FUNCTIONAL ALTERATIONS OF THE PITUITARY - ADRENAL AXIS - MORPHOLOGICAL STUDIES IN THE RAT.

by

MAHIR ABDUL GHANI KUBBA ©

B.V.M.& S. (Baghdad), MSc. (Baghdad).

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University Department of Pathology,

Royal Infirmary,

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TO MY PARENTS AND FAMILY FOR

THEIR HELP AND SUPPORT.

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Presentations

McNicol, A.M. & Kubba, M.A. Ultrastructural changes in rat pituitary corticotrophs following long-term corticosterone or dexamethasone therapy.
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Papers in Preparation

McNicol, A.M. & Kubba, M.A. Inhibition of corticotroph function in rat - ultrastructural studies.

McNicol, A.M., Kubba, M.A. & McTeague, E. Quantitative morphological studies in the rat pituitary following bilateral adrenalectomy.

McNicol, A.M., Kubba, M.A. & McTeague, E. A morphological study of the effects of CRF on the pituitary - adrenal axis of the rat.

SUMMARY

The factors involved in the regulation of corticotroph growth and differentiation in the anterior pituitary gland are not well understood. However, alterations in the function of these cells are known to result in changes not only in the morphology of individual cells, but also in the size of the corticotroph population. In the studies included in this thesis specific manipulations of corticotroph function were performed in the Sprague-Dawley rat. Qualitative and quantitative assessment of the morphological changes occurring in corticotrophs was made at light microscopic and ultrastructural levels. Because of sex differences in the pituitary - adrenal axis in the rat, and to allow comparison with previous work in specific areas some of the studies have been performed in both sexes, others in only one.

In Chapter one, relevant aspects of the control of corticotroph function are reviewed, concentrating mainly on stimulation by corticotrophin - releasing factor (CRF) and vasopressin (VP) and inhibition by glucocorticoid negative feedback. Current knowledge on the factors controlling corticotroph growth and differentiation is also reviewed.

In Chapter two, the quantitative changes in the corticotroph population of the female rat were monitored at 2 and 6 weeks after bilateral adrenalectomy, using the stereological measurement, volume density (Vv). This showed a three-fold increase, which is greater than the two-fold increase previously reported in the male rat.

Daily administration of CRF to male rats produced a dose-dependent increase in corticotroph Vv, although this

was much lower than that achieved by adrenalectomy. VP produced a further increase in Vv when given with lower doses of CRF, but not with high dose (50µg/Kg).

In Chapter three, corticotroph Vv was examined following functional inhibition by the synthetic glucocorticoid, dexamethasone. Daily administration in doses up to 500µg/Kg for six weeks to female rats did not produce a significant fall in corticotroph Vv, despite apparent inhibition of adrenocorticotrophin (ACTH) release. This is discussed in the light of biochemical data on the action of dexamethasone. The effects of long-term (26 weeks) inhibition of function were difficult to interpret because of the non-specific catabolic effects of the steroid. These problems are discussed. In addition, following withdrawal of dexamethasone, the recovery of the axis was followed up to 14 days.

In Chapter four, qualitative ultrastructural changes were examined. The reported changes occurring in male rats were confirmed and extended to 6 weeks after adrenalectomy. The changes in females were documented for the first time, and as with the light microscopic studies suggested a more marked response than in the male.

The changes following dexamethasone administration were monitored, and showed a general increase in granulation of corticotrophs in both sexes. These findings were different from the changes seen with corticosterone, where there was mainly a decrease in granulation in the male rat, and an increase in the female. The possible reasons for these findings are discussed.

Quantitative analysis of cell area was performed in

stimulated corticotrophs, to assess the degree of hypertrophy. The results were in keeping with a greater response in the female than in the male, and with a role for CRF in the production of hypertrophy. The subjective assessment of an increase in granule size in stimulated corticotrophs was confirmed. In dexamethasone inhibited corticotrophs, a reduction in granule diameter was demonstrated in both sexes but only in the male after corticosterone.

In Chapter five, the mitotic activity was measured in the normal pituitary, and following bilateral adrenalectomy and CRF administration using a metaphase arrest technique. A significant circadian rhythm was demonstrated with a peak at 1100 and a trough at 2300. Following adrenalectomy, a significant increase in general mitotic index was demonstrated only at 7 days, although mitoses in corticotrophs were increased at 2 days. The reasons for this apparent delay are discussed. CRF induced a more marked general increase than adrenalectomy at 2 days and levels were still significantly increased by 7 days. This appeared to be due mainly to an increase in mitoses in a non-corticotroph population. These findings are discussed.

Chapter 6 consists of a general discussion of the results.

CHAPTER 1

INTRODUCTION

INTRODUCTION

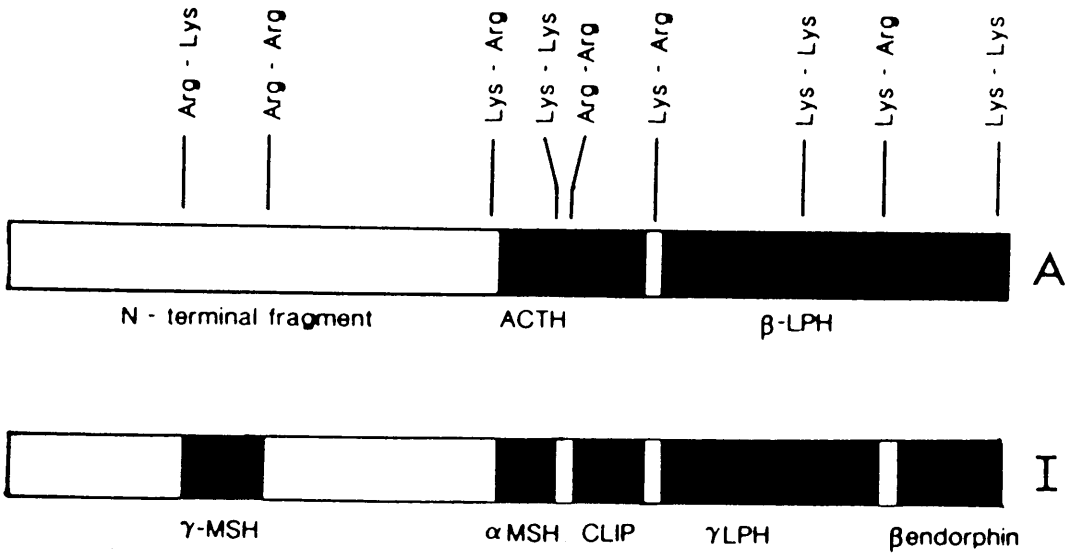
The corticotrophs of the anterior pituitary are the cells responsible for the synthesis and secretion of adrenocorticotrophic hormone (ACTH). The complex control of function of these cells has been investigated in some depth, but the factors involved in the regulation of corticotroph growth and differentiation, particularly in the adult gland, are poorly understood. There is evidence that alterations in the function of these cells results not only in changes in their morphological appearance, but also in the size of the corticotroph population within the gland. The studies contained in this thesis were designed to examine in more detail the morphological effects of altered corticotroph function in the adult Sprague-Dawley rat. The introduction, therefore, will outline the relevant aspects of the control of corticotroph function, and current knowledge on the factors controlling growth and differentiation of these cells.

