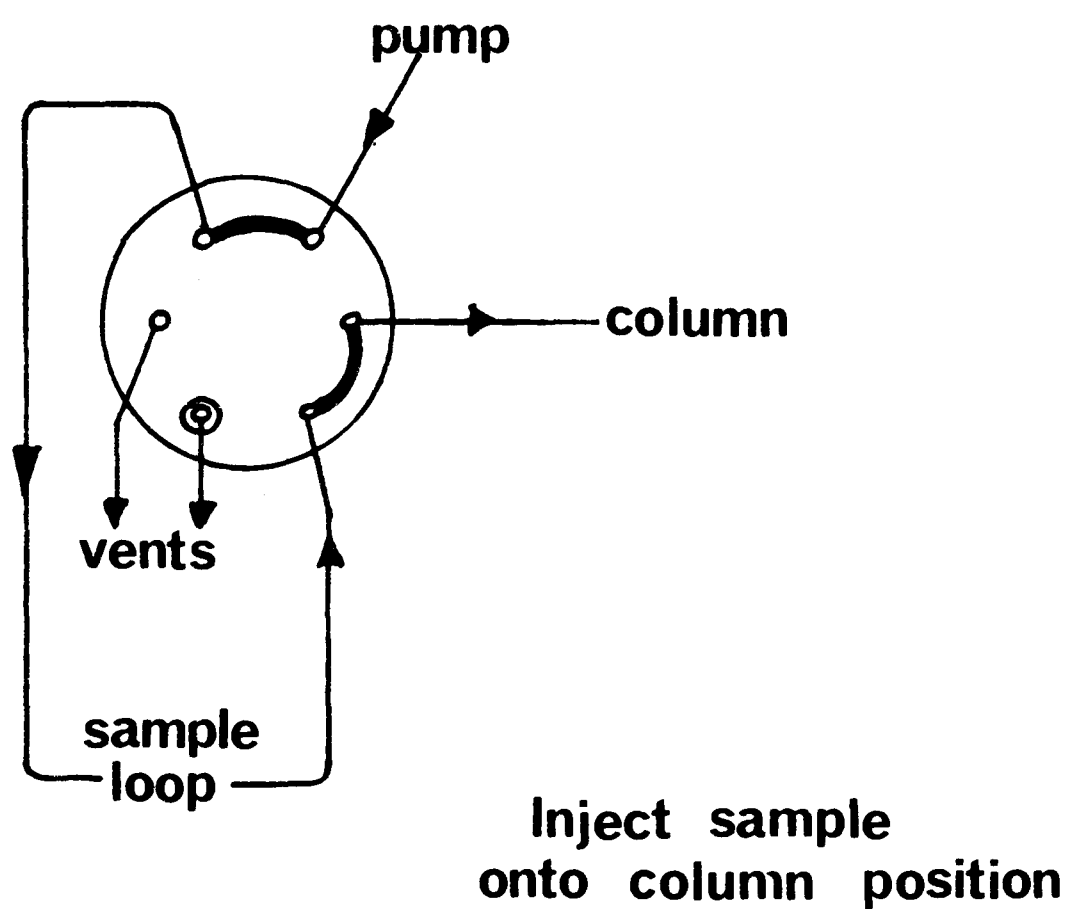
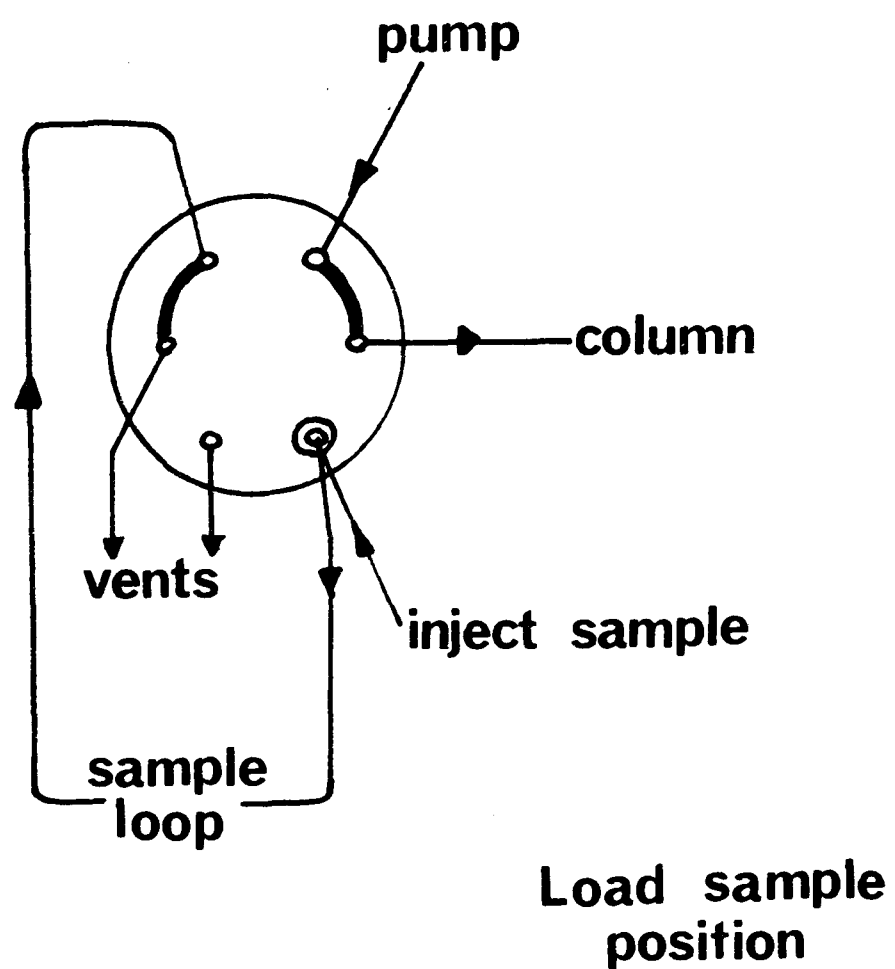


FIGURE 2-H: Flow switching arrangement of the Rheodyne 7125 HPLC injection valve.

Initial separation, purification, and protection of the analytical column from deterioration was carried out using a pre-column consisting of a 10 cm x 0.4 cm stainless steel column packed with Ultrasphere ODS (purchased prepacked), or Spherisorb 5 μ m ODS packed in this laboratory at a pressure of 4000 p.s.i., using a Magnus Slurry packer. No difference in performance was observed between Ultrasphere ODS and Spherisorb ODS pre-columns and these two types of columns were interchanged as necessary. The columns were fitted with zero dead volume stainless steel end fittings connected to the valve and analytical column using $\frac{1}{16}$ " O.D. x 0.008" I.D. type 316 stainless steel tubing. This type of tubing was used throughout the system unless otherwise stated.

The top of the pre-column was checked every 2-3 weeks for damage which was evident as a discoloration to the normally white packing material and an increase in back pressure registered on the Altex 110A pump. The damaged pre-column was repaired by removing the top 3-4 mm of packing material and replacing it with Ultrapac 5 μ m ODS. If the pre-column was found to be excessively worn (usually recognized by severe deterioration in performance), the whole column was repacked with Spherisorb 5 μ m ODS.

The major separation of the sample was carried out using a 25 cm x 0.46 cm Ultrasphere ODS column purchased prepacked. The Ultrasphere ODS packing material consisted of 5 μ m silica particles coated with hydrocarbon chains 18 CH_2 group in length. ODS packing material was reversed phase (i.e. hydrophobic) and allowed separation of ionic, non-ionic and ionizable water soluble compounds. The number of theoretical plates (calculated from the distance of a peak from the injection point, and the half peak height width (Knox, 1978) -

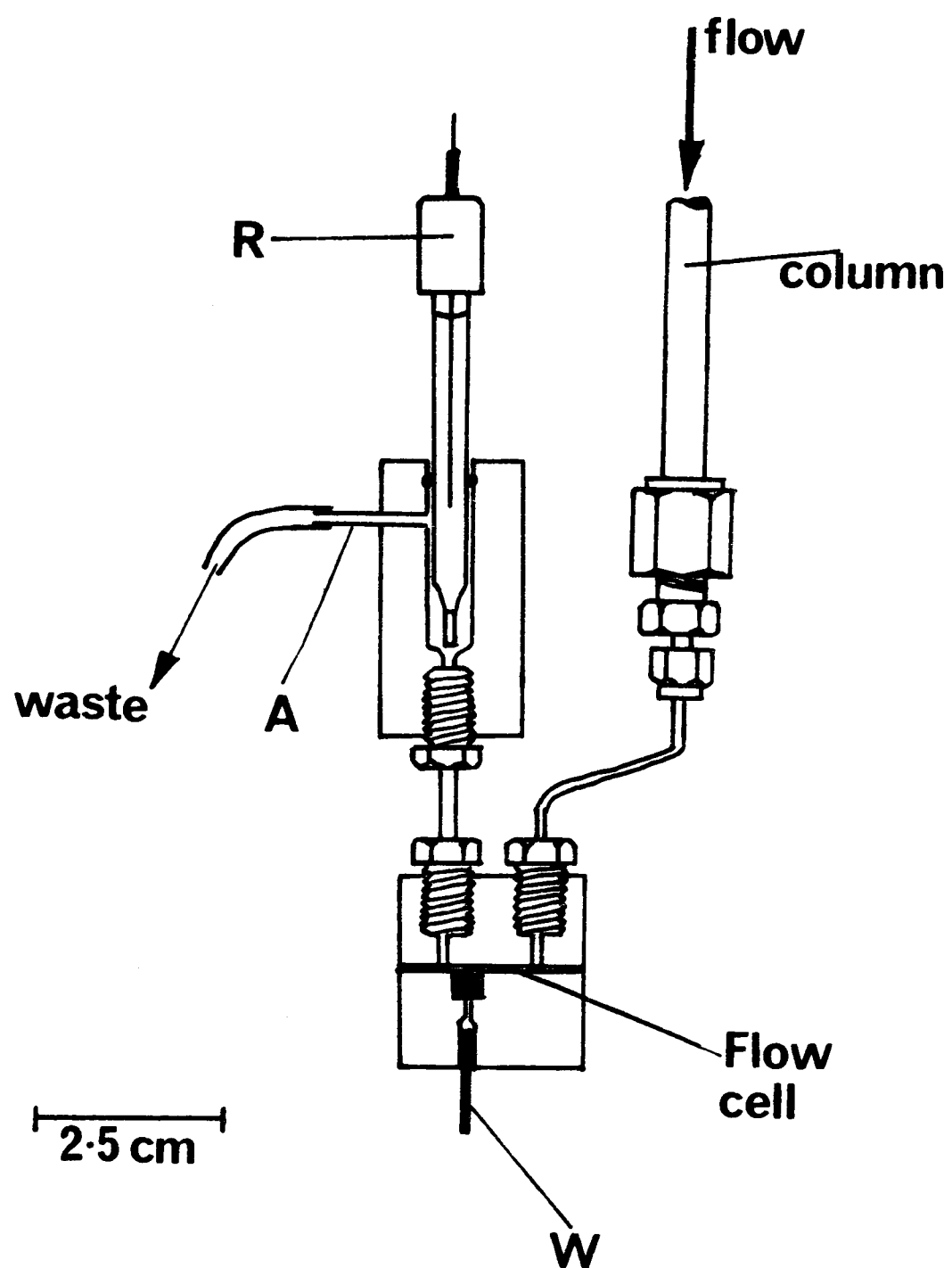
see Figure 2-P) specified by the manufacturers was in excess of $6.5 \times 10^5/\text{m}$. This was regularly achieved using the conditions described below (2.3.7c).

The outflow from the analytical column was connected to a Bioanalytical System (BAS Inc., West Lafayette, Indiana, U.S.A.) LC-17 glassy carbon transducer cell, using PTFE tubing (1.5 mm O.D. x 0.8 mm I.D.). The LC-17 cell (Figure 2-J) electrochemically detected compounds separated on the analytical column and consisted of a plexi-glass block separated into two halves by a thin (0.2 mm) teflon gasket. A slot (1.6 x 0.5 cm) cut in the gasket formed a flow cell of $<1 \mu\text{l}$ volume containing the glassy carbon working electrode. A potential was maintained across the cell (relative to a Ag/AgCl reference electrode) using a BAS LC-4A controller, which also amplified and converted the signal to a voltage for display on a Tarkan 600 chart recorder set at a range of 1 volt.

b) Principles of electrochemical detection:

The detailed theory of electrochemical reactions and continuous flow voltammetry has been described by Adams (1969) and Weber and Purdy (1978). Therefore, only the principles relevant to catecholamines and indoleamines are described here.

Electrochemical detection depends upon the ability of a molecule to undergo oxidation (i.e. loss of electrons) when subject to a fixed (oxidative) potential. The compound is exposed to the electrode surface in a suitable electrolyte solution and the limits of the oxidative potential are determined by the properties of the electrode material and the electrolyte solution. Figure 2-K shows the oxidative mechanisms of a catecholamine and indoleamine at a potential which would yield two electrons. Most catecholamines and indoleamines need oxidative



Electrodes

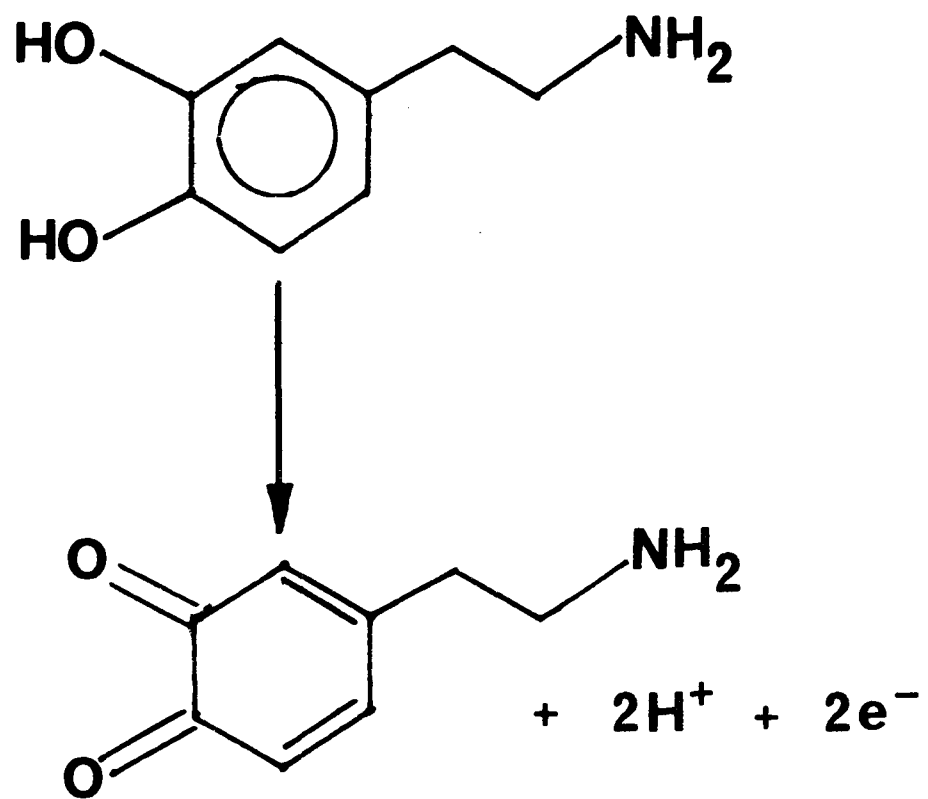
R- Reference

A- Auxiliary

W- Working

FIGURE 2-J: Cross-sectional diagram of the BAS LC-17 transducer cell.

a



b

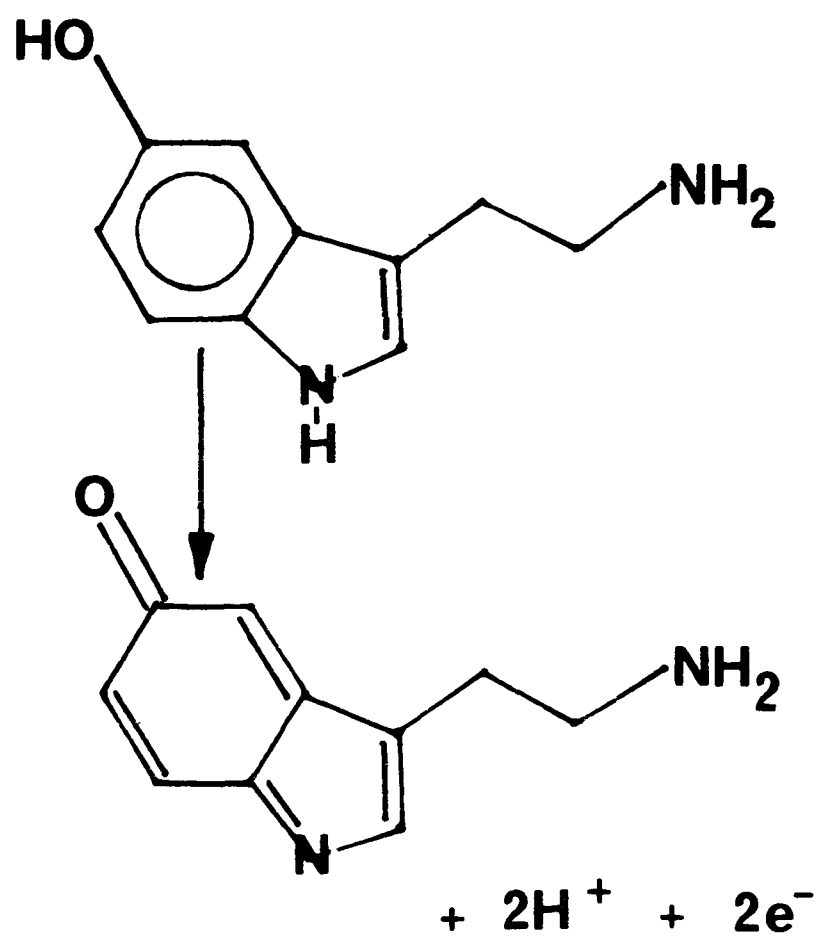


FIGURE 2-K: Two electron oxidative mechanism for a) catecholamines, b) indoleamines.

potentials between +0.5 and +0.7 volts with reference to a Ag/AgCl reference electrode. The current generated from the oxidative reaction of a compound is proportional to the molar concentration of that compound. The generated current is converted to a proportional voltage by the amperometric controller and then displayed on a chart recorder.

- *c) Mobile phases:*

Two mobile phases were used in the experiments described in this thesis. The studies described in Chapters VI and IX were carried out using an aqueous mobile phase (buffer A) containing 0.1M sodium acetate, 0.1M citric acid, 8-10% methanol, adjusted to pH 4.5 with sodium hydroxide (Mefford, 1981), which would resolve DA, DoPAC, 5-HT and 5-HIAA. The studies described in Chapter VIII used an aqueous mobile phase (buffer B) containing 0.1M sodium acetate, 0.1M citric acid, 5% methanol, 2% tetrahydrofuran, 100 mg/l sodium octanesulphonic acid adjusted to pH 4.9 - 5.1 with sodium hydroxide (R. Shoup, personal communication; Mayer and Shoup, 1982). Buffer B would resolve NA, DA, 5-HT and 5-HIAA. All reagents using in mobile phases were of 'Analar' grade. Chromatograms of standard mixtures using buffer A and buffer B are shown in Figures 2-L and 2-M.

- *d) Standards:*

All compounds used for standards and internal standard were obtained from Sigma (London, U.K.). Stock solutions of standards and internal standard were made up at a concentration of 0.1 - 0.2 mg/ml in 10^{-4} M cysteine solution in distilled water, added as an antioxidant. Cysteine was not retained on the columns and therefore did not interfere with other peaks using the elution conditions described. The stock

FIGURE 2-L: Standard chromatogram for a) 2 ng/20 μ l and b) 200 pg/20 μ l of dopamine (DA), 3,4-dihydroxyphenyl-acetic acid (DoPAC), serotonin (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) with 1 ng/20 μ l n-acetyl dopamine (IS - internal standard) using buffer A elution conditions (Section 2.3.7c)

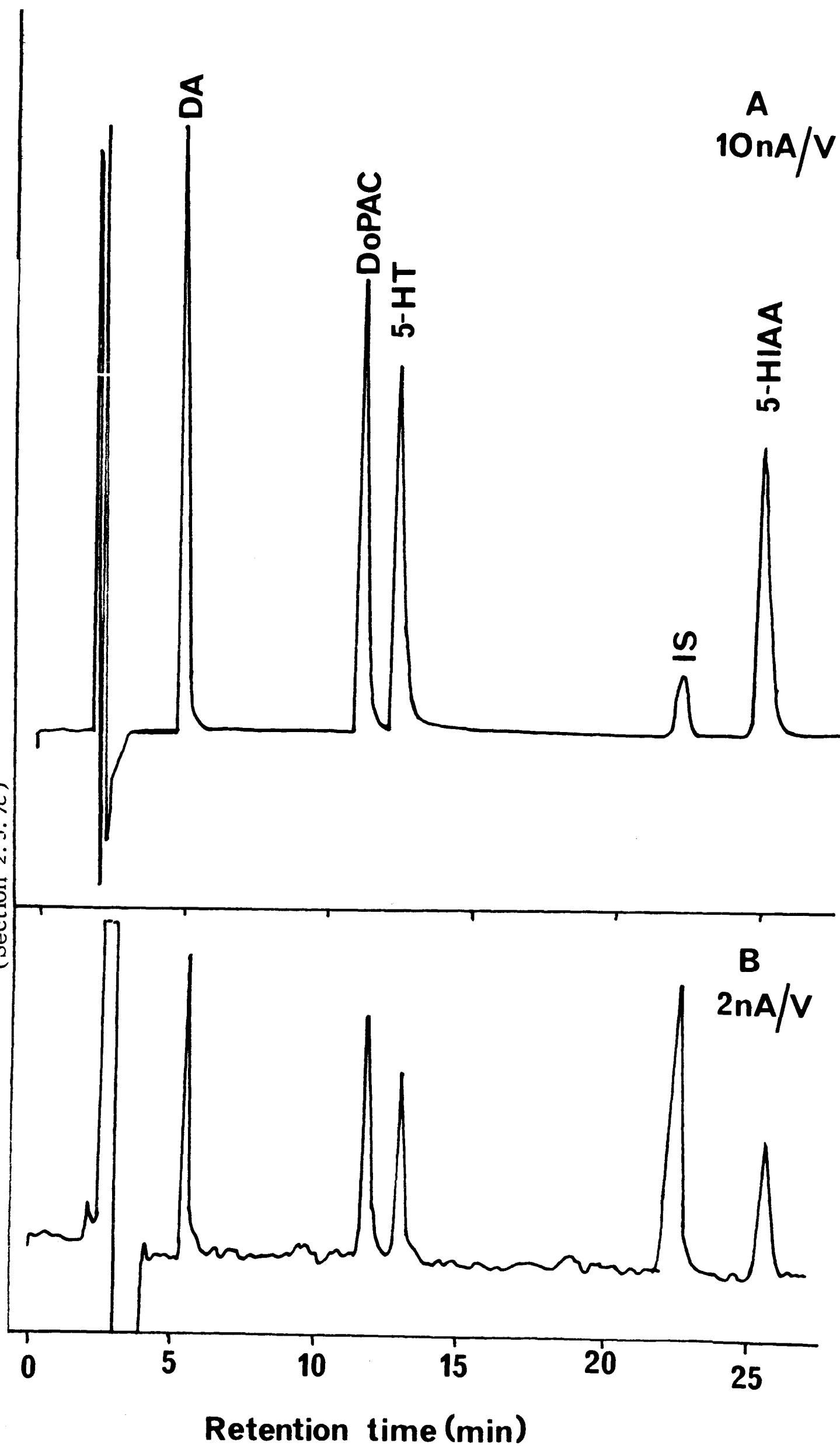
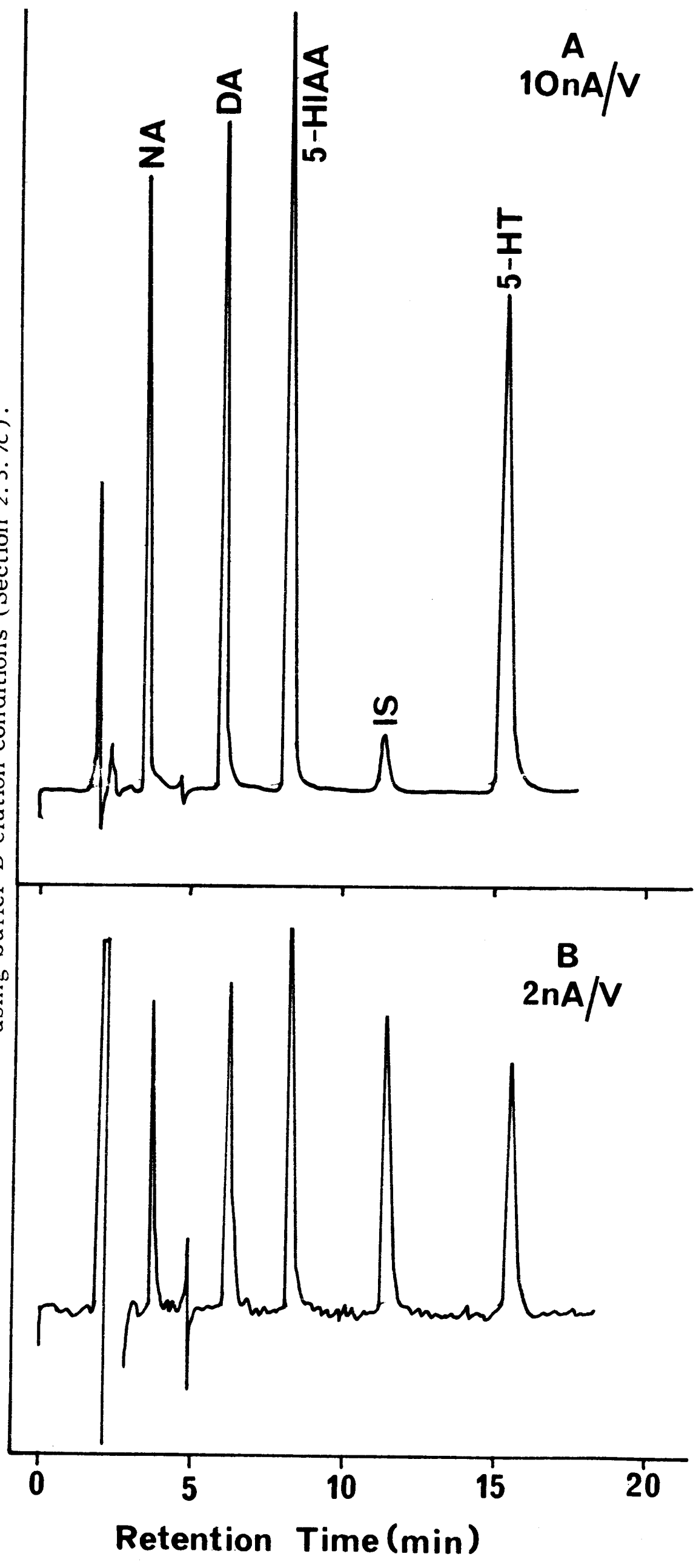


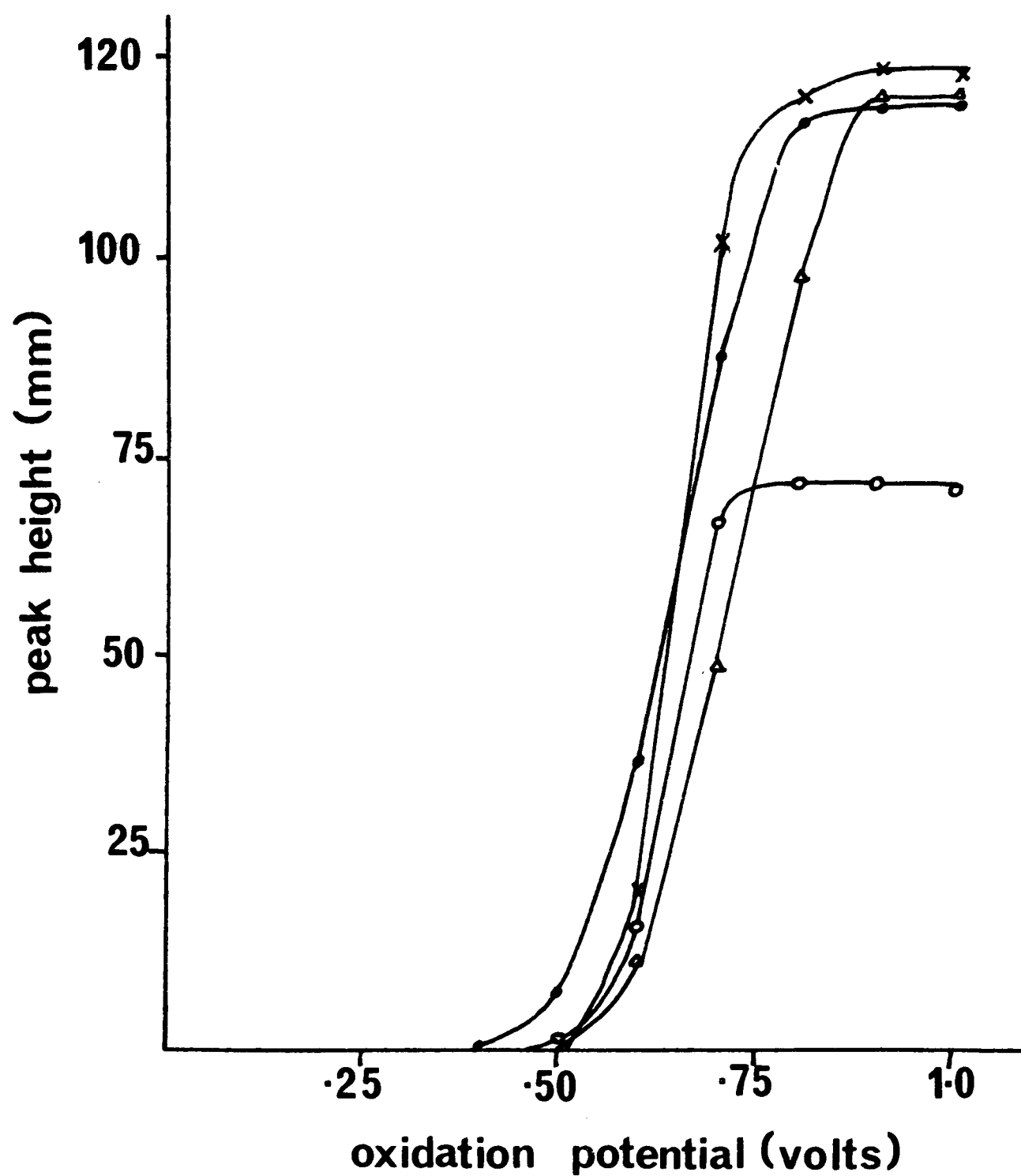
FIGURE 2-M: Standard chromatogram for a) 2ng/20 μ l and b) 200 pg/20 μ l of noradrenaline (NA), dopamine (DA), 5-hydroxy-indole acetic acid (5-HIAA) and serotonin (5-HT) with 1 ng/20 μ l of n-acetyl dopamine (IS - internal standard) using buffer B elution conditions (Section 2.3.7c).



solutions were stored in the dark at 4°C and in these conditions standards were stable for at least 2 months, and were replaced accordingly. Standard solutions for injection were diluted daily in 0.2M perchloric acid. Initially, the retention times of each compound were determined individually in the two mobile phases, but subsequently a single standard solution was prepared containing all the compounds required. Using this mixture standard curves for each compound could be prepared with a minimum number of injections. The range of concentrations of standards used routinely was 2 ng, 200 pg, 100 pg and 50 pg per 20 µl injection, however, the standard curve for each compound was linear over the range of 20 pg to 2 ng. The lower limit of sensitivity was defined as three times the baseline 'noise' and was between 15 and 25 pg per 20 µl injection for DA, NA, 5-HT, 5-HIAA and DoPAC. 1 ng/20 µl of n-acetyl dopamine was included as an internal standard in the homogenising buffer and the standard solutions. N-acetyl dopamine was chosen because, using the two mobile phases described above (2.3.7c), it did not interfere with any other peak found in all tissues studied in this thesis.

e) Identification of compounds in samples:

The differing ability of each compound to oxidize at two different oxidation potentials (Figure 2-N) was used to aid identification of compounds in samples (Blank, 1976; Mefford, 1981; Mayer and Shoup, 1982). A response ratio was calculated for each standard using its peak heights at +0.6 and +0.7 volts. An extracted sample (see 2.3.7f) from each tissue used in this thesis was analysed at +0.6 and +0.7 volts. and the response ratio was calculated for the peaks eluting at the same time as the standards. If the response ratios were the same for standards



x 5-HIAA

o 5-HT

Δ NA

• DA

FIGURE 2-N: The oxidative ability (hydrodynamic voltammogram) of noradrenaline (NA), dopamine (DA), serotonin (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) at different oxidative potentials using buffer B elution conditions (Section 2.3.7c).

TABLE 2.1: Response ratios (peak height at +0.6V/peak height at +0.7V) of compounds detected LCED in tissues analysed in this thesis. Elution conditions are described in Section 2.3.7c.

Compound	Buffer A elution conditions			Buffer B elution conditions			
	Std.	Hypothalamus	Retina	Std.	MPoA	SCA	ARA
Noradrenaline	-	-	-	0.31	0.29	0.30	0.29
Dopamine	0.58	0.60	0.57	0.55	0.50	0.56	0.51
DoPAC	0.32	0.30	0.33	-	-	-	-
5-HT	0.42	0.40	ND	0.29	0.30	0.31	0.29
5-HIAA	0.36	0.36	ND	0.20	0.19	0.19	0.20
n-acetyl dopamine	0.46	0.47	0.49	0.38	0.39	0.38	0.39

-, not detected

ND, below the level of assay sensitivity.

and corresponding peaks in the samples, this suggested that only a single compound was present in the peak and which behaved in a similar electrochemical manner to the corresponding standard.

Table 2-1 shows that for all tissue studied in this thesis the response ratio of the compounds in the samples were similar to the response ratios of the standards. Response ratios were only calculated at the beginning of an experiment and subsequently, retention times were used on a daily basis to identify compounds in the samples.

f) Extraction and preparation of samples:

Tissue samples were extracted by homogenisation in 0.2M perchloric acid and centrifugation (1000 x g) to precipitate the protein. The supernatant was directly injected onto the column (Mefford, 1981). However, to avoid blockage of the LCED tubing, small particulate matter was removed from the supernatant immediately before injection into the valve, by filtration through a 0.2 µm regenerated cellulose filter using a BAS MF1 centrifugal microfilter.

g) Calculation of results:

Peak heights were measured from a point on the baseline from which a perpendicular (relative to the direction of the chart paper movement) could be drawn through the maximum point of the peak. Figure 2-P shows peak height measurement for horizontal and sloping baselines. With 'noisy' baselines, the reference baseline was drawn midway between the 'noise' range (Figure 2-P).

The same mass of internal standard (1 ng/20 µl) was added to all standards and samples. For each standard concentration, the peak height ratio relative to the internal standard was calculated, that is:

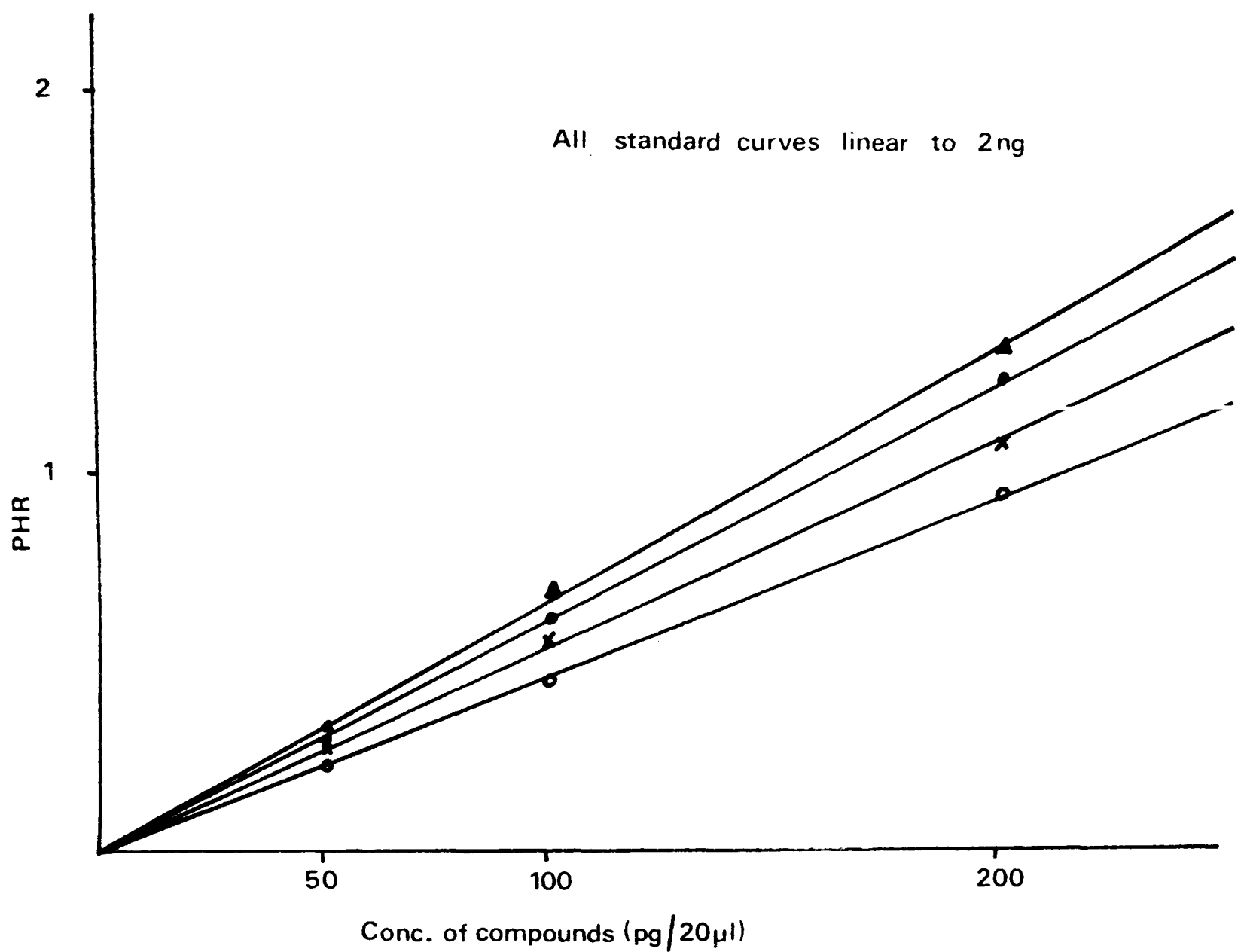


FIGURE 2-Q: Standard curves for DA, DoPAC, 5-HT and 5-HIAA (see Figure 2-L for abbreviations) using peak height ratios (PHR) (Section 2.3.7) - buffer A elution conditions (Section 2.3.7c).

- x 5-HIAA
- o 5-HT
- ▲ DoPAC
- DA

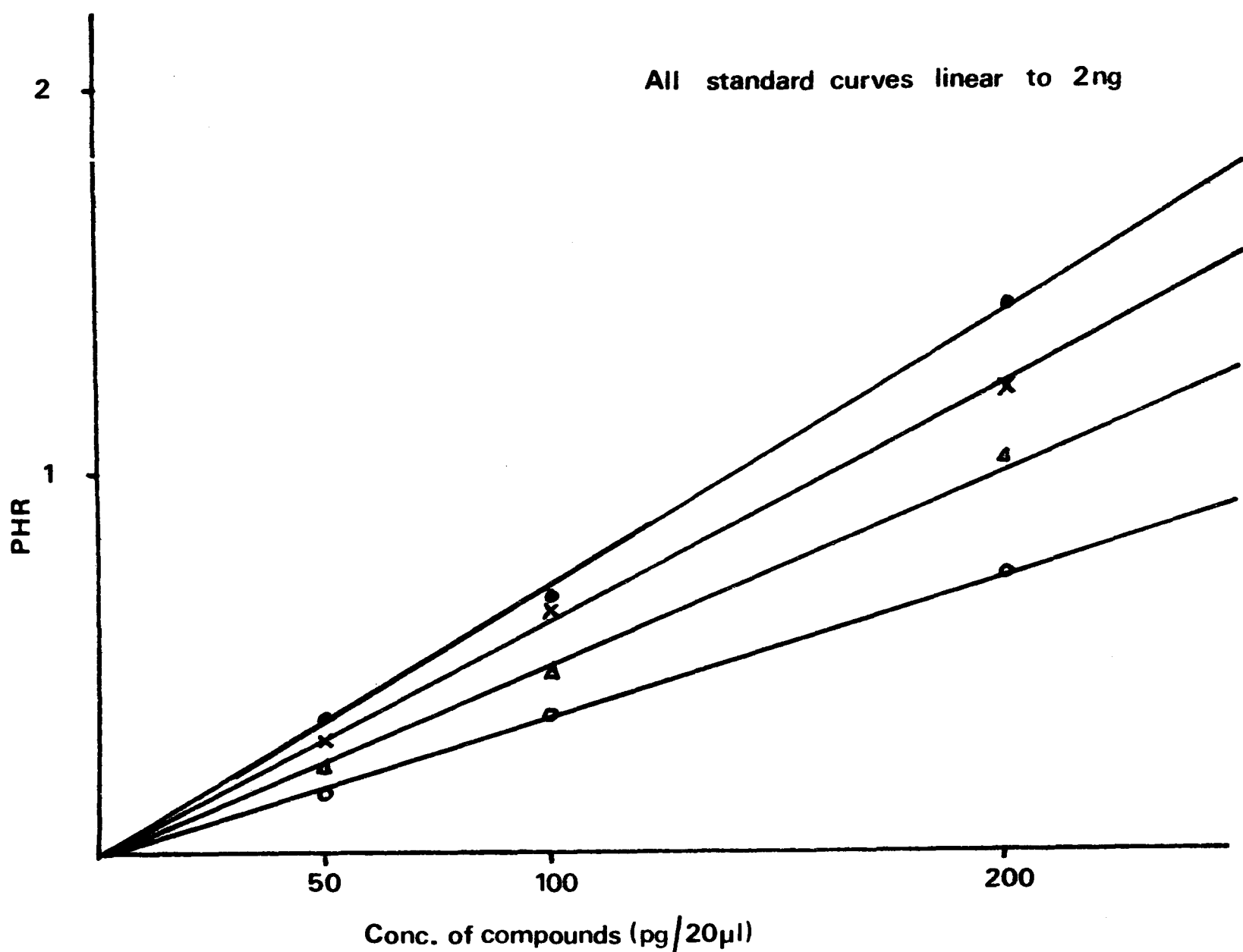


FIGURE 2-R: Standard curves for NA, DA, 5-HIAA and 5-HT (see Figure 2-M for abbreviations) using peak height ratios (PHR) (Section 2.3.7) - buffer B elution conditions (Section 2.3.7c).

- x 5-HIAA
- o 5-HT
- Δ NA
- DA

$$\frac{\text{Peak height of standard ('x' ng/20 } \mu\text{l)}}{\text{Peak height of internal standard (1 ng/20 } \mu\text{l)}}$$

A standard curve for each compound was drawn using the peak height ratio against concentration. Figures 2-Q and 2-R show the standard curves for each compound resolved by buffers A and B. The mass of each compound in each sample was determined by linear regression from the relevant standard curve using the peak height ratio of that compound relative to the internal standard. Peak height ratios relative to an internal standard allowed for correction of recovery and injection volume variation without further calculation.

2.3.8 Protein determination

The protein content of the perchloric acid precipitates in Chapter VIII were measured using the assay of Peterson (1977), modified from that of Lowry, Rosenbrough, Farr and Randall (1951). Precipitates were dissolved in 540 μl of 0.1N sodium hydroxide. The sample solution was mixed with 540 μl of a copper sulphate/sodium dodecyl sulphate buffer and then 270 μl of Folin-Ciocalteu solution. Standards (13.5 μg - 54 μg /tube) were similarly treated. The absorbance of the resulting solution was measured at 750 nm using a Gilford 250 spectrophotometer. The details of the assay are described in Appendix 4.

Results were calculated by linear regression from a standard curve (log absorbance vs log protein concentration).

2.4 Statistical Methods

All data in this thesis (except the LHRH concentrations in hypophysial portal blood in Chapter III) were analysed using parametric statistics. The significance of the difference between two means

was determined using Students unpaired 't' test, while the significance of differences between multiple means were determined using one-way analysis of variance and the multiple range test of Duncan (1955, 1957) using the tables compiled by Harter (1960). The LHRH concentrations in hypophysial portal blood in Chapter III were analysed using Mann-Witney U test (Siegal, 1960), because some of the values used for data analysis were below the limit of the LHRH RIA and therefore no absolute value could be assigned to them.

CHAPTER III

The Involvement of the Release and Priming Effect of Luteinizing Hormone Releasing Hormone in the Stimulation of Pituitary Responsiveness by Oestrogen

3.1 Introduction

An important factor in the development of the pro-oestrous increase in pituitary responsiveness to LHRH is the increasing concentration of oestradiol-17 β (Debeljuk, Arimura and Schally, 1972, 1973; Aiyer and Fink, 1974; Cooper, Fawcett and McCann, 1974; Fink and Henderson, 1977a) found on the morning of pro-oestrus (e.g. Brown-Grant, Exley and Naftolin, 1970; Shaikh and Shaikh, 1975; Henderson, Baker and Fink, 1977b). Henderson *et al.* (1977b) showed that pituitary responsiveness was significantly increased 12 h after the implantation of an oestradiol-containing capsule implanted on the morning of dioestrus, an effect which could be blocked by sodium pentobarbitone 8 h after capsule implantation. This suggested either that oestradiol might exert its effect by stimulating the release of LHRH and thereby invoke the priming effect of LHRH (Aiyer, Chiappa and Fink, 1974b; Fink, Chiappa and Aiyer, 1976) or that for the full effect of oestradiol, the pituitary gland had to be exposed to some LHRH. To investigate these possibilities, two experiments were carried out, both based on the model of Henderson *et al.* (1977b). First, LHRH was infused i.v. at concentrations too small to release significantly large amounts of LH to try to overcome the sodium pentobarbitone blockade of increased pituitary responsiveness. Secondly, the concentration of LHRH was measured in hypophysial portal blood either 4-6 h or 12-13 h after ovariectomy and the implantation of either an empty or oestradiol-17 β containing Silastic capsule. The timing of the portal blood collections was chosen to coincide with the times at which Henderson *et al.* (1977b) had found the LH response to LHRH to be either at a minimum (4-6 h) or maximum (12-13 h).

3.2 Materials and Methods

Female Wistar Cobs (200–300 g) with regular oestrous cycles were supplied and maintained in controlled lighting (LD) as described in Chapter II (2.1). Silastic capsules (4–5 mm length x 2.5 mm internal diameter) packed with oestradiol-17 β were prepared as described in Chapter II (2.2.8). Capsules of this size have been shown in this laboratory to produce plasma concentrations of oestradiol-17 β of between 30 and 50 pg/ml (Henderson *et al.*, 1977b). Control animals were implanted with an empty capsule of the same size.

3.2.1 Effect of intravenous infusion of LHRH on pituitary responsiveness

Five groups of rats were ovariectomized (Avertin anaesthesia, 1 ml/100 g b.w.) between 09.00 h and 10.00 h diestrus. An empty or oestradiol containing Silastic capsule was implanted immediately afterwards. Approximately 8 h after ovariectomy, 4 groups of rats (groups A, B, C, D) were anaesthetized with sodium pentobarbitone (36 mg/kg, b.w., i.p.) and then infused i.v. with various doses of synthetic LHRH or 0.9% saline for 4 h. An aliquot of the infusate was retained and assayed for LHRH (2.3.2). The infusion of LHRH was made through a 26 g hypodermic needle, which was connected to a 5 ml syringe by means of a polythene tube (Portex PP20). The piston of the syringe was driven by a 'six-speed continuous slow injector' (Model 6130, C.F. Palmer, Ltd., London) to give an infusion rate of 0.6 ml/hour. Blood samples (0.4–0.5 ml) were removed from the opposite external jugular vein (or rarely from the femoral vein) before and at various times during the infusion and the plasma stored at -20°C until assayed for LH and LHRH. At the end of the infusion period, pituitary responsiveness to LHRH was determined as described in

Chapter II (2.2.12) with the difference that blood samples were taken only at 20, 40 and 60 minutes after LHRH injection.

One group of rats (group E) was not injected with sodium pentobarbitone 8 h after ovariectomy and was not subsequently infused. Pituitary responsiveness to LHRH was determined in these animals as in the four other groups; that is, under sodium pentobarbitone anaesthesia injected 30 min. before the LHRH injection (12 h after capsule implantation).

At the end of the experiment the animals were killed and uteri dissected out and weighed (dry weight only).

3.2.2 Effect of ovariectomy and oestradiol on LHRH in pituitary stalk blood

Four groups of rats were ovariectomized under Avertin anaesthesia and immediately implanted with an oestradiol-containing or empty Silastic capsule. Blood from the cut pituitary stalk was collected under Althesin anaesthesia (2.2.7) for a period of 30 min either at 4-6 h or 12-13 h after capsule implantation. The stalk plasma was frozen at -20°C until assayed for LHRH. In some animals blood from the external jugular vein was taken for LHRH assay immediately after induction of anaesthesia.

Both LHRH and LH were determined by the radioimmunoassays described in Chapter II (2.3.2 and 2.3.3). For LHRH the values were expressed as concentrations (pg synthetic LHRH/ml) and contents (concentrations x plasma volume) for 30 min. collection periods. Values of 10-12 pg/ml (the calculated lower limit of the assay) were assigned to the few samples below the lower limit of detection of the assay.

The significance of the difference between 2 means was determined by the unpaired 't' test for plasma LH concentrations and uterine weights, and the Mann Whitney U test for LHRH concentrations and content.

3.3 Results

3.3.1 Effect of intravenous infusion of LHRH on pituitary responsiveness to LHRH

Tables 3-1 and 3-2 show the mean ($\pm$ S.E.M.) plasma concentrations of LH and LHRH before and during the infusion of LHRH. The plasma LH concentration before and the mean-maximal increments in plasma LH concentration after i.v. injection of LHRH are shown in Table 3-3. Table 3-1 shows that the two highest doses of LHRH infused (0.08 ng/100 g b.w./min and 0.04 ng/100 g b.w./min) resulted in elevated levels of LHRH being established within 30 min after the beginning of the infusion. This is in contrast to the lowest dose of LHRH (0.02 ng/100 g b.w./min) and saline infusions where there were no significant increases in plasma LHRH concentrations. Table 3-2 shows that plasma LH concentrations in animals that had been implanted with an oestradiol or an empty capsule, rose during the LHRH infusion in a dose dependent manner. No significant increase in the plasma concentration of LH occurred in animals infused with saline. Within each group infused with LHRH there were no significant differences between the plasma LH concentration at 240 min. in animals implanted with oestradiol capsules compared with those with empty capsules. However, the plasma LH concentrations at 240 min. were significantly greater in groups A and B ($p < 0.001$) and C ($p < 0.01$) than the plasma LH concentration before LHRH injection in group E

TABLE 3-1: The mean $\pm$ S.E.M. plasma concentrations (pg/ml) of LHRH before (0 min) and during the i.v. infusion of LHRH or 0.9% saline.

Group	Infusion	Capsule	n	Time of infusion (min)							
				0	30	60	120	180	210	240	
A	LHRH (0.08 ng)	OE	4	15*	31 $\pm$ 6	37 [†]	31 $\pm$ 5	34 $\pm$ 4	35 [†]	41 $\pm$ 2	
		empty	4	15**	45 $\pm$ 6	54 $\pm$ 9	54 $\pm$ 7	51 $\pm$ 3	47 [†]	55 $\pm$ 3	
B	LHRH (0.04 ng)	OE	2	16*	32	30	27	29	31	31	
		empty	3	17*	29 $\pm$ 1	32 $\pm$ 2	40 $\pm$ 6	32 $\pm$ 1	30 $\pm$ 3	29 $\pm$ 1	
C	LHRH (0.02 ng)	OE	3	18 $\pm$ 5	21*	21 $\pm$ 5	21 $\pm$ 5	18**	22 $\pm$ 3	21 $\pm$ 5	61.
		empty	3	13*	15 $\pm$ 1	14 $\pm$ 0	14*	19 $\pm$ 0	21 $\pm$ 7	16 $\pm$ 0	
D	Saline	OE	3	17*	15*	14**	15**	19 $\pm$ 2	17*	13**	
		empty	3	14*	18*	16 $\pm$ 1	12***	13**	14**	14**	

*1, **2, ***3 samples in the group were undetectable.

[†]n=2 at these times.

Figure in parentheses after LHRH indicates infusion dose/100 g b.w./min.

TABLE 3-2: The mean $\pm$ S.E.M. plasma concentrations (ng/ml) of LH before (0 min) and during the i.v. injection of LHRH or 0.9% saline.

Group	Infusion	Capsule	n	Time of infusion (min)							
				0	30	60	120	180	210	240	
A	LHRH (0.08 ng)	OE	4	1.8 $\pm$ 0.4	5.5 $\pm$ 0.7	8.0 $\pm$ 1.5	15.8 $\pm$ 4.5	24.2 $\pm$ 7.4	32.5 $\pm$ 7.5	30.9 $\pm$ 4.6	62.
		empty	4	1.4 $\pm$ 0.6	10.6 $\pm$ 5.9	10.1 $\pm$ 4.1	16.7 $\pm$ 3.3	40.0 $\pm$ 14.5	53.4 $\pm$ 23.6	54.5 $\pm$ 16.4	
B	LHRH (0.04 ng)	OE	2	2.6	4.8	5.5	8.5	9.9	8.1	8.8	62.
		empty	3	2.6 $\pm$ 0.4	4.7 $\pm$ 0.8	5.8 $\pm$ 0.6	12.3 $\pm$ 1.9	12.6 $\pm$ 2.9	13.5 $\pm$ 2.0	13.5 $\pm$ 1.9	
C	LHRH (0.02 ng)	OE	3	2.1 $\pm$ 0.4	3.3 $\pm$ 0.6	2.1 $\pm$ 0.1	3.4 $\pm$ 0.7	4.9 $\pm$ 0.8	5.4 $\pm$ 1.4	6.4 $\pm$ 1.9	62.
		empty	3	2.3 $\pm$ 0.1	5.2 $\pm$ 0.2	4.1 $\pm$ 0.4	11.1 $\pm$ 4.1	8.2 $\pm$ 1.8	8.0 $\pm$ 1.7	10.0 $\pm$ 3.5	
D	Saline	OE	3	2.3 $\pm$ 1.1	2.0 $\pm$ 0.5	1.6 $\pm$ 0.4	2.1 $\pm$ 0.4	1.8 $\pm$ 0.3	2.5 $\pm$ 1.1	1.3 $\pm$ 0.1	62.
		empty	3	3.1 $\pm$ 1.2	2.8 $\pm$ 0.7	4.2 $\pm$ 0.8	4.5 $\pm$ 0.5	4.4 $\pm$ 1.8	4.1 $\pm$ 1.5	3.1 $\pm$ 1.0	

Figures in parenthesis after LHRH indicates infusion dose/100 g b.w./min.

TABLE 3-3: The mean $\pm$ S.E.M. plasma LH concentrations (ng/ml) of LH 240 min after the start of the LHRH or saline infusion (pre-LHRH injection) and the mean maximal increments in plasma LH concentration (ng/ml) after the i.v. injection of 50 ng/100 g b.w. LHRH.

Group	Infusion	Capsule	n	Pre-injection*	Mean maximal increment
A	LHRH (0.08 ng)	OE	4	30.9 $\pm$ 4.6	130.4 $\pm$ 16.5
		empty	4	54.5 $\pm$ 16.4	112.8 $\pm$ 39.8
B	LHRH (0.04 ng)	OE	2	8.8	61.9
		empty	3	13.5 $\pm$ 1.9	63.3 $\pm$ 16.7
C	LHRH (0.02 ng)	OE	3	6.4 $\pm$ 1.9	63.4 $\pm$ 10.9
		empty	3	10.0 $\pm$ 3.5	80.6 $\pm$ 26.1
D	Saline	OE	3	1.3 $\pm$ 0.1	27.6 $\pm$ 6.7†
		empty	3	3.1 $\pm$ 1.0	23.2 $\pm$ 9.5†
E	No infusion	OE	7	2.0 $\pm$ 0.3	51.8 $\pm$ 8.3
		empty	6	2.9 $\pm$ 0.9	27.7 $\pm$ 4.4

*For groups A-D this was the LH value 240 min. after the beginning of the infusion.

†Based on 6 (OE) and 4 (empty) animals; the mean $\pm$ S.E.M. plasma concentrations before injecting LHRH were 1.7 $\pm$ 0.2 (OE) and 2.7 $\pm$ 0.8 (empty).

(not infused with LHRH or saline). Group E was the only group in which the maximal increments in animals implanted with an oestradiol capsule were significantly greater ($p < 0.05$) when compared with those in animals implanted with an empty capsule. The mean dry weight of the uteri in animals implanted with oestradiol capsules (329 ± 16 mg) was significantly greater ($p < 0.01$) compared with those in animals implanted with empty capsules (256 ± 12 mg).

3.3.2 Effect of ovariectomy and oestradiol on LHRH in pituitary stalk plasma

Table 3-4 shows that the concentrations and contents of LHRH in pituitary stalk ^aplasma 12-13 h after implantation of an oestradiol capsule were significantly greater ($p < 0.01$) than the corresponding values 12-13 h after the implantation of an empty capsule. Table 3-4 also shows that the concentrations of LHRH in pituitary stalk plasma were significantly lower ($p < 0.025$) 12-13 h after implantation with an empty capsule than the concentrations 4-6 h after implantation of an empty capsule. The LHRH content of pituitary stalk plasma was significantly greater ($P < 0.05$) 12-13 h after the implantation of an oestradiol capsule. The LHRH concentrations in pituitary stalk plasma 12-13 h after implantation of an oestradiol capsule were also significantly greater ($p < 0.001$) than those measured in peripheral plasma obtained under Althesin anaesthesia 12 h after ovariectomy and the implantation of either empty or oestradiol capsules. The concentration of LHRH in pituitary stalk plasma 12-13 h after implantation of an empty capsule did not significantly differ from that in peripheral plasma.

TABLE 3-4: Mean (S.E.M.) rat pituitary stalk (groups 1-4) or peripheral groups (5 and 6) plasma concentrations and contents of LHRH 4-6 h and 12-13 h after ovariectomy and implantation of an oestradiol-17 β containing or empty Silastic capsule.

Group	Capsule	Time after ovariectomy and capsule implantation (h)	n	Blood volume collection (μ l/30 min)	Plasma LHRH concentration (pg/ml)	content (pg/30 min)
<i>Pituitary stalk plasma</i>						
1	Empty	4-6	9	421 $\pm$ 94	34 $\pm$ 12	7.4 $\pm$ 2.3
2	Oestradiol	4-6	9	432 $\pm$ 102	29 $\pm$ 12	5.4 $\pm$ 1.9
3	Empty	12-13	13	459 $\pm$ 67	16 $\pm$ 2	4.0 $\pm$ 0.4
4	Oestradiol	12-13	12	680 $\pm$ 120	26 $\pm$ 5	8.5 $\pm$ 1.5
<i>Peripheral plasma</i>						
5	Empty*	12-13	7		13 $\pm$ 1**	
6	Oestradiol*	12-13	9		13 $\pm$ 1**	

*These groups were comprised of samples taken from some animals in groups 3 and 4 (see Materials and Methods).

**2 samples in group undetectable.

3.4 Discussion

Henderson, Baker and Fink (1977b) showed that the facilitation of pituitary responsiveness to LHRH found 12 h after oestradiol-17 β capsule implantation could be significantly reduced by sodium pentobarbitone injected 4 h but not 0.5 h before the LHRH injection. This was confirmed by the mean maximal increments in plasma LH concentrations of the saline infused group and the group which received no infusion (D and E, Table 3-3). The mean maximal increments in plasma LH concentrations in the groups infused with LHRH (A, B and C, Table 3-3) were all similar to or greater than those in the group that had been implanted with oestradiol capsules but not infused (group E). This shows that the sodium pentobarbitone blockade at 8 h in animals infused with saline (group D, Table 3-3) could be overcome by infusing LHRH for 4 h at concentrations which themselves only brought about relatively small changes in plasma LH concentrations and shows that this effect of sodium pentobarbitone is probably due at least in part to reduced LHRH release and the consequent blockade of the priming effect of LHRH (Aiyer, Chiappa and Fink, 1974; Fink, Chiappa and Aiyer, 1976; Fink and Pickering, 1980). Table 3-3 shows that there was no significant difference, within each group, between the mean maximal increments in plasma LH concentrations in animals infused with LHRH and implanted oestradiol or empty capsules and this shows that the priming effect of LHRH on the LH response to LHRH can override the facilitatory effect of oestradiol.

Table 3-4 shows that, except for the concentrations at 12-13 h after ovariectomy in animals bearing empty capsules, the stalk plasma LHRH concentrations were all significantly higher than the LHRH concentrations in jugular venous plasma taken from similarly treated

animals. The stalk plasma LHRH concentrations were also similar to or higher than the stalk plasma LHRH concentrations obtained by Sarkar, Chiappa, Fink and Sherwood (1976) from intact animals at times during the oestrous cycle other than between 15.00 h pro-oestrus and 04.00 h oestrus. The stalk plasma LHRH concentration at 12-13 h in animals bearing empty capsules was similar to those obtained from animals anaesthetized with sodium pentobarbitone (Sarkar, Chiappa, Fink and Sherwood 1976) and shows that by about 12 h after ovariectomy LHRH release has been reduced to a minimum (i.e. not significantly above peripheral plasma concentrations). Sarkar and Fink (1980) showed that stalk plasma LHRH concentrations, after a rise immediately after ovariectomy, declined to a relatively low level by 2 days. The stalk plasma LHRH concentrations in stalk plasma collected from animals 12-13 h after ovariectomy and implanted with an empty capsule suggest that this decline is probably complete by 12-13 h. As shown by the stalk plasma LHRH concentrations in animals ovariectomized 12-13 h earlier but bearing an oestradiol capsule, this decline in stalk plasma LHRH concentrations appeared to be prevented by oestradiol-17 β . Taken together with previous data (Cooper, Fawcett and McCann, 1975; Henderson, Baker and Fink, 1977b), the data in the present study suggested that the increase in the LH response to LHRH which follows shortly after ovariectomy is due to increased release of LHRH, and that the potentiating effect of oestradiol is partly due to a maintenance by oestradiol of the increased release of LHRH which occurs immediately after ovariectomy (Sarkar and Fink, 1980), thereby priming the pituitary gland.

From this it seems that the augmentation of pituitary responsiveness to LHRH by oestradiol involves two distinct mechanisms. First,

an effect at the central nervous system which leads to LHRH release at a rate which it is not sufficient by itself to produce an LH surge. Second, a direct action on the gonadotrophs has been shown by studies from animals in which the pituitary stalk had been sectioned (Greeley, Allen and Mahesh, 1975; Fink and Henderson, 1977b; Greeley, Volcan and Mahesh, 1978) and in dispersed pituitary cell systems (Drouin, Lagace and Labrie, 1976; Hsueh, Erickson and Yen, 1978; Speight and Fink, 1981). The action of oestradiol upon LHRH release mechanisms may explain the marked sex difference in the effect of manipulating steroid hormones on pituitary responsiveness to LHRH in the rat (Barracclough and Turgeon, 1974; Fink and Henderson, 1977b), and the effects of short-term constant light exposure on the pro-oestrous LH surge (discussed in Chapter IV).

CHAPTER IV

The Effects of Constant Light Exposure During the Oestrous Cycle

4.1 Introduction

The purpose of these studies was to investigate in detail the effect of constant light (LL) exposure during the three days prior to the pro-oestrous LH surge in cycling female rats. Hormonal studies during the period intermediate between normal cycling in control lighting (LD) and the eventual onset of persistent vaginal cornification and ovulation of the long-term LL exposed female rat, have been limited to the determination of the time of ovulation after short-term exposure to constant light (McCormack and Sridaran, 1978), the measurement of LH in single plasma samples from pro-oestrous animals exposed to LL for between 2-5 days (Daane and Parlow, 1971b) and the serum levels of gonadotrophins, oestradiol and progesterone, in the first two cycles in LL (Campbell and Schwartz, 1980). A more detailed study of the first cycle in LL was carried out here using serial sampling techniques to measure plasma LH concentration which were later correlated with subsequent ovulation. Since increased pituitary responsiveness to LHRH is a major determinant in the timing and magnitude of the pro-oestrous LH surge (Aiyer, Fink and Greig, 1974a; Cooper, Fawcett and McCann, 1974; Gordon and Reichlin, 1974; Fink, 1979), the effect of LL upon pituitary responsiveness during pro-oestrus was also investigated.

4.2 Materials and Methods

Female Wistar rats (200-300 g b.w.) were maintained as outlined in Chapter II (2.1). Oestrous cycles were assessed by daily inspection of vaginal smears, and the only animals used had shown at least two 4-day cycles immediately before experimentation. Animals were placed in LL on the morning of oestrus and were used for experiments before

the following oestrus. The pattern of vaginal smears in animals placed in LL up to and including the following oestrus was the same as that in animals in LD.

Blood was collected from both anaesthetized and conscious animals as described in Chapter II (2.2.5 and 2.2.6), however, insertion of the intra-atrial catheter was carried out under Avertin anaesthesia (0.5 ml of a 2% solution in 0.9% saline/100 g b.w.) supplemented with ether when necessary. The collected blood was treated as outlined in Chapter II (2.2.4).

Silastic capsules (4-5 mm length x 2.5 mm internal diameter) containing crystalline oestradiol-17 β were prepared and inserted as described in Chapter II (2.2.8). Steroids for subcutaneous injection (s.c.) were oestradiol benzoate (OB: Evans Medical Ltd., Liverpool) and progesterone (Evans Medical Ltd.). Each was administered in 0.25 ml arachis oil under light ether anaesthesia.

Pituitary responsiveness to LHRH was determined (as described in Chapter II, 2.2.12) in: a) intact pro-oestrous animals; b) animals ovariectomized between 10.00 h - 11.00 h dioestrus, injected immediately afterwards with 2.5 μ g OB (s.c.) and on the following day with 2.5 mg progesterone (s.c.) at 13.00 h and then LHRH between 17.00 and 18.00 h; c) animals ovariectomized between 10.00 h - 11.00 h dioestrus and immediately with an oestradiol capsule. The experimental design of b) was based on Aiyer and Fink (1974) and Fink and Henderson (1977a) while that of c) was based on the methods of Henderson *et al.* (1977b) and the results described in Chapter III.

Plasma LH was determined by radioimmunoassay (2.2.3). The significance of differences between means was determined using the unpaired 't' test.

4.3 Results

4.3.1 Effect of constant light on the pro-oestrous LH surge

An intra-atrial cannula was inserted between 06.00 - 08.30 h pro-oestrus into 13 animals; 7 had been exposed to LL since the previous oestrus while the remaining 6 had been exposed only to LD. Blood samples (0.2 ml) were withdrawn at intervals during the day as shown in Figure 4-A and the plasma LH concentration determined. Figure 4-A shows that the procedure of inserting an intra-atrial cannula early on the morning of pro-oestrus did not interfere with the occurrence of the LH surge later in the day in animals in LD. The mean time of the surge in LD animals was $18.00 \text{ h} \pm 1 \text{ h}$, while the mean peak plasma LH concentration (calculated from the maximal concentration in each animal) was $61.6 \pm 5.8 \text{ ng/ml}$. Exposure to LL, however, delayed and depressed the LH surge (Figure 4-A); the mean surge time was $21.30 \text{ h} \pm 1 \text{ h}$ and the mean peak plasma LH concentration was $36.9 \pm 7.2 \text{ ng/ml}$. The mean plasma concentrations at 18.00 h, 19.00 h and the mean peak plasma LH concentration in the animals exposed to LD were all significantly greater ($p < 0.01$, $p < 0.01$ and $p < 0.02$, respectively) than those in animals exposed to LL. All the animals from the experiment described in Figure 4-A were checked for the presence of ova on the following morning (oestrus). Ovulation occurred in 6 out of 7 rats exposed to LL, and in these the mean ($\pm \text{S.E.M.}$) number of ova per animal (11.8 ± 0.9) was similar to that in animals exposed to LD (12.3 ± 0.7), all of which ovulated. In a further experiment 16 animals (not cannulated) were checked for ovulation on the oestrus immediately following the oestrus in which they were placed in LL. Eleven animals had a normal number of ova (10.4 ± 1.0) while the remaining 5 failed to ovulate.

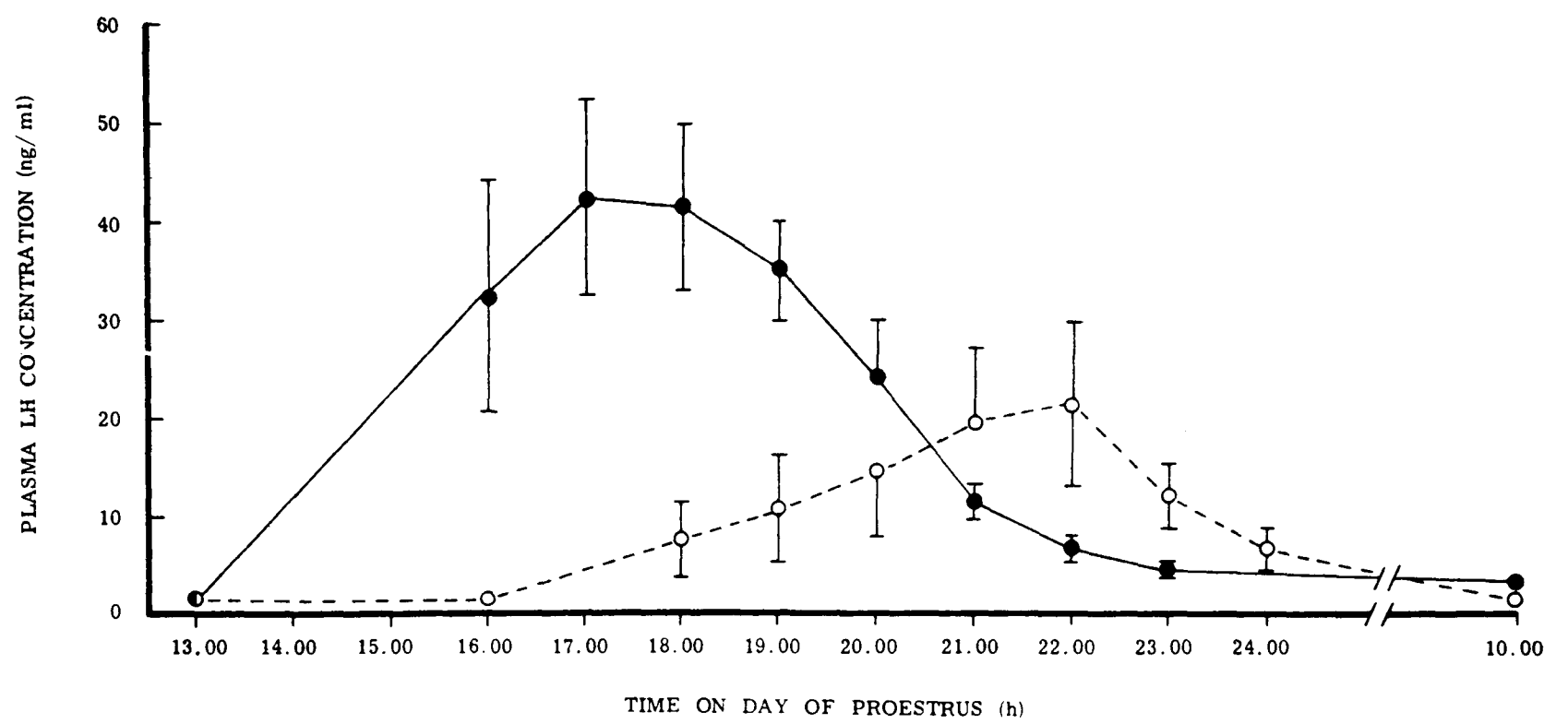


FIGURE 4-A: Plasma LH concentrations throughout the day of proestrus in rats exposed to LD (●—●; n=6) or LL (o---o; n=7). Values are means ($\pm$ S.E.M.).

4.3.2 Effect of constant light on pituitary responsiveness to LHRH during the LH surge

Table 4-1 shows that the pituitary responsiveness to LHRH in intact animals at about the time of the peak of the LH surge was markedly reduced by LL; the value at 21.00 h in animals exposed to LL was about six times lower than that found at 17.00 h in animals exposed to LD and maintained under the same conditions in this laboratory (Aiyer *et al.*, 1974a).

4.3.3 Effect of constant light on pituitary responsiveness to LHRH after acute ovariectomy and treatment with OB followed by progesterone

Animals exposed to either LL (n=19) or LD (n=7) were ovariectomized between 10.00 h and 11.00 h on the morning of dioestrus and treated with OB and progesterone, and injected with LHRH as described in materials and methods (group b). The mean plasma LH concentrations before and mean maximal increments in plasma LH concentrations after injecting LHRH are shown in Table 4-2. The pre-injection LH concentrations and the mean maximal increment in plasma LH were significantly lower ($p < 0.005$ and $p < 0.01$, respectively) in the animals exposed to LL (group LLa) when compared to those exposed to LD. However, 5 of the 19 animals exposed to LL gave responses in the same range as those in animals exposed to LD (see Figure 4-B); in these 5 animals the mean plasma LH concentrations before and the mean maximal increments in plasma LH after injecting LHRH were not significantly different from those in the animals exposed to LD. In the remaining 14 animals exposed to LL the mean pre-injection LH concentrations and maximal increments in plasma LH concentration were significantly reduced ($p < 0.001$) compared with

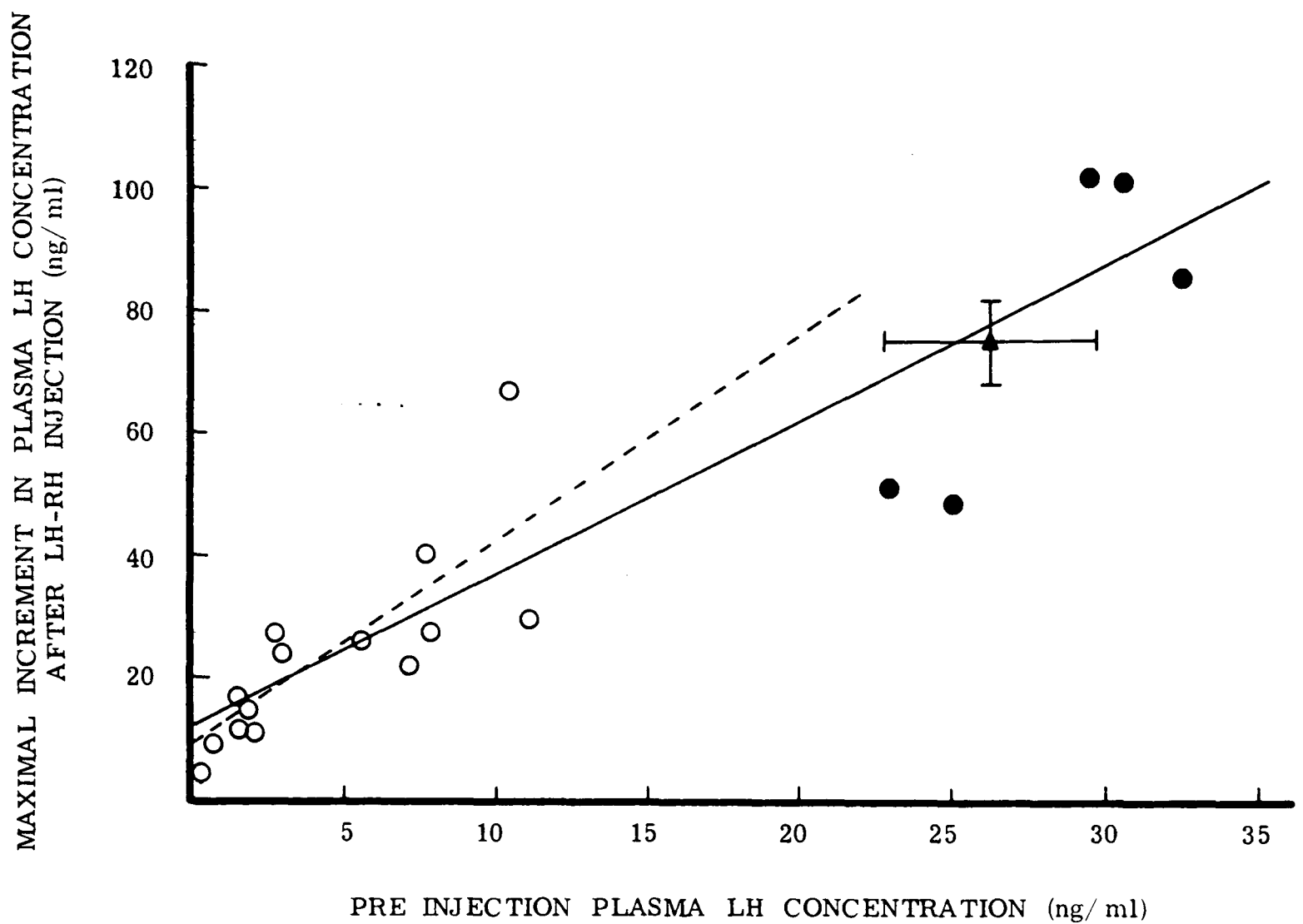


FIGURE 4-B: The correlation between the plasma LH concentrations (ng/ml) before and the maximal increments in plasma LH concentration after an intravenous injection of synthetic LHRH (50 ng/100 g b.w.) into animals exposed to LL, for all the animals (● and ○; —; $r=0.92$; $P < 0.001$, $n=19$) and for the animals which showed a significantly lower response to LHRH (○; ----; $r=0.76$, $p < 0.001$, $n=14$) compared with that in animals exposed to LD. The mean ($\pm$ S.E.M.) response of animals exposed to control lighting and given the same treatment as the LL exposed animals is also shown (▲). The animals were ovariectomized and immediately afterwards given an injection of 2.5 μ g oestradiol benzoate (s.c.) between 10.00 - 11.00 h dioestrus and then 2.5 mg progesterone (s.c.) at 13.00 h of the following day, followed by an injection of LHRH between 17.00 - 18.00 h.

TABLE 4-1: Mean ($\pm$ S.E.M.) concentrations of plasma LH (ng/ml) before and mean (S.E.M.) maximal increments in plasma LH after i.v. injection of LH-RH at times throughout pro-oestrus in intact animals exposed either to control lighting (lights on 05.00 - 19.00 h) or constant lighting for the 3 days preceding pro-oestrus.

Time	Constant lighting			Control lighting*		
	Before	After	n	Before	After	n
10.00 h	2.5 $\pm$ 0.3	6.6 $\pm$ 0.9	4	1.1	12.3 $\pm$ 3.2	6
13.30 h	2.5 $\pm$ 0.3	11.2 $\pm$ 0.6	7	1.2	19.6 $\pm$ 4.0	6
17.00 h	2.4 $\pm$ 0.2	9.0 $\pm$ 0.9	8	29.1 $\pm$ 5.0	128.2 $\pm$ 16.4	6
21.00 h	6.2 1 2.5	21.2 $\pm$ 6.6	8	10.8 $\pm$ 1.3	72.8 $\pm$ 19.9	6

n = number of animals/group.

Where no S.E.M. is given the concentration of LH in some samples of the group was below the level of detection.

*Values for control lighting were taken from Aiyer, Fink and Greig (1974a) and converted from NIH-LH-S13 to NIH-LH-S18 using a conversion factor of 0.83 (Aiyer, 1974).

TABLE 4-2: Mean ($\pm$ S.E.M.) concentrations of plasma LH (ng/ml) before and mean (S.E.M.) maximal increments in plasma LH after i.v. injection of LHRH in animals exposed to either control lighting (lights on 05.00 - 19.00 h; LD) or constant lighting (LL) for the 3 days preceding pro-oestrus then ovariectomized and treated immediately with 2.5 μ g oestradiol benzoate on the morning of dioestrus and 2.5 mg progesterone on the afternoon of pro-oestrus.

Lighting	Before	After	n
LD	26.1 $\pm$ 3.5	75.1 $\pm$ 6.9	7
LLa	10.7 $\pm$ 2.6	39.1 $\pm$ 7.0	19
LLb	4.5 $\pm$ 1.0	24.6 $\pm$ 4.3	14
LLc	28.0 $\pm$ 1.8	79.6 $\pm$ 10.3	5

n = number of animals/group.

LLa - mean ($\pm$ S.E.M.) of all animals exposed to LL.

LLb - mean ($\pm$ S.E.M.) of animals remaining from LLa, after excluding LLc animals.

LLc - mean ($\pm$ S.E.M.) of animals exposed to LL but giving responses the same as group LD.

those animals exposed to LD. Figure 4-B shows that there was a significant positive correlation, in all the animals exposed to LL, between the pre-injection LH concentrations and the maximal increments in plasma LH concentration ($r=0.92$, $p < 0.001$). In the 14 animals which showed a low response to LHRH, the correlation between the pre-injection and the maximal increments in plasma LH concentrations was also significant ($r=0.76$, $p < 0.001$).

4.3.4 Effect of constant light or pituitary responsiveness to LHRH after acute ovariectomy and the implantation of an oestradiol- 17β capsule

Thirty-one animals were ovariectomized on the morning of dioestrus and immediately implanted with an oestradiol capsule. Pituitary responsiveness to LHRH was determined at 8, 12, 16 or 20 h after implantation. Figure 4-C shows that in animals exposed to LL, oestradiol- 17β did not significantly increase the pituitary responsiveness to LHRH 12 h after capsule implantation. This contrasts with animals exposed to LD in which pituitary responsiveness to LHRH had increased markedly 12 h after implantation of an oestradiol-containing capsule.

4.4 Discussion

Figure 4-A shows that exposure to LL during the 3 days before pro-oestrus resulted in a 3-hour delay and a 40% decrease in the size of LH surge. This is consistent with the data of Daane and Parlow (1971) who found a decreased incidence of the LH surge in rats exposed to LL for periods of between 2-5 days as assessed by measuring LH in single plasma samples from individual animals between 15.15 - 16.55 h of pro-oestrus. Campbell and Schwartz (1980) also found reduced

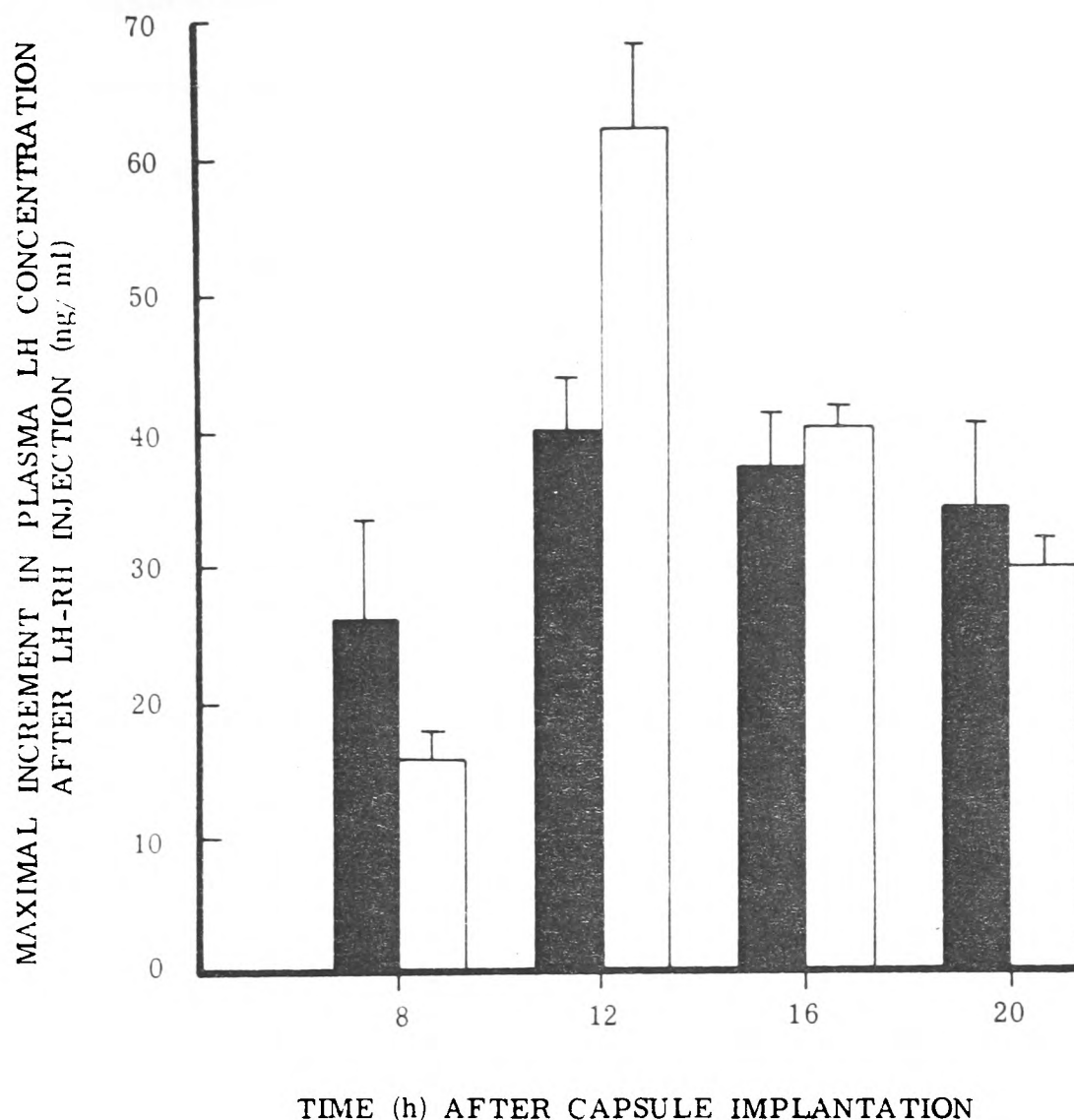


FIGURE 4-C: Mean ($\pm$ S.E.M.) maximal increments in plasma LH concentration after an intravenous injection of synthetic LHRH (50 ng/100 g b.w.). Animals were exposed either to constant (solid bars) or control lighting (open bars). The animals were ovariectomized between 10.00 - 11.00 h dioestrus immediately after which they were implanted with a Silastic capsule containing oestradiol-17 β and then given the injection of LHRH at various times after capsule implantation. There was no significant difference between the values from animals exposed to LL. The values for animals exposed to control lighting were taken from studies previously carried out in this laboratory using comparable conditions (Henderson, Baker and Fink, 1977b).

No. of animals/group: LL, 5 for 8 h, 9 for 12 h, 10 for 16 h, 7 for 20 h.
LD, all groups = 4.

serum LH concentrations at 17.00 h on the first pro-oestrus in LL (animals having been placed in LL at metoestrus). A delay in the LH surge was also suggested by the results of McCormack and Sridaran (1978) who found that exposure to LL beginning on metoestrus delayed ovulation by 3 hours on the subsequent oestrus. The timing of the LH surge depends upon a daily neural signal which is expressed only when the hypothalamic-pituitary system is exposed to increased plasma concentrations of oestradiol-17 β . One of the main lines of evidence for the occurrence of the daily neural signal for LH release is the LH surge which occurs at a precise time on each day in long-term ovariectomized rats exposed to oestrogen (e.g. Caligaris, Astrada and Taleisnik, 1971a; Legan, Coon and Karsch, 1975; Henderson, Baker and Fink, 1977a). The daily surge of LH in long-term ovariectomized rats exposed to oestrogen is blocked in rats exposed to LL (see results in Chapter V) and the data described here suggest that this block may begin with a delay (possibly a phase shift) of the LH surge. McCormack and Sridaran (1978) found a greater delay in ovulation in animals exposed to 'full LL' compared with 'dim LL', and suggested that the delay in 'dim LL' was caused by the induction of a free-running rhythm controlling LH release. The data on ovulation described in this chapter show that although the LH surge is reduced by LL it is still sufficient to result in full ovulation. The one animal from the cannulated LL group that did not ovulate was the only one which did not have a LH surge. The absence of a LH surge is presumably the explanation for the fact that ovulation did not occur in the 5 animals of the non-cannulated group. The fact that the quota of ova in LL animals which ovulated was similar to that in animals exposed to LD contrasts with the findings (Brown-Grant, Davidson and Greig, 1973; Fink, 1975;

data in Chapter VII) that after long-term exposure to LL the ovulatory quota is significantly reduced, presumably due to changes in responsiveness of ovarian follicles to gonadotrophins.

Table 4-1 shows that exposure to LL markedly reduced the responsiveness of the anterior pituitary to LHRH. This six-fold reduction in pituitary responsiveness was sufficiently large to account for the significant reduction in the magnitude of the LH surge (Figure 4-A). In rats exposed to LD, ovariectomy on dioestrus markedly reduced the LH-response to LHRH administered during the afternoon of expected pro-oestrus, an effect which could be reversed by administering oestrogen between 10.00 - 11.00 h of dioestrus followed by progesterone at 13.00 h of expected pro-oestrus (Aiyer and Fink, 1974; Fink and Henderson, 1977a). Table 4-2 shows that in the majority of animals exposed to LL (14 out of 19) there was a marked reduction in the efficacy of treatment with oestrogen followed by progesterone in restoring pituitary responsiveness, a finding which is supported by the results in Figure 4-3. Thus, the components of the hypothalamic-pituitary system that are responsible for the pro-oestrous increase in the LH response to LHRH are much less sensitive to the effects of oestrogen followed by progesterone in animals exposed to LL compared with those in animals exposed to LD. The data of Campbell and Schwartz (1980) (although not directly comparable because of strain differences and the use of 5-day cycling animals) do not support the idea of reduced oestrogen secretion during the pre-LH surge period of the first pro-oestrus in LL. They showed that the pre-ovulatory oestradiol surge was not inhibited by LL, but that it was of longer duration in LL than LD controls. Turgeon (1979) showed that if the decline in the surge of plasma oestradiol-17 β found during the morning

and early afternoon of pro-oestrus was prevented using oestradiol filled Silastic capsules, the magnitude of the afternoon surge of plasma LH was diminished. Taken together with the data of Campbell and Schwartz (1980), the data of Turgeon (1979) suggest that the diminution of the pro-oestrus LH surge found in LL treated rats in the present study, may be due to an extended surge of oestradiol-17 β during pro-oestrus morning. Campbell and Schwartz (1980) also suggested that the pre-LH surge increase in plasma progesterone may be delayed in animals exposed to LL, but their sampling procedure (single samples taken on the afternoon of pro-oestrus) could only record an absence of a progesterone surge at that particular time but could not exclude a surge later on.

In animals exposed to LD, the pro-oestrous increase in pituitary responsiveness depends upon the priming effect of LHRH (Aiyer, Chiappa and Fink, 1974b; Fink, Chiappa and Aiyer, 1976), and that this is also the case in animals exposed to LL is suggested by the significant positive correlation between the LH-responses to LHRH and the pre-LHRH injection plasma concentration of LH (Table 4-2, Figure 4-B; see also Fink, 1975). Indeed, the priming effect of LHRH may play a critical role in mediating the facilitatory effect of oestrogen on pituitary responsiveness. The results in Chapter III (Tables 3-1 to 3-4) support this idea in that the potentiation of pituitary responsiveness to LHRH by oestradiol administered immediately after ovariectomy is due, at least in part, to the maintenance by oestrogen of the increased release of LHRH which follows ovariectomy (Sarkar and Fink, 1980) and which presumably primes the pituitary gland. Thus, the results described in Figures 4-B and 4-C suggest that one action of LL might be to reduce the stimulatory effect of

oestrogen on the LHRH release mechanism. The fact that 5 out of 19 animals showed an apparently normal response to steroids (Table 4-2) is difficult to explain but previous studies have also shown that in terms of the LH response to progesterone (Brown-Grant, Davidson and Greig, 1973; Chapter VII of this thesis) and LHRH (Fink, 1975) rats exposed to LL can be divided into two distinct groups. Taken together, these data show that this split into two groups in terms of the LH response begins as early as 3 days after exposure to LL and continues for at least 2 months.

CHAPTER V

The Effects of Constant Light on Pulsatile and Diurnal Luteinizing Hormone in Ovariectomized Rats

5.1 Introduction

The LH surge on pro-oestrus in cyclic female rats is dependent upon the increase in plasma concentration of oestradiol-17 β ^{eq.} (Aiyer and Fink, 1974) which reaches a peak on the morning to early afternoon of pro-oestrus (Brown-Grant, Exley and Naftolin, 1970; Shaikh and Shaikh, 1975; Henderson, Baker and Fink, 1977a). If the plasma oestrogen concentration is continually elevated artificially in ovariectomized rats, either by repeated injections of oestradiol benzoate or the implantation of a silicone elastomer (Silastic) capsule containing oestradiol-17 β , a surge of LH is seen during late afternoon every day (Caligaris, Astrada and Taleisnik, 1971a; Legan, Coon and Karsch, 1975; Chazal, Faudon, Gogan, Hery, Kordon and Laplante, 1977; Henderson, Baker and Fink, 1977a). This diurnal rhythm disappears on removal of oestrogenic feedback and is eventually replaced by a pulsatile release of LH (Weick, Soper and Greenberg, 1981) with a peak of LH occurring every 20-30 mins (Gay and Sheth, 1972; Gallo, 1980a). In contrast to the diurnal rhythm of LH, pulsatile release of LH does not vary throughout the 24 h day (Soper and Weick, 1977). To investigate possible mechanisms by which LL modifies gonadotrophin secretion in the female rat, the effects of LL upon these two distinct rhythms were investigated.

Since Althesin anaesthesia has been shown not to block either the pro-oestrous surges of LH and LHRH (Sarkar, Chiappa, Fink and Sherwood, 1976) or the diurnal rhythm of LH release in long-term ovariectomized rats treated with oestrogen (Sarkar and Fink, 1980), the effects of this anaesthetic on pulsatile release of LH were also investigated.

5.2 Methods and Materials

Female Wistar Cobs (200-300 g, b.w.) were maintained either in control (LD) or constant light (LL) as outlined in Chapter II (2.1).