Stimulation of Corticotroph Function

ACTH is the peptide primarily responsible for the stimulation of glucocorticoid production by the adrenal cortex, and is derived by the enzymatic cleavage of a 31 kilodalton precursor molecule, pro-opiomelanocortin (POMC) (Fig.1:1) (Nakanishi et al, 1979). In addition to ACTH, several other peptides are produced from POMC, some of which have been shown to act on the adrenal cortex, stimulating either steroidogenesis (Pedersen et al, 1980) or adrenal growth (Estivariz et al, 1980). The stimulation of the anterior pituitary corticotroph is under complex control, (Fig.1:2) several factors including cortico-

Figure. 1:1

Pro-opiomelanocortin processing. The 31K precursor molecule is cleaved to produce various smaller peptides. The signal peptide is required for access to the cisternae of the rough endoplasmic reticulum. Enzymatic cleavage occurs at the sites of paired basic amino-acid residues as shown. In the rat anterior lobe, (A) the main products are ACTH and β lipotropin, (BLPH), whereas in the intermediate lobe, (I) further processing occurs to produce α-MSH, corticotrophin-like intermediate lobe peptide (CLIP) and β endorphin.
γ-MSH has been identified in both lobes.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CLIP
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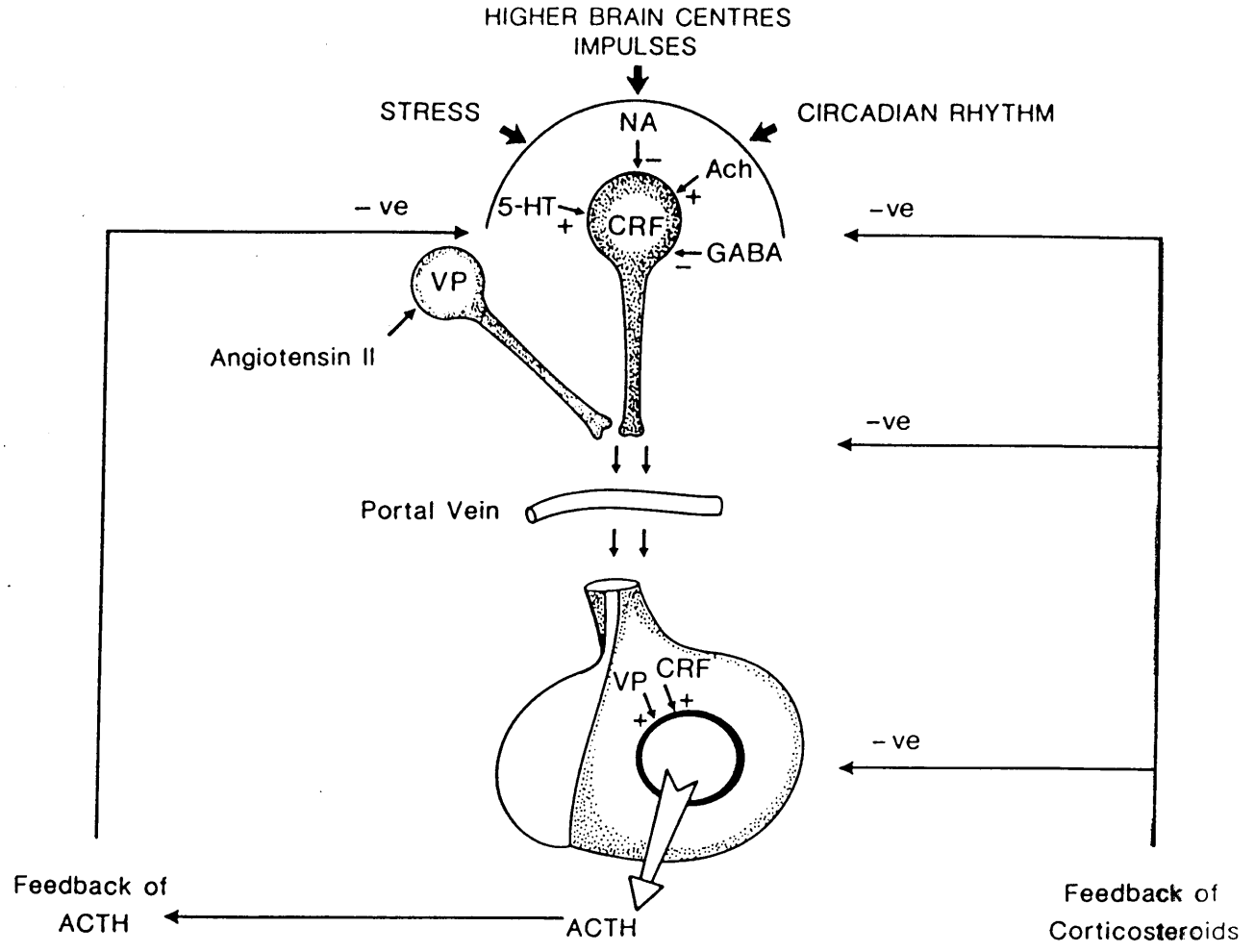
Figure. 1:2



Diagrammatic representation of the control mechanisms for ACTH release from the anterior pituitary gland. 5-Hydroxytryptamine, (5HT) Noradrenaline, (NA) Acetylcholine, (Ach) Gamma amino butyric acid, (GABA) Vasopressin, (VP) Corticotropin-releasing factor, (CRF).

trophin-releasing factor (CRF) vasopressin (VP) and a variety of neurotransmitters having been demonstrated to increase ACTH release either *in vivo* or *in vitro*.

Corticotrophin-Releasing Factor

Although the hormone CRF has only recently been characterised as a 41-amino acid peptide produced in the hypothalamus (Vale et al, 1981) the existence of such a factor was originally hypothesized by Harris in 1948. Since then ACTH-releasing properties have been demonstrated in various tissues including the brain, muscles, kidney, liver, small intestine, pancreas and adrenals (Yasuda and Greer, 1976; Kawaia et al, 1985). Using a variety of *in vivo* and *in vitro* assays, the majority of studies however clearly established highest activity in the hypothalamus (Yasuda, Greer and Aizawa, 1982). CRF-like activity is higher in the median eminence and pituitary stalk than in other parts of the hypothalamus or CNS in rats and there is evidence that female animals have higher levels than males (Krieger, Liotta and Brownstein, 1977). Conflicting data exist concerning alterations in hypothalamic CRF-like activity following manipulation of the HPA axis, with evidence of increased content (Vernikos-Dannellis, 1965; Hiroshige et al, 1969; Sato et al, 1975) or no change (Sirett and Purves, 1973) following acute stress. Similarly an increase has been demonstrated following adrenalectomy in some studies (Vernikos-Danellis, 1965; Buckingham, 1979) but not in others (Hillhouse and Jones, 1976; Yasuda and Greer, 1976).

It has now been established that the major component of the corticotroph stimulating activity of the hypothalamus is the 41 amino acid peptide characterised by Vale's group. This peptide was first isolated from the ovine hypothalamus, (Spiess et al, 1981) and similar peptides have since been characterized in man (Shibahara et al, 1983) and rat (Rivier, Spiess and Vale, 1983).

CRF has been shown to stimulate ACTH secretion in a dose-dependent manner both in vitro (Vale et al, 1983b; Widmaier and Dallman, 1984) and in vivo (Rivier and Vale, 1983) acting via cyclic AMP and protein kinase (Labrie et al, 1982; Vale et al, 1983b). CRF-receptors have been demonstrated on pituitary corticotrophs, (De Souza et al, 1984) and binding and internalisation of the peptide by corticotrophs has been demonstrated by autoradiography (Leroux and Pelletier, 1984). Some desensitisation of corticotroph response to CRF can be demonstrated with continued stimulation both in vitro (Reisine and Hoffman, 1983) and in vivo (Rivier and Vale, 1985) and this may be related to down-regulation of receptors. It is nevertheless not associated with either a reduced ACTH content in corticotrophs, or with normalisation of circulating ACTH or corticosterone levels (Vale et al, 1983). The stimulatory effect of CRF on the corticotrophs can be inhibited by glucocorticoid pre-treatment. It has been suggested that this is mediated either by a reduction in intracellular cAMP concentration (Vale et al, 1982a) or by suppression of cyclic AMP generating steps (Bilezikjian and Vale, 1983). However, there is evidence that CRF and glucocorticoids exert their effect at different sites, since even in the

presence of maximum inhibition of corticotroph function by dexamethasone *in vitro*, high doses of CRF are still able to stimulate ACTH release (Vale et al, 1983b).

CRF is produced mainly in the paraventricular (PVN) (Antoni et al, 1983) and, to a lesser extent, the supra-optic (SON) nuclei of the hypothalamus (Bloom et al, 1982). CRF containing nerve fibres arise predominantly in the parvocellular parts of the PVN (Swanson et al, 1983) and are concentrated in the external medial part of the median eminence, with few fibres in the lateral areas (Kawata et al, 1982). It is of interest that the PVN and SON are the nuclei which also produce VP (see below) and that the two peptides may be identified not only in the same neurone, but even in the same neurosecretory granule (Whitnall et al, 1985). CRF-containing neurones have also been identified in the pituitary stalk and posterior lobe in several species including the pig (Kawata et al, 1982b) and man (Nieuwenhuijsen-Kruseman et al, 1984; Coates, 1985) although the role of the peptide in these areas is unclear, since little evidence exists for significant transport of factors from the posterior to the anterior lobe of the gland.

Recent studies based on specific radioimmunoassay or gene probe analysis have demonstrated specific increases in hypothalamic CRF (or its precursor) following adrenalectomy (Moldow and Fischman, 1982; Suda et al, 1983; Jingami et al, 1985) and this is accompanied by an increase in the number of CRF immunopositive cell bodies in the para-ventricular nucleus (Antoni et al, 1983; Swanson et al, 1983; Merchenthaler et al, 1984), thus confirming the role

of these CRF producing pathways in the control of ACTH synthesis and release.

CRF immunoreactive neurones have been identified in other parts of the brain (Merchenthaler et al, 1982; Schipper et al, 1983) and in various peripheral tissues (Niewenhuijzen-Kruseman et al, 1984; Suda et al, 1984). It is assumed that since CRF receptors may also be identified in these areas (Udelsman et al, 1986) its effects are neurocrine or paracrine, with no relevance to the physiological control of ACTH secretion, but it may account for the ACTH-releasing abilities of extracts of these tissues as discussed above.

Vasopressin

VP has been demonstrated by many groups to stimulate ACTH secretion in vivo (Yasuda et al, 1978; Aizawa et al, 1982) but Saffran and Saffran (1959) showed that the dose of exogenous vasopressin required to stimulate such release was greater than that required for antidiuretic activity and this has been confirmed (Krieger, 1977). This raised doubts as to the physiological significance of its ACTH-releasing role. However, VP concentration in the median eminence was shown to rise after adrenalectomy and to decrease following glucocorticoid administration (Watkins et al, 1974; Zimmerman et al, 1977). In addition, the levels of VP in the pituitary portal blood were significantly greater than in the systemic circulation (Zimmerman et al, 1973), and were similar to those achieved by the exogenous administration of the peptide in doses which stimulate ACTH secretion. This suggests that it does, in fact, have a physiological role in the control of

corticotroph function. In support of this is the fact that the Brattleboro rat, which is genetically deficient in hypothalamic vasopressin shows impairment of some aspects of ACTH release (McCann et al, 1966; Buckingham and Leach, 1980) and that the ACTH releasing ability of its hypothalamus may be restored by the addition of VP (Buckingham, 1981). Recent studies both in vitro (Vale et al, 1983b) and in vivo (Rivier and Vale, 1983a) have indeed demonstrated that it acts synergistically with CRF in stimulating ACTH release, although on its own, its ACTH releasing capacity is relatively low. VP appears to bind to a specific receptor (Castel, 1978; Baertschi and Friedli, 1985) but does not act via cAMP (Giguère and Labrie, 1982).