5.2.1 Pulsatile release

Two groups of animals were ovariectomized under Avertin anaesthesia as described in Chapter II. The first group was maintained in LD for 7 days and then placed in LL for 21 days. The second group was maintained in LD for 28 days. Animals from both groups were then anaesthetized with Althesin (0.4 - 0.6 ml/100 g, b.w., i.p.), while some of the animals from the second group were anaesthetized with sodium pentobarbitone (36 mg/kg, i.p.). In all animals both external jugular veins were exposed and a tracheal cannula inserted to allow regular clearance of mucous from the airway. Thirty min later, 0.15 ml of blood was collected and the sampling repeated every 10 min for 2 h.

5.2.2 Diurnal release

Nine animals were ovariectomized under Avertin anaesthesia as described in Chapter II and 2 weeks later placed in LL. Four weeks after exposure to LL, an intra atrial cannula and a 20 mm oestradiol capsule were inserted at the same time under Avertin anaesthesia. Both procedures are described in Chapter II (2.2.5 and 2.2.8). Blood samples were collected immediately and at various times after capsule implantation and prepared and assayed for LH as described in Chapter II (2.2.4 and 2.3.3).

5.3 Results

Figure 5-A shows that pulsatile release of LH occurred in all animals that had been exposed to either LL or LD and anaesthetized with Althesin. However, in animals anaesthetized with sodium pentobarbitone the plasma concentrations of LH were low and did not fluctuate. In both groups of animals anaesthetized with Althesin, there was a pulse of LH about once every 35 mins. The peak LH values of the pulses in rats exposed to LL or LD were 38.9 ± 0.9 (S.E.M.) and 33.9 ± 0.7 ng/ml respectively while the lowest values were 20.0 ± 0.7 and 18.9 ± 0.6 ng/ml respectively. There was a significant difference ($p < 0.001$) between the peak and the trough values for rats exposed to either LL or LD, but there was no significant difference between the peak values for rats on LL and LD and between trough values for those on LL and LD. The mean change of LH concentrations between each sampling line (10 min intervals) was 1.6 ± 0.3 (sodium pentobarbitone), 7.0 ± 0.8 (Althesin, LD) and 8.7 ± 1.5 (Althesin, LL) ng LH/ml. The mean change in plasma concentrations of LH in the group anaesthetized with sodium pentobarbitone was significantly ($p < 0.001$) less than that in either of the other two groups.

Figure 5-B shows that there was a decline in plasma levels of LH over the first 48 h after capsule implantation, levels at both 24 h and 42 h were significantly lower than at 0 h ($p < 0.05$ and $p < 0.001$ respectively). After 48 h the levels remained constant, being 6.1 ± 0.4 at 52 h and 5.5 ± 0.3 ng/ml at 115 h. A value of 32.2 ng/ml at 97 h in one animal accounted for the high mean and S.E.M. at that time.

FIGURE 5-A: Plasma concentrations of LH in (a) individual rats
ovariectomized and then exposed to control lighting
(lights on 05.00 - 19.00 h) for 28 days, or (b) control lighting for 7
days then constant lighting for 21 days and anaesthetized with Althesin
(●—●) or sodium pentobarbitone (o---o).

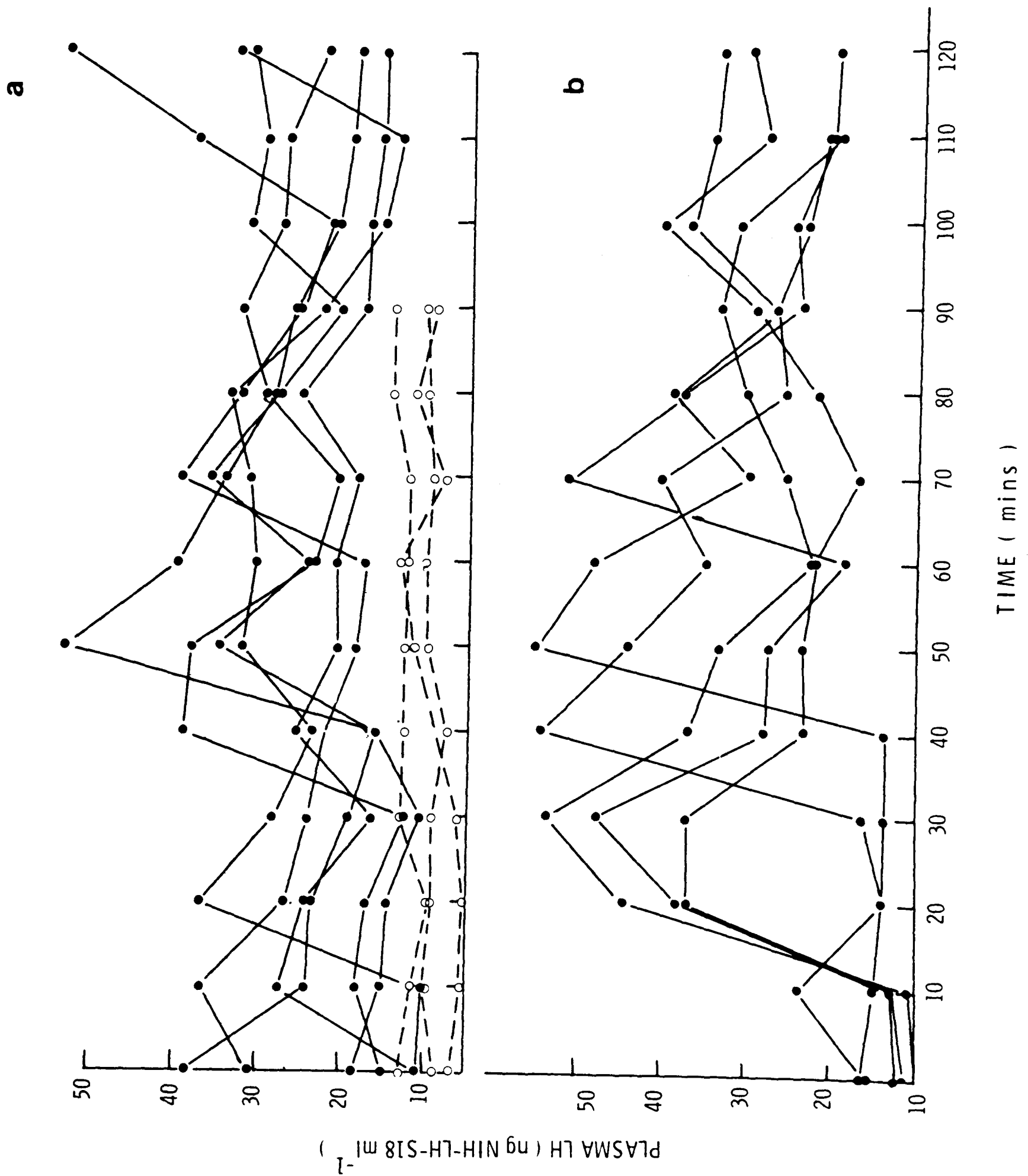
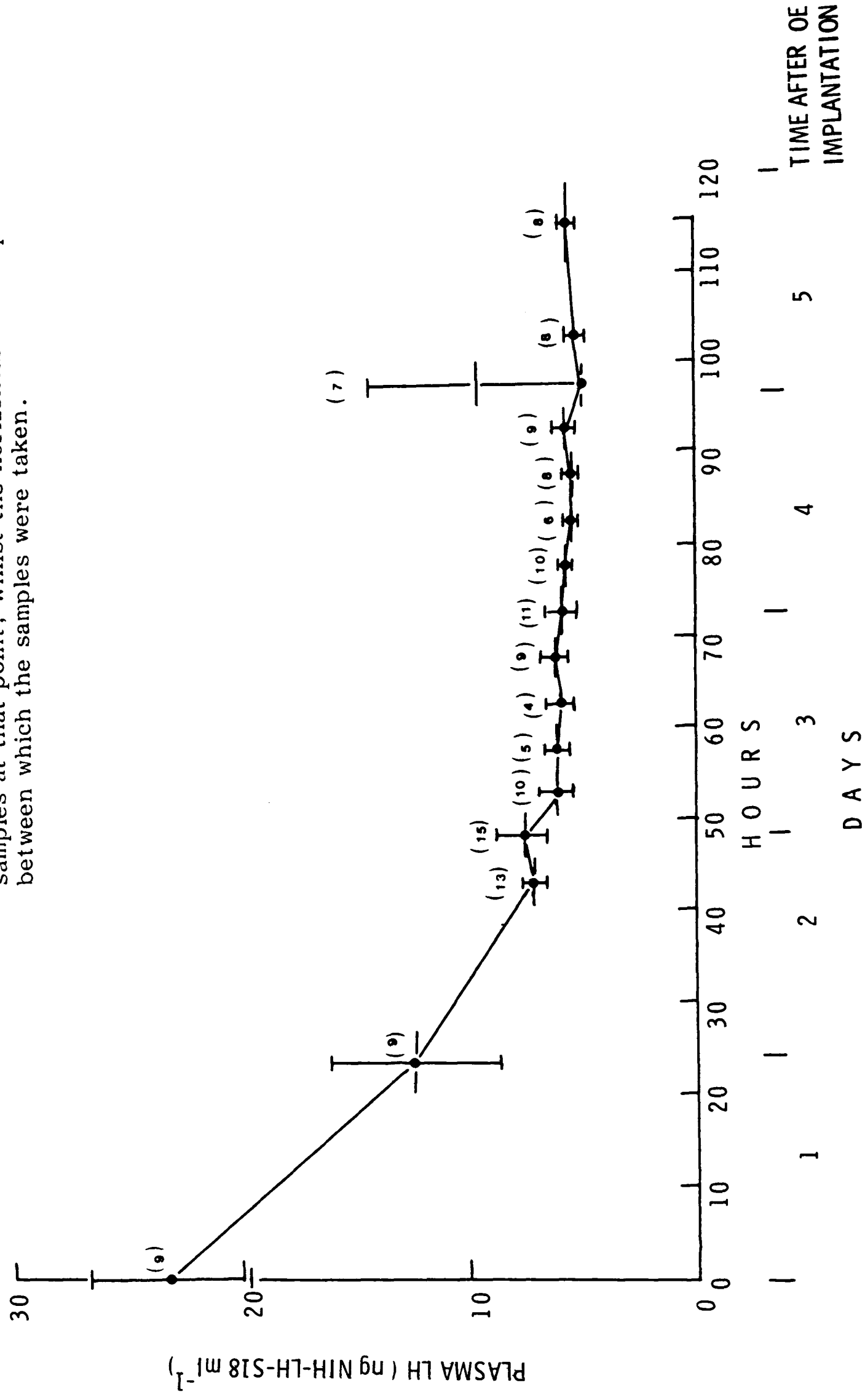


FIGURE 5-B: Plasma concentrations of LH in ovariectomized rats implanted with a capsule containing oestradiol-17 β (OE) crystals 4 weeks after exposure to constant light. The points shown are means $\pm$ S.E.M. The number above each point is the number of samples at that point, whilst the horizontal lines represent the interval between which the samples were taken.



5.4 Discussion

The height of the pulses from rats kept in both LD and LL was about twice that of basal concentrations of plasma LH. That is similar to the values found in conscious rats (Blake and Sawyer, 1974; Soper and Weick, 1980). Figure 5-A shows that the pulsatile release occurs without any significant loss of height under Althesin anaesthesia but not using sodium pentobarbitone. Arendash and Gallo (1978a) also found that sodium pentobarbitone anaesthesia inhibited pulsatile release for a period of 1.5 h. Figure 5-A also shows that LL did not affect the height or periodicity of LH release. The 35 minute periodicity found here was slightly longer than in previous studies in conscious animals from which blood samples were taken every 5 min (e.g. Soper and Weick, 1980) but was within the range reported by Gay and Sheth (1972) and Blake and Sawyer (1974) who took samples every 10 min.

The findings in Figure 5-B confirm and extend those of Mennin and Gorski (1975) who found that 24 h after injecting 20 μ g oestradiol benzoate (OB) into ovariectomized rats that had been exposed to LL there was a significant drop in the plasma concentration of LH. This shows that the inhibitory action of oestradiol is not affected by LL (a finding also confirmed by the results in Chapter VI). Mennin and Gorski (1975) also found that in rats kept in LL and given two successive injections of OB there was no afternoon increase in the plasma LH concentration. However, because of their sampling procedure, Mennin and Gorski (1975) could not exclude the presence of a diurnal type release with peaks occurring at other times of the day (which is what would be predicted if the rhythms were free-running but not abolished). Figure 5-B shows that there is no diurnal rhythm in rats kept in LL and that the LH release pattern contrasts markedly with that of females

kept in LD (Caligaris, Astrada and Taleisnik, 1971a; Legan, Coon and Karsch, 1975; Chazal, Faudon, Gogan, Héry, Kordon and Laplante, 1977; Henderson, Baker and Fink, 1977a) and is similar to that in oestrogen-treated castrated males exposed to LD (Henderson, Baker and Fink, 1977a). The absence of diurnal LH release in oestradiol-treated ovariectomized rats exposed to LL (Figure 5-B) agrees with the findings of Cheifetz, Gaffud and Dingman (1968) that the circadian release of adenocorticotrophic hormone (ACTH) also disappears after exposure to LL. Taken together, these results support the suggestion of Szafarczyk, Héry, Lapante, Ixart, Assenmacher and Kordon (1980) for a common (or linked) oscillatory mechanism in the daily release of ACTH, prolactin and LH.

The results in Figures 5-A and 5-B are consistent with the view that pulsatile and diurnal release of LH are mediated by different neuronal pathways. Blake and Sawyer (1974) and Soper and Weick (1980) showed that surgical isolation of the medial basal hypothalamus (MBH) was compatible with pulsatile release provided that the arcuate nucleus remained intact. That the suprachiasmatic nucleus (SCN) is essential for the maintenance of LH rhythms was demonstrated by the fact that oestrous cycles (Stetson and Watson-Whitmyre, 1976) and the oestrogen-induced diurnal release of LH in ovariectomized rats (Coen and MacKinnon, 1980; Kawakami, Arita and Yoshioka, 1980) are both abolished by discrete lesions of the SCN. A serotonergic mechanism appears to be essential for the diurnal rhythm as this is abolished by the inhibition of serotonin synthesis (Héry, Laplante and Kordon, 1976) or the destruction of the raphe nuclei (Héry, Laplante and Kordon, 1978), efferents of which are thought to be the main serotonergic input to the SCN (Fuxe and Jonsson, 1974,

Palkovits, Saavedra, Jacobowitz, Kizer, Zaborsky and Brownstein, 1977; Parent, Descarries and Beaudet, 1981). Serotonergic influence on basal (i.e. unstimulated) pulsatile LH release seems, however, to be minimal. This was suggested by Gallo (1980b), Rasmussen, Jacobs, Kissinger and Malvern (1981) and the results in Chapter VI of this thesis, where a depletion of 5-HT by PCPA did not lead to any change in the pattern of pulsatile LH release. The SCN does not seem to be essential for the continuation of basal pulsatile LH release as this is still seen in animals having lesions limited only to the SCN (Arendash and Gallo, 1979; Soper and Weick, 1980). Electrophysiological (Kawamura and Inouye, 1979) and biochemical (Schwartz and Gainer, 1977; Fuchs and Moore, 1980; Schwartz, Davidsen and Smith, 1980) evidence suggests that the SCN possesses an endogenous circadian rhythm which may persist even after surgical isolation and removal of visual stimuli by blinding or constant darkness (Kawamura and Inouye, 1980; Schwartz, Davidsen and Smith, 1980). The fact that LL suppresses the SCN-dependent diurnal LH release but not the relatively SCN-independent pulsatile release (Arendash and Gallo, 1979; Soper and Weick, 1980) suggests that LL may uncouple diurnal LH mechanisms from an endogenous circadian oscillator in the SCN. This uncoupling may involve suppression of serotonergic mechanisms which would result in the loss of diurnal release of LH (Héry, Laplante and Kordon, 1976, 1978) but not the pulsatile release of LH (Gallo, 1980b; Rasmussen *et al.*, 1981).

CHAPTER VI

The Pattern of Pulsatile LH Release
and Effects of Oestradiol-17 β on the Pulsatile
LH Release in Gonadectomized Male, Female,
Constant Light Exposed Female and Androgen
Sterilized Female Rats

6.1 Introduction

Removal of gonadal steroid feedback in adult rats leads to an elevation in plasma LH concentration (Taleisnik and McCann, 1961; Bognadove, Parlow, Bognadove, Bhargava and Crabill, 1964; Gay and Midgley, 1969). Gay and Sheth (1972) first demonstrated that in these circumstances LH was released in discrete pulses rather than in a tonic manner. Evidence from studies using anti-LHRH serum (Snabes and Kelch, 1979), direct measurement of LHRH concentrations in portal plasma (Sarkar and Fink, 1980), and data showing the response of the pituitary gland to constant or pulsatile infusions of LHRH in animals treated with phenobarbitone (Schuiling and Gnodde, 1976; Blake, Sridaran, Elias, Ashiru and Rush, 1980) suggested that pulsatile LH release was controlled centrally and reflected pulsatile LHRH release from the median eminence.

Work on the central mechanisms involved in the control of pulsatile LH release has been carried out mainly on female rats (e.g. Blake and Sawyer, 1974; Drouva and Gallo, 1976; Gallo, 1980a; Soper and Weick, 1980), while work on androgen-sterilized female rats has been limited to measuring the effects of sub-cutaneous oestradiol benzoate treatment on plasma LH (Turgeon and Barraclough, 1974). The aim of this study was to determine and compare the characteristics of pulsatile LH secretion in gonadectomized male and female rats and female rats rendered anovulatory by neonatal treatment with androgen or by long term exposure to continuous light.

Blake, Norman and Sawyer (1974) showed that an i.v. injection (3 μ g) of oestradiol-17 β (OE₂) would rapidly reduce plasma LH levels in ovariectomized rats. Sarkar and Fink (1980) also showed that LHRH concentrations in hypophysial portal plasma and LH concentrations in

peripheral plasma could be lowered quickly by injecting 1 μ g of OE₂. Therefore, in the present study an i.v. injection of 1 μ g of OE₂ was used in all 4 groups of animals to compare the inhibitory effect of OE₂ with that of sodium pentobarbitone on LH secretion.

Long term oestrogen treatment as achieved by s.c. implantation of Silastic capsules filled with OE₂ or by injections of oestradiol benzoate resulted in a diurnal rather than a pulsatile mode of LH secretion (Caligaris, Astrada and Taleisnik, 1971a; Ramirez and Sawyer, 1974; Legan, Coon and Karsch, 1975; Chazal, Faudon, Gogan, Héry, Kordon and Laplante, 1977; Henderson, Baker and Fink, 1977a). Complete inhibition of this rhythm after treatment with the tryptophan hydroxylase inhibitor para-chlorophenylalanine (PCPA) (Héry, Laplante and Kordon, 1976; Coen and MacKinnon, 1979) as well as partial inhibition after lesions of the serotonergic raphe nuclei (Héry, Laplante and Kordon, 1978) suggested that the oestrogen-induced diurnal release was dependent upon a serotonergic mechanism. However, serotonin (5-HT) did not seem to be involved in the maintenance of pulsatile release (Gallo, 1980b; Rasmussen, Jacobs, Kissinger and Malven, 1981). In this study investigations were carried out into the role of 5-HT in the initial rapid inhibitory action of OE₂ on LH secretion.

6.2 Methods and materials

6.2.1:

Male and female Wistar Cobs were maintained as described in Chapter II (2.1). Ovariectomy, orchidectomy, atrial cannulation and placement of sampling cannulae, radioimmunoassay of plasma LH, and determination of catecholamines and indoleamines by LGED, were all carried out using procedures described in Chapter II.

One group of females was injected 4 days after birth with 1.25 mg of testosterone propionate (TP) s.c. diluted in 100 μ l of arachis oil. At 3 months of age daily vaginal smears were examined and only those animals which showed at least 10 consecutive days of oestrous smears were used for subsequent experiments. A second group of female rats (200-220 g b.w.) were put on constant illumination (LL) as described in Chapter II (2.1). Eight weeks later, vaginal smears were inspected daily and only those animals that showed at least 10 consecutive oestrous smears were used for subsequent experiments. These 2 groups of animals together with a group of untreated female rats (220-280 g b.w.) maintained in control lighting (LD - 2.1) were all ovariectomized (2.2.2). Male rats (180-200 g b.w.) kept in LD were castrated (2.2.3). In all groups an intra-atrial catheter was inserted 3 weeks after gonadectomy.

6.2.2 The characteristics of pulsatile LH release

Four groups of animals were prepared as described above (6 2.1). Twenty-four hours after insertion of the intra-atrial catheter, a sample cannula was attached to the intra-atrial cannula (2.2.5) and left in position for the duration of the experiment. Thirty min later a 100 μ l blood sample was taken into a heparinised 1 ml syringe. Sampling was then repeated every 7 min for a further 18 samples (a total of 126 mins). Plasma LH concentrations were determined in the samples by radioimmunoassay. All the samples from one experiment were assayed in the same assay. A peak was included in the data if the difference between it and the preceding trough (pulse amplitude) was at least four times the standard deviation (SD) for the assay pools. In all cases, the SD was no greater than 0.4 and was equivalent to 1.7 ng/ml for a pool of 12 ng/ml and 2.2 ng/ml for a pool of 40 ng/ml.

For each animal the mean plasma LH concentration, mean peak plasma LH concentration, mean trough plasma LH concentration, the mean interpulse interval (time from peak to peak) and the mean pulse amplitude (the difference between a peak and its preceding trough) were determined. The group mean for each parameter was calculated from the means of each animal. Differences between groups were then determined using one-way analysis of variance and the multiple range test of Duncan.

6.2.3 The inhibitory effect of oestradiol-17 β

Four groups of animals were prepared as described above (6.2.1) and fitted with an intra-atrial cannula 24 h before sampling. Thirty min after fitting the sample cannula (6.2.1), blood samples (100 μ l) were taken every 10 min for 1 h. Then a single injection of either 1 μ g OE₂ (Sigma, London) in 0.5 ml ethanol/saline vehicle (1 : 50, v/v) or 0.5 ml vehicle alone, was injected through the intra-atrial cannula and blood samples (100 μ l) were taken every 10 min for the next 3 h. Plasma LH concentrations were determined by radioimmunoassay (2.3.3). Samples from any one experimental group were assayed together. For each group of animals plasma LH concentrations were averaged at each sampling time, irrespective of the presence of peaks or troughs. Significance of differences between means were determined at each sampling time using the unpaired 't' test.

To determine the secretion of LH after OE₂ treatment in each group, the mean plasma LH concentration was calculated from the plasma LH concentrations from the final 5 sampling times. The unpaired 't' test was used to determine the difference in the means of each group after pentobarbitone treatment and after OE₂ treatment.

6.2.4 The determination of pituitary responsiveness to LHRH and the secretion of LH after sodium pentobarbitone treatment

Four groups of animals were prepared as described above (6.2.1). Three weeks after gonadectomy, animals were anaesthetized using sodium pentobarbitone (Nembutal; 36 mg/kg). Thirty to forty-five min later both external jugular veins were exposed and a blood sample (250 μ l) collected into a 1 ml heparinized syringe. A further 4 samples were similarly taken at 10 min intervals. Immediately after the final blood sample each animal was given an i.v. injection of synthetic LHRH (50 ng/100 g b.w. in 0.9% saline) and blood samples were taken 10, 15, 20, 30 and 60 min after LHRH injection to determine pituitary responsiveness (as described in Chapter II, 2.3.3). The mean maximal increment was calculated for each group using the plasma LH concentration in the fifth pre-injection blood sample for the pre-injection value. The significance of differences between the group means was calculated using one-way analysis of variance and the multiple range test of Duncan.

To determine the effect of pentobarbitone anaesthesia on LH secretion in each group, the mean plasma LH concentration was calculated from the pre-LHRH injection samples. The mean plasma LH concentration from each group was compared to the corresponding plasma LH concentration after OE_2 injection as described in Section 6.2.3.

6.2.5 The effect of PCPA on the inhibitory effect of oestradiol-17 β

Untreated female rats maintained in LD were ovariectomized (Chapter II, 2.2.2). Two days before being fitted with the intra-atrial catheter, 5 rats were injected with 320 mg/kg b.w. of PCPA methyl ester (Sigma, London) in a solution of 80 mg/ml in 0.9% saline. A further 5 rats were injected with saline vehicle alone. All animals were

then fitted with an intra-atrial catheter as described previously. The sampling procedure and the injection of OE_2 were carried out as described in Section 6.2.3. Plasma LH concentrations were determined by radioimmunoassay (2.3.3) and were analysed in the manner described in Section 6.2.3 with significance of differences between means being determined by the unpaired 't' test.

At the end of the sampling period, animals were killed by decapitation and the brains dissected out and placed on glass microscope slides on ice. The hypothalamus and preoptic area were dissected as described in Chapter II (2.2.13) and homogenized in 300 μ l of 0.2M perchloric acid containing 10^{-4} M cysteine and 20 ng/ml n-acetyl dopamine. The homogenate was treated as described in Chapter II and the concentrations of 5-HT, 5-hydroxyindole acetic acid (5-HIAA) and dopamine (DA) determined by LCED (2.3.7).

6.3 Results

6.3.1 Measurement of the characteristics of pulsatile LH release

Table 6.1 shows the mean plasma LH concentration, mean peak plasma LH concentration, mean trough plasma LH concentration, mean interpulse interval and mean pulse amplitude for the 4 groups of animals. An animal having the characteristics closest to the group means was selected from each group and the plasma LH concentration profiles shown in Fig. 6-A.

The mean plasma LH concentrations in the untreated females and males were significantly greater (all $p < 0.01$) than those in the androgenized (TP) and LL rats. There were no significant differences between the mean plasma LH concentration in either the untreated males and females or between the TP and LL treated females. The pulse

TABLE 6-1: The mean ($\pm$ S.E.M.) peak, trough, amplitude (trough to peak) and overall mean plasma LH concentrations (ng/ml), and mean interpulse interval (peak to peak) (min) in conscious gonadectomized LD female, male, androgen-sterilized (TP) and LL female rats, bearing an intra-atrial cannula.

Group	n	Peak	Trough	Amplitude	Mean LH	Interpulse interval
LD female	10	33.7 $\pm$ 2.1	16.9 $\pm$ 1.3	16.7 $\pm$ 1.9	24.4 $\pm$ 1.5	20.3 $\pm$ 1.5
LD male	8	23.7 $\pm$ 2.2	17.4 $\pm$ 1.6	7.1 $\pm$ 0.8	20.6 $\pm$ 1.8	19.8 $\pm$ 0.6
LD TP female	15	11.6 $\pm$ 0.9	5.9 $\pm$ 0.5	5.6 $\pm$ 0.5	8.4 $\pm$ 0.7	26.8 $\pm$ 1.0
LL female	4	12.7 $\pm$ 0.4	5.4 $\pm$ 0.2	7.7 $\pm$ 0.2	8.8 $\pm$ 0.4	29.0 $\pm$ 4.4

n = number of animals/group.

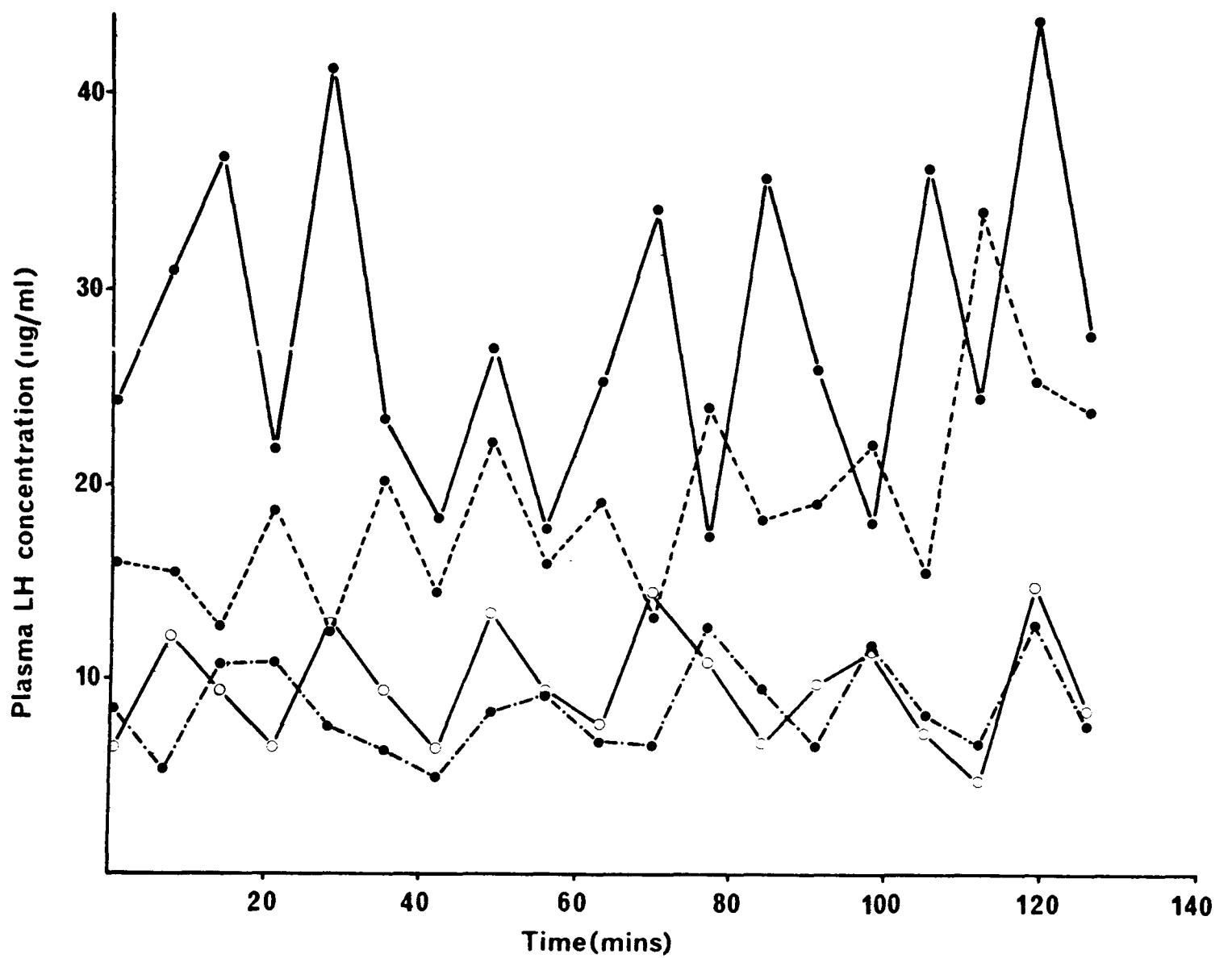


FIGURE 6-A: Plasma LH concentration (ng/ml) in individual female (●—●), male (●---●), androgen sterilized (o—o) rats maintained on LD, and female rats maintained on LL (●----●). All rats were gonadectomized and at least 21 days later implanted with an indwelling intra-atrial cannula. 24 h after cannula implantation, blood samples were taken every 7 min.

amplitude was significantly greater in the untreated females ($p < 0.01$) than in all other groups but there was no significant difference between the untreated males, TP females and LL treated females. The peak plasma LH concentration was significantly greater ($p < 0.01$) in the untreated female rats than in the TP and LL treated rats as well as in the untreated males ($p < 0.05$). The untreated males also had a significantly higher ($p < 0.05$) peak plasma LH concentration than the TP or LL rats. Both the untreated males and females had significantly higher ($p < 0.01$) trough plasma LH concentrations than the TP or LL treated rats, although there were no significant differences between the untreated males and females or between the TP and LL treated rats. Finally, the interpulse interval in the LL treated rats was significantly longer ($p < 0.05$) than in the untreated males or females.

6.3.2 The inhibitory effect of oestradiol-17 β

Figures 6-B to 6-E show the effects of an i.v. injection of vehicle or 1 μ g OE₂ on the pulsatile secretion of LH in all 4 groups. In all groups there were no significant differences at any time during the first hour between the animals subsequently treated with OE₂ or vehicle. After injection, animals in all groups treated with OE₂ showed a rapid decrease in plasma LH concentration, while those treated with vehicle showed no such decline. After OE₂ treatment, all groups first showed significantly lower plasma LH concentrations around 1 h after injection and thereafter remained significantly lower than the vehicle-treated group. In all groups sampling times later than 2 h occasionally showed no significant differences between the OE₂ and vehicle-treated animals. This was due to synchronous presence of troughs in the plasma LH concentration of the vehicle-treated animals.

FIGURE 6-B to 6-E:

Mean ($\pm$ S.E.M.) plasma LH concentrations (ng/ml) in gonadectomized rats. 21 days after gonadectomy each animal was implanted with an indwelling intra-atrial cannula. 24 h after cannula implantation, blood samples were taken every 10 min. 1 h after the first blood sample was taken, animals received ($\uparrow$) either 0.9% saline solution or 1 μ g OE₂ dissolved in 1 : 50 ethanol/0.9% saline solution (v/v) given through the intra-atrial cannula.

Significance of difference between saline and OE₂ animals at sample times for all diagrams.

+ p < 0.05, ++ p < 0.02, +++ p < 0.01,
* p < 0.005, ** p < 0.002, *** p < 0.001.

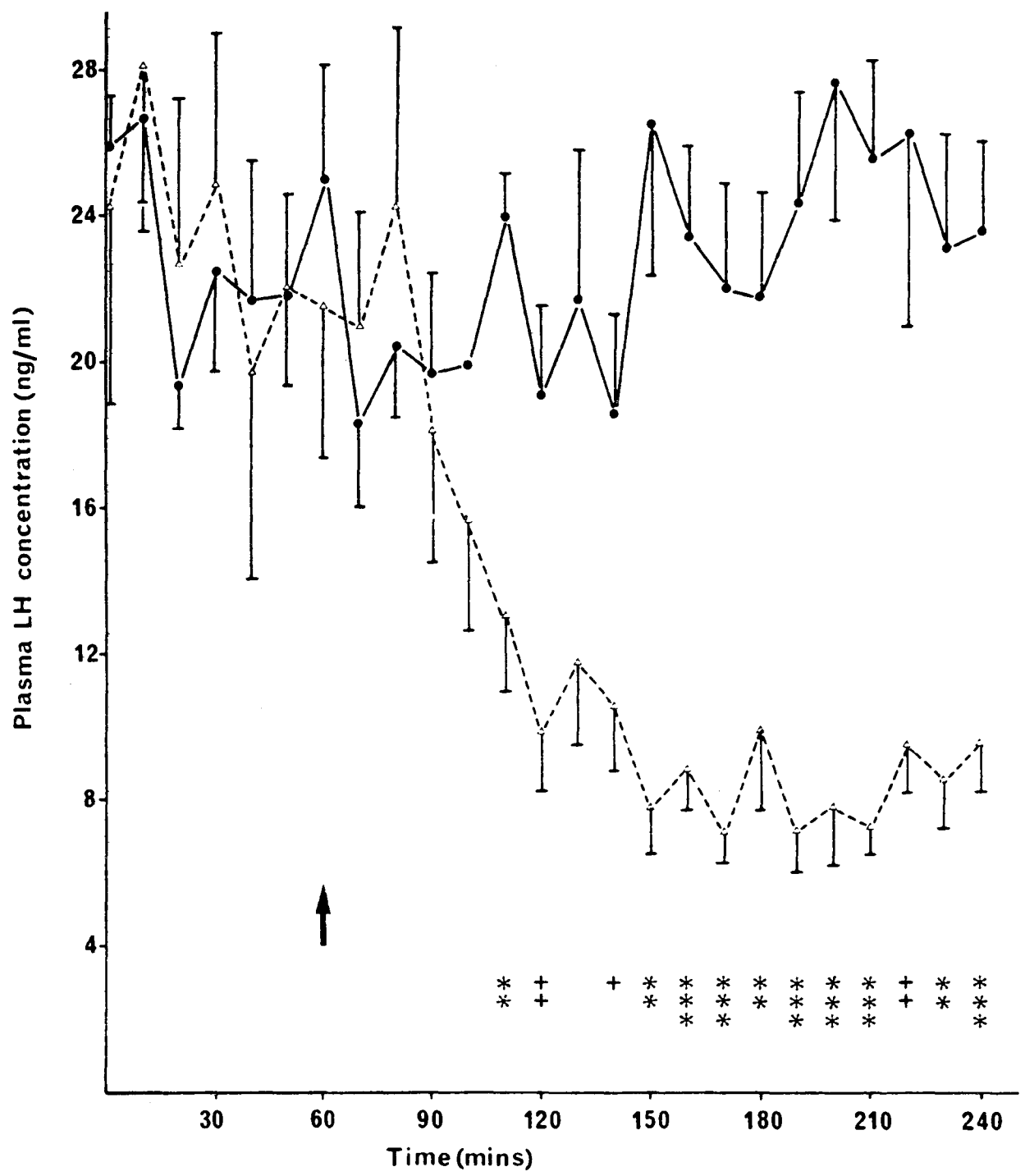


FIGURE 6-B: Saline (n=5, ● —●) or OE₂ (n=5, Δ --- Δ) injections into female rats maintained on LD. See p.94 for detailed description.

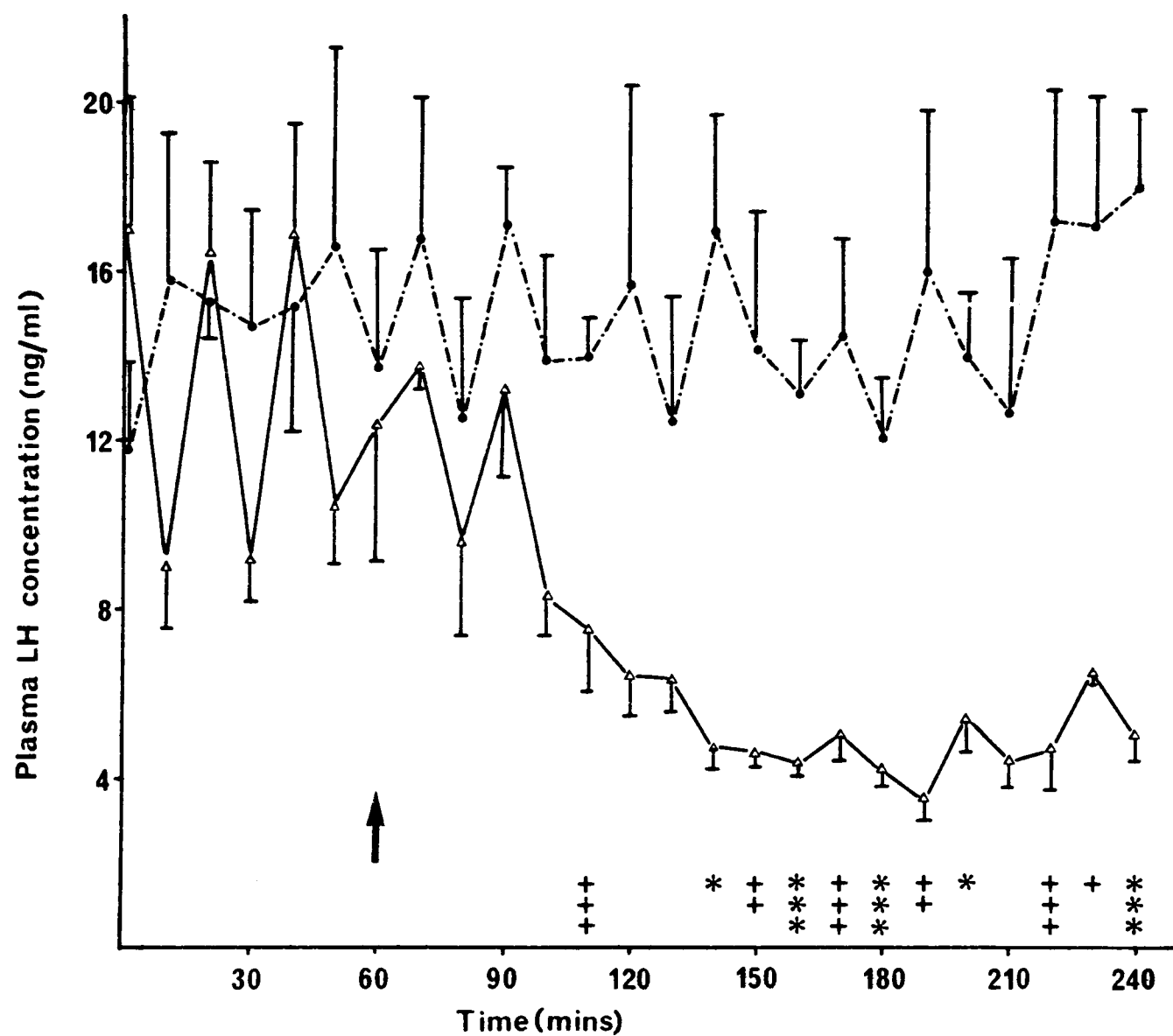


FIGURE 6-C: Saline (n=5, ●—●) or OE₂ (n=6, Δ—Δ) injections into male rats maintained on LD. See p.94 for detailed description.

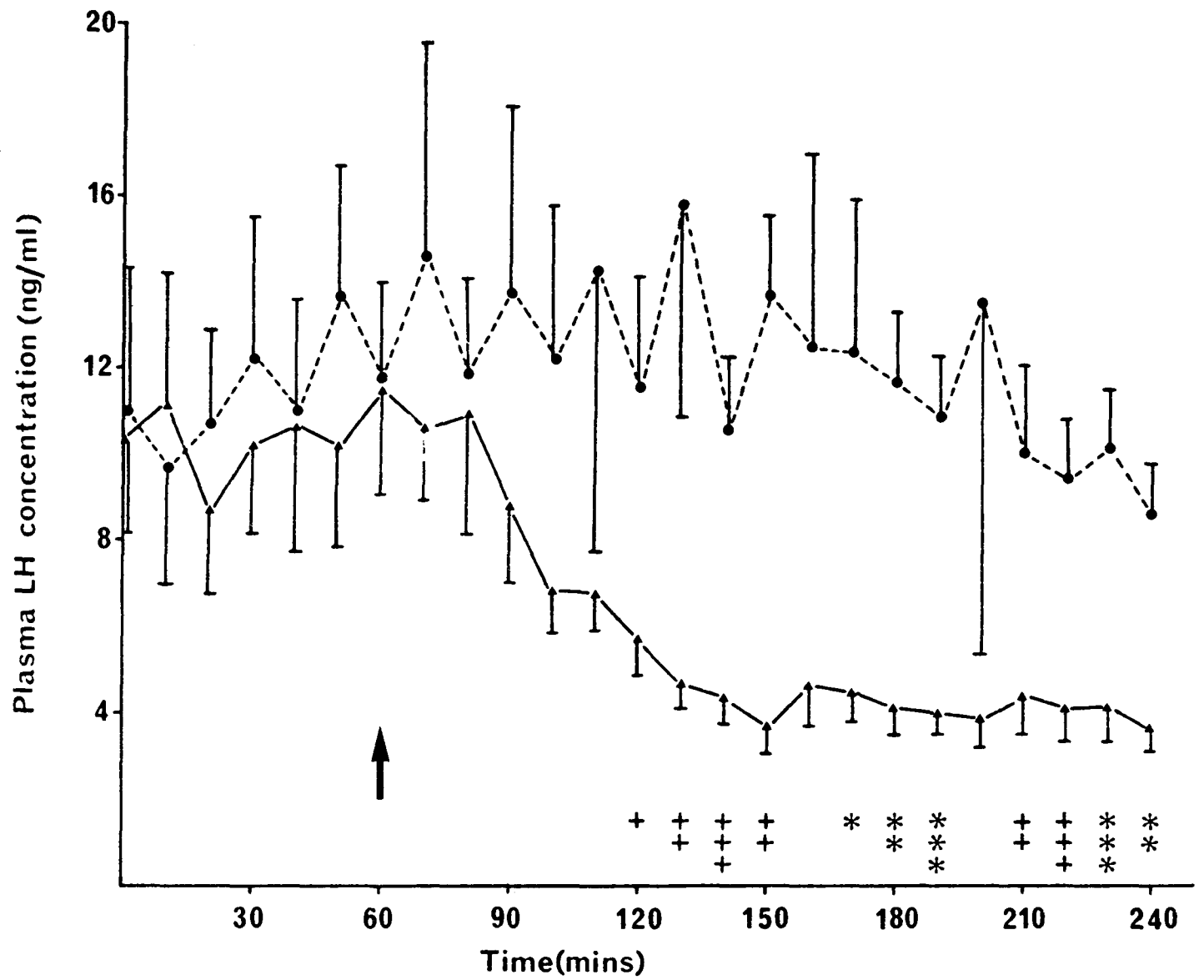


FIGURE 6-D: Saline (n=5, ● - - - ●) or OE₂ (n=4, ▲ —▲) injections into androgen-sterilized female rats maintained on LD. See p.94 for detailed description.

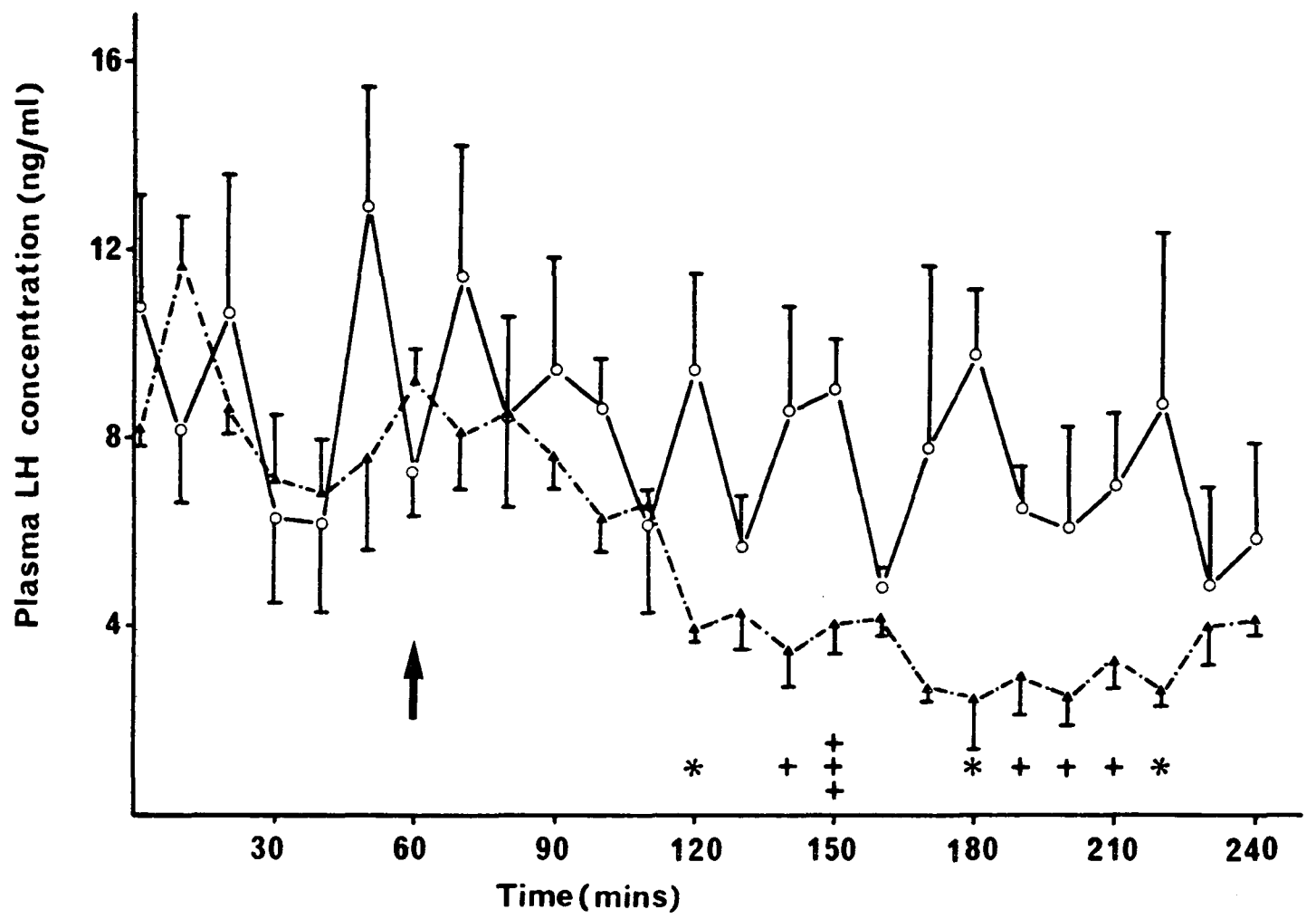


FIGURE 6-E: Saline (n=4, o—o) or OE₂ (▲-----▲) injections into female rats maintained on LL. See p.94 for detailed description.