There have been suggestions that VP also acts centrally to regulate ACTH secretion by facilitating the release of CRF (Hedge et al, 1966). In contrast, others have proposed that it has a central inhibitory role (van Dijk et al, 1981; Plotsky, Bruhn and Vale, 1984). There is evidence for direct vasopressinergic innervation of CRF-containing cells in the paraventricular nucleus (Leranth, Antoni and Palkovits, 1983) but the exact role for such a tract is unclear at present.

Vasopressin is produced in both the supraoptic and paraventricular nuclei (Dornhorst et al, 1981). The evidence is in favour of the latter being more important in the control of ACTH secretion, there being a tract running from it to the median eminence, which shows changes in VP concentration in states of altered HPA function (Silverman and Zimmerman, 1982).

It is of interest that Whitnall et al (1985) showed that following adrenalectomy the numbers of neurones in the median eminence showing co-localisation of VP and CRF (discussed above) increased from 44% to 100% again supporting the co-ordinate role of these two peptides in stimulating ACTH release.

Other Stimulating Factors

Minor stimulatory effects on ACTH release at pituitary level have been demonstrated for angiotensin II (Spinedi and Negro-Vilar, 1983) and C-terminal gastrin-releasing peptide (Hale et al, 1984). Serotonin has also been shown to stimulate ACTH release, although the site of action is uncertain, with some evidence supporting a direct pituitary action (Winkelmann et al, 1983) while other data suggest a more central action (Gibbs and Vale, 1983). A direct stimulatory effect of catecholamines has also been demonstrated (Giguère, Côté and Labrie, 1982), while acetylcholine appears to act centrally (reviewed in Jones, 1979).

Inhibition of Corticotroph Function

Glucocorticoid Negative Feedback

It is known that an increase in levels of circulating glucocorticoids exerts complex negative feedback on the HPA axis to inhibit further secretion. This is effected at several sites within the axis, including CNS, hypothalamus and pituitary gland. The mode of feedback is biphasic, with fast and delayed feedback components, the first occurring immediately (1-5 minutes) after steroid administration while plasma glucocorticoids are still

increasing. The second occurs 1-2 hours later after plasma levels have reached a plateau or have decreased (reviewed in Keller-Wood and Dallman, 1984).

The existence of fast-feedback was first postulated by Dallman and Yates (1969) who demonstrated that the injection of corticosteroids into rats inhibited the corticosterone response to histamine administration if the injection preceded the histamine by up to 5 minutes but not if it was given 15 minutes before or 2 minutes after it. Jones, Brush and Neame (1972) showed that this form of feedback is dependent on the rate of increase in plasma glucocorticoids in that it was linearly related to the rate of increase in plasma corticosteroid concentration. They also showed that a minimum rate of increase of plasma corticosterone of 1.3µg/100ml/minute was required in the male rat for fast feedback to occur. The rate of increase required to effect feedback is higher in female rats (Abe and Critchlow, 1977) which might be attributed to the proportionately lower free corticosterone concentrations and higher clearance rate of corticosterone in female rats.

Although the exact mechanism of this type of feedback is uncertain, it has been shown to have a rapid effect on both stimulated ACTH-releasing potency of hypothalamic extracts (Vermes, Mulder and Smelik, 1977) and stimulated ACTH secretion (Vale and Rivier, 1977; Buckingham and Hodges, 1977). The rapidity of the effect suggests a primary action on hormone release rather than synthesis. This is supported by the demonstration of an increase in hypothalamic CRF content and inhibition of the release of bioactive CRF (Jones and Hillhouse, 1976; Jones, Hillhouse and Burden 1977).

The delayed feedback effect of corticosteroids which requires between 45 and 120 minutes to develop (Dallman and Yates, 1969) appears to be dependent on the maximum level of steroid achieved, the interval since administration and the total dose of steroid administered. The period of inhibition is extremely prolonged after large doses of steroids and repeated administration (Jones et al, 1974). Such treatment has been shown to decrease pituitary sensitivity to CRF preparations (Rochefort, Rosenberger and Saffran, 1959) and pituitary content of ACTH (Fortier, 1959b).

Recent evidence has been presented that in the rat two types of delayed feedback exist (Keller-Wood and Dallman, 1984), an "intermediate type" which operates 2-10 hours after exposure to corticosterone and is of relatively short duration and a slow feedback which is associated with constant administration of corticosterone for 12 hours or more. Both types of delayed feedback depend on the total dose of steroid administered over the time. However, intermediate feedback appears after a relatively short duration of corticosteroid exposure or after repeated but discontinuous increases in circulating plasma corticoids whereas slow feedback appears after prolonged periods of high plasma concentrations of corticosteroids. Such feedback is thought to inhibit both ACTH release (Engeland, Shinsako and Dallman, 1975) and ACTH synthesis (Roberts et al, 1979) via a reduction in POMC messenger RNA (mRNA) (Nakanishi et al, 1977; Schachter et al, 1982). There is evidence however, that short periods ($<$4 hours) of exposure to high levels of corticosterone may not be sufficient to

cause a subsequent decrease in ACTH synthesis (Stark et al, 1962).

It is understood that fast and intermediate feedback occur in physiological conditions when plasma corticosterone increases in response to moderate stress. Delayed feedback, however, is thought to be associated with pathological conditions in which increased steroid levels are present (e.g. Cushing's syndrome) or in cases of prolonged steroid administration for therapeutic purposes.

It is also of interest that glucocorticoids may vary in their potency to exert various forms of feedback. It appears that this is related to their structure, both a 21-hydroxyl and 11-β-hydroxyl group being required for fast feedback whereas the presence of either hydroxyl group is sufficient for delayed feedback (Jones et al, 1974; Jones and Hillhouse, 1976; Jones and Tiptaft, 1977). This would suggest that different receptors may be involved in the two types of feedback. Glucocorticoid feedback action is effected at various sites along the HPA as well as in higher centres within CNS. The involvement of the pituitary gland, the hypothalamus and the limbic system of the brain has been examined in a large number of studies (reviewed in Keller-wood and Dallman, 1984) which suggest that the corticotrophs, the CRF neurones, hypothalamic neurotransmitter efferent neurones and other more central neuronal components of the axis may all co-operate in the control of the pituitary-adrenal response to glucocorticoids. These observations are further supported by the demonstration of glucocorticoids binding sites in the pituitary (Koch et al, 1978a), the hypothalamus

(Warembourg, 1975) and the limbic system (hippocampus) (McEwen, Magnus and Wallach 1972; McEwan and Wallach, 1973). However since steroids also produce behavioural changes, it is probable that not all central binding sites have a role in the control of ACTH secretion.

There is also evidence that a range of different glucocorticoid receptors exists, and that their distribution varies in different tissues. Firstly there is the classical glucocorticoid receptor (McEwen et al, 1972) which is present in many tissues. In addition, a trans-cortin-like receptor has been identified in a range of tissues, including pituitary. This appears to be in association with cell membranes in biochemical studies (de Kloet, Burback and Mulder, 1977; Koch et al, 1978b) and has recently been demonstrated by immunohistochemistry in the rat pituitary (de Kloet et al, 1984). An additional high affinity cytoplasmic receptor has also been demonstrated in pituitary (Krozowski and Funder, 1982). There are differences in the binding of specific steroids to these various receptors, and this may account, at least in part, for differences in their activity.

ACTH Negative Feedback

Whether there is physiological short-loop negative feedback of ACTH on the hypothalamus is uncertain. The demonstration of retrograde blood flow in the long pituitary vessels (Bergland and Page, 1978) and high levels of ACTH in these vessels (Oliver, Mical and Porter, 1977) would however support the data of Takebe, Sakakura and Brodish (1973) who suggested that it does operate.

Circadian Rhythm of HPA Axis

Circadian rhythmicity of the various hormones of the HPA is well established in both man (Krieger et al, 1971) and rat (Guillemin, Dear and Liebelt, 1959) with peak plasma corticosteroid levels occurring around the time of waking i.e. in the early morning in man and in the early evening in the nocturnally active rat. The primary site of control is not certain, but lesioning experiments suggest a central role for the suprachiasmatic nucleus (Moore and Eichler, 1972) and Krieger (1979) has suggested that a certain degree of neuronal maturation is necessary before such periodicity is established, since it appears to be age-related.

However, circadian rhythmicity has also been demonstrated in adrenal sensitivity to ACTH both in vivo (Krieger, 1977) and in vitro (Ungar and Halberg, 1962) and the former study also documented a circadian rhythm in cortisol metabolic clearance rate. In addition circadian variation has been reported in glucocorticoid output in hypophysectomised animals (Meier, 1976; Ottenweller and Meier, 1982) suggesting that there is, in addition to the effects of ACTH, some extra-pituitary regulation of adrenal rhythm. The study of Ottenweller and Meier (1982) suggested that this might be mediated by adrenal innervation, since disruption of adrenal nerves or atropine injection abolished such rhythmicity. However, others (Wilkinson, Shinsako and Dallman, 1981) have been unable to confirm such a role, and this would be supported by the demonstration of rhythmic secretion of corticosterone from adrenal cells in culture (Andrews, 1968).

Sex Differences in the Rat HPA Axis

Female rats have higher basal plasma corticosterone levels than males (Kitay, 1961) but much of this is buffered by the higher transcortin levels. As outlined above, negative feedback appears to operate at a higher level in the female due partly to the plasma buffering capacity and also probably to higher intrapituitary buffering related to the greater numbers of transcortin-like receptors in the female gland (Sakly and Koch, 1981). The adrenal glands of female rats are heavier than those of male animals and this may also reflect the greater steroid output.

Control of Corticotroph Growth

Corticotrophs are the first hormone-producing cells to develop in the anterior lobe of the pituitary gland being seen on day 13-16 in the rat (Setalo and Nakane, 1972). The factors controlling this are uncertain, but it has been suggested that locally-produced mesenchymal factors may be important (Svalander, 1974; Watanabe and Daikoku, 1979). A second possibility is that blood-borne factors, presumably of hypothalamic origin, are important and that these are transported in the blood vessels which grow in with the remainder of the mesenchyme. The work of Fink and Smith (1971) suggests that such factors are necessary for the normal growth and differentiation of these cells, although the exact nature of the stimuli is unclear. CRF cannot be important, at least in the early stages, since it is not identified in hypothalamic neurones until day 18 (Bugnon et al, 1982) after the initial development of the corticotrophs. In addition, important species variation may exist

in the role of these various factors. In the anencephalic human foetus, the presence of corticotrophs suggests that hypothalamic factors do not influence their early development in man. However, they are reduced in number (Osamura, 1977) and pituitary ACTH content is low (Pavlova et al, 1968) which would be in keeping with a role for these factors in the further development and differentiation of these cells.

Detailed studies on the post-natal growth of the corticotroph population have not been carried out, but the overall growth pattern of adenohypophyseal cells demonstrated by Friend (1979) have suggested that these cells undergo significant hyperplasia during the first 10 days of life. In that study the intensity of cellular multiplication was shown to diminish in the period between 10-25 days with a shift to cellular hypertrophy accounting for further increases in pituitary weight. However, in another study, the peak mitotic activity of gland has been demonstrated at 30 days (Shirasawa and Yoshimura, 1982) declining thereafter. These workers also reported that mitotic rate in different hormone producing cells peaked at different times, corticotrophs showing a maximal proliferation at day 5. The early post-natal development of corticotrophs appears to result in the formation of clusters which then break down and the small cells disperse through the gland, perhaps indicating a stem-cell population.