6.3.3 The pituitary responsiveness to LHRH in gonadectomized female, male, androgenized female and constant light treated female rats

Figure 6-F shows for all 4 groups the mean maximal increments in plasma LH concentrations in response to an i.v. injection of LHRH. There was no significant difference between the mean maximal increment in plasma LH concentration from gonadectomized female and male rats. However, both these groups had mean maximal increments in plasma LH concentration significantly greater ($p < 0.01$) than in either the ovariectomized TP rats or ovariectomized LL-treated rats. The mean maximal increment in plasma LH concentration from ovariectomized LL-treated rats were significantly higher ($p < 0.05$) than that found in ovariectomized TP rats.

6.3.4 The secretion of LH after sodium pentobarbitone or oestradiol- 17β injection

Table 6.2 shows that in both gonadectomized female and male rats the plasma LH concentration was significantly lower after OE_2 than after pentobarbitone ($p < 0.001$ females; $p < 0.01$ males). TP females had significantly higher ($p < 0.001$) plasma LH concentrations after OE_2 treatment than after pentobarbitone treatment. There were no significant differences between plasma LH concentrations from ovariectomized LL females injected with OE_2 or sodium pentobarbitone.

6.3.5 The effect of PCPA on the negative feedback of oestradiol- 17β

Figure 6-G shows the effect of PCPA or saline on the OE_2 -induced inhibition of LH secretion. The secretion pattern from two individual animals from each group is shown in Figure 6-H. At no time either before or after the OE_2 injection was there a significant difference between the 2 groups. Table 6.3 shows that the concentrations of dopamine, 5-HT and 5-HIAA in the hypothalami were all significantly

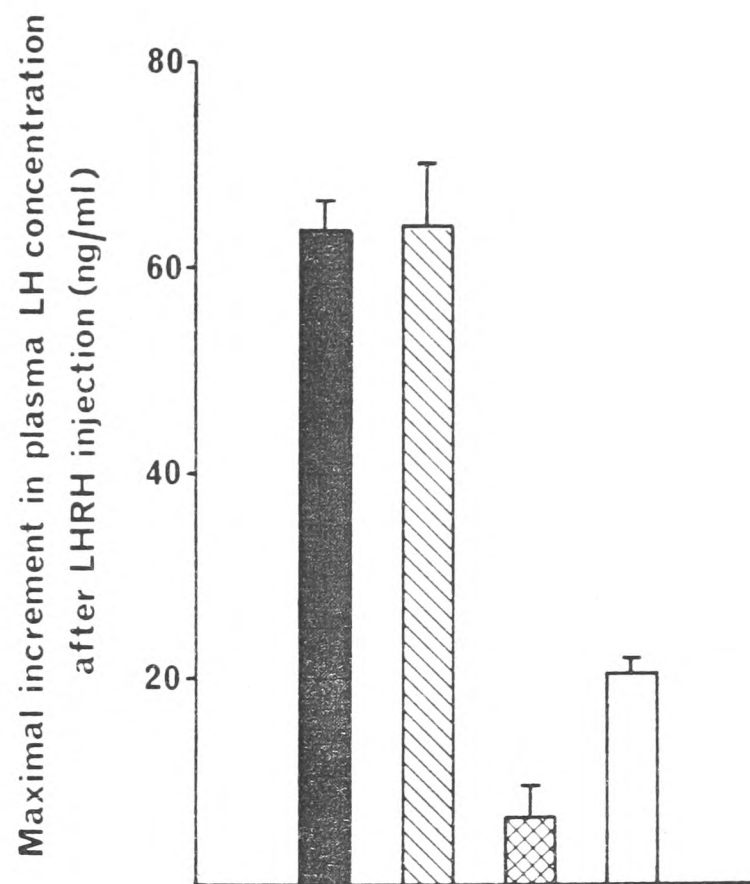


FIGURE 6-F: Mean (+ S.E.M.) maximal increment in plasma LH concentration (ng/ml) in female (solid bar, n=6), male (single hatched bar, n=5), androgen sterilized female (cross hatched bar, n=7) rats maintained in LD, and female rats maintained on LL (open bar, n=6). All rats were gonadectomized and 21 days later given an i.v. injection of 50 ng/100 g b.w. synthetic LHRH after anaesthesia with sodium pentobarbitone (36 mg/kg b.w.). Blood samples were taken 10, 15, 20, 30 and 60 min after LHRH injection and plasma LH concentration determined.

TABLE 6-2: The mean ($\pm$ S.E.M.) plasma LH concentration (ng/ml) in LD females, males and androgen-sterilized (TP) and LL female rats treated with oestradiol-17 β or sodium pentobarbitone.

Group	n	Oestradiol-17 β	n	Sodium pentobarbitone
LD female	5	7.0 $\pm$ 0.3**	6	12.3 $\pm$ 1.3
LD male	5	4.1 $\pm$ 0.1*	5	5.3 $\pm$ 0.2
LD TP female	4	5.2 $\pm$ 0.4**	7	2.3 $\pm$ 0.1
LL female	4	3.3 $\pm$ 0.4	6	4.4 $\pm$ 0.2

*p < 0.01, **p < 0.001 when compared with rats treated with sodium pentobarbitone. n = number of animals/group.

TABLE 6-3: The concentration (pg/mg wet weight) of dopamine (DA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the preoptic area and hypothalamus of LD female rats after para-chlorophenylalanine (PCPA) or saline treatment.

	n	DA	5-HT	5-HIAA
Saline	5	746.1 $\pm$ 45.8*	998.9 $\pm$ 81.1**	144.5 $\pm$ 11.3
PCPA	5	497.3 $\pm$ 29.1*	77.0 $\pm$ 16.9**	ND
% depletion		33%	92%	100%

*p < 0.005, **p < 0.001, ND - not detectable when comparing PCPA with saline.

n = number of animals/group.

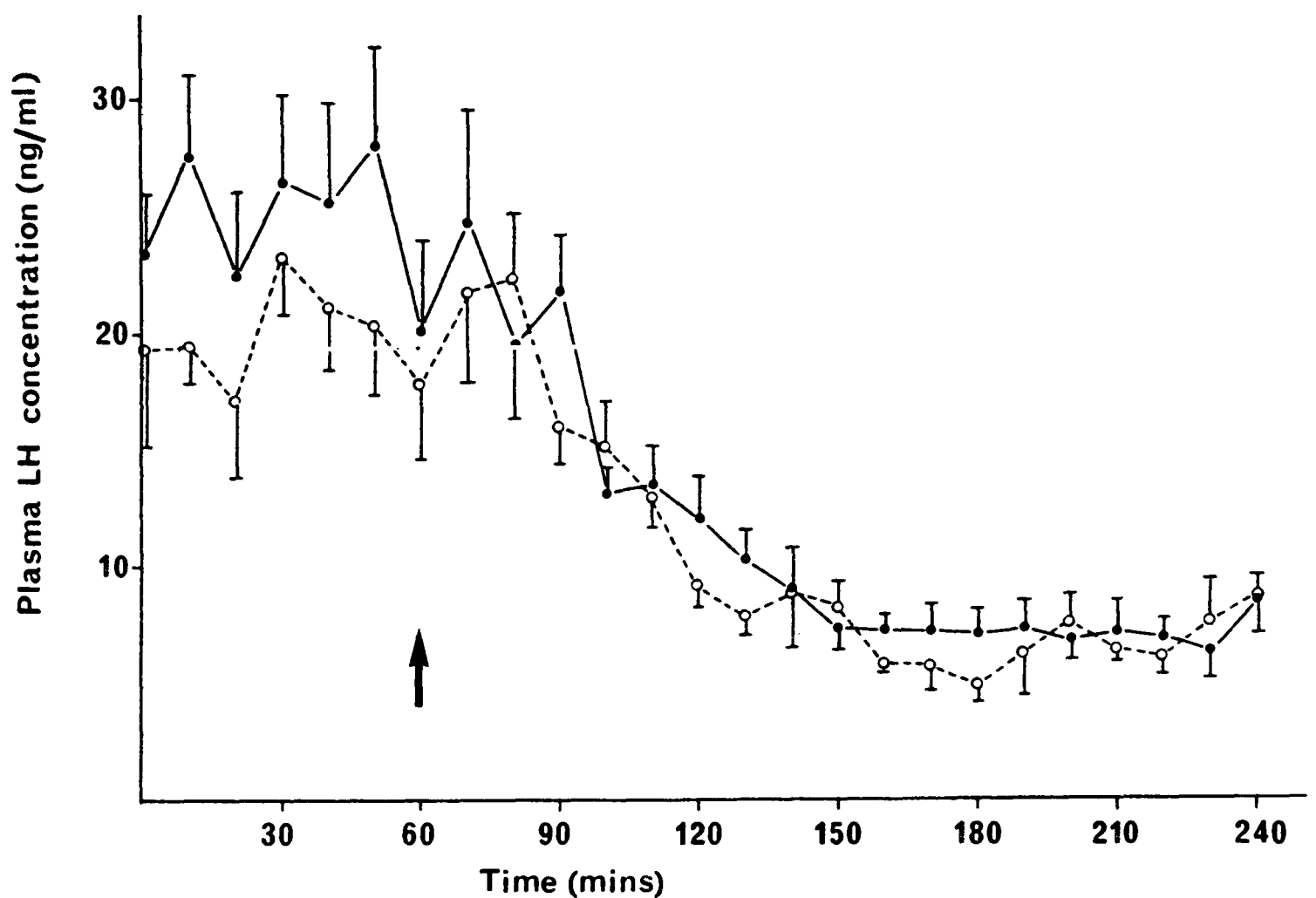


FIGURE 6-G: Mean ($\pm$ S.E.M.) plasma LH concentration (ng/ml) in female rats maintained on LD which were gonadectomized and 21 days later given an i.p. injection of 320 mg/kg b.w. para-chlorophenylalanine (PCPA) in 0.9% saline solution (n=5, o---o) or 0.9% saline solution only (n=5, ● — ●). 48 h after PCPA injection each animal was implanted with an intra-atrial cannula. 24 h after cannula implantation blood samples were taken through the cannula, and 1 h after the first blood sample was taken each animal received (↑) 1 μ g of OE₂ dissolved in ethanol : 0.9% saline solution (1 : 50 v/v) through the cannula.

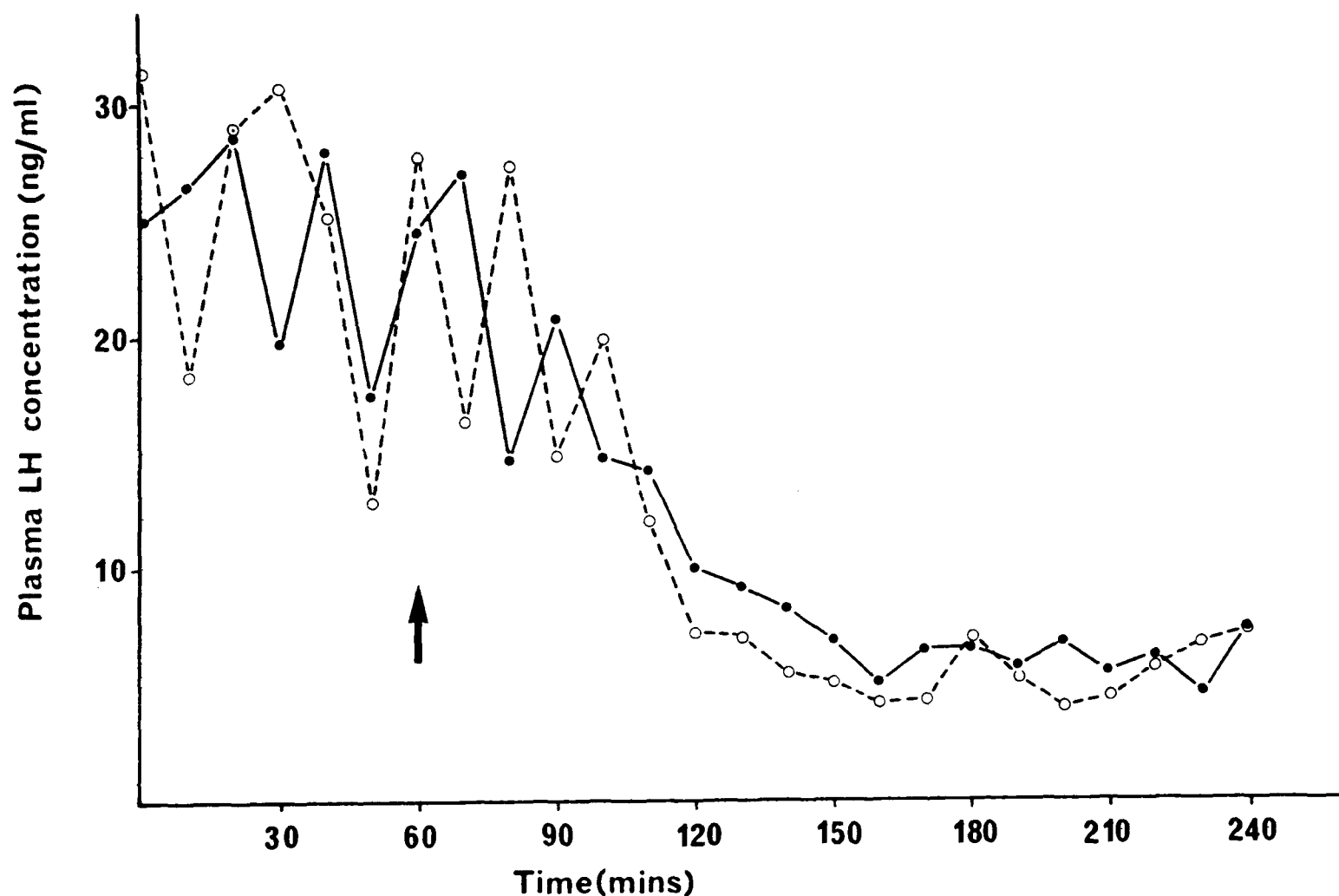


FIGURE 6-H: Plasma LH concentrations (ng/ml) in individual female rats maintained on LD which were gonadectomized and 21 days later given an i.p. injection of 320 mg/kg b.w. parachloro-phenylalanine (PCPA) in 0.9% saline solution (o---o) or 0.9% saline solution only (●—●). 48 h after PCPA injection each animal was implanted with an intra-atrial cannula. 24 h after cannula implantation blood samples were taken through the cannula, and 1 h after the first blood sample was taken each animal received (↑) 1 μ g of OE₂ dissolved in ethanol : 0.9% saline solution (1 : 50 v/v) through the cannula.

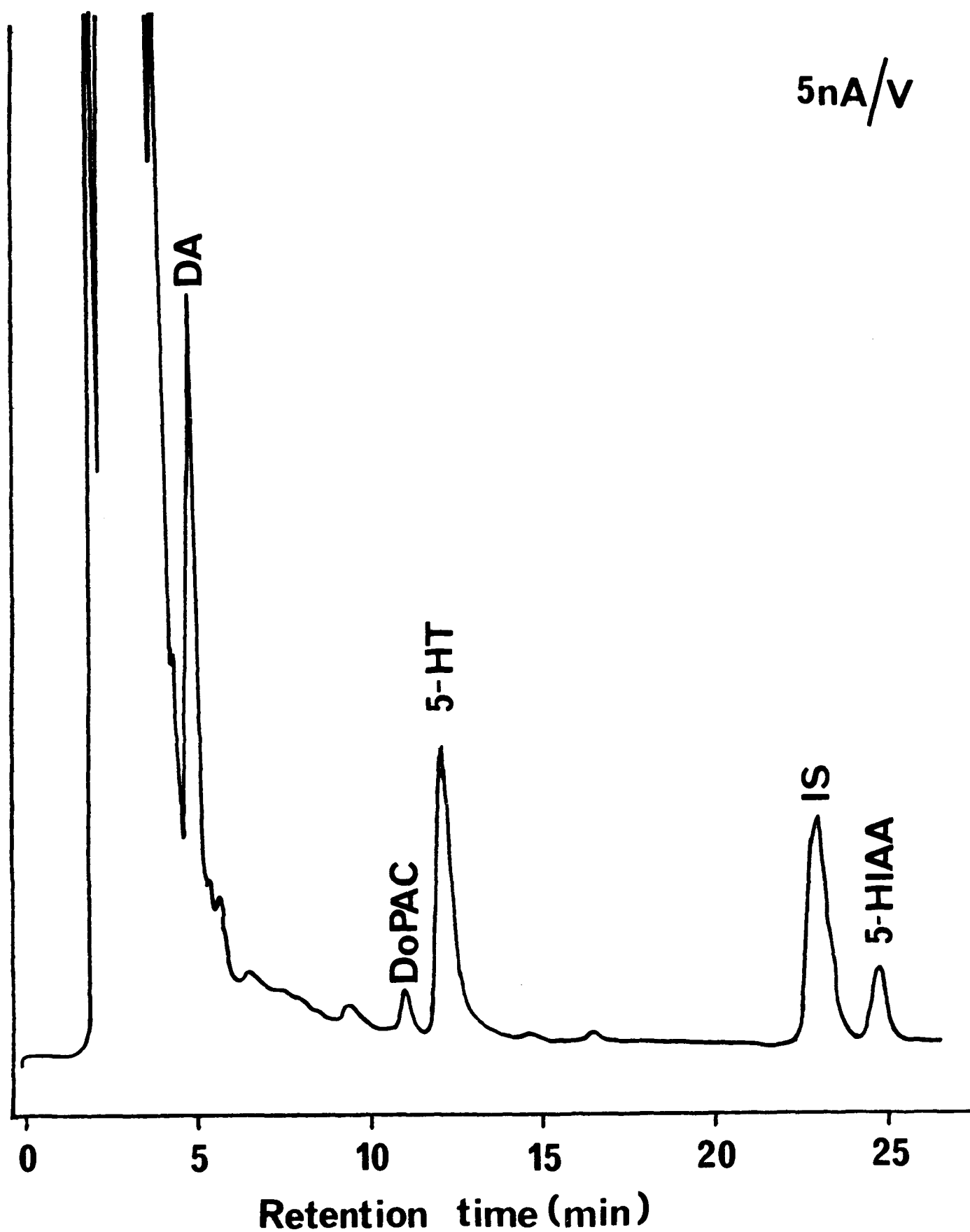


FIGURE 6-Ja: LCED chromatogram (buffer A) of hypothalamic extract from one of the animals described in Figure 6-G and injected with 0.9% saline solution. DA - dopamine, DoPAC - 3,4-dihydroxyphenylacetic acid, 5-HT - serotonin, 5-HIAA - 5-hydroxyindole acetic acid, IS - internal standard (n-acetyl dopamine).

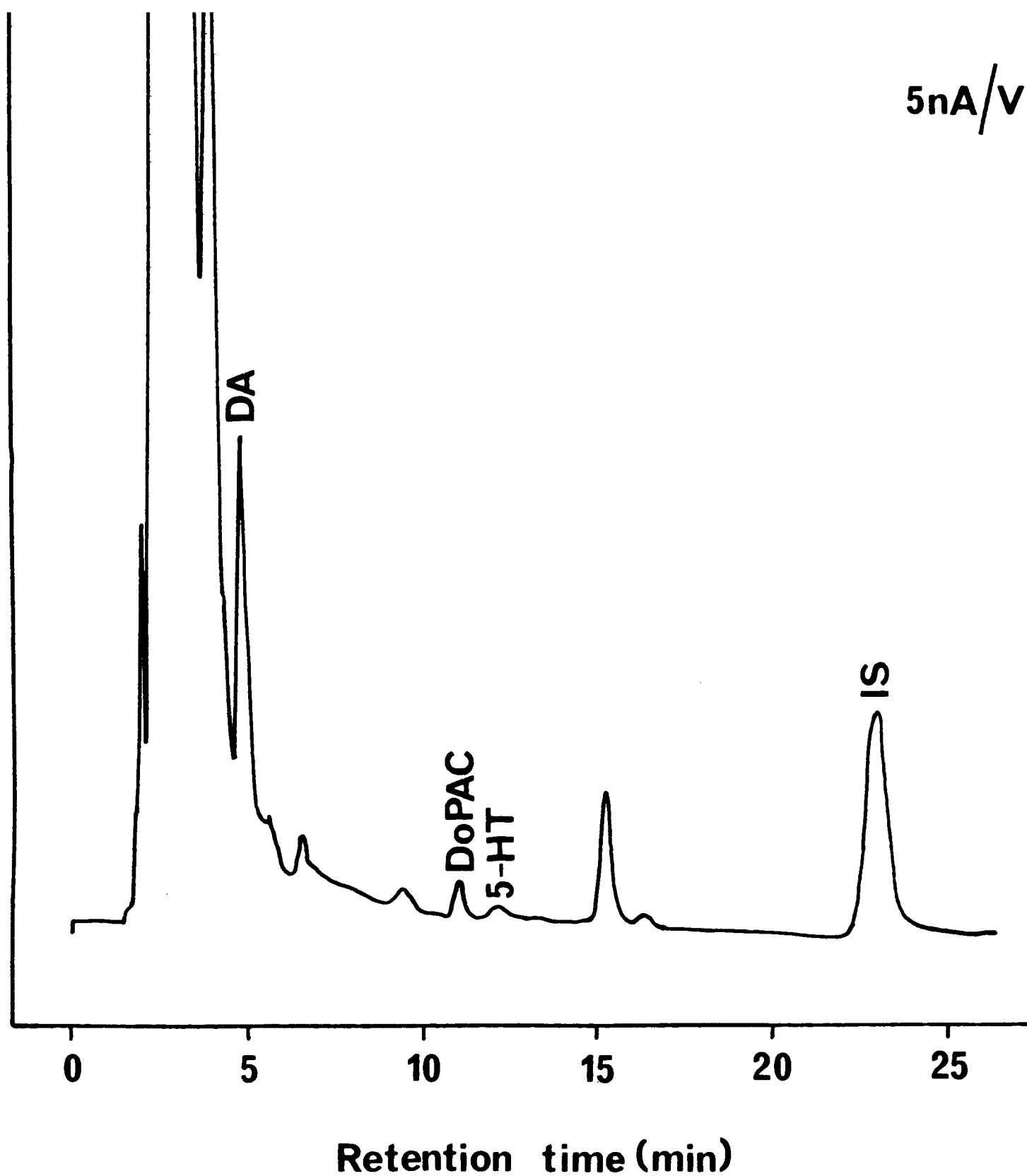


FIGURE 6-J: LCED chromatogram (buffer A) of hypothalamic extract from one of the animals described in Figure 6-G and injected with 320 mg/kg b.w. parachlorophenylalanine in 0.9% saline solution. Abbreviations as in Figure 6-Ja.

reduced by PCPA treatment. Figure 6-J shows the LCED profile obtained from the hypothalamic extracts of one animal from each group.

6.4 Discussion

The presence of LH pulses in ovariectomized rats is well documented (Gay and Sheth, 1972; Blake and Sawyer, 1974; Drouva and Gallo, 1976; Gallo, 1980a; Soper and Weick, 1980; Weick, 1981; Sylvester, Van Vugt, Aylsworth, Hanson and Meites, 1982). Pulses begin to increase in amplitude two days after ovariectomy and by eight days are occurring every 20-30 mins (Weick, Soper and Greenberg, 1981). No variation in pulse frequency and amplitude occurs throughout the 24 h day (Blake, 1974; Soper and Weick, 1977). Gallo has recently demonstrated pulsatile LH release in intact female rats on metoestrus, dioestrus, pro-oestrus morning, oestrus (Gallo, 1981a) and during the LH surge on pro-oestrus afternoon (Gallo, 1981b). Evidence from *in vitro* pituitary perfusions (Osland, Gallo and Williams, 1975) and from LHRH infusions in pentobarbital treated rats (Blake, 1976b) suggested that the pituitary only remained responsive when it received discrete pulses of LHRH rather than continually elevated concentrations. Taken together, this evidence suggested that LH is preferentially secreted in a pulsatile manner in normal (e.g. the oestrous cycle) and altered (e.g. ovariectomy) physiological circumstances and that this release reflected pulsatile LHRH release from the median eminence (Schuiling and Gnodde, 1976; Snabes and Kelch, 1979; Blake, Sridaran, Elias, Ashiru and Rush, 1980; Sarkar and Fink, 1980).

Tables 6.1 and 6-A showed that although pulsatile release of LH was present in female, male, TP and LL rats 3 weeks after gonadectomy, the characteristics of the pulses were different in each group.

The mean plasma LH concentrations of male and female rats were significantly greater than the TP female and LL female rats, and this probably reflected the difference in the pituitary responsiveness to LHRH of these animals (Figure 6-F) and showed that pituitary responsiveness probably represented a major determinant in their pulse amplitude and mean plasma LH concentration. Because of these differences in pituitary responsiveness to LHRH it was not possible to conclude that the amount of LHRH released per pulse in gonadectomized male and female rats was different from TP females and LL female rats. However, gonadectomized male and female rats had similar interpulse intervals and pituitary responsiveness to LHRH but female rats had a significantly greater pulse amplitude than male rats. This indicated that LHRH was released at similar intervals but in female rats more LHRH may have been released per LH pulse than in male rats. Conversely, in the TP and LL rats there was no significant difference in the pulse amplitude but the pituitary responsiveness to LHRH was significantly higher in the LL rats than in the TP rats and may have indicated greater LHRH secretion per LH pulse in TP female rats than in LL female rats. The interpulse intervals in LH release were similar in gonadectomized female, male and TP rats but LL rats showed a longer interpulse interval and probably represented a lower frequency of LHRH pulses.

The finding that the mean plasma LH concentration in TP rats was 65% less than that of normal female rats (Table 6.1) was similar to the 50% reduction found by Turgeon and Barraclough (1974). However, Turgeon and Barraclough (1974) found an extended interpulse interval in TP rats; a finding not confirmed here. This could have been a consequence of the less frequent sampling procedure used

by Turgeon and Barraclough (1974) (9 samples in 90 min as opposed to the 19 samples in 120 min taken here), a lower group number (6 against 15) or differences in the strains of animals. The mean plasma LH concentration found in LL rats in Table 6.1 was in contrast to that found in the ovariectomized LL rats in Chapter V. In Chapter V, pulsatile release of LH was found to be unaffected by LL (Figure 5-A). The difference observed between the animals in Chapter V and those in this study could have been due to differences in pituitary responsiveness similar to those shown in Table 6.2. However, a further possibility was different plasma steroid environments resulting from the different periods of exposure to LL before ovariectomy. The rats in the study described in Chapter V were (in contrast to the animals used in the present study) ovariectomized before being placed in LL and, as a consequence, were not exposed to continually elevated plasma oestrogen concentrations found in intact female rats in LL (see Table 7-1, Chapter VII). Brawer, Naftolin, Martin and Sonnenschein (1978) have shown that the long term elevation of plasma concentrations of oestrogen led to morphological changes in the arcuate nucleus similar to those seen after LL exposure (Brawer, Schipper and Naftolin, 1980). The arcuate nucleus was thought to be important controlling pulsatile LH release (Blake and Sawyer, 1974; Gallo and Osland, 1976; Gallo and Moberg, 1977; Soper and Weick, 1980). Therefore, the differences found between the mean plasma LH concentrations of the 2 groups of LL-rats used in Chapter V and this chapter could have been due to oestrogen-induced changes in the arcuate nucleus. Such changes may also have contributed to the extended interpulse interval found in LL-rats in Table 6.1.

Yamaji, Dierschke, Bhattacharya and Knobil (1972) showed that OE_2 given intravenously rapidly lowered plasma LH concentrations and inhibited pulsatile LH release in ovariectomized rhesus monkeys. This observation was confirmed in the ovariectomized rat by Blake, Norman and Sawyer (1974), Sarkar and Fink (1980) and Higuchi and Kawakami (1982). The results presented in Figures 6-B to 6-E supported and extended these findings, which differed from those of Weick (1977), who found a much slower response to an intravenous injection of 10 μ g OE_2 with significant pulses still occurring 2 h after injection. The similarity of the 4 groups of animals in the response of their plasma LH concentration to 1 μ g OE_2 suggested that at least some of the centres involved in the inhibitory action of OE_2 are neither sexually differentiated nor affected by perinatal androgen or chronic LL treatment. The observation that females, males and TP-rats had similar temporal responses to OE_2 agrees with the data obtained by s.c. injection of oestrogen (Kalra, Fawcett, Krulich and McCann, 1973; Mennin and Gorski, 1975; Henderson, Baker and Fink, 1977a) and intrahypothalamic implants of oestradiol benzoate (Danguy, Ectors and Pasteels, 1977a; Danguy, Pasteels and Ectors, 1977b). The data from LL-rats contrasts with that of Mennin and Gorski (1975) who showed LL-rats had a slower response than normal females to subcutaneous oestrogen treatment. However, the regime used by Mennin and Gorski (1975) differed from used here in that they did not specify the period of exposure to LL or the timing of ovariectomy in relation to the beginning of exposure to LL. Therefore, their results are difficult to compare to the present data.

The data presented here on TP-rats and those of Mennin and Gorski (1975) contrasted with those of Turgeon and Barraclough (1974)

who concluded that the TP-female rat was less sensitive to oestrogen than the normal female. The sampling procedure of Turgeon and Barraclough (1974) (samples taken 72 h after oestrogen treatment) did not allow examination of plasma LH concentrations during the first few hours after oestrogen treatment and their results may have represented a difference in the time taken for the animals to clear oestrogen from the circulation (Gustafsson and Stenberg, 1976).

Data are confusing as to the location of all the areas involved in the inhibitory action of OE_2 , and interaction probably occurs both in the brain and pituitary. A central locus was suggested by the data of Sarkar and Fink (1980) where an i.v. injection of OE_2 led to a parallel decrease in portal plasma LHRH concentration and peripheral plasma LH concentration. Blake *et al.* (1974) concluded from experiments using deafferentation of the medial basal hypothalamus and similar blood sampling techniques to those used here, that extra hypothalamic neurones did not mediate this effect. However, Rethyly, Vigh, Setalo, Merchenthaler, Flerko and Petrusz (1981) and Kawano and Daikoku (1981) suggested that some LHRH neurones may escape this type of deafferentation, and therefore, extra-hypothalamic areas should not be totally excluded from consideration. A preoptic locus for oestrogen negative feedback was suggested by Danguy *et al.* (1977a,b) but Wiegand, Terasawa, Bridson and Goy (1980) found that lesions in a number of locations within the suprachiasmatic and preoptic areas did not interfere with the inhibitory action of OE_2 . Oestradiol probably also acts at the pituitary level to reduce LH secretion (Negro-Villar, Orias and McCann, 1973; Blake *et al.*, 1974; Libertun, Orias and McCann, 1974). Higuchi and Kawakami (1982) have recently used ovariectomized rats deafferentated at the anterior hypothalamus to show

that OE_2 acted rapidly to reduce pituitary responsiveness to LHRH. That this also occurred in gonadectomized males as well as ovariectomized rats was suggested by the data in Table 6.2 where OE_2 reduced plasma LH concentrations in both groups to a level significantly lower than that produced by sodium pentobarbitone. In TP-rats, pentobarbitone reduced plasma LH concentrations more than OE_2 . This suggested that a different mechanism may have operated at the pituitary of these animals. No such conclusion could be reached in the LL-rats because, although plasma LH concentrations tended to be higher in the pentobarbitone-treated than OE_2 -treated animals, the differences were not significant.

The mechanism of interaction between OE_2 and neurones involved in controlling pulsatile LH release is not well understood. The data in Figures 6-G and 6-H showed that the modulation of LHRH neuronal activity by OE_2 probably did not involve serotonergic neurones. Evidence from studies using naloxone (Sylvester, Van Vugt, Aylsworth, Hanson and Meites, 1982) showed that opioid peptide neurones may be involved in the inhibitory effect of OE_2 . However, the data in Figures 6-G and 6-H do not support the suggestion of Sylvester *et al.*, 1982, that serotonergic neurones may be affected by opioid neurones, and that this interaction was involved in inhibiting LH release.

Figures 6-G and 6-H showed that massive depletion of hypothalamic 5-HT (Table 6.3) had no effect on the pre-injection pulsatile LH release pattern. This observation agreed with the data of Gallo (1980b) and Rasmussen *et al.* (1981) and suggested that 5-HT was not involved in basal pulsatile LH release.

Taken in conjunction with the data of Héry *et al.* (1976) and Coen and MacKinnon (1979), these data showed that 5-HT only seemed

to be involved in the OE_2 -induced diurnal release of LH and not in its initial inhibitory action on pulsatile LH release. This could not be directly concluded from Héry *et al.* (1976) and Coen and MacKinnon (1979) because OE_2 was administered either before (Héry *et al.*, 1976) or at the same time (Coen and MacKinnon, 1979) as PCPA. The initial reduction in plasma LH concentrations therefore occurred before any significant depletion in 5-HT concentrations.

CHAPTER VII

The Effects of Progesterone on the
Pituitary Responsiveness to, and
Priming Effect of, Luteinizing Hormone
Releasing Hormone in Long Term Constant
Light Exposed Female Rats

7.1 Introduction

Ovulation can be induced in a significant number of female rats exposed to long-term LL, by mating (Brown-Grant, Davidson and Greig, 1973), LHRH (Fink, 1975), human chorionic gonadotrophin (Singh and Greenwald, 1967) or progesterone (Singh and Greenwald, 1967; Brown-Grant *et al.*, 1973). Progesterone injection results in an increase in LHRH concentrations in hypophysial portal plasma (Sarkar and Fink, 1979) and in a corresponding increase in plasma LH concentrations (Brown-Grant *et al.*, 1973), but details of the mechanisms involved in progesterone induced LH release are not clear.

Similarly, the mechanisms by which mating stimulates a surge of LH in LL exposed rats (Brown-Grant *et al.*, 1973; Davidson, Smith and Bowers, 1973) are not known. Therefore, in the present study the plasma concentration of oestradiol-17 β (OE₂) and progesterone were measured in mated and non-mated LL female rats to investigate the role played by these steroids in the mating-induced LH surge.

The increase in pituitary responsiveness to LHRH found during pro-oestrus (Aiyer, Fink and Greig, 1974a; Cooper, Fawcett and McCann, 1974; Gordon and Reichlin, 1974) is a critical factor in the development of LH surge in female rats maintained on LD. The increase in the magnitude of the priming effect during the afternoon of pro-oestrus (Aiyer, Fink and Chiappa, 1974b; Fink, Chiappa and Aiyer, 1976) contributes to the abrupt increase in pituitary responsiveness to LHRH found during the afternoon of pro-oestrus. The aim of the present study was to determine the effect of progesterone on the magnitude of the priming effect of LHRH and on the pituitary responsiveness to LHRH in female rats exposed to long-term LL.

7.2 Materials and Methods

Female Wistar rats (250–300 g, b.w.) were maintained as described in Chapter II (2.1). Animals were kept in LL for at least 60 days, and daily inspection of vaginal smears was started about 14–16 days before the experiment. Only animals which had shown at least 10 days of full vaginal cornification were used. Ovariectomy (2.2.2), the collection (2.2.6) and treatment of peripheral blood samples, and determination of ovulation (2.2.10) were all described in Chapter II. Progesterone (2.5 mg) and oestradiol benzoate (OB) were diluted in arachis oil. Progesterone was injected intra-muscularly (2.2.9) and OB injected s.c. as described in Chapter II (2.2.9). All steroid and arachis oil injections were 0.25 ml.

The effect of mating on the plasma concentrations of OE_2 and progesterone was determined in long-term LL rats using a mating cage (a semi-circular cage with a transparent front) containing two sexually experienced male Wistar rats. The experiment was carried out in subdued red lighting between 11.00 h and 12.00 h, with males kept in reverse lighting conditions (lights on 17.00 and off 07.00). One female at a time was introduced into the mating cage and the number of times mounted and the lordotic response noted. The period of exposure for each female was between 10 and 20 min (mean 15 min). The lordosis quotient (LQ; $100 \times \text{number of mounts with a lordotic response} / \text{the total number of mounts}$) was calculated for each female tested. Non-mated females were placed singly for 20 min into the mating cage with no males present. Two h after the mid-point of the mating or control exposure, female rats were killed by decapitation and the trunk blood collected. Plasma was separated and stored at -40°C until assayed for LH, OE_2 (Chapter II, 2.3.4) and progesterone (2.3.5). For comparison

untreated LL-females were decapitated between 09.00 - 10.00 h, and LD females showing regular oestrous cycles, were decapitated at dioestrus (10.00 - 11.00 h), pro-oestrus AM (10.00 - 11.00 h), pro-oestrus PM (17.00 - 18.00 h) and oestrus (10.00 - 11.00 h). Blood samples were treated in the same way as samples from the mated-LL females.

The pituitary responsiveness to LHRH was determined by measuring the LH response to an injection of 50 ng/100 g b.w. LHRH (Section 2.2.12) at either 1, 3, 5 or 7 h after the injection of progesterone or at 1 or 5 h after the injection of oil. In a second experiment, LL rats were given sodium pentobarbitone 15 min before an injection of oil or progesterone. Pituitary responsiveness to LHRH was tested 5 h later.

The magnitude of the priming effect of LHRH was determined using the methods of Aiyer *et al.* (1974b) in 4 groups of animals: a) untreated LL rats; b) intact LL rats injected with oil or progesterone 2 h before the first LHRH injection; c) intact LL rats injected with sodium pentobarbitone (36 mg/kg i.p.) 4 h before an injection of oil or progesterone, and then injected 2 h later with the first LHRH injection; d) LD rats ovariectomized at least 21 days before an injection of 20 µg oestradiol benzoate (OB) s.c., and 72 h later given an injection of 2.5 mg progesterone. Animals were injected 2 h later with the first LHRH injection. To determine the magnitude of the priming effect of LHRH, animals were anaesthetized with sodium pentobarbitone (36 mg/kg, i.p.) and given 2 injections (i.v.) of LHRH (50 mg/100 g b.w.) separated by an interval of 60 min. Blood samples (0.45 ml) were taken before and at 20, 40, 60, 80, 90 (in most cases), 100 and 120 min after the first injection of LHRH. The second injection of LHRH was given immediately after the 60 min blood sample. In some untreated LL rats, the second LHRH injection was replaced by a 0.5 ml injection of 0.9%

saline solution. The mean maximal increment in plasma LH concentration was calculated for both injections of LHRH by subtracting the plasma LH concentration in the pre-LHRH injection sample from the maximum plasma LH concentration in the post-injection samples. The 60 min sample was used as the pre-injection sample to calculate LH increment in response to the second LHRH injection. In both the responsiveness and priming experiments animals were allowed to recover, returned to constant light and subsequently examined for the occurrence of ovulation. Plasma LH concentrations were determined by the RIA described in Chapter II (2.3.3).

The significance of differences between means was determined using the unpaired 't' test (all data except from the pituitary responsiveness to LHRH experiment) or analysis of variance and the multiple range test of Duncan, after transformation of primary data to a logarithmic scale (base 10) to normalize variances (the data from the pituitary responsiveness to LHRH experiment).

7.3 Results

7.3.1 The effects of mating on the plasma concentrations of luteinizing hormone, oestradiol-17 β and progesterone

Table 7.1 shows that 2 h after mating or control exposure, the plasma concentration of LH and progesterone were significantly greater ($p < 0.002$, $p < 0.05$ respectively) in mated females than in non-mated females. The LQ (93.5 ± 4.2 ; mean $\pm$ S.E.M., $n=5$) showed that the mated females were highly receptive. There was no significant difference between the plasma concentration of OE₂ in mated and non-mated females.

TABLE 7-1: Plasma concentrations of OE_2 , LH and progesterone in LL exposed female rats killed 2 h after mating or control exposure (non-mated), or untreated LL female rats. Also plasma concentrations of OE_2 , LH and progesterone in female rats maintained on LD and killed during dioestrus morning (D; 10.00 - 11.00 h), pro-oestrus morning (P-AM; 10.00 - 11.00 h), pro-oestrus evening (P-PM; 17.00 - 18.00 h) and oestrus morning (O; 10.00 - 11.00 h). n = number of animals/group.

Group	n	Plasma concentration of		
		OE_2 (pg/ml)	LH (ng/ml)	progesterone (ng/ml)
LL-mated	5	51.3 $\pm$ 3.5	10.5 $\pm$ 2.0	30.2 $\pm$ 6.7
LL-non-mated	5	54.0 $\pm$ 4.2	2.8 $\pm$ 0.8	14.6 $\pm$ 5.8
D	11	32.1 $\pm$ 11.6	1.7 $\pm$ 0.2	13.6 $\pm$ 1.6
P-AM	9	119.1 $\pm$ 27.6	1.9 $\pm$ 0.3	10.6 $\pm$ 1.7
P-PM	15	37.6 $\pm$ 8.9	30.2 $\pm$ 4.7	34.9 $\pm$ 3.3
O	7	26.1 $\pm$ 8.7	2.5 $\pm$ 1.0	11.8 $\pm$ 1.6
LL-untreated	8	-	2.4 $\pm$ 0.5	16.1 $\pm$ 3.5

7.3.2 The effects of progesterone on the pituitary responsiveness to LHRH in long-term LL exposed rats

Table 7-2 shows that not all animals responded to the oil or progesterone injections by ovulating.

- *Animals which did not ovulate in response to progesterone or oil injection:*

Table 7-2 shows that in animals which did not ovulate, neither the pre-injection plasma LH concentration nor the mean maximal increment in plasma LH concentration (LH response to LHRH) were significantly different at any time after oil or progesterone injection.

- *Animals injected with oil or progesterone which subsequently ovulated:*

Table 7-2 shows that no significant increase occurred in either the pre-LHRH injection plasma LH concentration or the LH response to LHRH during the first 3 h after progesterone injection. However, between 3 h and 5 h after progesterone injection there was a significant increase in the pre-LHRH injection plasma LH concentration ($p < 0.01$) and the LH response to LHRH ($p < 0.01$). Seven hours after progesterone injection only the pre-LHRH injection plasma LH concentration was significantly greater than at 3 h ($p < 0.01$). Table 7-2 shows that 5 h after progesterone the pre-LHRH injection plasma concentration and the LH response to LHRH were significantly greater in ovulating animals than in either non-ovulating animals 5 h after progesterone (both $p < 0.01$) or in animals 5 h after oil injection (both $p < 0.01$). Seven hours after progesterone the pre-LHRH injection plasma LH concentration and the LH response to LHRH were significantly greater in ovulating animals than in either non-ovulating animals 7 h after progesterone (both $p < 0.01$) or in animals 5 h after oil injection (both $p < 0.01$).

TABLE 7-2: Mean ($\pm$ S.E.M.) plasma LH concentration (ng/ml) before, mean (S.E.M.) maximal increment in plasma LH concentration (ng/ml) after an i.v. injection of 50 ng/100 g b.w. synthetic LHRH and ovulatory response in female rats exposed to long-term LL and given either 2.5 mg progesterone, oil, 2.5 mg progesterone + 36 mg/kg sodium pentobarbitone, or oil + 36 mg/kg sodium pentobarbitone (SP).

Treatment	Time after treatment of LHRH injection	Ovulating animals			Non-ovulating animals		
		n	pre	increment	no. of ova/ovulating animal	n	pre increment
Progesterone	1 h	1	1.6	16.2	4	6	1.6 $\pm$ 0.1 14.9 $\pm$ 3.1
	3 h	4	1.3 $\pm$ 0.2	18.0 $\pm$ 5.1	3.5 $\pm$ 1.0	5	1.0 $\pm$ 0.1 15.0 $\pm$ 2.5
	5 h	11	24.6 $\pm$ 8.9	45.0 $\pm$ 9.5	6.5 $\pm$ 1.0	6	1.0 $\pm$ 0.2 9.0 $\pm$ 1.9
	7 h	7	12.9 $\pm$ 3.1	22.8 $\pm$ 4.0	5.1 $\pm$ 0.7	7	1.2 $\pm$ 0.4 8.5 $\pm$ 2.9
Oil	1 h	0				5	1.9 $\pm$ 0.5 11.0 $\pm$ 1.3
	5 h	2	1.8, 3.1	7.3, 21.2	5.0, 2.0	8	2.0 $\pm$ 0.3 8.6 $\pm$ 1.2
Progesterone + SP	5 h	2	5.4, 9.6	9.8, 13.0	3.0, 15.0	6	1.8 $\pm$ 0.7 9.7 $\pm$ 3.3
Oil + SP	5 h	6	1,8 $\pm$ 0.2	8.9 $\pm$ 2.5	9.7 $\pm$ 1.6	2	1.1, 1.8 5.6, 20.3

The pre-LHRH injection plasma LH concentration and the LH response to LHRH were significantly lower (both $p < 0.01$) in animals given pentobarbitone and progesterone 5 h before LHRH injection than in animals given only progesterone 5 h before LHRH. Six out of 8 animals given sodium pentobarbitone and oil 5 h before LHRH subsequently ovulated. However, the pre-LHRH injection LH concentration and LH response to LHRH in these oil-treated ovulating animals were not significantly different from animals not ovulating after being given sodium pentobarbitone and progesterone.

7.3.3 The effect of progesterone on the priming effect of LHRH

Figure 7-A and Table 7-3 show that the LH response to the second injection of LHRH was significantly greater than the LH response to the first injection of LHRH in untreated ($p < 0.01$), oil treated ($p < 0.01$) and progesterone treated ($p < 0.001$) groups. In the untreated group given LHRH/saline there was no significant difference between the two responses. However, the response to the second injection of LHRH in the progesterone treated group was significantly greater than the corresponding response in the oil or untreated group given LHRH/LHRH injections (both $p < 0.001$). Table 7-3 shows that in oil or untreated animals given two doses of LHRH, ovulation occurred in 60% and 75% of animals respectively. Ovulation occurred in all the animals treated with progesterone, but in none of the animals given LHRH/saline.

Figure 7-B and Table 7-3 show that the sodium pentobarbitone given 4 h before a progesterone injection did not affect the magnitude of the priming effect of LHRH or the ovulatory response. Sodium pentobarbitone given 4 h before an oil injection increased the magnitude of the priming effect to a level which was not significantly different from that seen in progesterone-treated animals.