In the mature animal at least, there also appears to be a circadian rhythm of mitotic activity (Noüet and Kujas, 1975) with two peaks reported at 0600 and 0800. While this

might indicate some relationship to the rhythmic activity of hypothalamic stimulation, it should be noted that many tissues with no such control (e.g. skin) also show a circadian rhythm of mitoses (Scheving and Pauly, 1967). The effects of hypothalamic stimulation on corticotroph growth are again incompletely documented. Indirect induction of stimulation by abolishing glucocorticoid negative feedback is known to induce striking morphological changes. Adrenalectomy has been demonstrated to induce hypertrophic changes in anterior pituitary corticotrophs in many investigations (Siperstein and Miller, 1970; Moriarty and Halmi, 1972; Caselitz and Saeger, 1979). However, data on changes in the mitotic activity have been conflicting. Tritiated thymidine administration demonstrated no change one month after adrenalectomy (Gosbee et al, 1970) and a significant increase (Nakane, Sétálo and Mazurkiewicz, 1977) in labelling index in sections of the pars distalis of rats six days after adrenalectomy. In vitro studies supported the latter results (Rappay and Makara, 1981) by demonstrating a labelling index of 3.56% in pituitary cell suspensions from adrenalectomised rats compared with 2.22% in controls. Baker and Drummond (1972) on the other hand, noted hypertrophic changes and an increase in corticotroph numbers but dividing cells were not found. Hypertrophy of corticotrophs has been suggested to be the first step in cellular reaction to adrenalectomy, reflecting the higher synthesis of hormones at the level of the single cell, while prolonged stimulation elicits hyperplasia.

Similar changes, though less prominent, have also been demonstrated in corticotroph growth following long term

application of pharmacological inhibitors of glucocorticoid synthesis such as metyrapone (Caselitz and Saeger, 1979) and aminoglutethimide (Caselitz and Saeger, 1979; Zak et al, 1985). However, no data exist on the specific proliferative response to such treatment. The role of individual hypothalamic factors is even less well known. Westlund et al (1984) using a biotin-conjugated CRF analogue noticed rapid internalisation of CRF by these cells coupled with the mobilisation of ACTH stores and the formation of cellular processes. The increase in corticotroph cell area after such treatment has been quantified in another study (Westlund, Aguilera and Childs, 1985) which showed that in CRF treated animals it was twice that of controls. These changes were dose dependent, indicating that the hypertrophy was indeed a direct effect of CRF, and McNicol (1985) has demonstrated that such a dose dependent effect can be seen with more long term administration of CRF. However, again no data exists on the proliferative effects of this peptide.

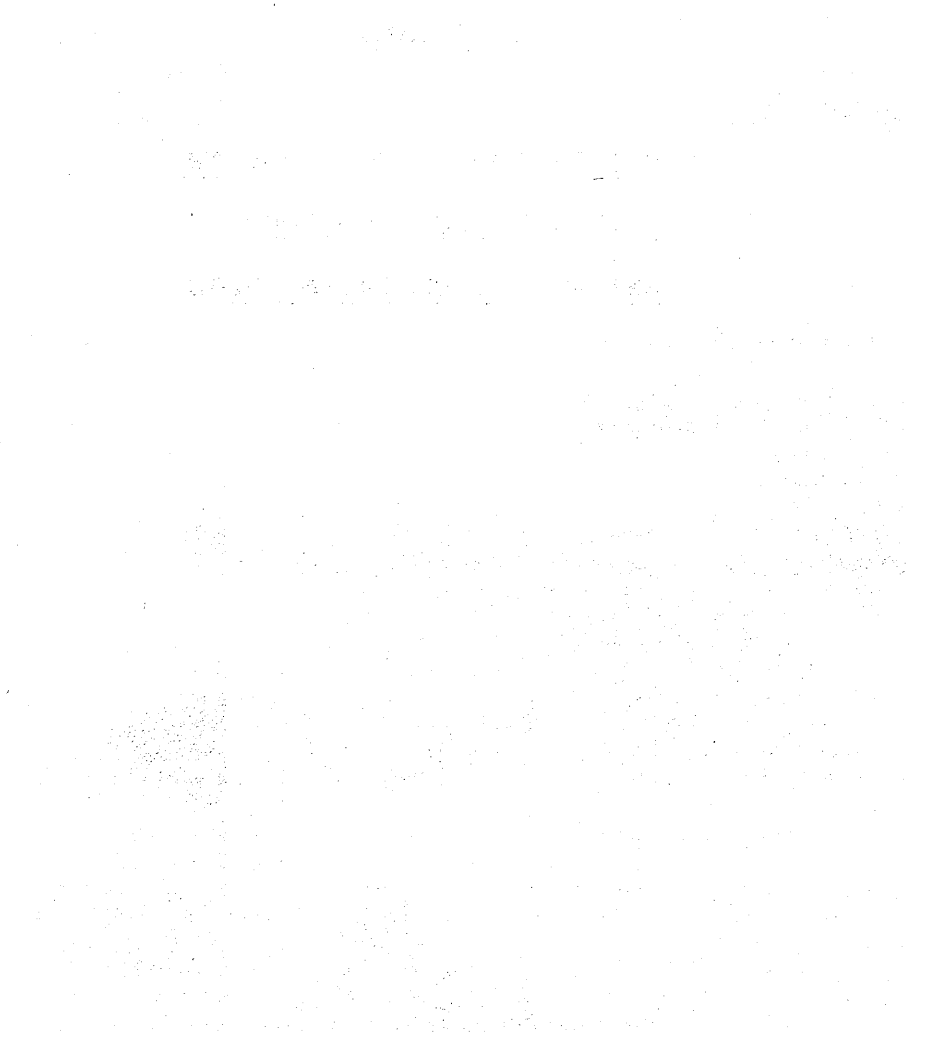
Vasopressin administration has also been shown to increase the corticotroph population of the anterior pituitary (Caselitz and Saeger 1979; McNicol, 1985) but again its mode of action is unclear although it has been shown to have a mitogenic action in a range of other tissues (Miller et al, 1977; Rozengurt, Legg and Pettican 1979).