TABLE 7-3: Mean ($\pm$ S.E.M.) maximal increments in plasma LH concentration (ng/ml) and ovulatory response to 2 successive i.v. injections of 50 ng/100 g b.w. synthetic LHRH given 60 min apart in untreated LL exposed female rats, or LL exposed female rats given either 2.5 mg progesterone or arachis oil 2 h before the first LHRH injection. Further groups of LL exposed female rats were injected with 36 mg/kg sodium pentobarbitone (SP) 4 h before either oil or 2.5 mg progesterone injections, and a group of untreated LL exposed female rats was injected with 50 ng/100 g b.w. synthetic LHRH and 1 h later with 0.9% saline solution. n = number of animals/group.

Significance of difference between means, * p <0.01; ** p <0.001 when compared to this mean maximal increment in response to first LHRH injection.

Group	n	Maximal increments in plasma LH concentration (ng/ml) after		No. of animals ovulating	No. of ova/ ovulating rat
		first injection	second injection		
Untreated	12	9.4 $\pm$ 1.4	26.3 $\pm$ 5.7*	9	7.2 $\pm$ 0.6
Untreated (LHRH/saline)	5	5.5 $\pm$ 0.7	1.2 $\pm$ 0.8	0	-
Oil	5	6.9 $\pm$ 1.0	33.0 $\pm$ 7.6*	3	6.3 $\pm$ 1.2
Progesterone	10	12.5 $\pm$ 2.0	82.7 $\pm$ 12.0**	10	8.9 $\pm$ 0.6
Oil + SP	5	9.8 $\pm$ 1.2	104.6 $\pm$ 10.9**	5	7.6 $\pm$ 0.7
Progesterone + SP	5	10.7 $\pm$ 2.1	90.7 $\pm$ 2.2**	5	8.1 $\pm$ 0.9

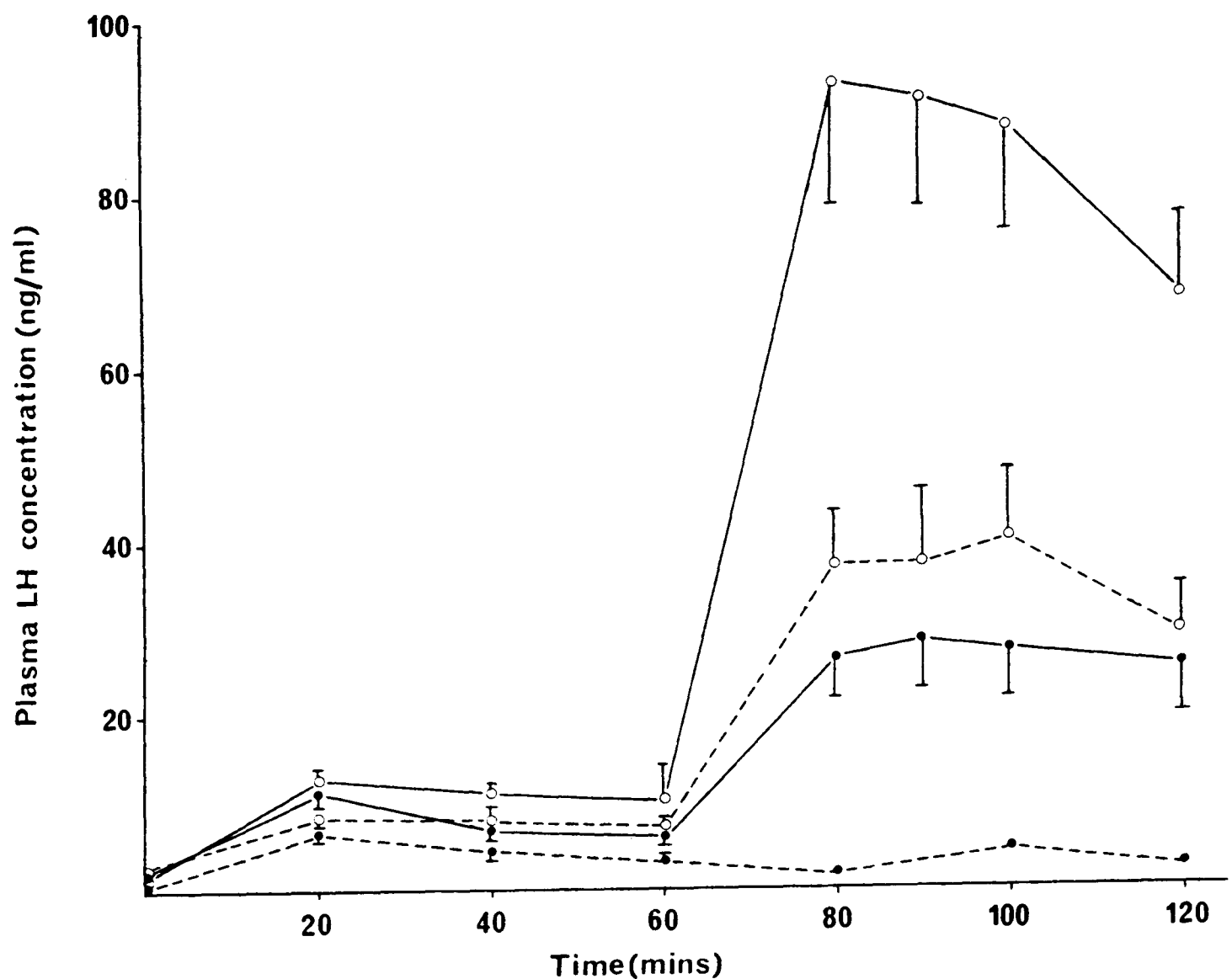


FIGURE 7-A: Mean ($\pm$ S.E.M.) plasma LH concentrations (ng/ml) in untreated female rats maintained on LL (n=12, ●—●), and female rats maintained on LL and injected i.m. with arachis oil (n=5, o---o) or 2.5 mg progesterone (n=10, o—o). Animals were injected with 2 i.v. injections of 50 ng/100 g b.w. synthetic LHRH 1 h apart (at 0 min and 60 min on the abscissa) beginning 2 h after oil or progesterone injection. Some untreated animals (n=5, ●---●) were injected with 50 ng/100 g b.w. synthetic LHRH (0 min) and 1 h later with 0.5 ml 0.9% saline solution.

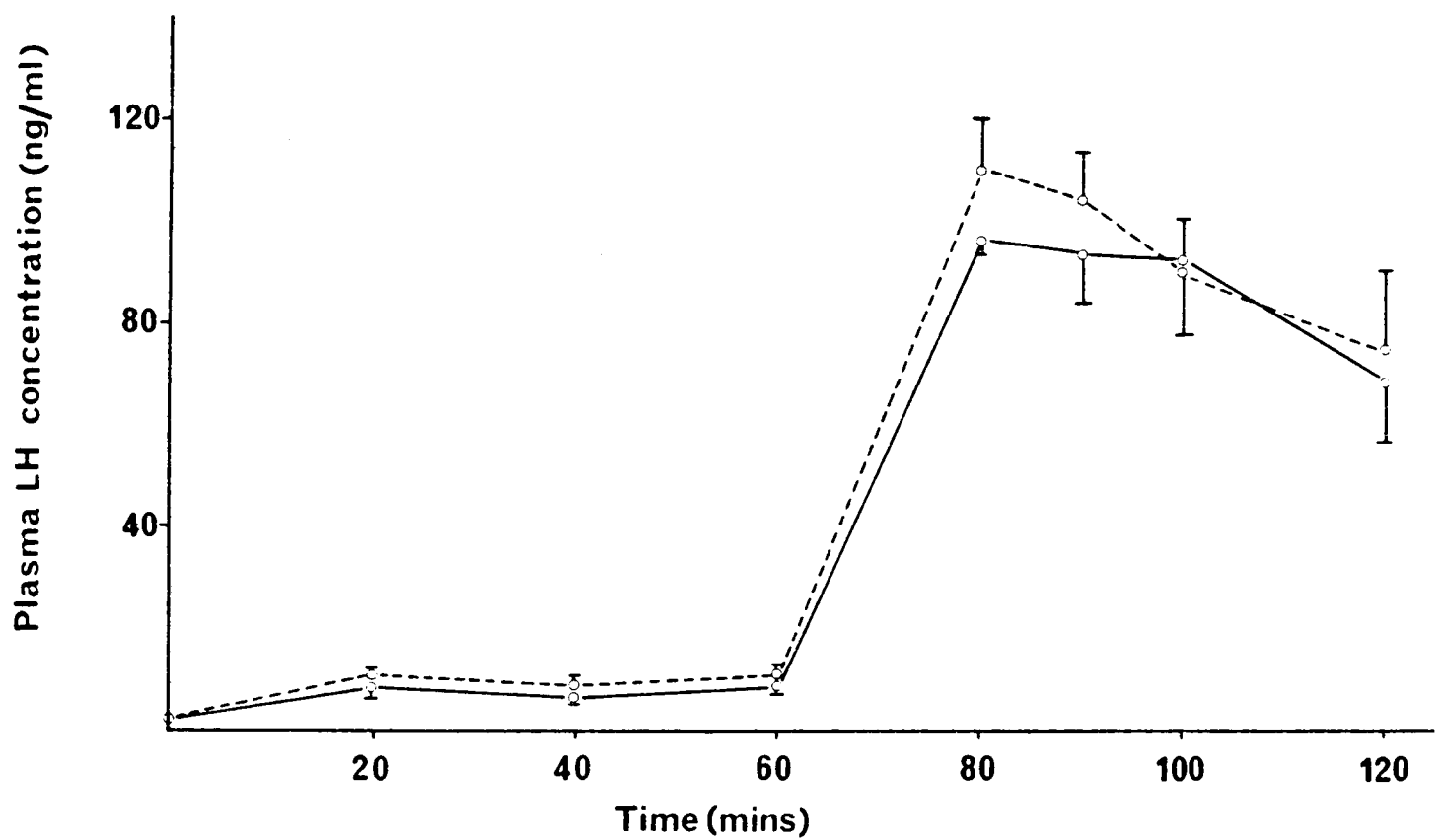


FIGURE 7-B: Mean ($\pm$ S.E.M.) plasma LH concentration (ng/ml) in female rats maintained on LL, injected i.p. with 36 mg/kg b.w. sodium pentobarbitone and 4 h later injected i.m. with 2.5 mg progesterone in 0.25 ml arachis oil (n=4, o—o) or 0.25 ml arachis oil (n=4, o---o). All animals were injected i.v. with 50 mg/100 g b.w. synthetic LHRH 2 h (at 0 min on the abscissa) and 3 h (at 60 min on the abscissa) after progesterone or oil injection.

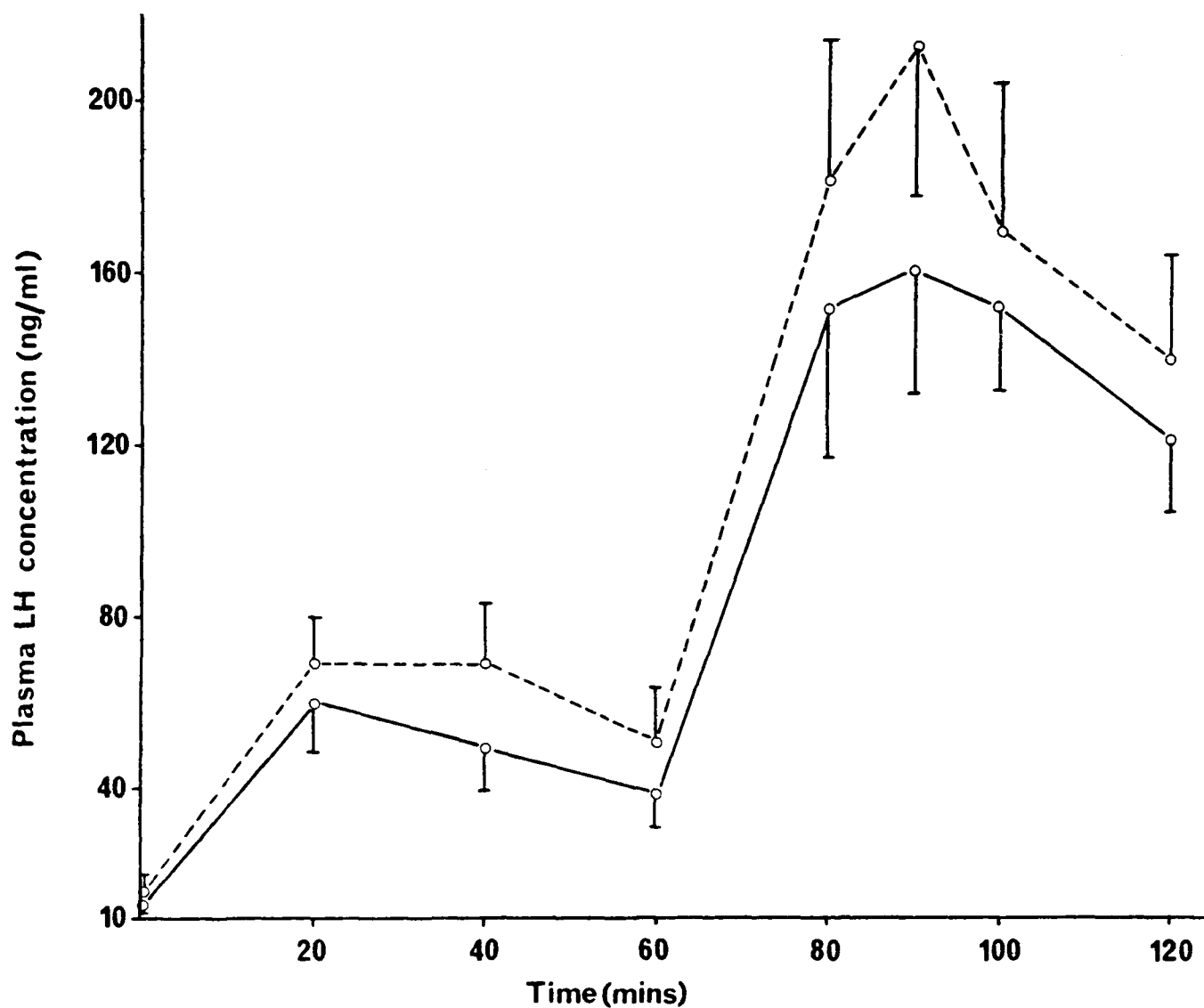


FIGURE 7-C: Mean ($\pm$ S.E.M.) plasma LH concentrations in female rats maintained on LD, ovariectomized and 21 days later given an s.c. injection of 20 μ g oestradiol benzoate in 0.25 ml arachis oil. 72 h later animals were given either 2.5 mg progesterone in 0.25 ml arachis oil (n=5, o—o) or 0.25 ml arachis oil (n=5, o---o). 2 h (0 min on the abscissa) and 3 h (60 min on the abscissa) after progesterone or oil injections, all animals were injected with 50 ng/100 g b.w. synthetic LHRH.

Figure 7-C shows that in LD long-term ovariectomized animals given oil or progesterone 72 h after a s.c. injection of 20 μ g OB, there was no significant difference in the magnitude of the priming effect of LHRH 2 h after the oil or progesterone injection.

7.4 Discussion

The data in Table 7-1 show that the plasma concentrations of OE₂ in female rats showing regular oestrous cycles were higher than those found by Henderson *et al.* (1977b) who used RIA, and Naftolin *et al.* (1972), who used competitive protein-binding assay. However, the plasma concentrations of OE₂ found in the present study were comparable to those found by Shaikh and Shaikh (1975). The pattern of OE₂ secretion found in the present study, with peak concentrations occurring during the morning of pro-oestrus, was comparable to that by Naftolin *et al.* (1972), Shaikh and Shaikh (1975) and Henderson *et al.* (1977b). The plasma concentration of OE₂ found in female rats exposed LL was found (Table 7-1) to be intermediate between those found on the morning of dioestrus and the morning of pro-oestrus in female rats with regular oestrous cycles maintained on LD. Naftolin *et al.* (1972) who measured OE₂ in peripheral plasma, and Takahashi, Ford, Yoshinaga and Greep (1977) who measured OE₂ in ovarian vein plasma, also found OE₂ concentrations in female rats exposed to LL to be intermediate between those found in female rats at dioestrus and pro-oestrus morning. The present data, taken in conjunction with those of Naftolin *et al.* (1972) and Takahashi *et al.* (1977), suggested that the elevated plasma OE₂ concentrations found in LL exposed rats was ovarian in origin. Brown-Grant *et al.* (1973) and Fink (1975) found that the plasma FSH concentrations in long-term LL exposed rats

were higher than those in animals exposed to LD, and these increased plasma concentrations of FSH probably contributed to the increased plasma oestradiol concentration. Table 7-1 shows that the plasma concentration of OE_2 was not significantly increased 2 h after mating. This suggested that OE_2 was not involved in mediating the mating induced LH surge, but a potentiating role for OE_2 cannot be excluded.

Treatment with progesterone, or mating stimulated LH release and ovulation in female rats exposed to LL (Singh and Greenwald, 1967; Brown-Grant *et al.*, 1973; Davidson *et al.*, 1973). The increased plasma concentrations of LH found 2 h after mating in the present study (Table 7-1) agrees with the findings of Brown-Grant *et al.* (1973). Taken together with the data of Singh and Greenwald (1967), Brown-Grant *et al.* (1973) and Davidson *et al.* (1973), the increased plasma concentrations of progesterone found 2 h after mating (Table 7-1) suggest that an increased secretion of progesterone contributed to the mating-induced LH surge and ovulation. The data in Table 7-2 show that progesterone only induced ovulation in a limited number of LL rats, and agree with the findings of Singh and Greenwald (1967) and Brown-Grant *et al.* (1973). The magnitude of the ovulatory response to progesterone (Tables 7-2 and 7-3) supported previous findings (Brown-Grant *et al.*, 1973; Fink, 1975) that the ovary of LL rats was less responsive to LH stimulation than the ovary of animals with regular oestrous cycles maintained under LD.

Sarkar and Fink (1979) showed that in contrast to LD females, progesterone would stimulate LHRH release into the hypophysial portal vessels of LL rats, with maximum concentrations occurring 3 h after injection. This correlated with the maximum plasma LH concentrations which also occurred 3 h after progesterone injection (Brown-Grant *et al.*

1973). The data in Table 7-2 show that during the first 3 h after progesterone injection in animals which subsequently ovulated, there was no increase in pituitary responsiveness, and as indicated by the pre-LHRH injection plasma LH concentration, sodium pentobarbitone completely inhibited the LH surge. Taken together, the data of Brown-Grant *et al.* (1973), Sarkar and Fink (1979) and the data in Table 7-2, suggested that during the first 3 h after injection, progesterone acted on the brain to stimulate LHRH release and thereby LH release from the pituitary.

The priming effect of LHRH probably contributes to the pro-oestrus LH surge in female rats in LD with regular oestrous cycles (Aiyer, Chiappa and Fink, 1974b; Fink, Chiappa and Aiyer, 1976). Figure 7-A showed that in untreated and in oil-treated intact LL-exposed female rats, a single injection of LHRH given 2 h after progesterone injection would prime the pituitary gland to a second injection of LHRH. Aiyer *et al.* (1974b) showed that the priming effect was potentiated, although not mediated by OE_2 in rats maintained on LD, and, in view of the elevated plasma OE_2 concentrations found in LL exposed rats (Table 7-1), OE_2 may have played a similar potentiating effect in LL exposed rats.

Although not directly comparable to the present studies because of differences in the time interval between progesterone and LHRH injections, Aiyer *et al.* (1974b) found that in intact LD females at metoestrus, progesterone did not increase the magnitude of the priming effect. Figure 7-C in the present study, showed that in oestrogen-primed ovariectomized LD females, an injection of progesterone did not increase the magnitude of the priming effect when compared to oil. Figure 7-A and Table 7-3, however, show that in contrast to LD females, an injection of progesterone in LL rats would markedly increase

the magnitude of the priming effect when given 2 h before the first LHRH injection. Table 7-3 and Figure 7-B show that in LL rats, sodium pentobarbitone given 4 h before a progesterone injection did not block the subsequent potentiation of the priming effect by progesterone. Therefore, the potentiating effect of progesterone on the priming effect of LHRH was probably directed at the pituitary and did not involve the increase in the release of LHRH found during the first 3 h after progesterone injection (Sarkar and Fink, 1979). The increase in the magnitude of the priming effect of LHRH in animals given pentobarbitone and oil (Table 7-3 and Figure 7-B) is difficult to explain, but may have been due to increased secretion of progesterone in these animals. Since Lawson and Gala (1974) showed that sodium pentobarbitone increased plasma concentrations of prolactin for at least 2 h after injection, the animals in Figure 7-B given oil may have had sufficiently increased plasma prolactin concentrations to stimulate adrenal progesterone secretion, in a manner similar to that reported by Piva, Gagliano, Motta and Martini (1973).

Taken together, the data in Table 7-2 from animals given progesterone and subsequently ovulating, and the data in Table 7-3, Figures 7-A and 7-B suggest that during the first 3 h after injection, progesterone acts both at the brain and pituitary to stimulate LH release. The significant enhancement of the priming effect by progesterone may partly explain why the LHRH surge induced by progesterone in LL rats will stimulate a LH surge twice the height of the spontaneous LH surge on pro-oestrus (Aiyer and Fink, 1974; Brown-Grant *et al.*, 1973), even though the progesterone-induced LHRH surge is less than 60% of the height of that found during the spontaneous LH surge on pro-oestrus (Sarkar, Chiappa, Fink and

Sherwood, 1976; Sarkar and Fink, 1979) and the pituitary responsiveness to LHRH in LL rats is about 9% of that found during the afternoon of pro-oestrus in LD rats (Aiyer *et al.*, 1974a; Smith and Davidson, 1974; Fink, 1975).

Brown-Grant *et al.* (1973) showed that the LH response to progesterone was correlated with the ovulatory response. The data in Table 7-2 confirm and extend these findings to show that the ovulatory response to progesterone was correlated with increased pituitary responsiveness to LHRH 5 h and 7 h after progesterone injection, and suggest that this increase in pituitary responsiveness contributed to the later development of the LH surge. Plasma LH concentrations, in animals which subsequently ovulated, were significantly increased 5 h after progesterone injection (Brown-Grant *et al.*, 1973), a time at which LHRH concentrations in hypophysial portal plasma were not significantly increased (Sarkar and Fink, 1979).

The data in Table 7-2 show that 5 h and 7 h after progesterone, the pituitary responsiveness to LHRH in animals which subsequently ovulated, was significantly increased when compared to animals which ovulated after treatment with LHRH 1 h or 3 h after progesterone. Plasma LH concentrations (as indicated by pre-LHRH injection plasma LH concentrations) in animals given progesterone 5 h and 7 h and subsequently ovulating, were less sensitive to the effects of sodium pentobarbitone than in animals subsequently ovulating after treatment with LHRH 1 h or 3 h after progesterone treatment. This suggests that 5 h and 7 h after injection, progesterone maintains LH secretion, mainly through increased pituitary responsiveness to LHRH, a situation also found in the long-term ovariectomized rat treated with oestrogen followed by progesterone (Caligaris, Astrada and Taleisnik, 1968;

Brown-Grant, 1974; Aiyer, Sood and Brown-Grant, 1976; Sarkar and Fink, 1980). The studies described in Chapter III of this thesis showed that in LD rats, OE_2 increased pituitary responsiveness to LHRH by a mechanism which involves the release and priming effect of LHRH, an effect which could be blocked by sodium pentobarbitone given 4 h but not 0.5 h before the injection of LHRH (Henderson, Baker and Fink, 1977b; Chapter III of this thesis). In the present studies, since sodium pentobarbitone given at the same time as progesterone inhibited the increase in pituitary responsiveness 5 h later, the increase in the release of LHRH during the first 3 h after progesterone may have been necessary for the increase in pituitary responsiveness to LHRH.

Taken together with previous studies (Singh and Greenwald, 1967; Brown-Grant *et al.*, 1973; Davidson *et al.*, 1973; Sarkar and Fink, 1979), the present study suggests that post-mating increases in progesterone secretion influence both brain and pituitary function to facilitate LH secretion. The source of post-mating increase in plasma progesterone secretion is not known. Brown-Grant *et al.* (1973) showed that the adrenal gland was not essential for mating-induced ovulation. However, Brown-Grant *et al.* (1973) did not measure plasma LH or progesterone concentrations after mating in adrenalectomized animals, and therefore could not exclude changes to the size of LH surge or alternative sources of progesterone.

CHAPTER VIII

The Effect of Constant Light on the Metabolism of Serotonin in the Preoptic, Suprachiasmatic and Arcuate Areas of the Female Rat

8.1 Introduction

The data presented in Chapter V (Figure 5-B) showed that LL exposure completely inhibited the oestrogen-induced diurnal LH surge and probably reflected central changes involved in the loss of regular oestrous cycles. The aim of the present study was to investigate the effect of LL exposure on the activity of the serotonergic system in the medial preoptic area (MPoA), the area of the suprachiasmatic nuclei (SCN) and the area of the arcuate nuclei (ARC) without pharmacological manipulation.

Studies in pro-oestrous female (Coen, Franklin, Laynes and MacKinnon, 1980) and ovariectomized rats have suggested that 5-HT (Héry, Laplante and Kordon, 1976, 1978; Coen and MacKinnon, 1979) and the SCN (Coen and MacKinnon, 1980; Kawakami, Arita and Yoshioka, 1980; Wiegand and Terasawa, 1982) play an important role in the phasic release of LH. The SCN contain a high concentration of 5-HT containing nerve terminals (Fuxe, 1965; Saavedra, Palkovits, Brownstein and Axelrod, 1974; Chan-Palay, 1977; Descarries and Beaudet, 1978; Steinbusch, 1978; Consolazione and Cuello, 1982) and possibly an endogenous circadian pacemaker (Inouye and Kawamura, 1979; Fuchs and Moore, 1980). However, the SCN play an essential role in controlling many behavioural and neuroendocrine circadian rhythms (Moore, 1978; Rusak and Zucker, 1979). The MPoA and the septum contain the cell bodies of LHRH neurons (Kawano and Daikoku, 1981; Réthelyi, Vigh, Sétáló, Merchenthaler, Flerkó and Petrusz, 1981), are essential for cyclic gonadotrophin release (Barraclough, 1966; Szentogothai, Flerkó, Mess and Halasz, 1968; Butler and Donovan, 1971) and are where important catecholaminergic/LHRH neuronal interactions occur during the oestrous cycle (Honma and Wuttke, 1980; Wise, Rance,

Selmanoff and Barraclough, 1981). Similarly, the ARC have been shown to possess important dopaminergic pathways (Dahlström and Fuxe, 1964; Hökfelt and Fuxe, 1972; Björklund, Moore, Nobin and Stenevi, 1973) and possibly 5-HT containing terminals (Saavedra *et al*, 1974; Descarries and Beaudet, 1978).

5-HT is oxidized to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase in 5-HT terminals. There are no other major metabolites and the molar ratio of 5-HIAA/5-HT is thought to be a reliable index of the turnover rate of 5-HT in terminals (Tozer, Neff and Brodie, 1966). In the present study, 5-HT and 5-HIAA concentrations in each sample were determined simultaneously by high-performance liquid chromatography with electrochemical detection (LCED).

By using LCED it was not only possible to measure 5-HT and 5-HIAA, but also DA and NA in the same sample. Therefore, although the main aim of this study was to investigate the effects of LL on 5-HT activity, LL effects on the concentrations of DA and NA in the MPoA, SCN and ARC were also reported.

8.2 Materials and Methods

Female Wistar rats (250-300 g b.w.) were maintained either in LD, or in LL for at least 60 days (Chapter II, 2.1). The only animals used had showed at least two consecutive oestrous cycles in LD immediately before use. Animals on LL had showed at least 10 consecutive days of vaginal oestrous smears immediately before use.

Animals exposed to LD were killed by decapitation at the following times of the oestrous cycle: dioestrus (D) morning (10.00 - 11.00 h), pro-oestrus morning (P-AM) (10.00 - 11.00 h), pro-oestrous afternoon (P-PM) (17.00 - 18.00 h) and oestrus (O) morning (10.00 - 11.00 h).

Animals exposed to LL were killed by decapitation between 09.00 h and 10.00 h. Brains of all animals were rapidly dissected and immediately frozen in a stainless steel dish containing iso-pentane precooled to -35°C using a Dewar flask containing ethanol and dry ice. After freezing, brains were removed from the iso-pentane and trimmed using a razor blade by removing the cerebellum and brain stem. The brains were mounted on circular cork discs (2 cm x 0.4 cm) using 'Cryo-M-Bed' (Brights Instrument Co. Ltd., Huntingdon, Cambs., U.K.) so the ventral surface of the brain was perpendicular to the cork disc. This was so that the areas of the SCN and ARC could be dissected with minimal loss of tissue from these nuclei (see Figure 8-A). Brains were then stored at -45°C . A freezing microtome (MSE, London) was used to cut four 700–800 μm thick sections from each brain as shown in Figure 8-A. The anterior commissure^S and optic chiasm were used as landmarks to start the cutting sequence. Sections were immediately placed on pre-cooled glass microscope slides and stored at -45°C until dissected. Slides with the sections were placed on dry ice and, with the aid of dissecting microscope, punches were taken from each section as shown in Figure 8-B using a hollow stainless steel needle of 600 μm internal diameter. The punches were immediately blown into homogenising buffer pre-aliquoted in 100 μl glass microhomogenisers ('Uniform', Jencons Ltd., London) cooled on ice. Because of the thickness of the sections and the diameter of the punches it was not possible in the case of the SCN and ARC to limit the punches to these small nuclei. Therefore, although the punches for these two nuclei contained the designated nucleus, they probably also contained tissue which was outside their identified limits. For this reason the punches were referred to have come from the suprachiasmatic area (SCA) and arcuate area (ARA).

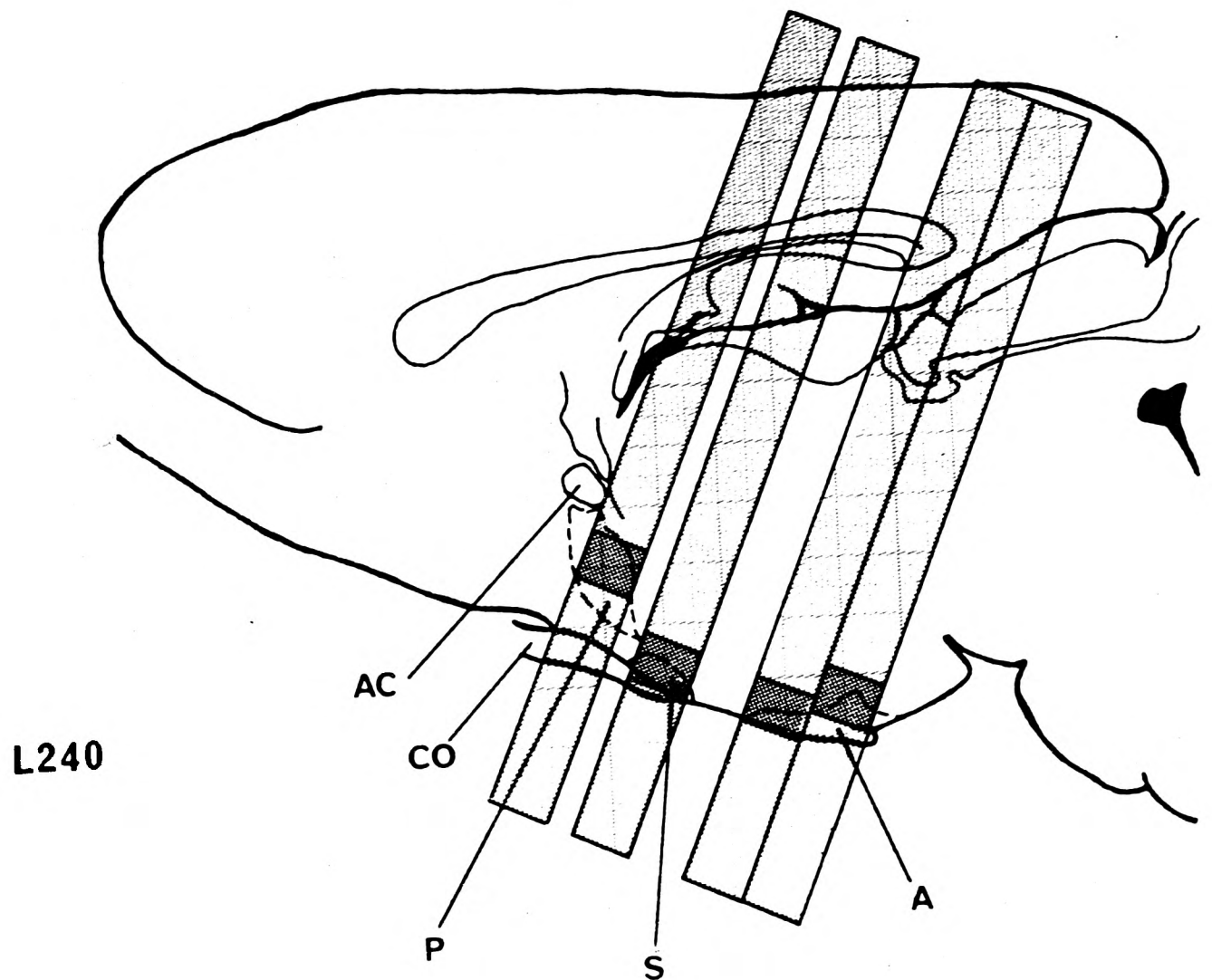


FIGURE 8-A: Longitudinal section through rat brain (level L-240, Konig and Klippel, 1963) showing position and size of slices (hatched) and punches (stippled) taken for dissection. AC - anterior commissure, CO - optic chiasm, P - medial preoptic area, S - suprachiasmatic nucleus, A - arcuate nucleus.

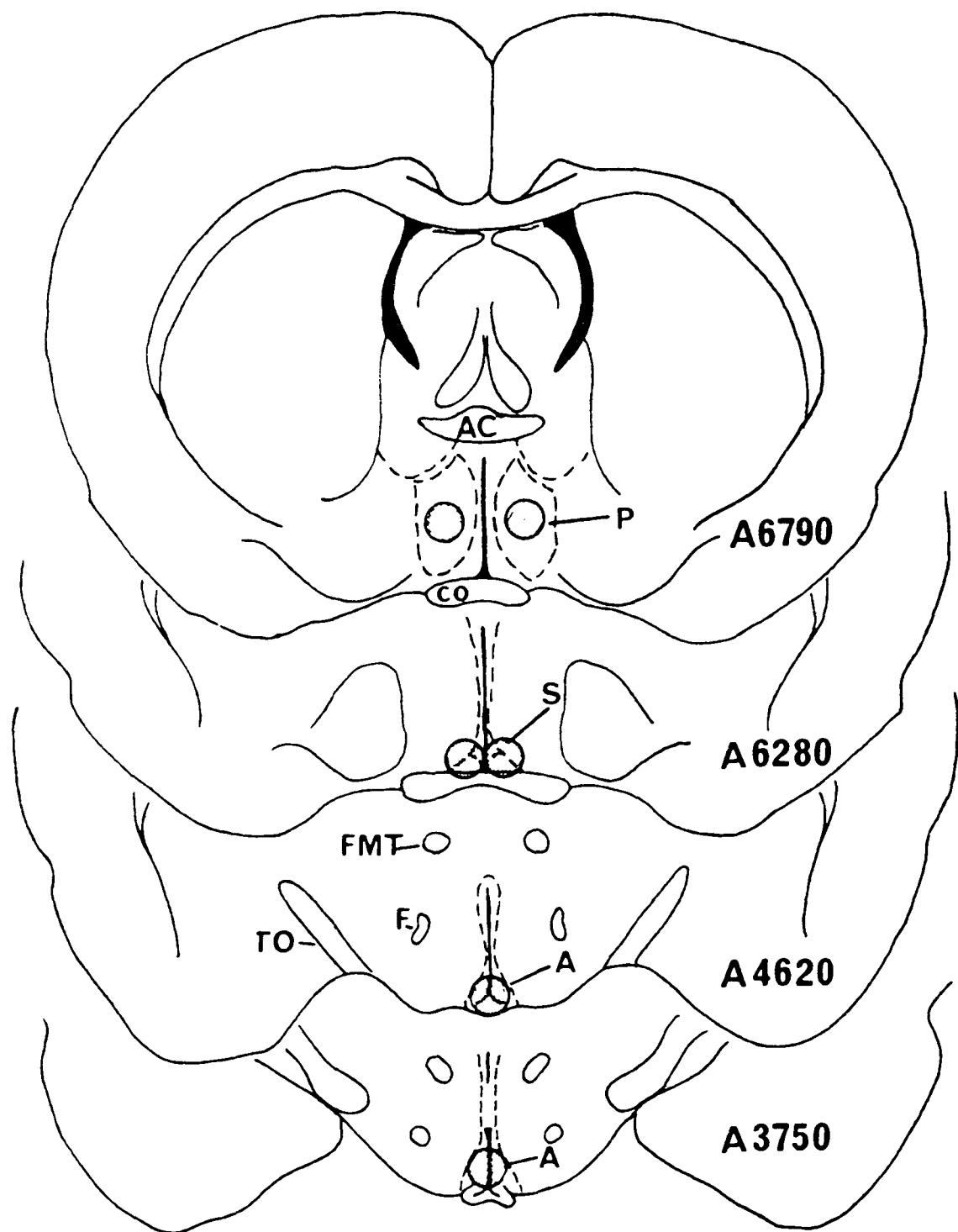


FIGURE 8-B: Transverse sections (numbers on sections refer to sections of Konig and Klippel, 1963) showing position and size of punches for medial preoptic area (P), supra-chiasmatic nuclei (S) and arcuate nucleus (A). CO - optic chiasm, AC - anterior commissure, TO - optic tract, FMT - fasciculus mammillothalamicus, F - columna fornicus.

Tissue from each animal was immediately homogenised in either 40 μ l (SCA and ARA) or 50 μ l (MPoA) of 0.2M perchloric acid containing 10^{-4} M cysteine and 20 ng/ml n-acetyl dopamine. The homogenates were centrifuged in the homogenisers at 4°C for 10 min at 1000 x g (Mistral 2L, MSE). The supernatant was analysed by LCED, while the pellet was analysed for protein content. The pellet was resuspended in 100 μ l of the homogenizing buffer, transferred to Eppendorf tubes and centrifuged (10,000 x g) for 10 min at 4°C using an Eppendorf 'microfuge'. The supernatant was discarded and the pellet stored at -45°C until assayed for protein (2.2.8).

The supernatant used for LCED analysis was either assayed immediately or frozen at -45°C and assayed within 48 h of dissection. Immediately before injection onto the HPLC column, samples were thawed (if necessary) and filtered using 0.2 μ m regenerated cellulose filters (BAS Inc.). Extracts were analysed using the LCED methods described in Chapter II (2.3.7). The mobile phase used was 0.1M sodium acetate, 0.1M citric acid, 100 mg/l sodium octylsulphonic acid, 2% tetrahydrofuran and 5% methanol. The pH was adjusted to 5.0 using sodium hydroxide. The working electrode was set at a potential of +0.7 V and the detector sensitivity at either 2 nA/V or 5 nA/V. Using these conditions, NA, DA, 5-HIAA and 5-HT could all be analysed in a single sample. All retention times were between 4 min (NA) and 16 min (5-HT). The internal standard eluted after 5-HIAA and before 5-HT.

The mass of each compound in the samples was calculated using peak height ratios with respect to the internal standard (2.3.7). The concentration of each compound in the dissected areas were expressed as pg/ μ g of protein.

Significance of differences between means of the concentrations of compounds within the oestrous cycle, and the 5-HIAA/5-HT in all animals, were determined using one-way analysis of variance and the multiple range test of Duncan. The significance of differences between the concentration of compounds from LL-exposed animals and LD-exposed animals was by the unpaired 't' test.

8.3 Results

8.3.1 Activity of the serotonergic system

Figures 8-C to 8-G showed the concentrations of 5-HT and 5-HIAA and the 5-HIAA/5-HT molar ratio (5-HIAA/5-HT) in the MPoA, SCA and ARA. There were no significant differences in the concentrations of 5-HT and 5-HIAA during the oestrous cycle in any area (Figures 8-C and 8-D). Therefore the values from each area were pooled and compared with the values from LL-exposed animals (Figures 8-F and 8-G).

Medial preoptic area (MPoA):

In LL-exposed animals the concentration of 5-HT was significantly higher ($p < 0.05$) than in LD animals. There was no significant difference in the concentration of 5-HIAA in LD and LL-exposed animals. 5-HIAA/5-HT was significantly lower during the afternoon of pro-oestrus than during the morning of oestrus ($p < 0.01$), the morning of dioestrus ($p < 0.01$) or the morning of pro-oestrus ($p < 0.05$).

Suprachiasmatic area (SCA):

The concentration of 5-HT was not significantly different in LL and LD-exposed animals. However, the concentration of 5-HIAA was significantly reduced ($p < 0.05$) in LL-exposed animals when compared

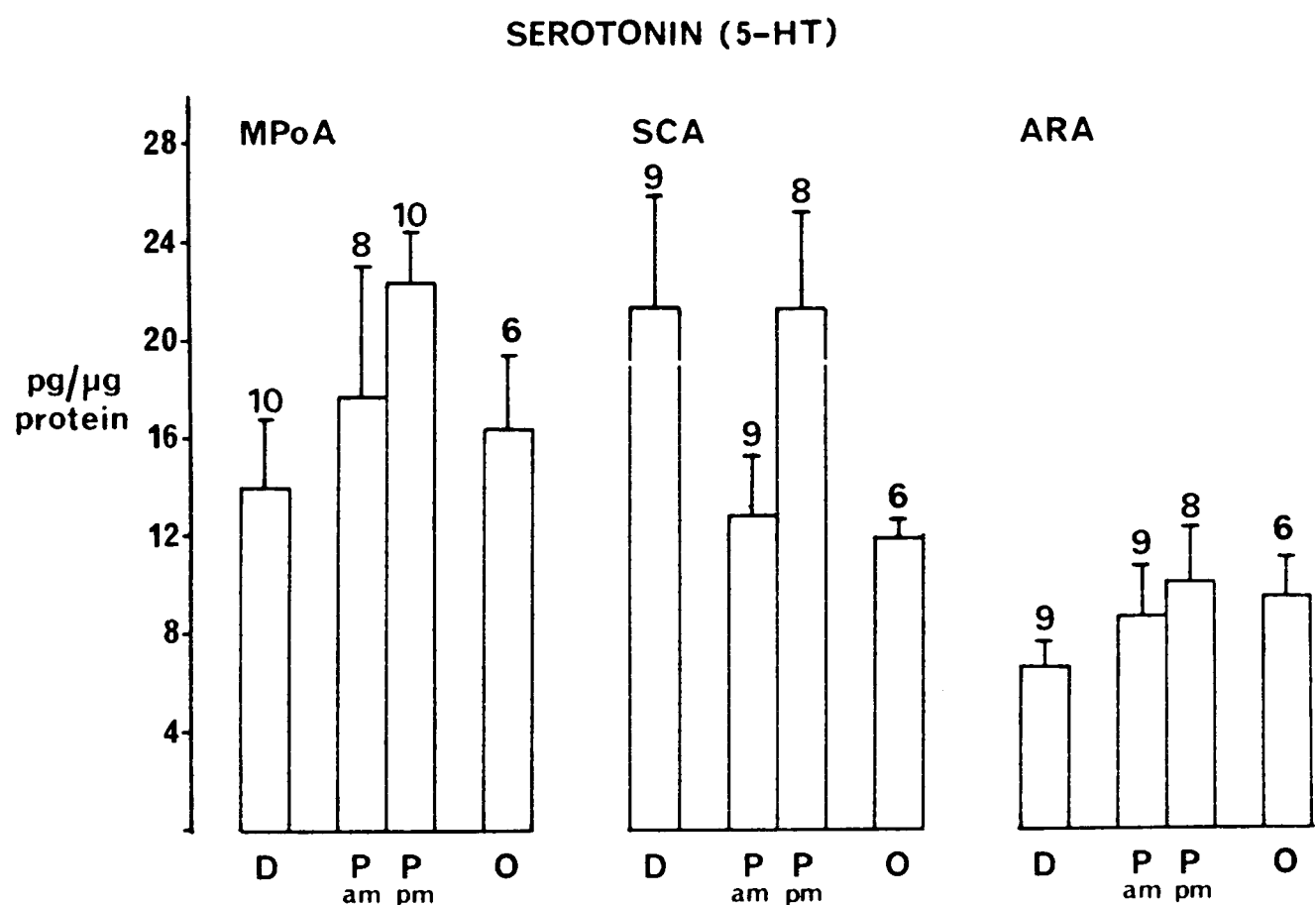


FIGURE 8-C: Mean (+ S.E.M.) concentration (pg/μg protein) of 5-HT in medial preoptic (MPoA), suprachiasmatic area (SCA) and arcuate area (ARA) from female rats maintained on LD and killed during dioestrus morning (D), pro-estrus morning (P-AM), pro-oestrus evening (P-PM) or oestrus morning (O). Numbers above blocks = number of samples/group.

5-HYDROXYINDOLE ACETIC ACID (5-HIAA)

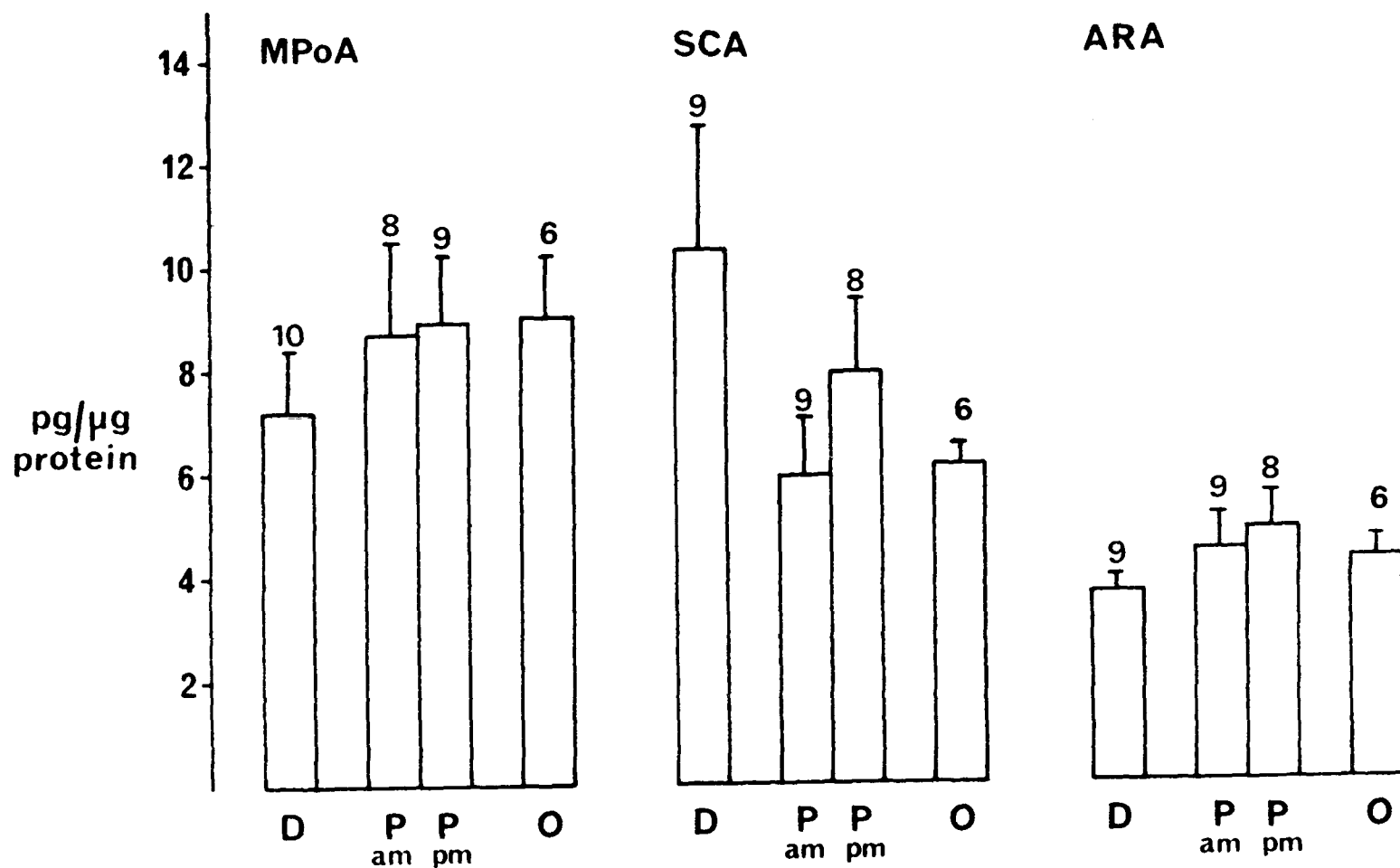


FIGURE 8-D: Mean (+ S.E.M.) concentration (pg/μg protein) of 5-HIAA in the MPoA, SCA and ARA (see Figure 8-C for abbreviations) from female rats maintained on LD and killed during dioestrus morning (D), pro-oestrus morning (P-AM), pro-oestrus evening (P-PM) or oestrus morning (O). Numbers above blocks = number of samples/group.

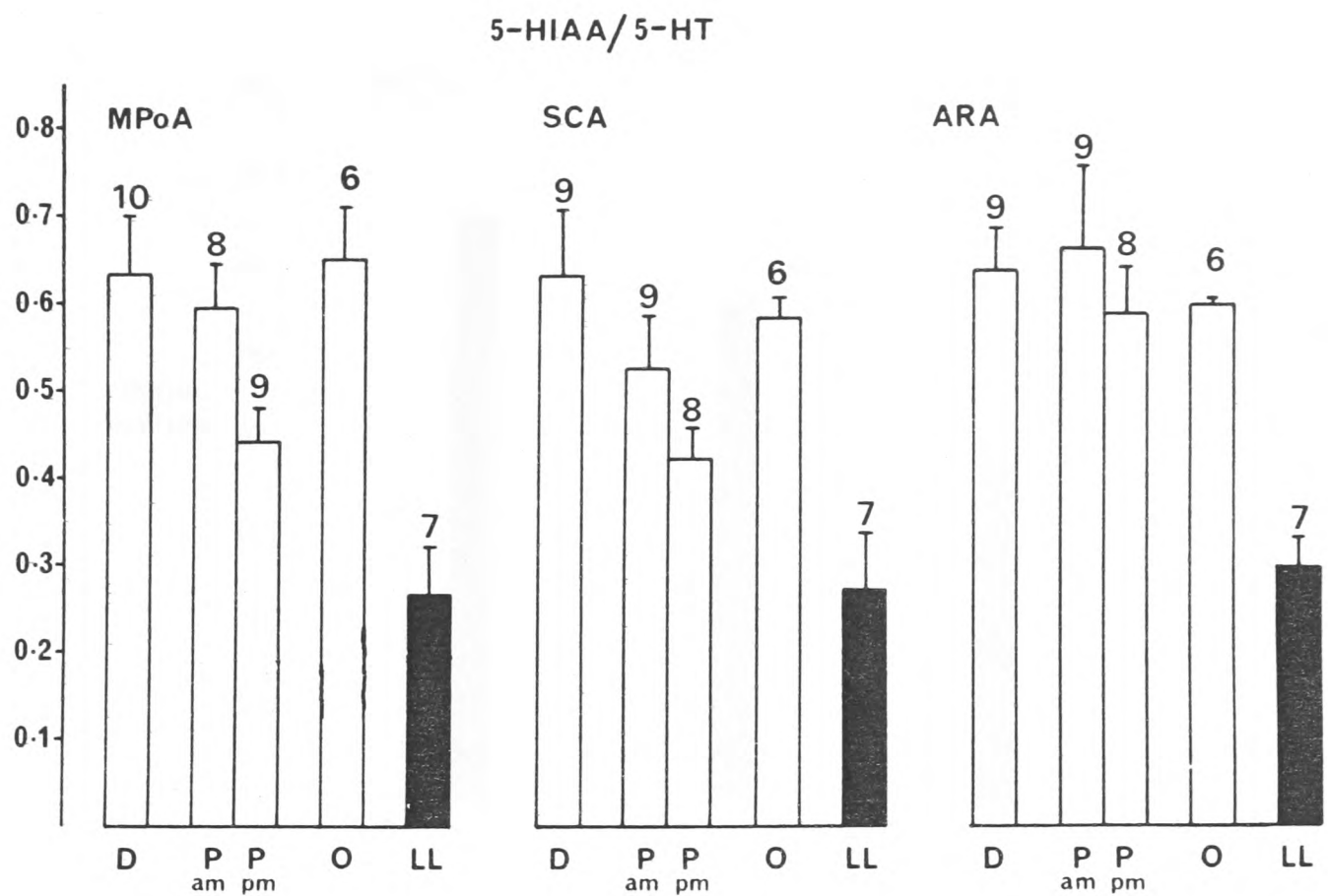


FIGURE 8-E: The mean molar ratio (+ S.E.M.) of 5HIAA/5HT in the MPoA, SCA and ARA (see Figure 8-C for abbreviations) of female rats maintained on LD and killed during dioestrus morning (D), pro-oestrus morning (P-AM), pro-oestrus evening (P-PM) and oestrus morning (O), or female rats maintained on LL (LL). Numbers above blocks = numbers of samples/group.

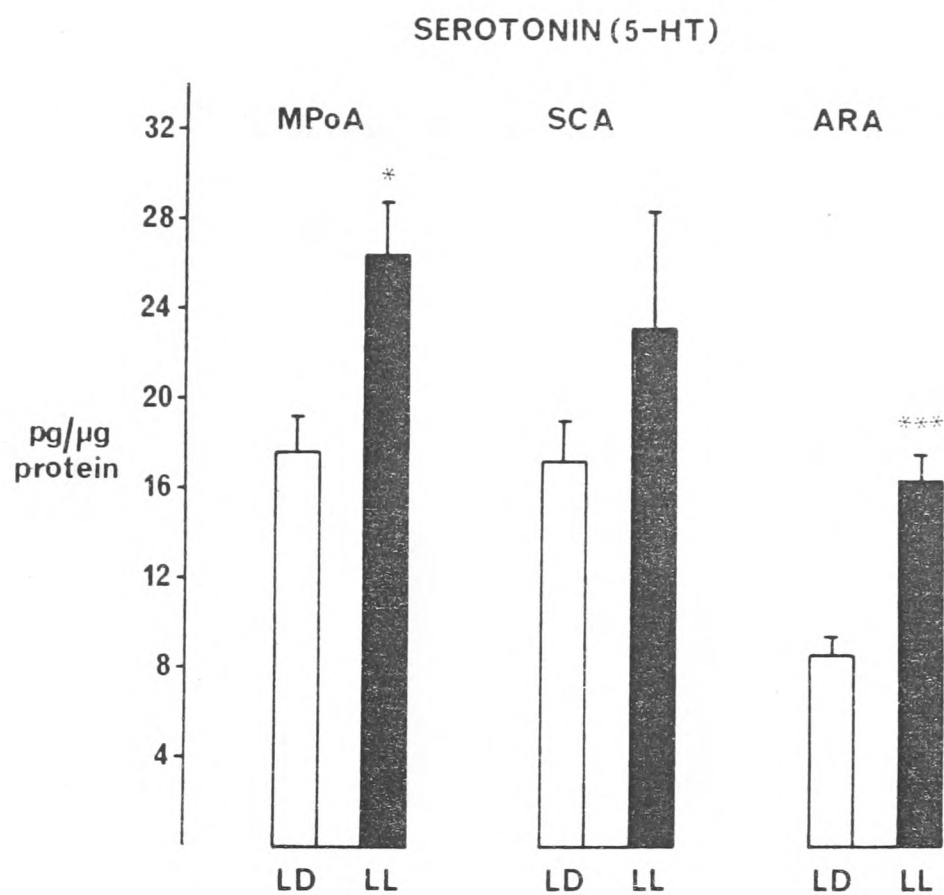


FIGURE 8-F: Mean (+ S.E.M.) concentration (pg/μg protein) of 5-HT in the MPoA (LD, n=33; LL, n=6), SCA (LD, n=32; LL, n=7) and ARA (LD, n=32; LL, n=7) of female rats maintained on LD and killed throughout the oestrous cycle (LD, data pooled from Figure 8-C) and female rats maintained on LL.

* $p < 0.05$, *** $p < 0.001$ when compared to animals maintained on LD.

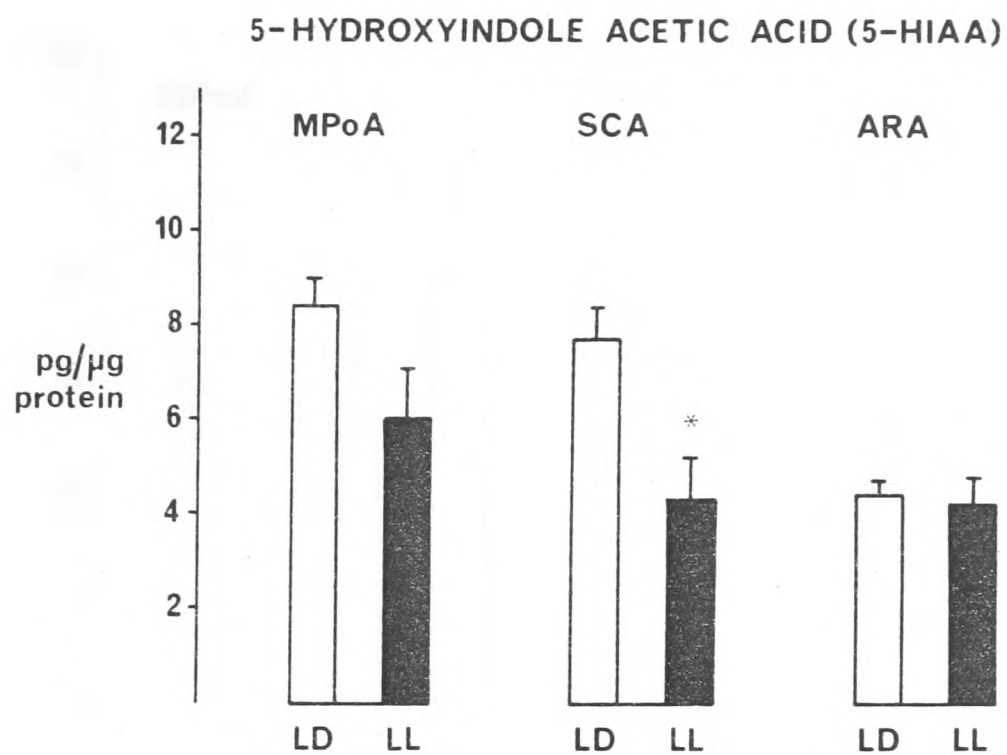


FIGURE 8-G: Mean (+ S.E.M.) concentration (pg/μg protein) of 5-HIAA in the MPoA (LD, n=33; LL, n=6), SCA (LD, n=32; LL, n=7) and ARA (LD, n=32; LL, n=7) (see Figure 8-C for abbreviations) of female rats maintained on LD and killed throughout the oestrous cycle (LD, data pooled from Figure 8-D) and female rats maintained on LL.

* $p < 0.05$ when compared to animals maintained on LD.

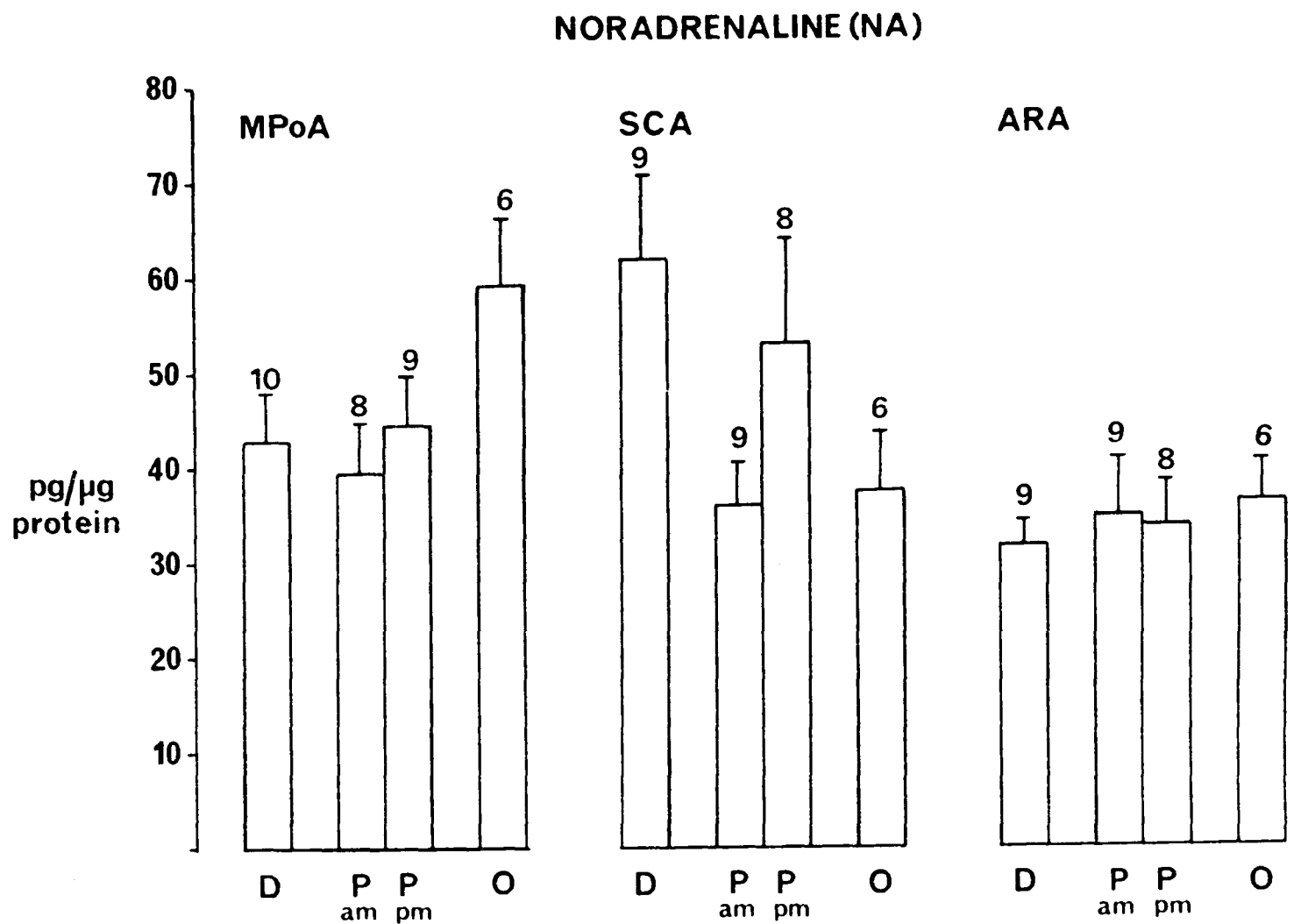


FIGURE 8-H: Mean (+ S.E.M.) concentration (pg/μg protein) of NA in MPoA, SCA and ARA (see Figure 8-C for abbreviations) from female rats maintained on LD and killed during dioestrus morning (D), pro-oestrus morning (P-AM), pro-oestrus evening (P-PM) or oestrus morning (O). Numbers above blocks = number of samples/group.

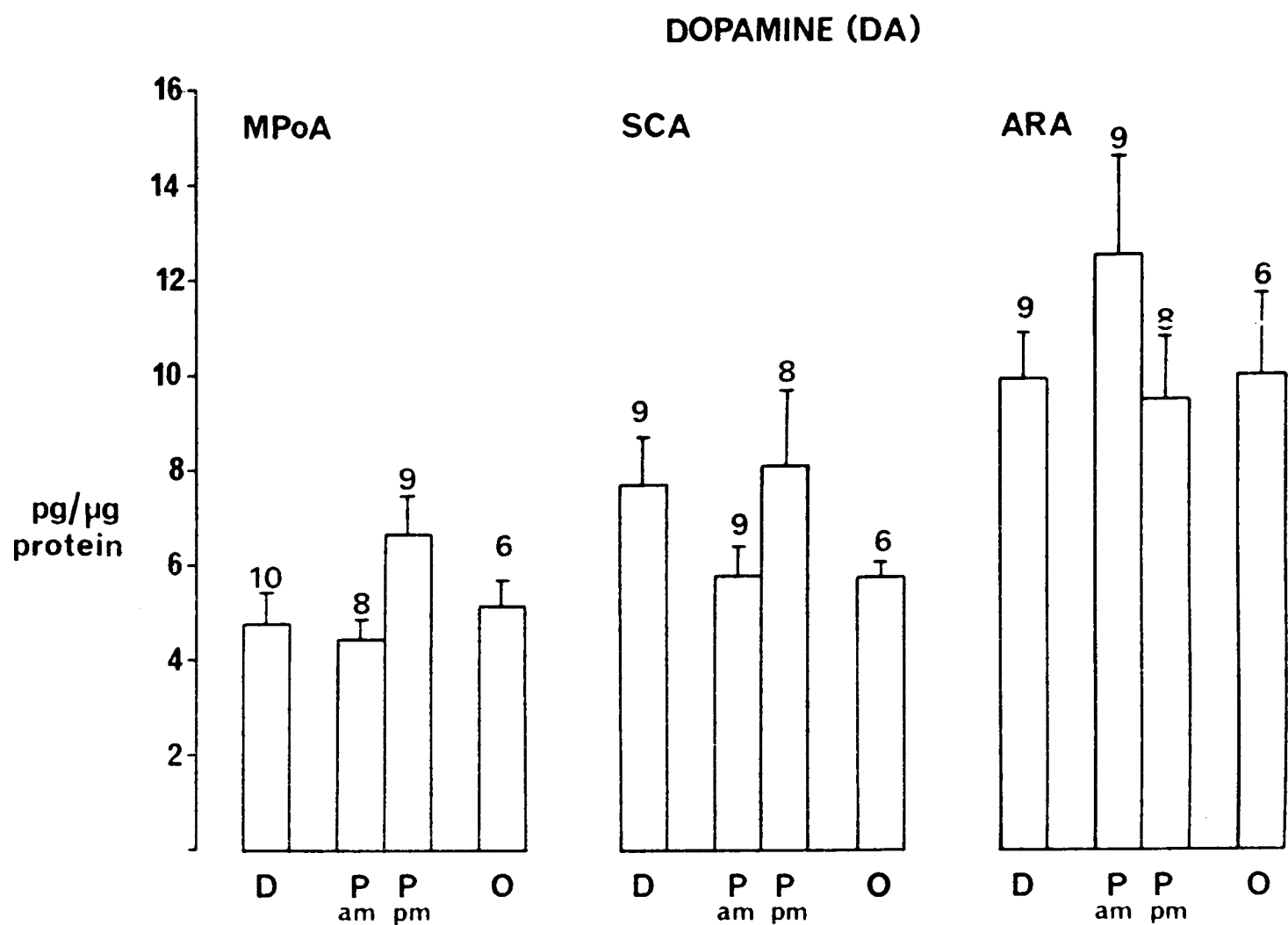


FIGURE 8-J: Mean (+ S.E.M.) concentration (pg/μg protein) of DA in MPoA, SCA and ARA (see Figure 8-C for abbreviations) from female rats maintained on LD and killed during dioestrus morning (D), pro-oestrus morning (P-AM), pro-oestrus evening (P-PM) or oestrus morning (O). Numbers above blocks = number of samples/group.

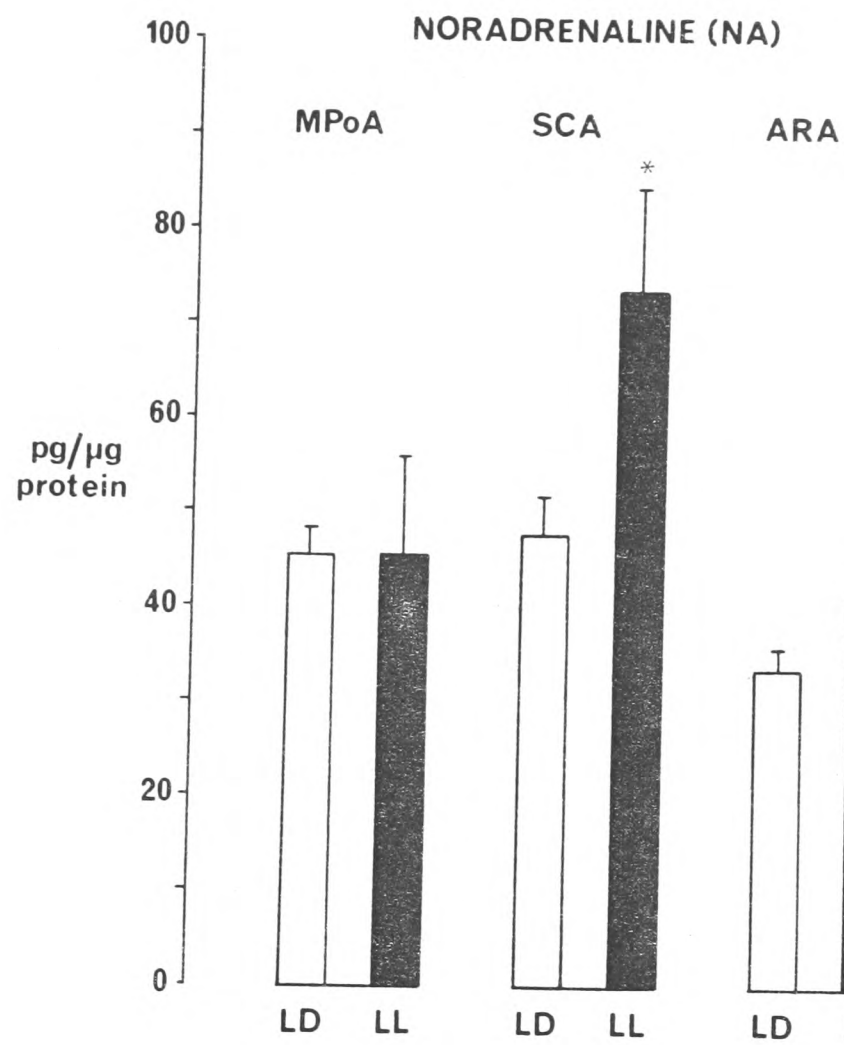


FIGURE 8-K: Mean (+ S.E.M.) concentration (pg/μg protein) of NA in the MPoA (LD, n=33; LL, n=6), SCA (LD, n=32; LL, n=7) and ARA (LD, n=32; LL, n=7) (see Figure 8-C for abbreviations) of female rats maintained on LD and killed throughout the oestrous cycle (LD, data pooled from Figure 8-H) and female rats maintained on LL.

*p<0.05 when compared to animals maintained on LD.

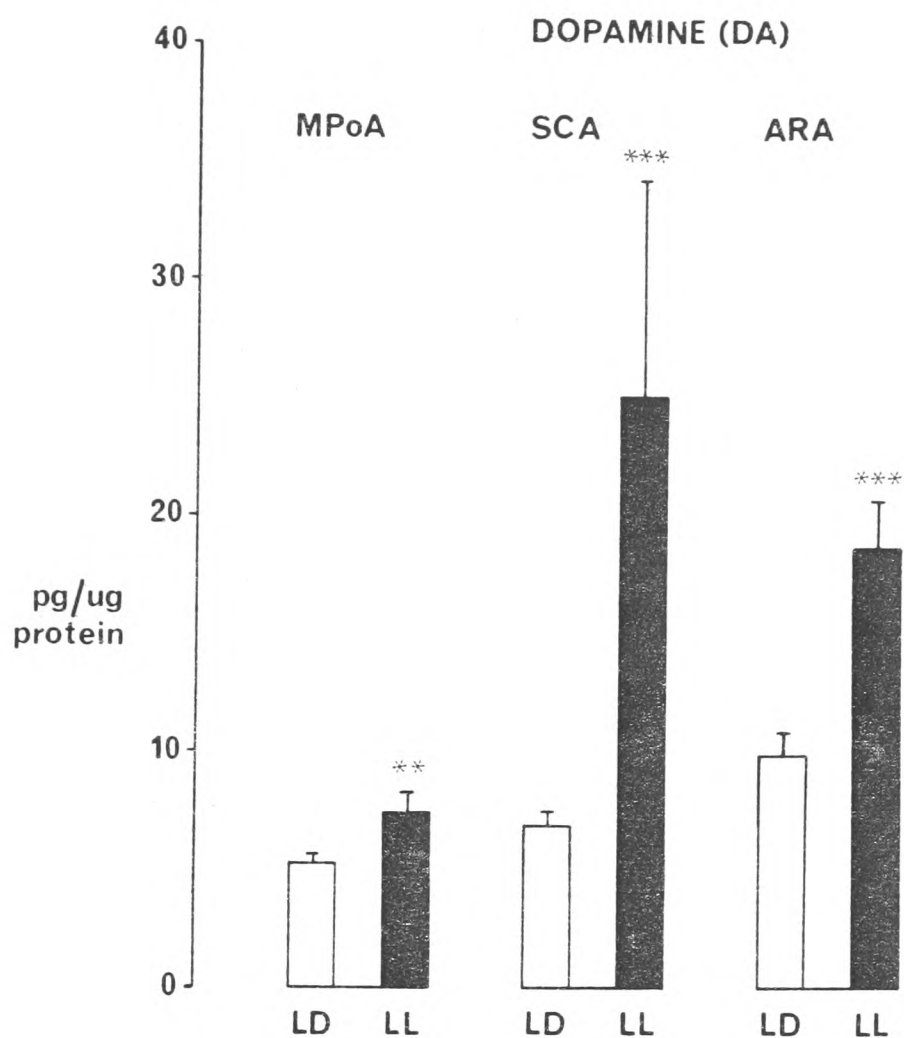


FIGURE 8-L: Mean (+ S.E.M.) concentration (pg/ μ g protein) of DA in the MPoA (LD, n=33; LL, n=6), SCA (LD, n=32; LL, n=6) and ARA (LD, n=32; LL, n=7) (see Figure 8-C for abbreviations) of female rats maintained on LD and killed throughout the oestrous cycle (LD, data pooled from Figure 8-J) and female rats maintained on LL.

** $p < 0.02$, *** $p < 0.001$ when compared to animals maintained on LD.

to LD exposed animals. 5-HIAA/5-HT was significantly lower during the afternoon of pro-oestrus than during the morning of dioestrus ($p < 0.01$) or the morning of oestrus ($p < 0.05$). 5-HIAA/5-HT was significantly reduced ($p < 0.01$) in LL exposed animals when compared to any stage of the oestrous cycle.

Arcuate area (ARA):

The concentration of 5-HT was significantly greater ($p < 0.001$) in LL exposed animals than in LD exposed animals. 5-HIAA/5-HT was unchanged throughout the oestrous cycle, but was significantly lower ($p < 0.01$) in LL exposed animals than LD exposed animals at any stage of the oestrous cycle.

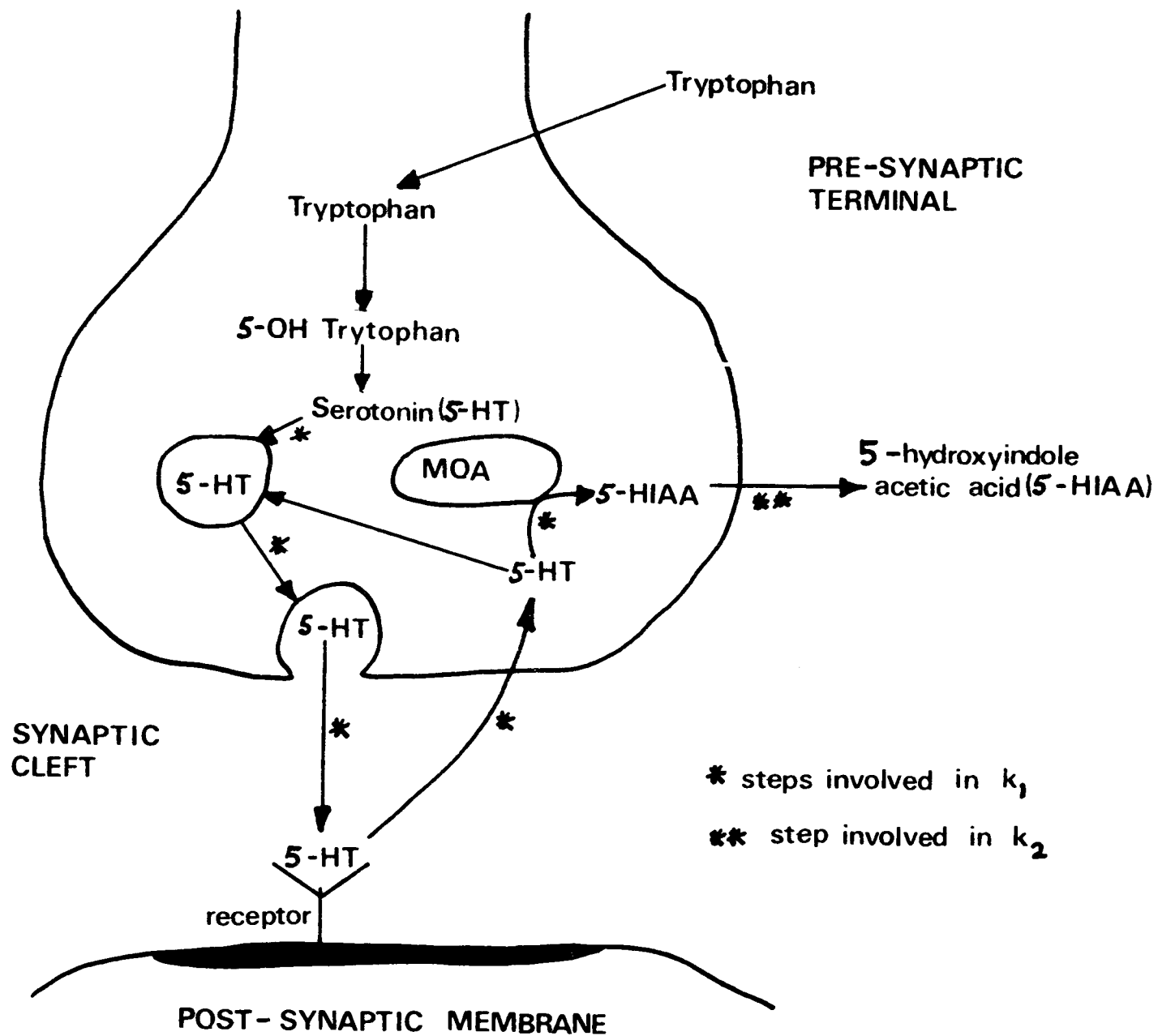
8.3.2 Catecholamines

The concentrations of NA (Figure 8-H) were unchanged throughout the oestrous cycle. After LL exposure the concentration of NA (Fig 8-K) was significantly increased in the SCA ($p < 0.05$).

The concentrations of DA (Figure 8-J) were unchanged throughout the oestrous cycle. After LL exposure the concentration of DA was (Fig 8-L) significantly increased in the MPoA ($p < 0.02$), SCA ($p < 0.001$) and ARA ($p < 0.001$).

8.4 Discussion

The concentrations of 5-HT in the SCA and MPoA in this study were in the same range as reported by Saavedra *et al* (1974), Crowley, O'Donohue, Muth and Jacobowitz (1979) and Cone, Davis and Goy (1981). The concentration of 5-HT in ARA was slightly lower than was reported by Saavedra *et al* (1974), probably because of differences in the area dissected. All the above authors used a radioenzymatic assay. The



$$i) \quad [5-HT] \cdot k_1 = [5-HIAA] \cdot k_2$$

$$ii) \quad \frac{[5-HIAA]}{[5-HT]} = \frac{k_1}{k_2}$$

FIGURE 8-M: Schematic diagram of a 5-HT neurone, showing rate equation of the conversion of 5-HT to 5-HIAA during steady state conditions (Tozer *et al.*, 1966), and steps involved in rate constants.

concentrations of 5-HIAA in MPoA and SCA were in the same range as reported by Bradshaw, Erskine and Baumm (1982), who used LCED.

Figure 8-E showed that LL resulted in a decrease of 5-HIAA/5-HT in the MPoA, SCA and ARA. However, the data in Figures 8-F and 8-G showed that the reduction of 5-HIAA/5-HT was not due to similar changes in the concentrations of 5-HIAA or 5-HT in each area. This suggested that LL affected the metabolism of 5-HT of each area in a different manner.

Tozer *et al* (1966) suggested that the ratio of the molar concentrations of 5-HIAA and 5-HT was equal to the ratio of the rate constants of 5-HT efflux and 5-HIAA loss (Figure 8-N). Therefore, a decrease in 5-HIAA/5-HT would indicate a decrease in the rate constant for 5-HT efflux (k_1), or an increase in the rate constant for 5-HIAA loss (k_2) or a combination of both. An increase in k_2 would indicate a change in the blood/brain barrier mechanisms for excretion of acid metabolites and would probably affect all areas of the brain equally. Therefore, an increase in k_2 by LL would have resulted in a reduction in the concentration of 5-HIAA in all areas. The data in Figure 8-G showed this was not the case. Therefore, LL must have caused a decrease in k_1 ; but whether this was combined with a change in acid metabolite excretion cannot be concluded from the experiments carried out here.

A decrease in k_1 could have been the result of one of, or a combination of the following possibilities.

1. *A decrease in the amount of 5-HT available for breakdown to 5-HIAA.* Reinhard and Wurtman (1977) showed that 5-HIAA was only formed from 5-HT which had been previously released and undergone reuptake into the pre-synaptic terminal. Therefore,

a decrease in the concentration of 5-HIAA would accompany a decrease in the amount of 5-HT released into the synaptic cleft and/or a decrease in the activity of the mechanisms of reuptake. Therefore, a change in k_1 accompanied only by a decrease in the concentration of 5-HIAA would indicate changes in the mechanisms of release and reuptake of 5-HT.

2. *An increase in the total (i.e. non-releasable and releasable) pool of 5-HT due to increased synthesis.* Increased activity of either of the synthetic enzymes (tryptophan hydroxylase and 5-hydroxytryptophan [5-HTP] decarboxylase) would result in an increase in the concentration of 5-HT. If this were not accompanied by changes in other components of k_1 (Figure 8-M), then the concentration of 5-HIAA would remain unchanged (see 1. for rationale).
3. *A decrease in the activity of monoamine oxidase (MAO).* This would result in an increased concentration of 5-HT and a decreased concentration of 5-HIAA if all components of k_1 were unchanged.

The unchanged concentrations of 5-HIAA in the ARA and MPoA of LL-rats suggested that the decreased 5-HIAA/5-HT was not due to an alteration in release and reuptake mechanisms of 5-HT or to a significant decrease in the activity of MAO. The increased concentration of 5-HT in the ARA and MPoA in LL-rats suggested that the decrease in 5-HIAA/5-HT was due to an increase in synthesis of 5-HT. The data in this study suggest that the plasma oestradiol concentrations in LL-rats were not sufficiently elevated to induce the significant decrease in the activity of MAO reported by Luine and McEwen (1974) in the mediobasal hypothalamus of the rat.

In the SCA the decreased 5-HIAA/5-HT was combined with a significant decrease in the concentration of 5-HIAA and this suggested that LL altered the mechanisms of release and reuptake of 5-HT from the presynaptic terminal. However, small changes in the rate of synthesis cannot be excluded. Taken together these data suggested that LL led to a greater alteration in the mechanisms of release and reuptake of 5-HT in the SCA than in the ARA and MPoA. However, further experiments measuring the turnover rates of 5-HT using enzyme inhibitors would help clarify these points.

The SCN and 5-HT are thought to be important in the maintenance of the diurnal rhythm of LH release in oestrogen-treated ovariectomized rats (Coen and MacKinnon, 1979, 1980; Kawakami *et al.*, 1980) and in the mechanism involved in the surge of LH on pro-oestrus in rats on LD (Coen *et al.*, 1980). Taken in conjunction with the data of the above workers and data showing the abolition by LL exposure of the diurnal LH surge in ovariectomized rats (Chapter V of this thesis), the data in the present study suggest that alterations in the mechanisms of release and reuptake in the SCA are contributory factors in the LL-induced loss of regular oestrous cycles (Critchlow, 1963) and the diurnal surge of LH.

The data in Figures 8-C to 8-E did not allow a conclusion about the mechanistic changes in serotonergic neurones which would lead to modulation of LH release during the oestrous cycle. However, there was a significant decrease in 5-HIAA/5-HT from the morning of dioestrus to the afternoon of pro-oestrus in the MPoA and SCA but not the ARA. Coen *et al.* (1980) have shown that 5-HTP was only effective in triggering an LH surge in 5-HT depleted pro-oestrous rats when 5-HTP was given between 10.00 h and 12.00 h. They suggested this

represented a 'critical period' for 5-HT involvement in triggering an LH surge. Taken together with the data of Coen *et al.* (1980), the data in the present study suggest that the area of interaction between serotonergic and LHRH neurones lies in the rostral hypothalamus and that changes in serotonergic function occur before and during the pro-oestrous LH surge.

The concentrations of NA and DA in the areas described in Figures 8-H and 8-J were consistent (allowing for the likely differences in the dissections) with those reported by Palkovits, Brownstein, Saavedra and Axelrod (1974), Crowley, O'Donohue and Jacobowitz (1978) and Honma and Wuttke (1980).

The data in Figure 8-K showed that concentrations of NA were not altered by LL in the ARA and MPoA. However, in the SCA there was a significant increase in NA concentrations. The data in Figure 8-K contrasted with the constant oestrus associated with ageing. Walker, Cooper and Timiras (1980) found the concentrations of NA and DA in the hypothalamus of aged female rats were significantly lower than in the hypothalamus of young female rats showing regular oestrous cycles. Aged female rats exhibiting persistent vaginal oestrus did not show an LH response to exogenous gonadal steroids after ovariectomy (Lu, Damassa, Gilman, Judd and Sawyer, 1980). Since exogenous gonadal steroids have been associated with increased turnover rates of catecholamines in hypothalamic areas of ovariectomized rats (Crowley, O'Donohue, Wachslicht and Jacobowitz, 1978; Honma and Wuttke, 1980; Wise, Rance and Barraclough, 1981), the loss of steroid induced LH surges in aged constant oestrus female rats could have been related to their reduction of hypothalamic NA and DA. However, female rats exposed to LL responded with LH surges to both

mating (Brown-Grant, Davidson and Greig, 1973; Davidson, Smith and Bowers, 1973) and to oestrogen and progesterone treatment after ovariectomy (Mennin and Gorski, 1975). The data of Brown-Grant *et al.* (1973), Davidson *et al.* (1973) and Mennin and Gorski (1975) suggested that in contrast to aged female rats in persistent vaginal oestrus, the centres involved in releasing LHRH were still intact after LL exposure. The data in Figure 8-K (although not conclusive because no turnover rates were determined) suggest that the noradrenergic system was still functional in the hypothalamus of LL exposed female rats. The finding that both NA and DA concentrations increased in the SCA, and DA concentrations increased in the ARA of LL exposed animals (Figures 8-K and 8-L), cannot be explained at present. It may have been related to the serotonergic changes in the SCA perhaps involved in the disruption of diurnal neuroendocrine rhythms, and perhaps with the morphological changes occurring in the ARA after LL exposure (Brawer, Schipper and Naftolin, 1980), but further experiments are necessary using enzyme blockers to determine turnover rates, to explore these possibilities.

CHAPTER IX

The Role of the Retina in the Reproductive Function of the Female Rat

9.1 Introduction

In animals which show oestrous cycles, the regularity of the light/dark cycle and the relative length of the light period within the light/dark cycle play essential roles in the maintenance of regular spontaneous ovulation (Browman, 1937; Hammond, 1951; Harris, 1955; Critchlow, 1963; Everett, 1977). The results of Critchlow (1963) and Brown-Grant (1974) suggested that the massive loss of photoreceptors caused by LL (Noell, Walker, Kang and Berman, 1966; O'Steen, 1970; O'Steen and Anderson, 1972; O'Steen and Donnelly, 1982) was not sufficient to inhibit regular oestrous cycles in animals that were subsequently returned to LD. However, Critchlow (1963) and Brown-Grant (1974) only monitored vaginal smears and therefore could not exclude possible changes in the timing and size of the LH surge on pro-oestrus or the number of ova released every oestrus. Degenerative changes similar to those found in Wistar rats in LL, occur spontaneously in LD in RCS and Hunter rats (Bourne, Campbell and Tansley, 1938; Lucas, Attfield and Davey, 1955; Yates, Dewar, Wilson, Winterburn and Reading, 1974). In RCS and Hunter rats, retinal degeneration is detectable at 12 days of age, is complete by 8 weeks of age and is accompanied by progressive changes in cyclic nucleotide metabolism (Dewar, Barron and Richmond, 1975; Lolley and Farber, 1982). In the present study, the size of the LH surge on pro-oestrus, the number of ova released the following oestrus, the timing of the critical period during pro-oestrus and the regularity of the oestrous cycle were measured in female rats with and without inherited retinal degeneration. The contribution of photoreceptor loss to the mechanisms necessary for regular spontaneous ovulation could therefore be assessed in female rats that had experienced no exposure to LL.

To determine whether the reproductive changes in female Wistar rats exposed to LL were due to degenerative changes in the retina, studies were carried out to determine the rate at which persistent vaginal cornification occurred in female rats with retinal degeneration but with no previous exposure to LL.

Dopamine (DA) is thought to be a functional neurotransmitter in the retina (Nicols, Jacobowitz and Hottenstein, 1967; Kramer, 1971; Da Prada, 1977; Iuvone, Galli, Garrison-Gund and Nett, 1978b; Proll, Kamp and Morgan, 1982) and is found in greatest concentration in the amacrine cells of the inner plexiform layer (Kramer, Potts and Magnall, 1971). Morgan and Kamp (1980) have recently shown that amacrine cell layer of the retina in rats exposed to LL can still respond to light, possibly through a dopaminergic mechanism. Investigations were carried out here to determine the effects of light upon the concentration of DA and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) in the retina of RCS and Wistar rats exposed to LD and LL.

9.2 Materials and Methods

All surgical and analytical techniques used were described in Chapter II. Animals used were PVG, Wistar (with normal retinae), RCS and Hunter rats (with inherited retinal dystrophy). Wistar and RCS strains were congenic, with RCS rats selected from Wistar rats which showed retinal degeneration to produce a pure breeding strain of non-pigmented rats homozygous for the blind characteristic (Barron *et al.*, 1938). PVG and Hunter rats were similarly congenic, with Hunters outbred from PVG rats by cross-breeding with RCS rats, to produce Hunter rats homozygous recessive for the 'blind' allele (Yates *et al.*, 1974).

9.2.1 Comparison of reproductive function in normal and dystrophic rats

Three parameters were studied: first, the pattern of oestrous cycles in all 4 strains of rats. Daily vaginal smears were taken between 09.00 h and 10.30 h (2.1). At least 5 consecutive cycles were recorded for each rat. Second, the timing of the 'critical period' during the day of pro-oestrus was determined for all 4 strains. Third, the size, timing of, and ovulatory response to the LH surge during the afternoon of pro-oestrus was determined in Wistar and RCS rats.

The timing of the pro-oestrous critical period was determined by observing the ability of sodium pentobarbitone to block subsequent ovulation when given between 14.00 h and 16.00 h of pro-oestrus (Everett, 1964; Aiyer, Fink and Greig, 1974). Sodium pentobarbitone (Nembutal, 36 mg/kg, i.p.) was given to all 4 strains of rats either during (15.00 h) or after (18.00 h) the pro-oestrous critical period. The following morning (oestrus), animals were killed by cervical dislocation and the oviducts excised. Ovulation was determined by checking for the presence of ova, as described in Chapter II (2.2.10).

The size and timing of the pro-oestrous LH surge was determined in RCS female rats using the method described in Chapter IV (4.3.1). Briefly, this involved the insertion of an intra-atrial cannula (2.2.5) early on the morning of pro-oestrus. Blood samples (200 µl) were taken every hour, starting at 15.00 h during the afternoon of pro-oestrus. Plasma samples were assayed for LH using the RIA described in Chapter II (2.3.3). Animals were checked for the presence of ova the following morning (2.2.10). The data from Wistar females were taken from data in Chapter IV (Figure 4-A). The animals used in the experiment described in Figure 4-A were maintained in the same lighting

conditions and were subject to the same experimental regime as the RCS females used in the present study.

9.2.2 Effects of constant light on the vaginal smear pattern and on the dopamine and 3,4-dihydroxyphenylacetic acid concentrations in the retina

The effects of LL on the retinal concentrations of DA and DOPAC were determined in Wistar and RCS female rats. Three groups of each strain were used. First, a group maintained in LD used after showing at least 2 consecutive 4-day oestrous cycles (group A). Second, a group (group B) having shown at least 2 consecutive 4-day oestrous cycles in LD were moved to LL. Vaginal smears were taken after transference to LL until at least 10 consecutive days of oestrous smears had been shown. Animals were left in LL for at least 60 days, but no longer than 90 days before being used. Third, a group of animals (group C) which had been treated in the same manner as group B, but were moved back into LD after 60 days in LL. Vaginal smears were not examined for the 7 days immediately after transfer back into LD, but then were started and continued until at least 2 consecutive 4-day oestrous cycles had been shown. Smears were discontinued until 4 days immediately before killing, when cycles were again checked.

Eyes from animals in groups A, B and C were excised and fixed overnight in 10% formalin solution, dehydrated in alcohol and mounted in paraffin blocks. 5 μ m sections were cut and stained using haematoxylin/eosin blue. (Figures 9-C - 9-H)

After vaginal smear criteria had been satisfied for both strains in groups A, B and C, animals were killed by decapitation, their eyes were quickly excised and placed in ice-cold saline. Connective tissue and ciliary muscles were removed and the cornea removed with a razor

blade. The lens and the aqueous humor were carefully removed, and the retina of each eye was removed by exerting pressure on the sclera with a small metal seeker. Each retina was placed on aluminium foil, blotted dry with filter paper (Whatman No. 1) and weighed on a torsion balance. Each retina was homogenized in 100 μ l 0.2M perchloric acid containing 10^{-4} M cysteine and 20 ng/ml n-acetyl dopamine, using glass microhomogenizers ('Uniform', Jencons Ltd., London). Homogenates were centrifuged at 1000 x g for 15 min at 4°C (MSE, Mistral 2L). The pellet was discarded, while the supernatant was filtered using 0.2 μ m regenerated cellulose filters (BAS, Inc.) and kept for LCED analysis. Samples were analysed immediately, or frozen and assayed within 48 h of dissection. DA and DOPAC were assayed using LCED as described in Chapter II (2.3.7). The mobile phase was 0.1M sodium acetate, 0.1M citric acid, 8% methanol with the pH adjusted to 4.5 using sodium hydroxide. The working electrode was set at +0.7V and the detector sensitivity at 5 nA/V. Concentrations were calculated using peak height ratios with respect to the internal standard (2.3.7). Concentrations were expressed as pg/mg wet weight of tissue and significance of differences between means was determined using the unpaired 't' test.