Changes in the size of the corticotroph population have also been demonstrated in certain diseases associated with abnormal ACTH secretion. The variation in pituitary morphology in Cushing's disease demonstrated in McNicol's

study (1981) was thought to be consistent with a variety of pathogenetic mechanisms, one suggestion being that cases associated with corticotroph hyperplasia were the result of increased hypothalamic stimulation. This has not been confirmed functionally as yet but it is known that in Addison's disease, where increased hypothalamic stimulation is present, corticotroph hyperplasia does occur (Scheithauer, Kovacs and Randall, 1983). Which hypothalamic factors are important in these situations has not been investigated, but corticotroph hyperplasia has recently been reported as the result of a CRF producing tumour of prostate (Carey et al, 1984) suggesting that, as in the experimental models, it plays some part in the regulation of corticotroph growth in man. Steroid feedback has been reported to induce variable degrees of atrophy in corticotrophs. Siperstein and Miller (1970) noticed a slight reduction in the size of ACTH cells after acute cortisol administration (14 hrs), while longer application (7 days) induced remarkable atrophic changes along with a significant reduction in their number. Similiar inhibitory effects were demonstrated by Bowie et al (1973) who noticed a marked reduction in the size of corticotrophs after corticosterone administration for 19 days. Methylprednisolone has also been reported to induce corticotroph atrophy in the rat (Caselitz and Saeger, 1979).

In addition, glucocorticoids were reported to inhibit cellular growth response to adrenalectomy (Siperstein and Miller, 1970; Moriarty and Halmi, 1972) or aminoglutethimide administration (Zak et al, 1985). A reduction in corticotrophs has also been demonstrated in association

with increased negative feedback in man particularly in the context of Cushing's syndrome with an ACTH-secreting adenoma in the pituitary gland (Tyrrell et al, 1978; Schnall et al, 1980; McNicol, 1981). Whether the exogenous administration of steroids produces a similar result is unclear although Phifer, Spicer and Orth (1970) suggested that it does occur.



CHAPTER 2

STIMULATION OF CORTICOTROPH FUNCTION - QUANTITATIVE LIGHT MICROSCOPIC STUDIES.

INTRODUCTION

As discussed in Chapter 1, the factors controlling the size of the anterior pituitary corticotroph population in the adult rat are unclear, and, in particular the specific role of hormonal factors altering function has been incompletely assessed.

Bilateral adrenalectomy removes negative feedback from the HPA axis and results in an increase in the synthesis and secretion of ACTH. There is an increase in pituitary ACTH content (Gemzell et al, 1951; Fortier, 1959a; Kraicer, Herlant and Duclos, 1967) which has been reported to be accompanied by corticotroph hypertrophy and hyperplasia (Moriarty and Halmi, 1972; Caselitz and Saeger, 1979). Quantitative assessment of these changes has been performed on few occasions, usually based on total cell counts (Kraicer, Gosbee and Bencosme, 1973; Rappay and Makara, 1981) and few studies have examined the time course of such changes. McNicol (1985) assessed the use of the stereological measurement, volume density (Vv) (Anderson and Dunnill, 1965) as an estimate of the corticotroph population in the normal adult Sprague-Dawley rat pituitary and then applied this method to measure the changes occurring at 2 and 6 weeks after adrenalectomy in the male rat. In view of the differences in the male and female HPA axis discussed in Chapter 1, the present studies set out to examine the changes occurring in the female rat at similar time intervals.

Although the role of individual hypothalamic stimulating factors in the genesis of the above changes is unclear, CRF appears to cause an early hypertrophy of

corticotrophs (Westlund et al, 1984) and to result in a dose-dependent increase in corticotroph Vv in the male rat (McNicol, Kubba and McTeague, submitted for publication). In view of the synergistic effect of VP and CRF on corticotroph function, a small pilot study was also performed to examine whether the chronic administration of a combination of these two peptides produced a greater change in corticotroph Vv than CRF alone. This was studied only in male rats.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 175-250gm at the start of the experiments and females weighing 150-180gm were used. Animals were assigned to control and treatment groups on a random basis and were housed 3-4 rats in each cage, in a constant temperature (21°C) and light (0800-2000) controlled environment. The rats were allowed to acclimatise for at least 7 days before the start of an experiment. Their weights were checked at weekly intervals. Animals were housed in separate cages 24 hours before they were sacrificed by decapitation within seconds of removal from their cages. Extreme care was taken in handling at this point to minimise stress. All animals were sacrificed between 1000 and 1130.

Adrenalectomy

Female rats (6 or 7/group) underwent bilateral adrenalectomy by the dorsal approach under ether anaesthesia. Control animals were sham operated with skin and muscle

incision and handling of the glands. Adrenalectomised animals received 0.9% saline as drinking water post-adrenalectomy. Animals were sacrificed at 2 and 6 weeks after surgery. At the time of sacrifice, the abdomen was examined for any signs of regenerating or ectopic adrenal tissue, as such animals would be discarded from the analysis.

CRF

Male rats (9/group) received synthetic ovine CRF (Sigma) in acid saline by daily intraperitoneal (i.p.) injection at a dose of 25 or 50µg/Kg body weight for 2 weeks. Two further groups (6-8/group) received 0.4 IU/Kg BW lysine vasopressin (VP) (Sigma) in addition to CRF. Control animals received acid saline.

Tissue Preparation

The adrenal glands were cleared of fat, weighed in pairs (to the nearest mg) and fixed in 10% buffered formalin, embedded in paraffin wax and 5µm sections were cut through the cortex and medulla and stained with haematoxylin and eosin (H & E). Pituitary glands comprising anterior and neurointermediate lobes were dissected, weighed (to 0.1mg) and fixed in Bouin's solution for 24 hours. In some cases, a piece of the anterior lobe was sampled for E.M. processing (see Chapter 4). Horizontal sections containing the three lobes were prepared in the same way as for the adrenal glands.

Immunocytochemistry

Sections from several levels through the pituitary gland were immunostained by a modification of the per-

oxidase-antiperoxidase method (PAP) of Sternberger et al (1970) using a primary antibody raised in rabbit to synthetic ACTH 1-24 (Synacthen, Ciba, Basle, Switzerland).