9.3 Results

9.3.1 Reproductive function in dystrophic rats

Table 9-1 showed the percentage of animals which exhibited regular 4-day oestrous cycles, and the ability of sodium pentobarbitone to block ovulation when given at 15.00 h or 18.00 h on the afternoon of pro-oestrus. At least 75% of animals of each strain exhibited regular 4-day oestrous cycles. Sodium pentobarbitone when injected at 15.00 h blocked ovulation in all PVG, Wistar and RCS rats. However,

TABLE 9-1: The percentage of Wistar, RCS, PVG and Hunter rats showing 4-day oestrous cycles, and the ovulatory response (mean $\pm$ S.E.M.) of Wistar, RCS, PVG and Hunter rats to an injection of sodium pentobarbitone (36 mg/kg, i.p.) given at 15.00 h or 18.00 h during the day of pro-oestrus.

Strain	% rats showing 4-day cycles	n	Time of pentobarbitone injection			
			15.00 h		18.00 h	
			n	Number ovulating	n	No. of ova/ ovulating rat
Wistar	85	33	6	0	6	12.6 $\pm$ 0.5
RCS	75	32	8	0	4	13.0 $\pm$ 0.2
PVG	77	35	6	0	5	12.4 $\pm$ 0.2
Hunter	84	33	8	1	6	9.8 $\pm$ 0.7

n = number of animals per group.

1 Hunter rat ovulated when injected with sodium pentobarbitone at 15.00 h. Sodium pentobarbitone, when injected at 18.00 h did not block ovulation in any Hunter or Wistar rats, but blocked ovulation in 1 out of 6 PVG and 2 out of 6 RCS rats.

Figure 9-A showed the LH surge on pro-oestrus in RCS and Wistar females. At no time was there any significant difference in the concentration of plasma LH between RCS and Wistar rats. No significant difference occurred in the mean maximum concentration of plasma LH (the mean of the maximum plasma LH concentration from each animal) between RCS (64.7 ± 6.7) or Wistar (61.6 ± 5.8) rats. No significant difference occurred either in the mean times at which the peak plasma LH concentrations occurred (RCS - 18.00 ± 0.25 h; Wistar - $18.00 \text{ h} \pm 1 \text{ h}$) or the number of ova found on the morning of oestrus (RCS 11.5 ± 0.5 ; Wistar 12.3 ± 0.7).

9.3.2 Effects of constant light on the vaginal smear pattern and on retinal dopamine and 3,4-dihydroxyphenylacetic acid

Figure 9-B shows that there was no difference in the time taken for RCS and Wistar rats moved from LD to LL to reach persistent vaginal cornification. When animals were moved back into LD both strains lost persistent oestrous smears within 7 days. Persistent oestrous was replaced by irregular oestrous cycles for 13-21 days, after which both strains showed at least 2 regular 4 day oestrous cycles.

Table 9-2 shows the retinal wet weights and the concentration of DA and DoPAC in the retina of Wistar and RCS rats in groups A, B and C. The retinal wet weight of Wistar rats in groups B and C were significantly lower ($p < 0.05$, $p < 0.001$ respectively) than the retinal wet weights of Wistar rats in group A. There was no significant difference between the retinal weights of any groups of RCS

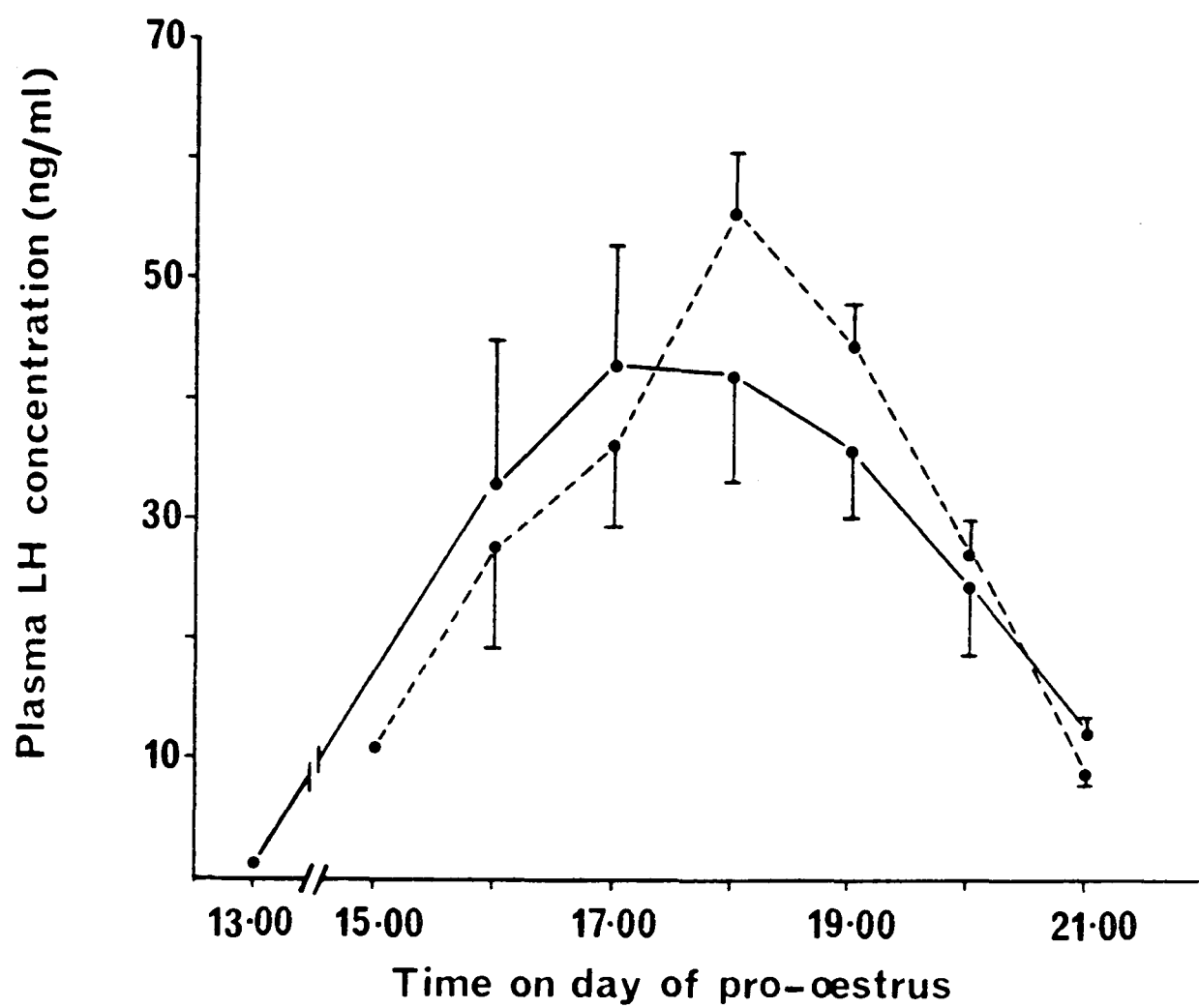


FIGURE 9-A: Mean ($\pm$ S.E.M.) plasma LH concentration during the day of pro-oestrus in RCS ($n=5$, $\bullet$ ---- $\bullet$) and Wistar ($n=6$, $\bullet$ — $\bullet$) female rats fitted with an intra-atrial cannula between 06.00 - 0.8.30 h pro-oestrus.

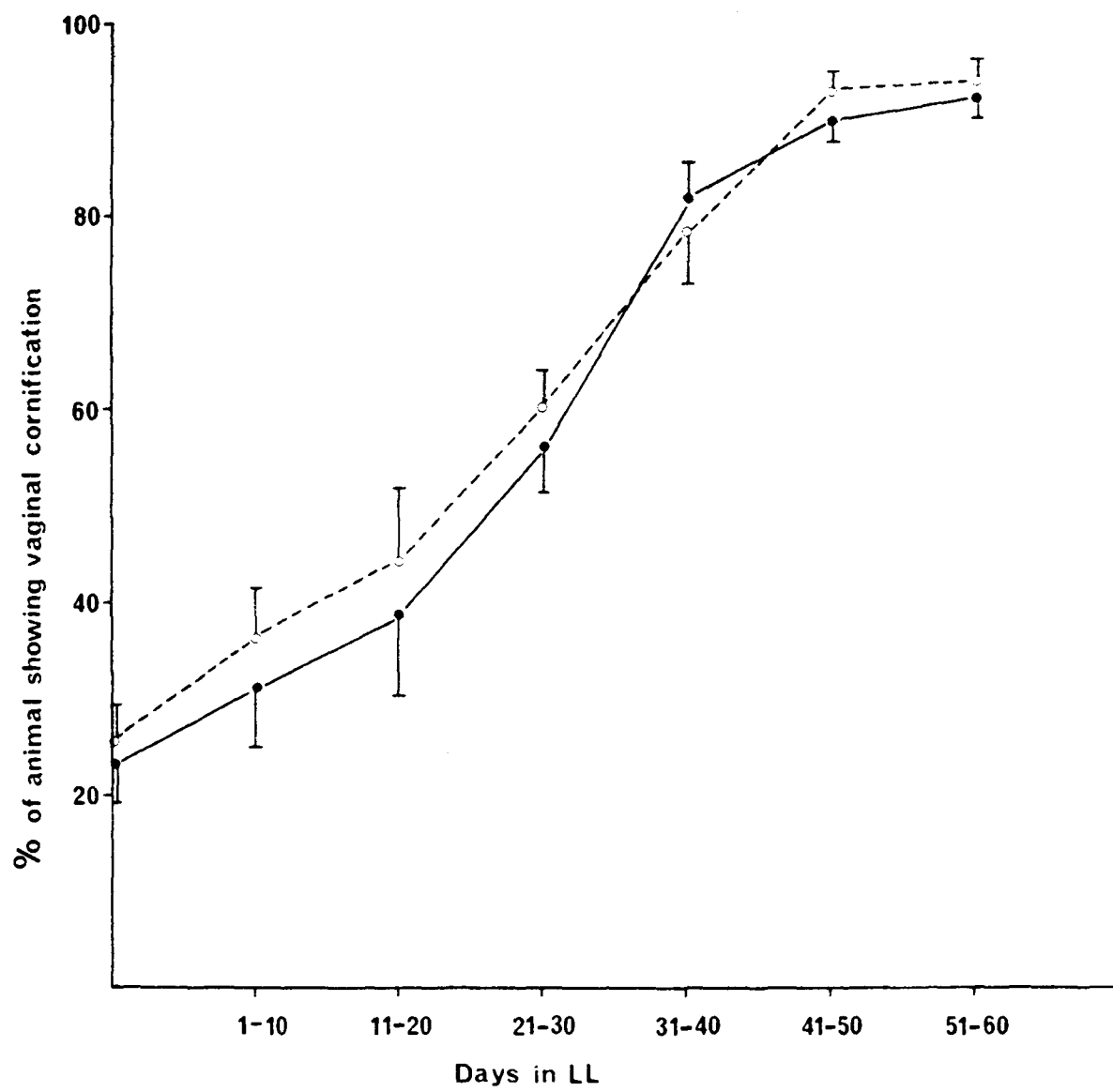


FIGURE 9-B: Mean ($\pm$ S.E.M.) percentage of Wistar (n=8, ● —●) and RCS (n=6, o----o) female rats showing vaginal cornification after various times on LL.

TABLE 9-2: The mean ($\pm$ S.E.M.) retinal wet weight (mg), content (pg) and concentration (pg/mg wet weight) of dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DoPAC) in female Wistar and RCS rats maintained in LD (group A); moved from LD to LL and maintained in LL for 60 days (group B); moved from LD to LL, maintained in LL for 60 days and then moved back into LD (group C).

Strain	Treat- ment	n	Retinal wet wt (mg)	DA content of retina (pg)	Retinal concentra- tion of DA (pg/mg wet weight)	DoPAC content of retina (pg)	Retinal concentra- tion of DoPAC (pg/ mg wet weight)
Wistar	A	12	9.0 $\pm$ 0.3	3597 $\pm$ 95	401.6 $\pm$ 12.3	353.6 $\pm$ 61.6	39.8 $\pm$ 7.1
	B	10	7.8 $\pm$ 0.8	1664 $\pm$ 325	221.0 $\pm$ 36.0	155.0 $\pm$ 28.5	19.0 $\pm$ 2.3
	C	20	5.2 $\pm$ 0.2	2109 $\pm$ 232	405.6 $\pm$ 17.6	333.7 $\pm$ 52.3	38.7 $\pm$ 6.2
RCS	A	14	6.3 $\pm$ 0.1	4429 $\pm$ 192	725.3 $\pm$ 36.6	189.5 $\pm$ 17.1	30.2 $\pm$ 2.5
	B	10	6.4 $\pm$ 0.1	3710 $\pm$ 188	605.2 $\pm$ 29.7	157.6 $\pm$ 7.7	25.1 $\pm$ 2.2
	C	10	6.5 $\pm$ 0.3	4680 $\pm$ 228	729.5 $\pm$ 23.9	218.6 $\pm$ 25.3	31.5 $\pm$ 2.6

n = number of animals/group.

FIGURES 9-C to 9-H:

Transverse sections through retinae, which were fixed, mounted in paraffin blocks, sectioned (5 μ m) and stained with haemotoxylin/eosin. All sections are x 400.

Abbreviations:

- PR - photoreceptor layer
- ON - outer nuclear layer
- OP - outer plexiform layer
- IN - inner nuclear layer
- IP - inner plexiform layer
- G - ganglion cell layer

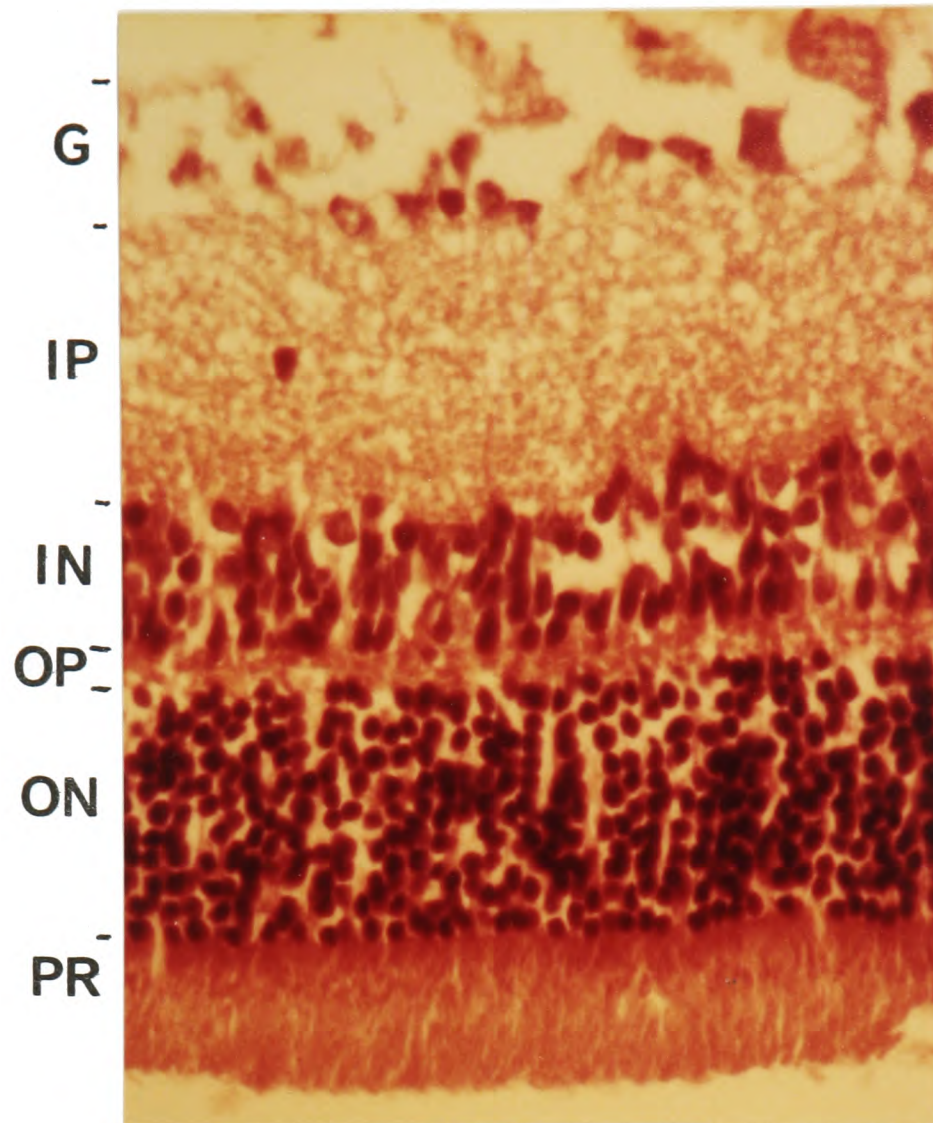


FIGURE 9-C: Wistar - maintained on LD. See p.138 for abbreviations.

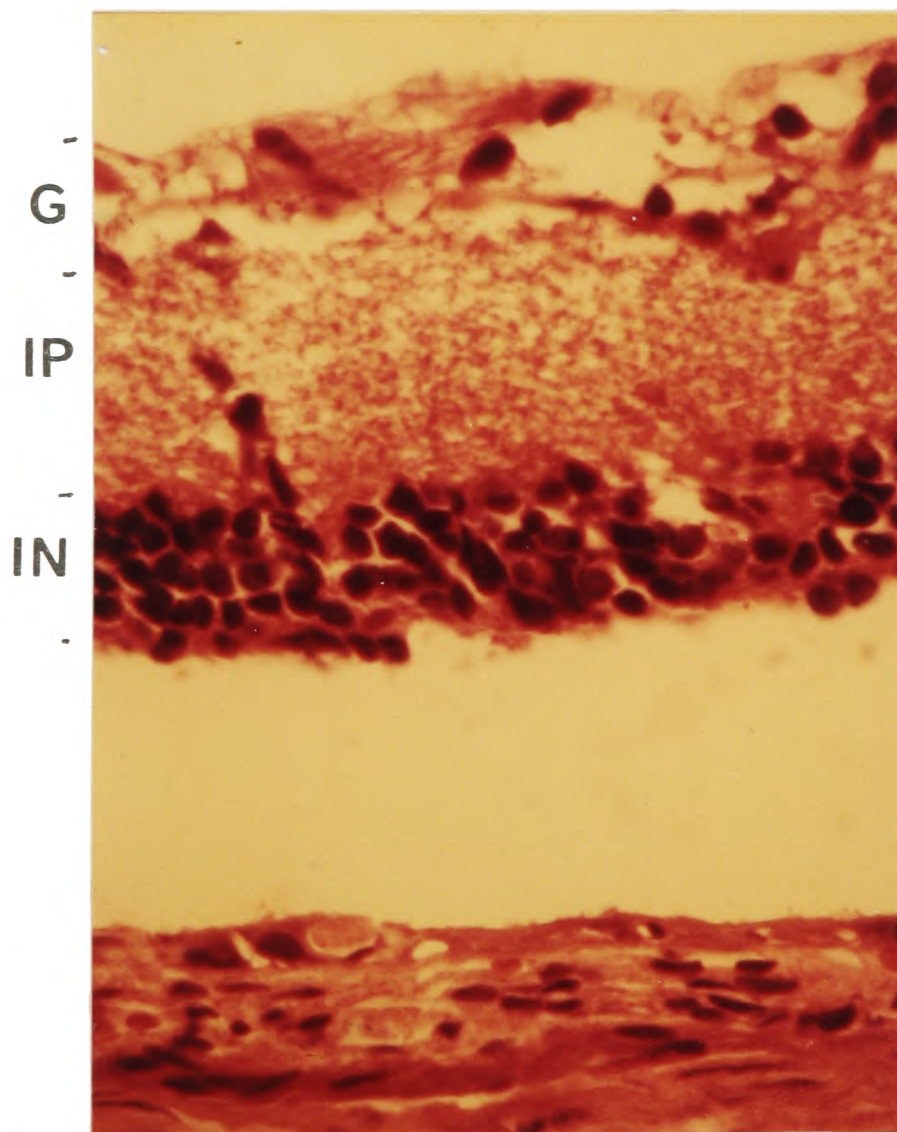


FIGURE 9-D: Wistar - maintained on LL. See p.138 for abbreviations.

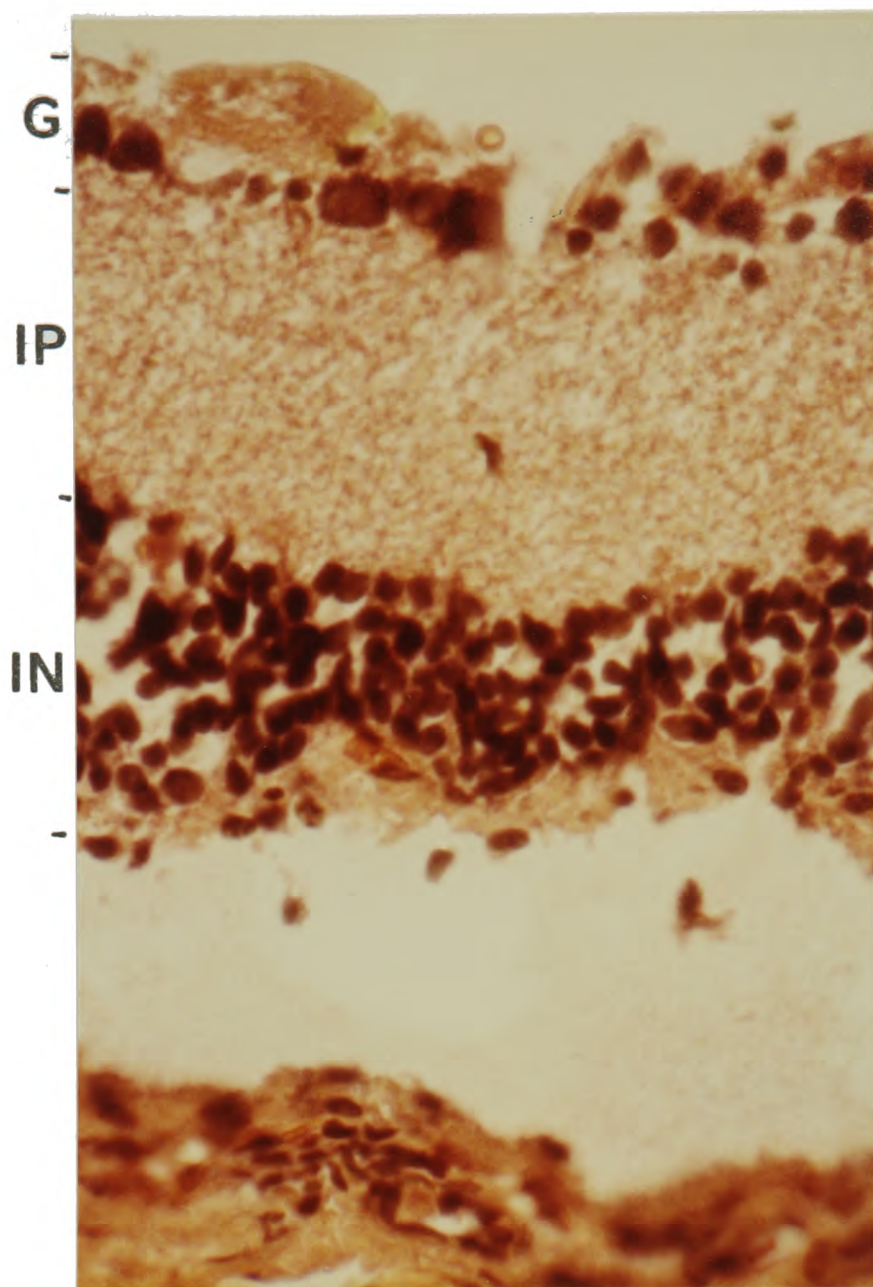


FIGURE 9-E: RCS - maintained on LD. See p.138 for abbreviations.

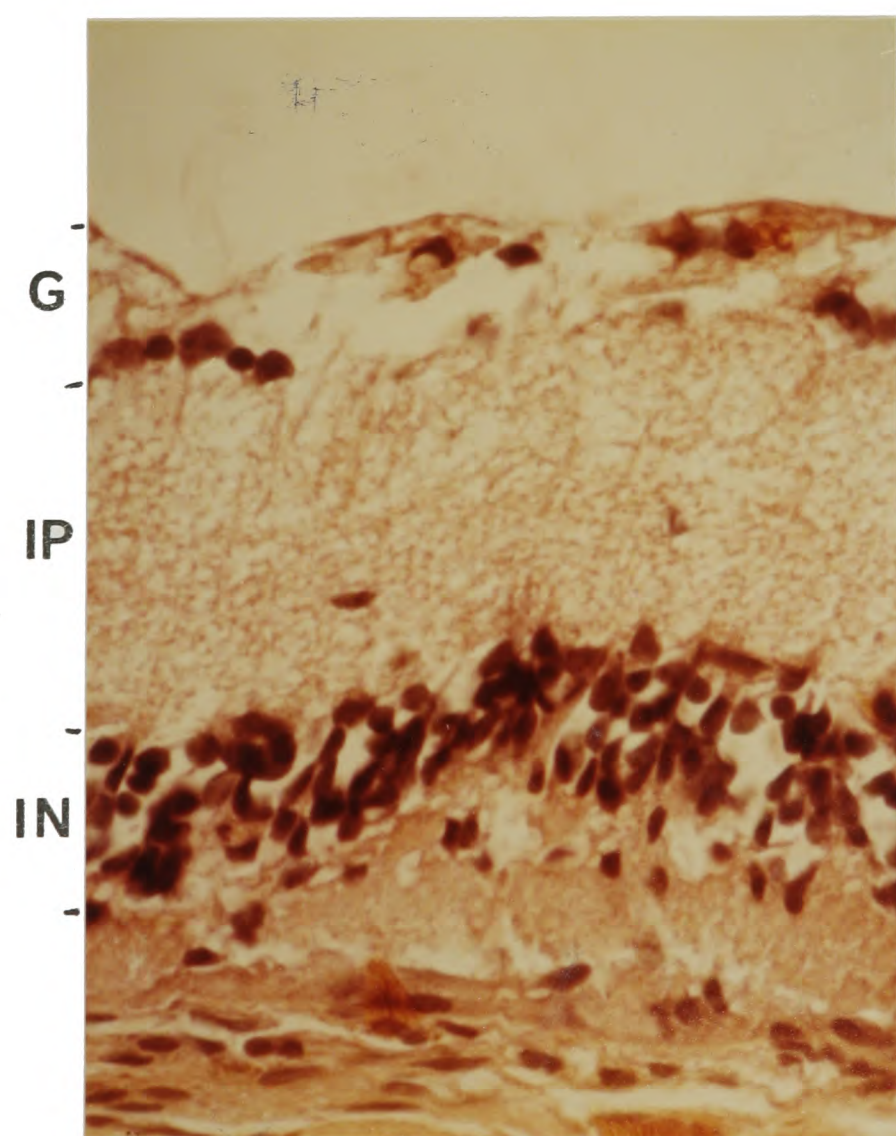


FIGURE 9-F: RCS - maintained on LL. See p.138 for abbreviations.

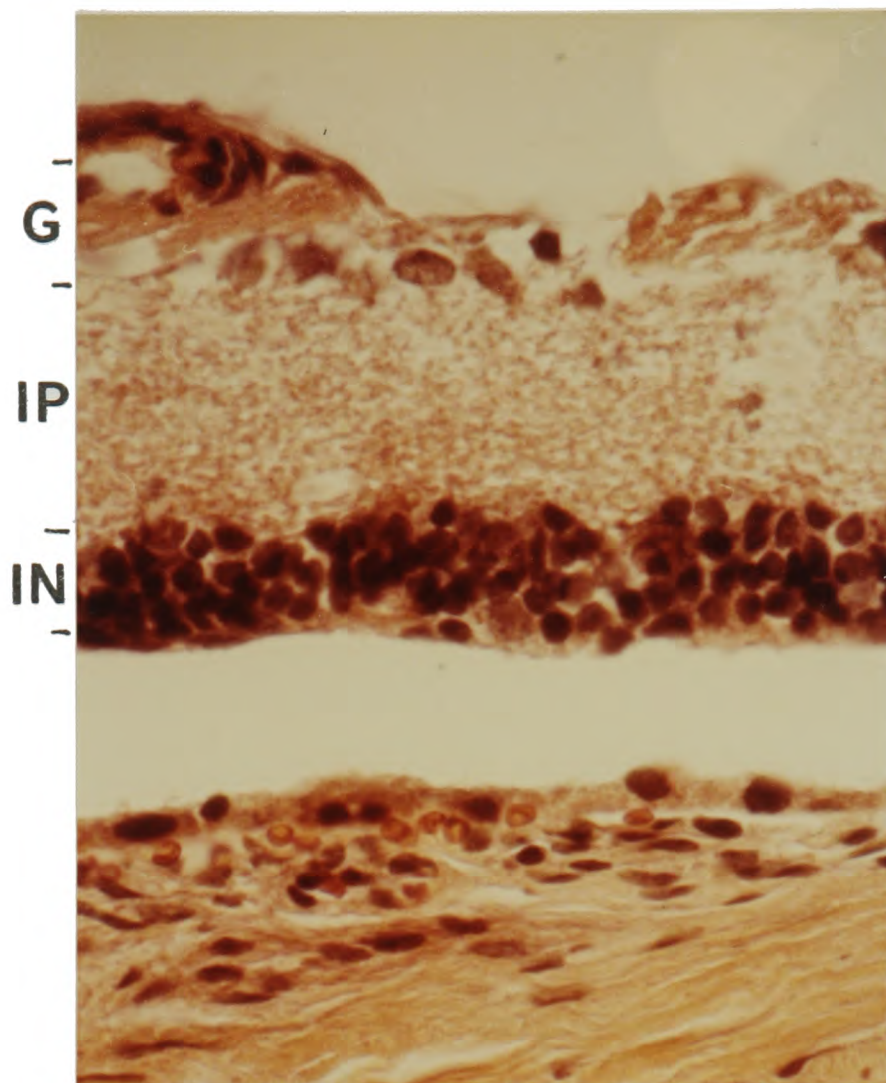


FIGURE 9-G: Wistar - maintained on LL, then moved to LD. See p.138 for abbreviations.

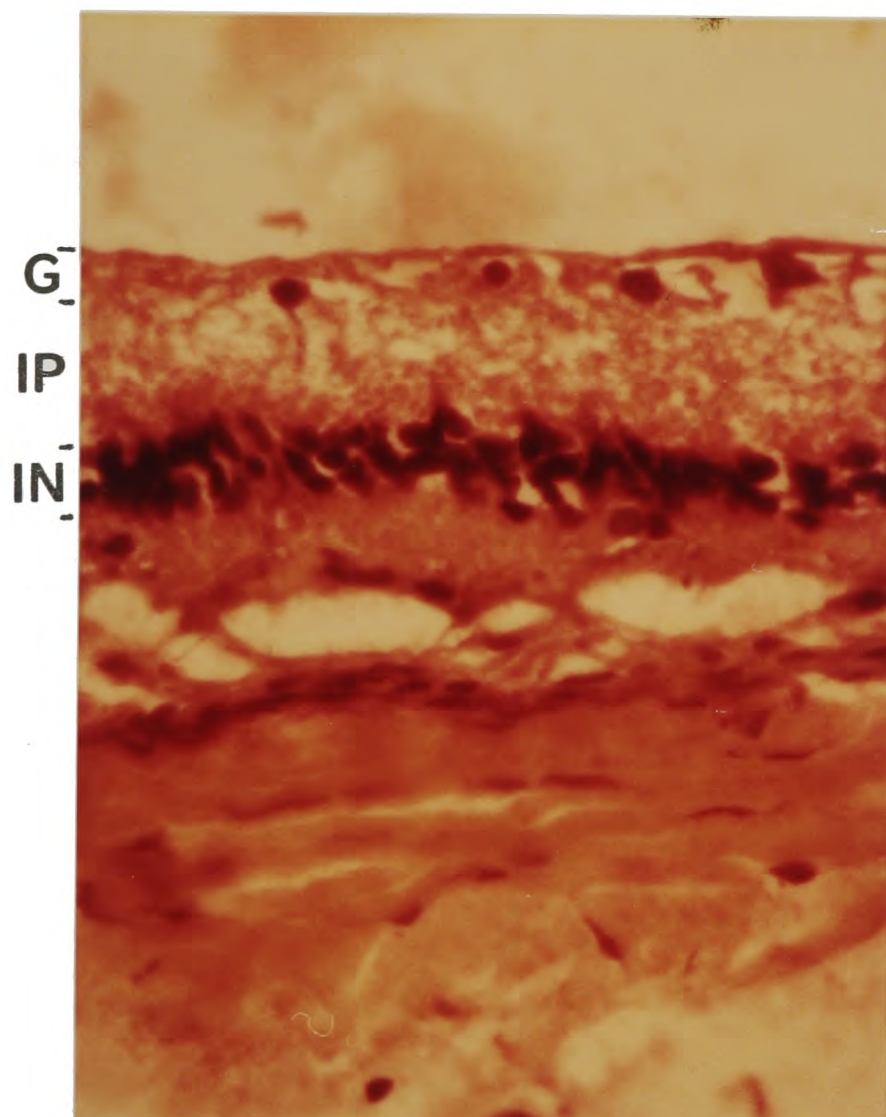


FIGURE 9-H: RCS - maintained on LL, then moved to LD. See p.138 for abbreviations.

rats. Both strains showed significant decreases in the retinal concentrations of DA ($p < 0.001$ Wistar, $p < 0.05$ RCS) and DoPAC ($p < 0.001$ Wistar, $p < 0.05$ RCS) when moved from LD to LL. When animals were moved back into LD the concentration of DA and DoPAC increased in both strains to levels which were not significantly different from untreated LD animals.

9.4 Discussion

The data in Figure 9-A and Table 9-1 showed that in RCS female rats kept in LD, there was no impediment in the mechanisms associated with regular spontaneous ovulation. The studies of Everett (1964), Everett and Tejasen (1967) and Hofmann (1970) showed that the timing of the pro-oestrous critical period was dependent on discrimination between light and dark. The data in Table 9-1 showed that there was no difference in the timing of pro-oestrous critical period between RCS and Wistar females in LD. However, the finding that pentobarbitone, when given at 15.00 h, was unable to block ovulation in all animals, and when given at 18.00 h, blocked ovulation in a small number of animals, agreed with the findings of Naftolin, Brown-Grant and Corker (1972) and Aiyer, Fink and Greig (1974a). The results in Figure 9-A and Table 9-1 therefore showed that a retina with extensive degeneration of the photoreceptor layer was capable of transmitting the information necessary for maintenance of regular and normally sized pro-oestrous LH surges.

Figure 9-B showed that there was no difference in the time taken for female RCS and Wistar rats to reach persistent vaginal cornification when exposed to LL. Figures 9-C and 9-D, and the reduction in the wet weight of the retina (Table 9-2), showed that during this period

Wistar rats suffered severe degeneration of the photoreceptor layer. However, Figures 9-E and 9-F showed that when RCS were exposed to LL for the same period of time as Wistar rats, there was no change to the gross morphology of their retina. Therefore, Figures 9-C, 9-D, 9-E and 9-F suggested that the induction of persistent vaginal cornification in Wistar rats (Figure 9-B) was due to the response of the brain to the light regime and not to parallel degenerative changes in the retina. A similar conclusion was reached by Brown-Grant (1974), who showed that retinal degeneration in Wistar rats caused by a period of LL did not interfere with the rate at which the onset of vaginal cornification was attained during a second exposure to LL. The observations that Wistar rats when returned to LD (after having been kept in LL) quickly lost persistent vaginal cornification and eventually returned to regular oestrous cycles, agreed with the findings of Critchlow (1963) and Brown-Grant (1974). The observations in the present study showed RCS rats behaved in a similar manner to Wistar rats when moved from LL to LD. Figures 9-G and 9-H (when compared to Figures 9-D and 9-F) showed that there was no change to the retinal morphology of female Wistar and RCS rats when moved from LL back to LD.

Evidence from behavioural studies suggested that albino rats exposed to LL (Anderson and O'Steen, 1972; Bennett, Dyer and Dunn, 1973a,b; Lemmon and Anderson, 1979a; Anderson and Lemmon, 1980) and RCS rats (LaVail, Sidman, Rausin and Sidman, 1974) were able to discriminate between light and dark in a number of complex situations. The present study supports the view that some portion of the retina remains photoreceptive after the destruction of the photoreceptor layer. LaVail and co-workers have demonstrated that a small number of

photoreceptor cells survive in the retina of RCS (LaVail *et al.*, 1974) and LL exposed albino rats (LaVail, 1976), although the nature of these cells is unclear (Lemmon and Anderson, 1979b). These surviving cells may have been sufficient to support normal oestrous cycles in RCS rats, although, the mechanism by which such surviving cells would function in both LL exposed Wistar and RCS rats is still unclear.

In contrast to the degeneration in the outer layer, the cells of the inner layers remain morphologically intact after LL exposure (Fifkova, 1973), although some synaptic reorganisation may occur (Fifkova, 1973). Among these intact cells are the dopamine containing amacrine cells (Kramer *et al.*, 1971) of the inner plexiform layer. DA may play an important role in the function of the retina. Light stimulated tyrosine hydroxylase activity (Iuvone, Galli and Neff, 1978a; Iuvone *et al.*, 1978b), the turnover and synthesis rates of DA in dark adapted retina (Da Prada, 1977; Iuvone *et al.*, 1978b; Morgan and Kamp, 1980; Proll *et al.*, 1981), and the release of DA from perfused rabbit retina (Kramer, 1971). Morgan and Kamp (1980) showed that amacrine cells of LL-exposed rats also (after a period of dark adaption) responded to light with an increase turnover rate of DA. In addition, Reading (1982) showed that light increased the activity of Na^+K^+ -dependent ATP-ase (a membrane bound enzyme important in the retinal response to light - Hagins, 1972) in a retinal preparation free of photoreceptors, whereas a preparation consisting of photoreceptors only, showed no change in Na^+K^+ -dependent ATP-ase after light or dark adaption. Taken together, the findings by other workers described above, suggest that the inner layers of the retina retain the capacity to biochemically respond to light in the absence of photoreceptors.

The data in Table 9-3 showed that the retina of Wistar and RCS rats both responded to LL with a significant reduction in the retinal concentration of DA and DoPAC. The magnitude of the DA decrease was greater (45%) in Wistar rats than in RCS rats (17%) and showed that a proportion of the decrease found in Wistar rats may have been related to the degenerative process occurring in Wistar rats (but not in RCS rats). The different magnitude of the response of the 2 strains when placed in LL (Table 9-2), and the differences between the retinal concentrations of DA and DoPAC in the 2 strains kept only in LD (Table 9-2) may also have been related to differences which occur in the number and K_d of retinal DA receptors in the 2 strains (Reading and Watts, 1982). Since the retinal DA concentrations in the group C Wistar rats (which showed a reduction in retinal wet weight compared to Wistar group A) and the group C RCS rats (which showed no change in retinal wet weight compared to RCS group A) were no different to the retinal concentrations of DA in group A animals of their respective strains, a proportion of the reduction in DA concentrations caused by LL was probably due to alterations in DA metabolism. Since the decreases and increases in retinal DA concentration in groups A, B and C (Table 9-2) were paralleled by changes in retinal DoPAC concentrations, the light induced changes in DA concentrations were possibly due to alteration in DA turnover in the retina. However, further experiments to determine turnover rates would help clarify these points.

CHAPTER X

Summary and Hypothesis

10.1 Summary

Constant light (LL) exposure during the 3 days preceding pro-oestrus in animals with regular oestrous cycles, delayed and diminished the height of the LH surge on the afternoon of pro-oestrus. However, the ovulatory response to the LH surge was the same in animals exposed to LL or control lighting (LD). Ovariectomy during the morning of dioestrus immediately followed by oestradiol treatment, and then by progesterone treatment at the beginning of the afternoon of the following day, resulted in an increase in the pituitary responsiveness to LHRH to levels found in sham ovariectomized and oil treated females. Ovariectomy during the morning of dioestrus followed by oestrogen and progesterone treatment in animals maintained on LL resulted in a significant reduction in pituitary responsiveness to LHRH when compared to animals maintained on LD. Similarly, the facilitation of pituitary responsiveness to LHRH found 12 h after ovariectomy and implantation of an oestradiol (OE₂) containing Silastic capsule into females on LD during the morning of dioestrus, was not seen in animals maintained on LL. Studies using infusions of LHRH into rats ovariectomized and implanted with empty or OE₂ containing capsules during the morning of dioestrus, showed that OE₂ increased pituitary responsiveness to LHRH by a mechanism which involved the release and priming effect of LHRH. Taken together, these results suggested that the centres in the brain concerned with LHRH release were less sensitive to the effects of OE₂ after LL exposure.

The daily neural signal for gonadotrophin secretion which can be seen in oestrogen-treated ovariectomized rats maintained on LD, was not evident in oestrogen-treated ovariectomized rats maintained on LL. However, pulsatile LH release most easily seen in ovariectomized rats,

was not abolished by LL, and this finding suggests that diurnal and pulsatile LH release are driven by different neural mechanisms. The suprachiasmatic nucleus (SCN) and serotonergic (5-HT) neurones have been shown by other workers to be essential for the maintenance of diurnal but not pulsatile LH release. LL exposure may therefore influence interactions in the SCN between LH releasing mechanisms and elements concerned with circadian timing. The concentration of 5-HT and its metabolite, 5-hydroxyindole acetic acid (5-HIAA) were measured in the medial preoptic area (MPoA) and the areas of the SCN (SCA) and arcuate nucleus (ARA) of female rats maintained on LD and LL. No significant changes occurred in the concentrations of 5-HT or 5-HIAA in any of the 3 areas throughout the oestrous cycle in rats maintained on LD. However, the molar ratio of 5-HIAA/5-HT decreased between dioestrus morning and pro-oestrus evening in the MPoA and SCA but not the ARA, and this finding suggested that interactions occurred between 5-HT neurones and centres in the rostral hypothalamus controlling gonadotrophin secretion. The decrease in 5-HIAA/5-HT, found in all 3 areas of female rats exposed to LL when compared to female rats exposed to LD, was due to a significant increase in the concentration of 5-HT in the MPoA and ARA. However, in the SCA, the reduced 5-HIAA/5-HT was the result of a significant reduction in the concentration of 5-HIAA. Therefore, the changes in 5-HIAA/5-HT found in LL exposed rats, were probably due to increases in the synthesis rate of 5-HT which were greater in the MPoA and ARA than in the SCA, and to changes in the release and reuptake mechanisms of 5-HT which were greater in the SCA than in the MPoA and ARA. The changes in the synaptic metabolism of 5-HT found in the SCA of LL exposed female rats may have contributed to the loss of diurnal LH release.

The pattern of pulsatile LH release was studied in gonadectomized male, female, and female rats rendered anovulatory by neonatal androgen (TP) or 60 days exposure to LL. The mean plasma LH concentration was greater in gonadectomized male and female rats than female rats treated with TP or maintained on LL. Pituitary responsiveness to LHRH was significantly greater in gonadectomized male and female rats, than in gonadectomized females treated with TP or maintained on LL for 60 days. These findings show that pituitary responsiveness to LHRH is a major determinant in the patterns of pulsatile LH release. The inter-pulse interval of pulsatile LH release was significantly longer in female rats maintained on LL, than in any of the 3 groups, and suggested that LL affected the centres concerned with the timing of LHRH release.

The centres upon which oestradiol acts to inhibit pulsatile LH release, were shown to be neither sexually differentiated nor affected by TP or LL. Ovariectomized rats with a 90% reduction in hypothalamic 5-HT levels, also showed oestradiol-induced inhibition of pulsatile LH release.

Progesterone induced ovulation in a limited number of female rats exposed to long-term LL, and in these animals, there was a significant increase in pituitary responsiveness to LHRH 5 h after progesterone injection. The magnitude of the priming effect of LHRH (already demonstrable in untreated LL exposed rats) was markedly increased 2 h after progesterone treatment. Treatment with sodium pentobarbitone (SP) at the same time as progesterone, blocked the increase in pituitary responsiveness to LHRH 5 h later, but treatment with SP 4 h before progesterone injection did not block the increased magnitude of the priming effect of LHRH. Taken together, these results suggest that progesterone acts both at the brain and pituitary to facilitate LH release.

Measurements of plasma concentrations of oestradiol and progesterone after mating, showed that plasma oestradiol concentrations, although not increased after mating, were increased when compared to female rats on LD during dioestrus, pro-oestrus evening or oestrus. Since plasma concentrations of progesterone were significantly increased after mating, progesterone may have been involved in mating-induced ovulation.

The timing of the critical period and LH surge during the afternoon of pro-oestrus, was unchanged in female rats with degenerated retinal photoreceptors (Hunter and RCS strains), when compared to female rats with intact retinae (PVG and Wistar strains). Both RCS and Wistar strains responded to LL exposure by attaining persistent vaginal cornification at the same rate. In addition, both RCS and Wistar strains regained normal oestrous cycles when moved from LL to LD. The photoreceptor layer of the retina therefore, is not essential for normal reproductive function. The results also show that the effects of LL on gonadotrophin secretion are mediated through the brain and are not the result of retinal degeneration.

Measurements of retinal concentrations of dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DoPAC), showed that concentrations of both compounds in the retina were reduced by LL, but concentrations then increased when animals were moved back into LD. These results suggest that dopaminergic mechanisms in the amacrine cell layer of the retina respond to different light regimes, perhaps by changes in turnover rates.

10.2 Hypothesis

A central factor in many of the effects of LL reported in this thesis, is the extended and elevated plasma concentrations of OE_2 . The delay in the pro-oestrous LH surge found after 3 days exposure to LL, may have been the result of an extended surge of OE_2 during the morning of pro-oestrus. The decrease in the mean plasma LH concentration found in rats ovariectomized after 60 days in LL, may have been related to inhibitory effects of prolonged periods of elevated plasma OE_2 concentrations acting on the brain to alter LHRH release, and on the pituitary to reduce LH responsiveness to LHRH. Finally, the effects of progesterone upon pituitary responsiveness to, and the priming effect of, LHRH may have required OE_2 to 'prime' both the brain and pituitary before progesterone could exert its effects. The mechanism by which plasma OE_2 concentrations are kept elevated may involve increased FSH secretion, and if so may represent an interesting dichotomy in the action of LHRH at the pituitary, where LH secretion is kept low, and plasma FSH concentrations are elevated.

The mechanism by which progesterone acts at the brain of LL exposed rats to stimulate LHRH release, and at the pituitary to increase the magnitude of the priming effect, is still not known. However, since progesterone seems to stimulate the release of more LH than either mating or 1000 ng/100 g b.w. LHRH, an increase may occur in the size of the readily releasable pool of LH in the pituitary after progesterone treatment.

LL reduces the sensitivity of centres in the brain upon which OE_2 acts, as well as increasing the ovarian (and from other sources?) secretion of OE_2 . LL probably acts by a mechanism which involves interactions between 'photosensitive' neurones in the SCN (and possibly

the preoptic area [POA]) and neurones in the POA and septum which control LHRH release. The desensitizing effect of LL is shown by its inhibition of oestrogen-induced diurnal LH release, an effect which probably involves 5-HT neurones.

The importance of the SCN in controlling many behavioural and neuroendocrine circadian rhythms is well known. However, its exact role is still the subject of much investigation. Some workers suggest that the SCN contains an endogenous pacemaker which continues to oscillate after surgical isolation. Other workers have shown that many behavioural and neuroendocrine circadian rhythms are abolished by lesions of the SCN (Moore, 1978; Chapter V for references). However, studies have been reported which suggest that circadian oscillations in corticosterone and locomotor activity persist after bilateral SCN lesions (Szafarczyk, Ixart, Malaval, Nouquier-Soulé and Assenmacher, 1979; Szafarczyk, Alonso, Ixart, Malaval, Nouquier-Soulé and Assenmacher, 1980b). The SCN, therefore, does not seem to act as a master clock but possibly as an integrator for neuroendocrine rhythms which use light as an entraining stimulus. The data in Chapter VIII, and that of other workers (see Chapter VIII for references) suggest that 5-HT is involved in this integrator function. LL may therefore act to reduce the sensitivity to OE_2 of neurones involved in this integrator function. This hypothesis could be extended to suggest that the neurones which control the diurnal release of LH are switched off by short (i.e. constant darkness and 'winter' photoperiods) and extended (LL) photoperiods, through a diminished action of OE_2 on 'integrator' neurones. With short photoperiods the diminished action of OE_2 may involve reduced plasma concentrations of OE_2 perhaps combined with reduced neuronal sensitivity to OE_2 , however, with LL this may only

involve reduced neuronal sensitivity to OE_2 . Consequently, the light period in summer photoperiods (e.g. 14 h light : 10 h dark) probably stimulates maximal neuronal sensitivity to OE_2 and thereby allows increased concentrations of OE_2 to trigger the mechanisms which lead to cyclic spontaneous ovulation.

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APPENDICES

APPENDIX 1: Iodination of LH and LHRH.

	<u>LH</u>	<u>LHRH</u>
Column	- 12 x 1 cm Biogel P-60	- 20 x 0.75 cm Sephadex G-25
Column coated with	- 0.01M PBS/5% egg white	- 0.01M PBS/0.1% gelatine
Hormone concentration and volume	- 5 µg in 10 µl dist. H ₂ O	- 2 µg in 20 µl dist. H ₂ O
¹²⁵ I, activity and volume	- 1 mCi in 10 µl	- 1 mCi in 10 µl
Reaction started with	- 10 µl of 5 mg/ml chloramine T solution	- 20 µl of 1 mg/ml chloramine T solution
Reaction time	- 2 min	- 15 seconds
Reaction stopped with	- 25 µl of 5 mg/ml sodium metabisulphide	- 20 µl of 2 mg/ml sodium metabisulphide
Column elution buffer	- 0.01M PBS	- 0.01M PBS/0.1% gelatine
Elution volume of hormone	- 3.5 - 4.5 ml	- 16-19 ml
Elution volume of ¹²⁵ I	- 11-13 ml	- 11-13 ml

APPENDIX 2: Assay protocol for RIA of LH and LHRH.

Buffers

0.01M PBS	8.17 g NaCl 0.1 g sodium merthiolate 0.25 g NaH_2PO_4 1.193g Na_2HPO_4 990 ml dist. H_2O pH to 7.6
Antiserum (A/S) buffer	500 ml 0.01M PBS 9.306g EDTA 1.7 ml normal rabbit serum pH to 7.0
Assay buffer for LHRH RIA	0.01M PBS/0.1% gelatine
Assay buffer for LH RIA	0.01M PBS/1% bovine serum albumin

Timetable

	<u>LH</u>	<u>LHRH</u>
Day 1	200 μl sample/standard 200 μl assay buffer 200 μl antiserum diluted in A/S buffer	200 μl sample/standard 200 μl assay buffer 200 μl antiserum diluted in A/S buffer 200 μl ^{125}I -LHRH (5000 cpm) in assay buffer
Day 2	200 μl ^{125}I -O-LH (10000 cpm) in assay buffer	200 μl ARGG diluted in 0.01M PBS
Day 3		1 ml 0.01 PBS, then centrifuged for 45 min at 2000 x g aspirated and the pellet counted
Day 4	200 μl ARGG diluted in 0.01M PBS	
Day 5	Tubes centrifuged for 30 min at 2000 x g, aspirated and pellet counted	

Both assays incubated at 4°C

Standards

0.25, 0.5, 0.75, 1.2, 2, 4, 8, 16 ng/ml	7.8, 15.5, 31, 62, 125, 250, 500 pg/ml
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APPENDIX 3: Assay protocol for RIA of oestradiol-17 β (OE₂) and progesterone (P₄)

OE₂ RIA

Recovery solution - 500 cpm ³H-OE₂ blown down to dryness, taken up in 50 μ l 0.01M PBS/1% gelatine and added to each 0.5 ml plasma sample.

Sample extraction - 0.5 ml plasma + 2 x 5 ml 10% diethyl ether/petroleum ether at 50°C. Ether extracts combined, blown down to dryness and taken up in 300 μ l 0.01M PBS 1%. 2 x 100 μ l taken for assay. 50 μ l counted for % recovery.

Assay - 100 μ l sample/standard.
100 μ l assay buffer (0.01M PBS/1% gelatine).
500 μ l antiserum diluted (1 : 300,000) in 0.01M PBS/1% gelatine.
100 μ l - 5000 cpm ³H-OE₂
Tubes mixed and then incubated overnight at 4°C.
500 μ l dextran/charcoal solution. Incubated for 15 min on ice, and then centrifuged 2000 x g for 30 min, 500 μ l of supernatant taken for counting.

Standards - 4.5, 9, 18, 36, 72, 144 pg/tube.

P₄ RIA

Recovery solution - 500 cpm ³H-P₄ blown down to dryness taken up in 20 μ l 0.01M PBS/1% gelatine and added to each plasma sample.

Sample extraction - 50 μ l or 200 μ l plasma extracted 2 x 2 ml petroleum ether at room temperature, blown dry and taken up in 300 μ l 0.01M PBS/1% gelatine. 2 x 100 μ l taken for assay. 50 μ l counted for % recovery.

Appendix 3 (cont.)

- | | |
|-----------|---|
| Assay | - Assay as for OE ₂ except 500 µl antiserum (diluted 8 : 30 from WHO stock) and 100 µl - 5000 cpm ³ H-P ₄ added. |
| Standards | - 6.6, 13.2, 26.4, 52.8, 105.6, 211.2, 422.2, 660 pg/tube. |

- 0, 13.5, 27, 54 μg bovine albumin (Sigma, fraction v)/540 μl 0.1N NaOH.

- 100 g NaCO_3 /500 ml H_2O	}	A 1
1 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 2.4 g Na, K tartrate 4 H_2O /500 ml		
100 g lauryl sulphate (SDS)/l		A 2
16 g/l NaOH		A 3

- 100 ml A1 + 200 ml A3 + 100 ml A2

- 100 ml Folin - Cocalteau solution + 500 ml H₂O

- 540 μ l standard/sample
540 μ l reagent A
Mixed and allowed to stand for 10 min.
270 μ l reagent B
Mixed immediately and allowed to stand
for 30 min - 2 h.
Absorbance then read at 750 nm.

Appendix 5

Dopamine receptor content and retinal degeneration

Reading H.W & Watts A.G.

Certain strains of rat, mice and dogs, exhibit progressive hereditary retinal degeneration which shows similarities to human retinitis pigmentosa (Reading 1980). The condition is characterised by abnormally high levels of cyclic nucleotides in the retina at a crucial stage in differentiation. (Lolley et al. 1977). Recently Hanbauer et al. (1979) demonstrated a relationship between stimulation of dopaminergic (DA) receptors and control of cyclic nucleotide metabolism through release of calmodulin and activation of phosphodiesterase.

We investigated the possibility that abnormal cyclic nucleotide metabolism in mutant rat retina could be associated with defects in the DA system by comparing congenic strains of rat, affected and normal.

Radioligand binding assays were carried out on washed retinal membranes using ^3H spiroperidol (Amersham International 21 Ci/mmol). Non specific binding was determined by incubation with non-radioactive spiroperidol (10^{-7}M) or + butaclamol (10^{-6}M). Membranes were collected and washed on Whatman GF/B discs, radioactivity on the discs was determined by liquid scintillation spectroscopy using 10mls of a micellar scintillant containing 0.1% Triton X-100. Details of procedure followed those described by Bennett (1978).

Table 1 shows the overall binding data for pigmented rats, normal, unaffected (P.V.G.) and affected (Hunter).

Table 2 gives similar data for albino, (pink-eyed) rats, normal (C.O.B.S. Wistar) and affected (R.C.S.).

Most striking difference between affected and normal animals, is the lower receptor density (B_{max}) in the former. It also appears that affected animals show a lower K_D for spiroperidol.

The character of the DA receptor as defined by ^3H spiroperidol binding was examined in more detail by the use of competitor antagonists having differential specificity. Mianserin ($5 \times 10^{-6}\text{M}$) was used to determine the fraction of 5HT sites labelled by ^3H spiroperidol, which in all retinae, amounted to $20 \pm 4\%$. This agreed well with the figures for haloperidol (10^{-6}M) which gave a figure for DA sites as $87 \pm 1\%$ of ^3H spiroperidol sites.

Differentiation of DA sites into D_1 and D_2 was achieved by the use of -sulpiride (10^{-5}M) as competitor. -Sulpiride, an antipsychotic, binds to DA, D_2 sites with some degree of selectivity (Kebabian & Calne 1979). Scatchard plots for D_2 receptors in comparison with plots of combined D_1 D_2 receptors are shown in Figs. 1, 2, 3 and 4. The K_D of the D_2 receptors for ^3H spiroperidol was always lower than that of the combined D_1 D_2 . The overall percentage distribution of D_1 and D_2 sites is shown in Fig. 5. Total sites ($D_1 + D_2$) = 100%. In both pigmented and albino strains proportion of D_1 sites was always lower in affected animals.

DA and DOPAC were estimated in albino retina by HPLC of 0.2M perchloric acid/0.004% ascorbic acid extracts, using electrochemical detection.

Table 3 gives the results for albino strains. There was a significantly higher DA content in affected (RCS) rat retina at 12 weeks on both a concentration and content per retina basis, and the metabolite DOPAC was lower in the affected retina suggesting a lowering of DA turnover. No such differences were detectable at 2-3 weeks of age.

Conclusions

1. A reduction in receptor density (B_{max}) is associated with the retinal dystrophic condition in both albino and pigmented rats. This is more marked in albino animals, in which the lesion develops more rapidly.
2. Changes in K_D values suggest a differential receptor affect. Characterisation of the DA receptors into D_1 and D_2 classes by the use of -Sulpiride as antagonist showed receptor loss to be associated with the D_1 (adenylcyclase linked) type.
3. DA levels were higher in dystrophic retina at 12 weeks of age but no differences were found at 2-3 weeks.
4. Reduction in receptor density and high levels of neurotransmitter both suggest a state of retinal DA receptor hyposensitivity in the dystrophic rat. It appears that the receptor anomaly precedes the increase in DA levels and the reduction in DA turnover.

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Table 1.

³H Spiroperidol binding to Retinal membranes ;
Congenic strains of Pigmented Rats

AGE	K_D (nM)		B_{max} (fmols mg ⁻¹)	
2 - 3 weeks	<u>NORMAL</u>	0.21±0.01 n=5	335±35 n=5	P = 0.01
	<u>AFFECTED</u>	0.25±0.14 n=5	128±18 n=4	
4 - 6 weeks	<u>NORMAL</u>	0.17±0.05 n=9	208±14 n=5	P=0.01
	<u>AFFECTED</u>	0.11±0.03 n=9	137±16 n=5	
8 - 10 weeks	<u>NORMAL</u>	0.16±0.02 n=12	221±27 n=10	P<0.01
	<u>AFFECTED</u>	0.09±0.01 n=12	93±5 n=10	

NORMAL RATS: P.V.G STRAIN, AFFECTED: HUNTER STRAIN.

VALUES ± S.E.M.

Table 2.

³H SPIROPERIDOL BINDING TO RETINAL MEMBRANE

CONGENIC STRAINS OF ALBINO RATS.

AGE (WEEKS)	K _D (nM)		B _{MAX} (f.mols mg ⁻¹)	
2-3	NORMAL	0.08 ± 0.01 n=9	112 ± 4 n=7	N.S.
	AFFECTED	0.07 ± 0.02 n=9	132 ± 14 n=7	P = 0.2
4-6	NORMAL	0.23 ± 0.09 n=7	133 ± 25 n=7	P = 0.02
	AFFECTED	0.08 ± 0.01 n=7	81 ± 21 n=7	P = 0.02
8-10	NORMAL	0.16 ± 0.03 n=10	175 ± 19 n=10	P < 0.01
	AFFECTED	0.07 ± 0.01 n=10	86 ± 12 n=10	P < 0.01

NORMAL RATS; COBS WISTAR., AFFECTED, R.C.S.

VALUES ± S.E.M.

Table 3.

DA & DOPAC in Rat RetinaAlbino animals

Age	Type	Wet Wt(mg)	Concn. pg/mg	Amount pg/retina
2-3weeks	N (Cobs Wistar) n = 5 *	7.7 $\pm$ 0.6	47.4 $\pm$ 4.0(DA) 22.5 $\pm$ 3.5(DOPAC)	715.2 $\pm$ 62.8(DA) 166.6 $\pm$ 22.2(DOPAC)
	A (RCS) n = 4	6.6 $\pm$ 0.3	48.4 $\pm$ 5.8(DA) 22.3 $\pm$ 4.8(DOPAC)	632.5 $\pm$ 64.9(DA) 144.9 $\pm$ 27.3(DOPAC)
12 weeks	N (Cobs Wistar) n = 6	9.0 $\pm$ 0.3	** 401.6 $\pm$ 12.3 (DA) ° 39.8 $\pm$ 7.1(DOPAC)	*** 3597.1 $\pm$ 94.6(DA) ° 353.6 $\pm$ 61.6(DOPAC)
	A (RCS)	6.28 $\pm$ 0.13	** 725.3 $\pm$ 36.6(DA) ° 30.2 $\pm$ 2.5(DOPAC)	*** 4429.7 $\pm$ 192.4(DA) ° 189.5 $\pm$ 17.1(DOPAC)

5HTP, 5HIAA undetectable

5HT on or below sensitivity level

* No significant difference between normal or affected rats

** p = << 0.01

*** p = 0.01

° Not significant

°° p = 0.02

Normal pigmented

Rat Retina;

(P.V.G)

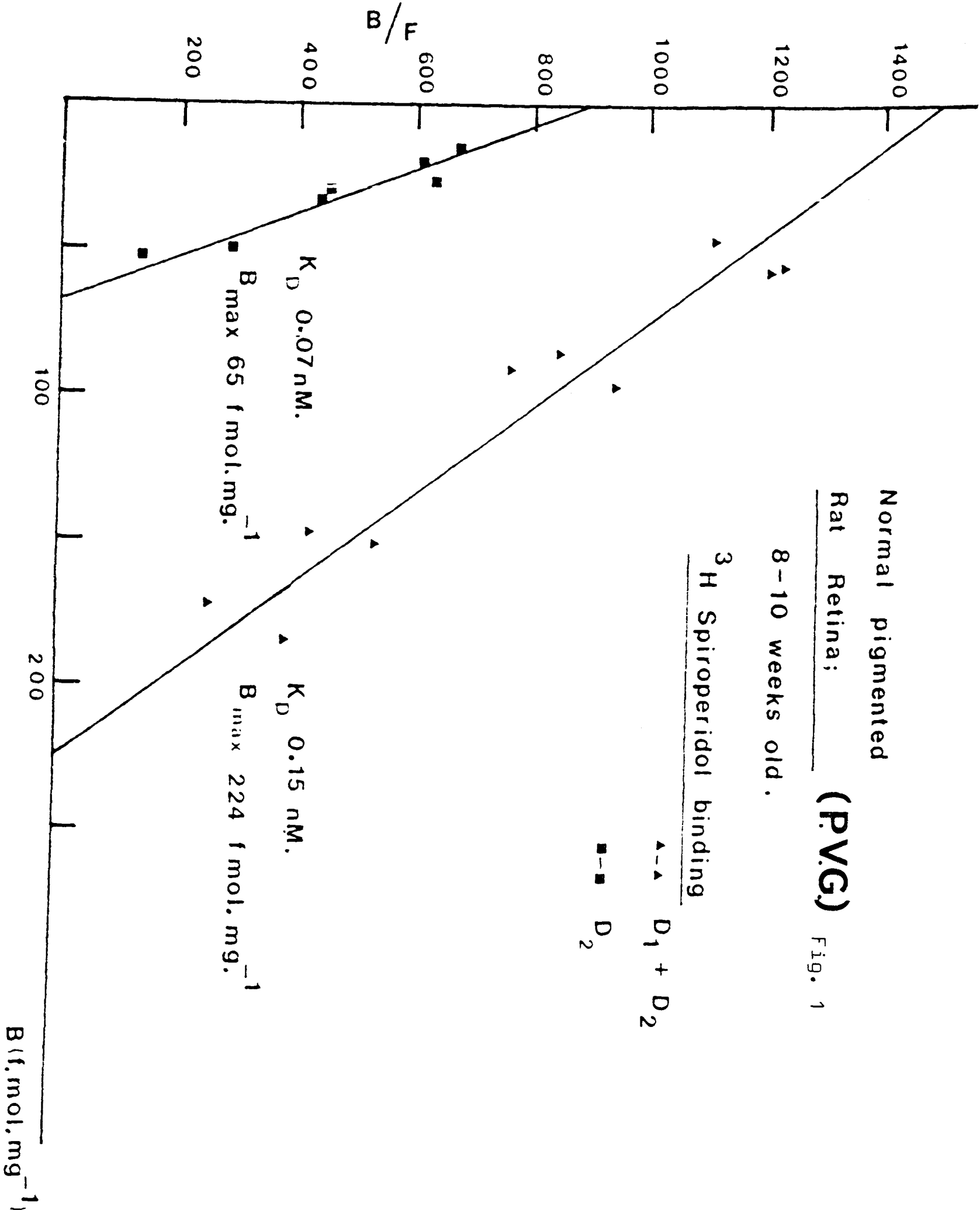
Fig. 1

8-10 weeks old.

³H Spiroperidol binding

▲-▲ D₁ + D₂

■-■ D₂



AFFECTED, PIGMENTED RAT RETINA. (HUNTER)

Fig. 2

8-10 weeks old.

³H Spiroperidol binding

Δ D₁ + D₂

■ D₂

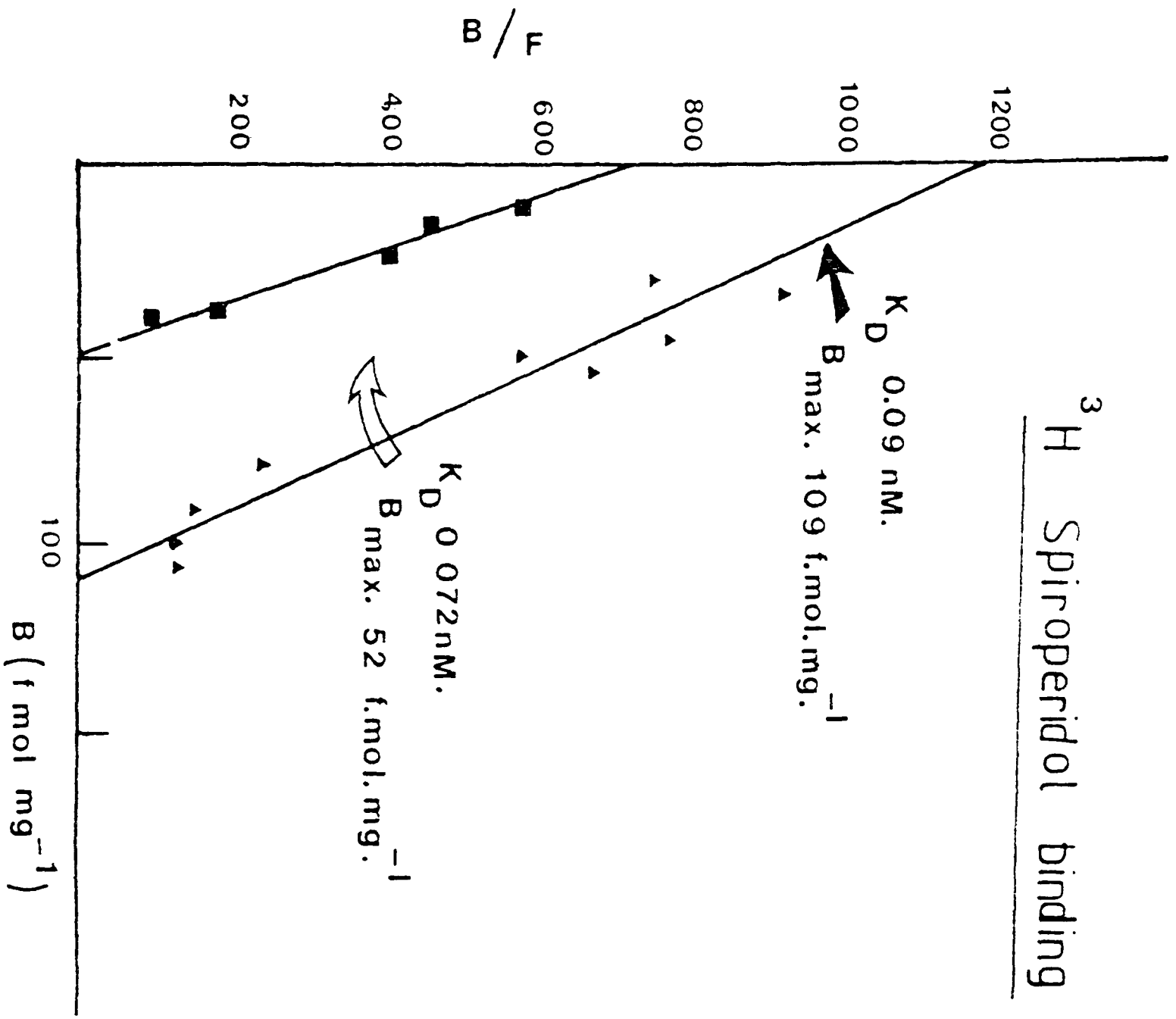
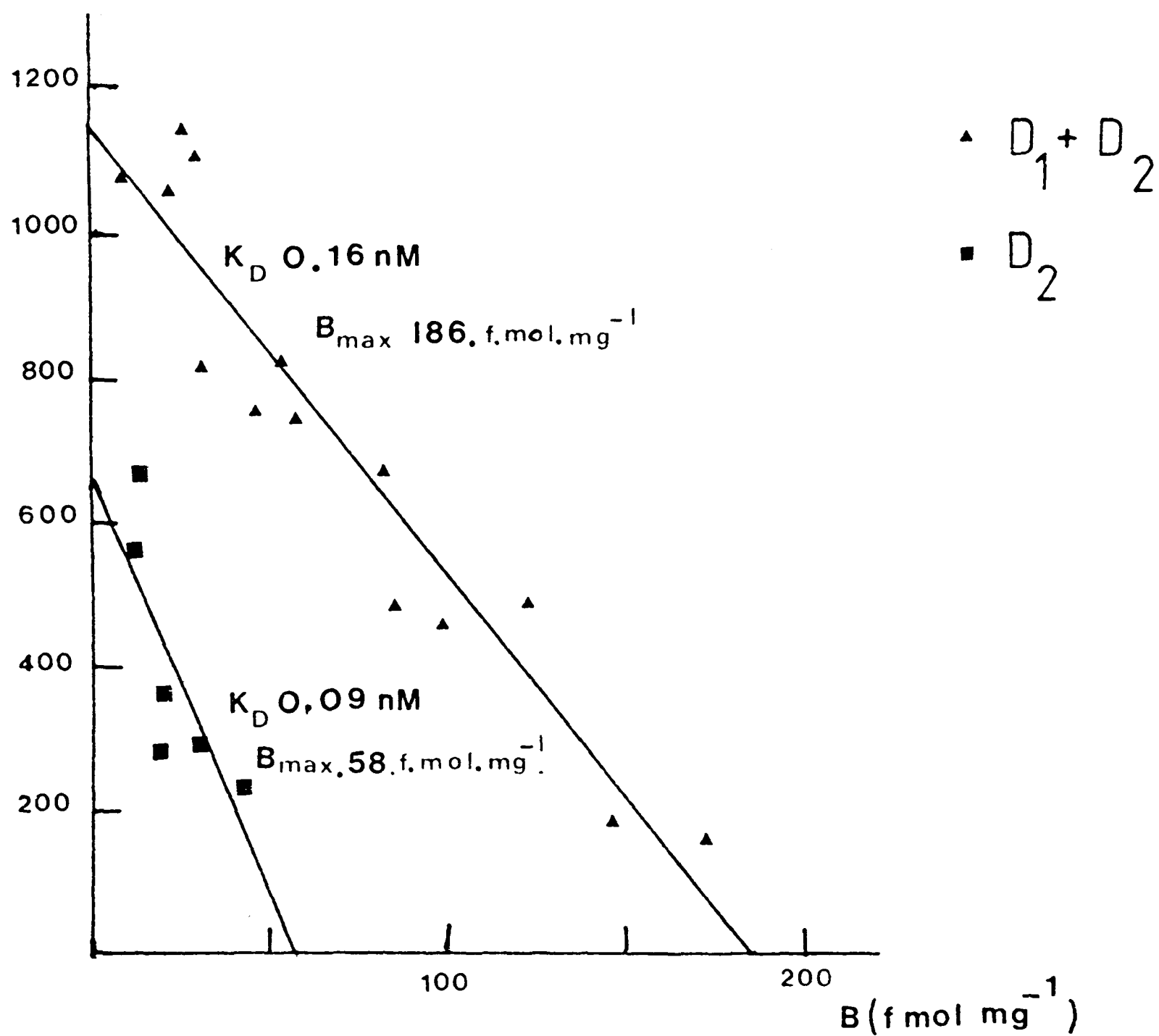


Fig. 3

NORMAL ALBINO RAT RETINA.
(C.O.B.S. WISTAR.)

8 - 10 weeks old.

^3H Spiroperidol binding

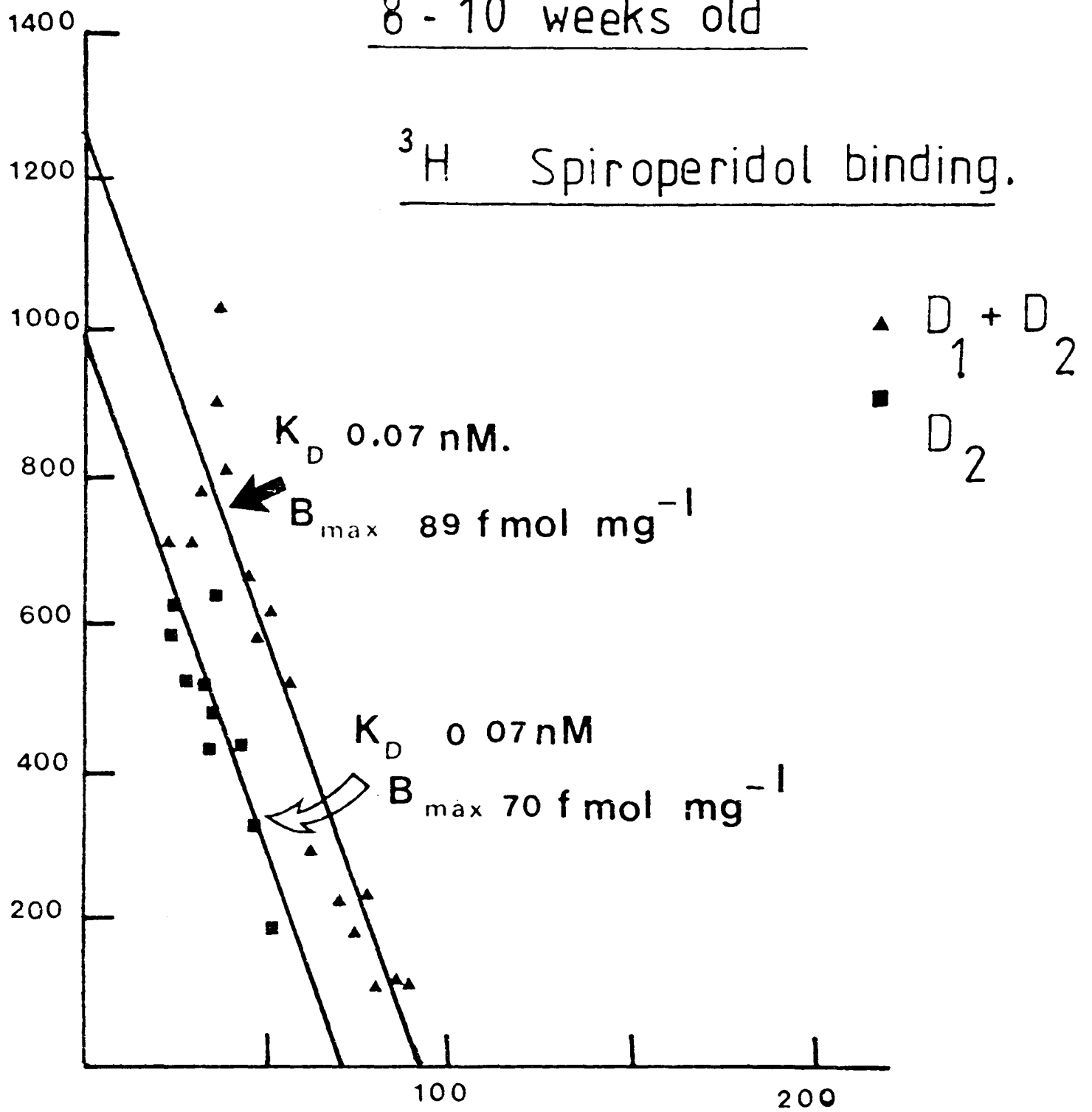


AFFECTED ALBINO RAT RETINA

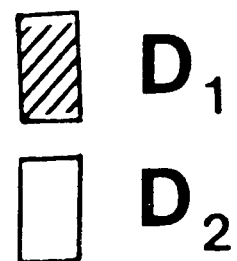
(R.C.S.)

8 - 10 weeks old

^3H Spiroperidol binding.



DOPAMINE RECEPTOR TYPE



N = NORMAL A = AFFECTED

Pigmented rats

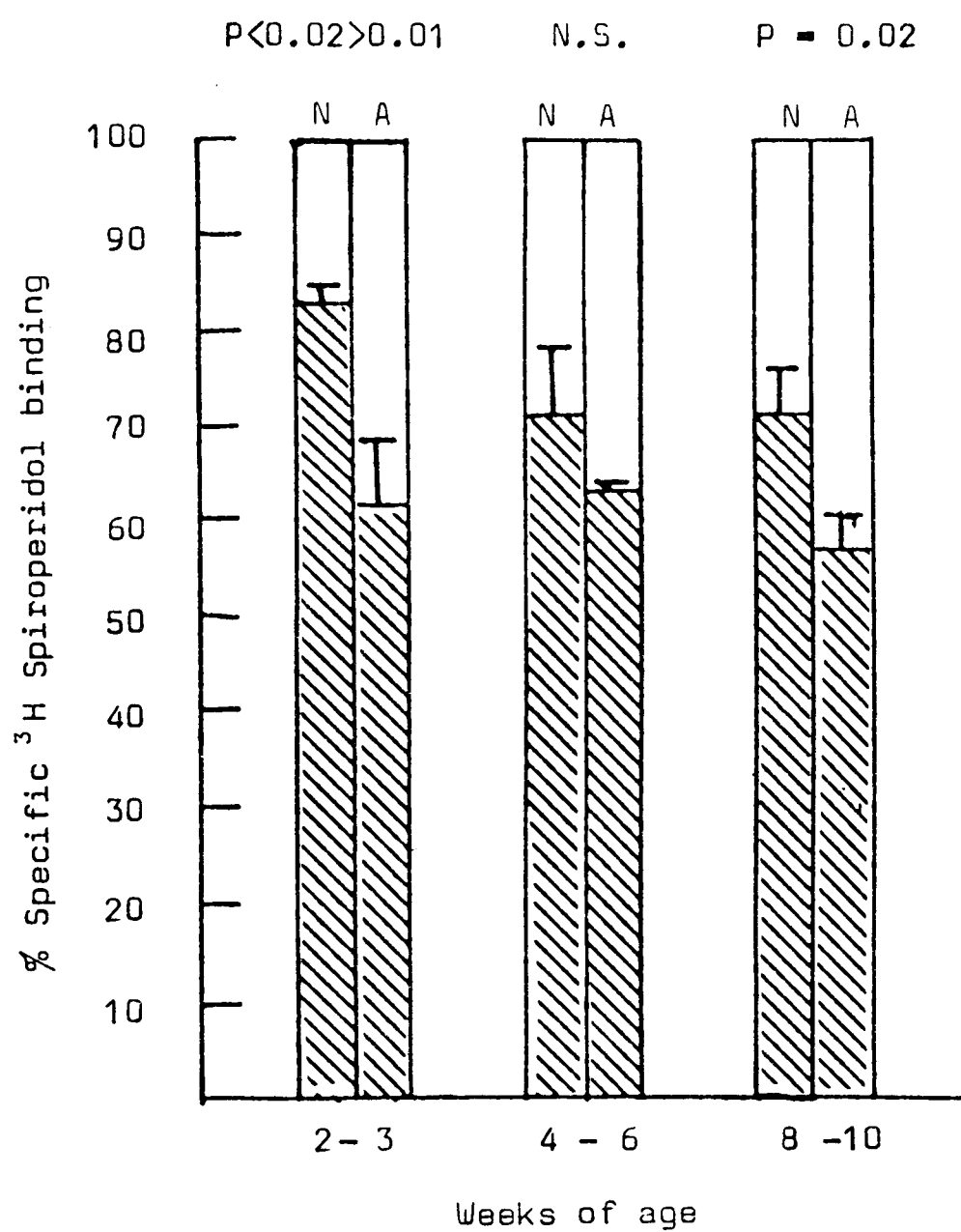


Fig. 5.

DOPAMINE RECEPTOR TYPE

N = NORMAL A = AFFECTED

 **D₁**
 **D₂**

Albino rats

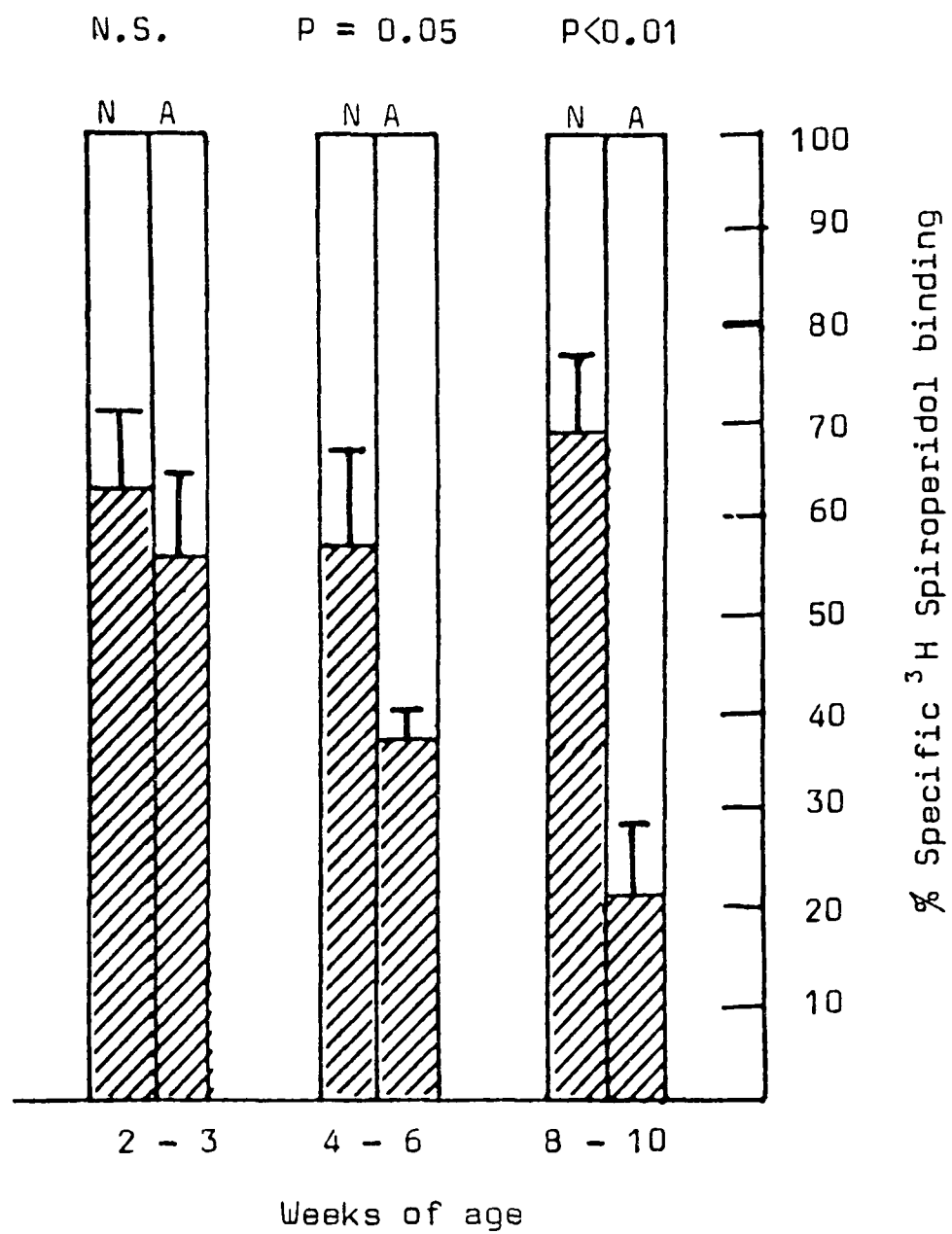


Fig. 5.

Appendix 6

ATPase and Phototransduction in Bovine Retina

Reading H.W.

ATPase and Phototransduction in Bovine Retina

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In dark-adapted vertebrate retina, an inwardly-directed Na^+ current exists across the plasma membrane of the rod outer segment (ROS); light absorption closes the Na^+ channels either by release of Ca^{2+} or calmodulin from the discs. (Hubbell and Bounds, 1979). How this inward Na^+ current is balanced electrochemically is undecided. One suggestion, that Na^+ is actively pumped out by the inner segments, is supported by the fact that metabolic poisons abolish the dark current (Hagins, 1972). The involvement of the sodium pump mechanism implies changes in such activity associated with light and dark adaptation to balance the 'switching' on and off of the Na^+ current. There are few reports of direct biochemical estimation of retinal ATPases under these conditions.

Retinae were carefully dissected from bullock eyes into ice-cold Krebs's bicarbonate medium, within one hour of slaughter. Retinal tissue was homogenised in 0.32M sucrose containing 0.1mM Mg^{2+} in a Teflon-glass homogeniser and then diluted with incubation medium, followed by centrifugation at 4°C (800g for 10 min.). The pellet was re-suspended in the original volume of buffer, and fractions called '800g supernatant' and 'pellet'. Examination of the fractions by light microscopy showed the 800g supernatant to consist mainly of detached photoreceptor cells in vesicular form, with only a few nuclei present. The pellet comprised the photoreceptor nuclei, bipolar and ganglion cells (inner layers). Light and dark adaptation was achieved by leaving portions of the preparations at room temperature for 2 hrs. either within 30cm of a double bank of 40 watt fluorescent white lamps or else in complete darkness in light-tight containers. Na^+K^+ -dependent ATPase activity

(E.C.3.6.1.3.) was estimated as the difference between specific activities estimated in the absence of ouabain ('total' ATPase) presence of 0.1mM ouabain ('ouabain-insensitive ATPase'). Endogenous ATPase activity (without added ATP.) was never more than 9% of maximal activity after addition of exogenous ATP.

The '800g pellet' suspension containing fragments of the inner retinal layers, showed significant differences in Na^+K^+ ATPase activity between light and dark adaptation conditions. In light, Na^+K^+ ATPase showed about twice the specific activity of that in dark. On the other hand, the '800g supernatant' fractions, containing the photoreceptor elements, showed little difference between light/dark conditions.

These results indicate that most of the retinal ATPase activity resides in the inner retinal layers, and that Na^+K^+ -dependent ATPase (ouabain-sensitive) is reduced in activity in the dark-adapted retina. On the other hand, ouabain-insensitive ATPases are not affected by changes in dark/light adaptation.

Radio-ligand binding assays using ^3H -labelled ouabain of high specific activity were carried out on the 800g supernatant and pellet fractions. Non-specific binding was determined by incubation with both ^3H ouabain and non-radioactive ouabain in large excess. Scatchard plots were constructed and values of K_D and B_{max} determined. The photoreceptor layer fractions gave values of K_D $4.67 \pm 1.23 \times 10^{-8}\text{M}$ and B_{max} $679 \pm 7\text{ f.mol mg}^{-1}$ and the inner retinal fractions, K_D $4.85 \pm 1.01 \times 10^{-8}\text{M}$ and B_{max} $3192 \pm 440\text{ f.mol mg}^{-1}$. The B_{max} differ significantly ($P = 0.001$). The kinetic constants for ouabain binding, therefore support the evidence that Na^+K^+ -dependent ATPase sites are five times denser in the inner than the photoreceptor layer. The dissociation constants are similar, and are obviously of a similar physical nature. These experiments, using radio-ligand binding and direct enzymatic assays, show that most of the Na^+K^+ ATPase activity resides in the inner retinal layers and not in the photoreceptors. In addition, Na^+K^+ ATPase activity has been shown to be affected by changes in light/dark adaptation. In the dark adapted state, Na^+K^+ ATPase activity is reduced. These changes in Na^+K^+ ATPase activity are the opposite to what would be expected if, in the dark adapted state, Na^+ was

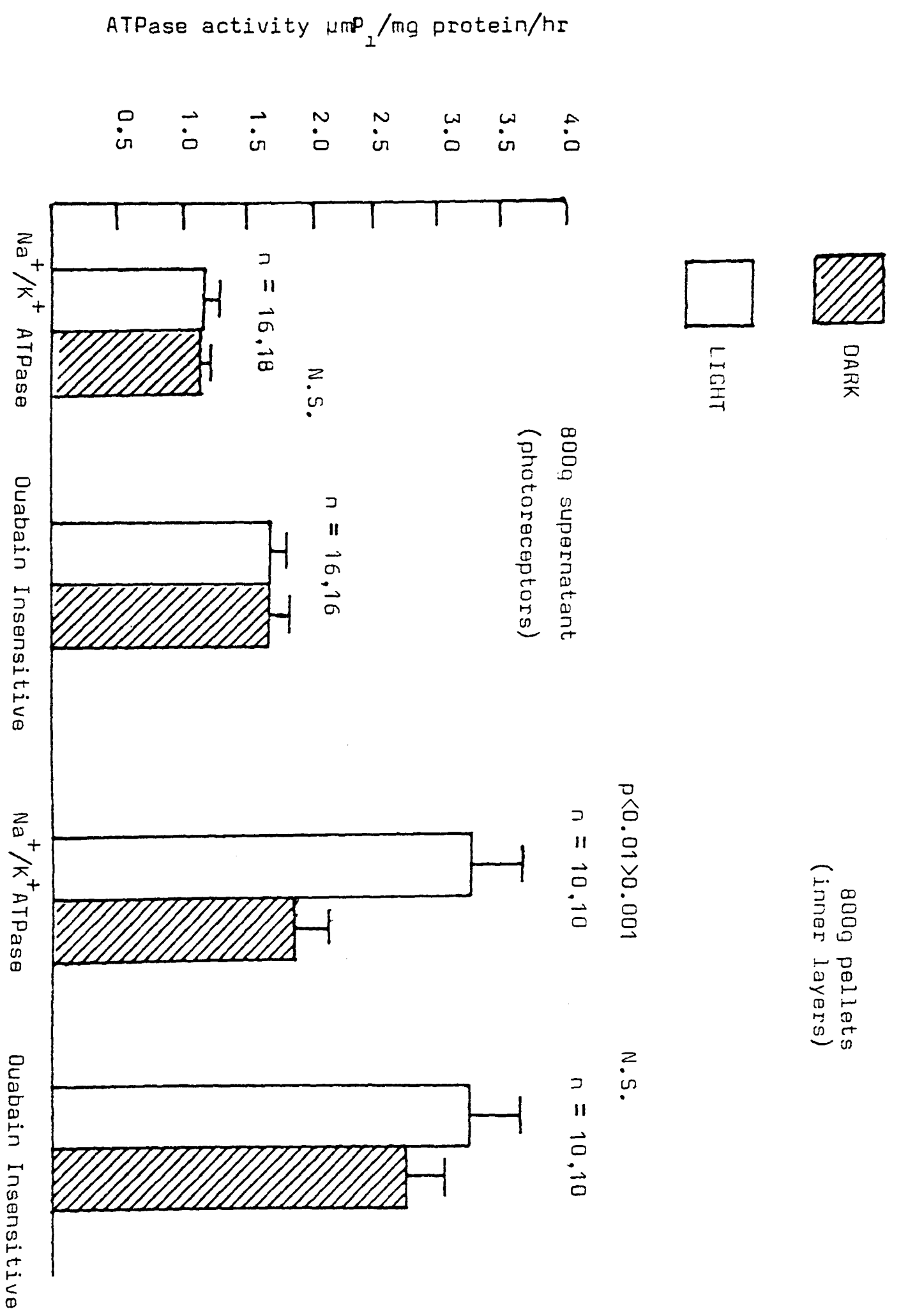
being actively pumped out of the inner layers, and in the light-adapted state this sodium pumping was switched off, in order to maintain electrochemical equilibrium of the photoreceptors.

REFERENCES

- Hagins, W.A., (1972). Ann. Rev. Biophys. Bioeng. 1 131-158.
- Hubbell, W.L. and Bounds, M.D., (1979). Ann. Rev. Neurosci. 2 17-34.
- Lebel, D., Poivier, G.G. and Beaudoin, A.R., (1978). Anat. Biochem. 85 86-89.

Fig. 1 Effect of light/dark adaptation on ATPase activities in
photoreceptor and inner retinal layer suspensions

Suspensions prepared as described in text and kept under light or dark conditions for 2 hrs. at room temperature. Suspensions (3ml aliquots) were incubated at 37°C with shaking. 'Dark-adapted' suspensions were kept in light-tight containers, and additives made through light-tight rubber closures. Ouabain (0.1mM) was added where necessary and incubation continued for 15 min. Reaction was commenced by adding ATP (3mM) and incubation continued for 60 min. The vials were then rapidly cooled in crushed ice, and 1.0ml aliquots added to 0.5ml 15% trichloroacetic acid. After centrifugation, phosphate estimations on clear supernatants were carried out by the method of Lebel (1976). Lowry protein estimations were done on separate aliquots.



Appendix 7

Publications

Speight, A., Popkin, R., Watts, A.G. & Fink, G. (1981)

Oestradiol-17 β increases pituitary responsiveness by a mechanism which involves the release and the priming effect of luteinizing hormone releasing factor.

J Endocr. 88, 301-308.

Watts, A.G. & Fink, G. (1981)

Constant light blocks diurnal but not pulsatile release of ovariectomized rat.

J Endocr. 89, 141-146.

Watts, A.G. & Fink, G. (1981)

Effects of short-term constant light on the pro-estrous luteinizing hormone surge and pituitary responsiveness in the female rat.

Neuroendocr. 33, 176-180.