Quantitation

Quantitative assessment of the adenohypophyseal corticotroph population was made by point-counting. Based on an analysis of a pilot study a total of 4-5000 points per gland was counted from 6-8 sections sampled through the gland, using a 25 point ocular grid at X400 magnification. Points falling on immunostained cells and on nuclei of cells whose cytoplasm was stained were counted as positive. Volume density (Vv) of corticotrophs was calculated as the number of points falling on immunopositive cells divided by the total number of points counted and was expressed as a percentage.

RESULTS

(i) Adrenalectomy

Body weight. Adrenalectomised rats lost weight (-2%) at 2 weeks compared to an increase of 5.6% in controls. In the 6 weeks group, however, although the final weights were lower in test animals, the rise after the third week paralleled that of the sham group.

Pituitary weight. Pituitary weights were heavier in test animals at 2 (13.0 ± 0.6 vs 11.7 ± 1.1mg, (mean ± SEM)) and 6 (14.0 ± 0.5 vs 11.6 ± 0.8mg) weeks. This achieved significance ($p < 0.05$) only at 6 weeks.

Volume density (Vv) of corticotrophs. Results are shown in Fig.2:1.

At both 2 and 6 weeks, test animals showed an approximately three-fold increase in Vv ($p<0.001$). There was subjective increase not only in cell size and the number of cell processes, but also in cell numbers (Figs.2:4 and 5) compared to control gland (Figs.2:2 and 3).

(ii) CRF and VP Administration

Pituitary weight. There was no significant difference in pituitary weight between any test group and the control animals (Table.2:1).

Corticotroph Vv. Results are shown in Fig.2:6.

There was a subjective increase in corticotrophs, (Fig.2:7) though not so marked as that following adrenalectomy.

The administration of CRF produced a dose-dependent increase in Vv (25µg/Kg, $p<0.01$; 50µg/Kg, $p<0.001$). The addition of VP increased the Vv only when given with the lower dose.

Adrenal weight. Results are shown in Table.2:2.

There was a significant dose-dependent increase in adrenal weight compared to controls with all treatment regimes. There was no difference between the CRF alone and CRF plus VP groups.

DISCUSSION

The use of Vv as a measurement of the corticotroph population in the anterior pituitary of the normal rat has been reported infrequently (Dada, Campbell and Blake, 1984; Haüsler et al, 1984; Zak et al, 1985). In addition, Tankosic et al (1982) used Vv in a study of the corticotrophs of the Brattleboro rat, which has an abnormal

HPA axis. The method of assessment in the present study was slightly different from those above, since mesenchyme was excluded from the analysis. The method used here corresponded to that of Cronin et al (1982) in a study of gonadotrophs and mammotrophs. In the case of Dada et al (1984), it is possible to recalculate their data to give corticotroph Vv as a percentage of the total immunostained cell population, yielding a value of 6.9% for males and 5.5% for females. These are similar to the control values in this study and in the separate studies of McNicol (1985) where a similar method of analysis was used. As in those studies corticotroph Vv in control female rats has been consistently lower than in males, and that in older animals lower than younger. Since Vv is not an absolute measurement, but may be altered by changes in other cell populations within the anterior pituitary, these differences may be due to the greater numbers of prolactin cells in the female pituitary and the increasing numbers of these cells with age (Takahashi and Kawashima, 1982) as discussed by McNicol (1985). The main benefit of Vv is its relative ease of estimation compared to cell counting. The main drawback is that it does not differentiate between hypertrophy and hyperplasia. However, particularly in adrenalectomized animals, there was an obvious subjective increase in corticotroph numbers.

Most studies on the pituitary following adrenalectomy have examined the male rat. Rappay and Makara (1981) reported 9.3% corticotrophs in adrenalectomized rats after 2 weeks compared to 5.1% in sham-operated animals, but did not comment on cell size. Nakane, Setalo and Mazurkiewicz

(1977) reported a two-fold increase in the numbers of corticotrophs 4 weeks after adrenalectomy. Using Vv to assess changes in the population in the male rat, a similar two-fold increase was demonstrated at 2 and 6 weeks after adrenalectomy (McNicol, 1985). Baker and Drummond (1972) examined pituitaries from both male and female rats, and while they noted that the maximum change in cell size and number occurred at 2-3 weeks after adrenalectomy, they gave no figures and made no comment on differences between male and female.

In the present study, the approximately three-fold increase in Vv in the female rat was considerably greater than the two-fold increase in Vv in the male rat described above. This would be in keeping with the withdrawal of a higher physiological level of negative feedback in the female. Again, as with the male, the major increase is achieved within the first two weeks following adrenalectomy. However, a rough estimation of total corticotroph mass at 2 and 6 weeks, based on the anterior lobe accounting for approximately 80% total pituitary weight (Gosbee et al, 1970) suggests that there is indeed a further increase in the corticotroph population between 2 and 6 weeks. This differs from the male (McNicol, 1985) where no real difference was identified. These differences might be investigated further by studying the cell kinetics of the response in both sexes.

Since the major increase is achieved at a time when pituitary ACTH content is still rising (Fortier, 1959a) if hypothalamic factors are involved in the induction of

these changes, their functional and growth-promoting effects must eventually become dissociated. An alternative explanation is that adrenalectomy causes the release of specific growth factors, and that their time course of action is different from the functional stimulating factors.

The increases in adrenal weights seen following CRF administration are consistent with stimulation of ACTH release. The results of the Vv measurement would be in keeping with a role for CRF in the control of the size of the anterior pituitary corticotroph population, and confirm the data of McNicol (1985) where the peptide was administered for up to 6 weeks. However, it is not possible to define the mode of action of CRF from this study. Westlund et al (1984) in an in vitro study demonstrated a very short-term increase in numbers of corticotrophs in response to CRF and suggested that this might represent either increased ACTH content of existing corticotrophs, making a greater number of them immuno-positive or recruitment of an uncommitted cell population. The subjective increase in corticotroph numbers seen in the present study may be due to either of these factors, or may represent true proliferation of corticotrophs or of a stem cell population with subsequent differentiation. This is investigated further in Chapter 5. As discussed in Chapter 1, it has now been shown that VP and CRF act synergistically to stimulate ACTH release, and that the release of CRF and VP from the same hypothalamic neurones is increased following adrenalectomy. Vasopressin has been shown to be mitogenic for several tissues (Payet and Isler,

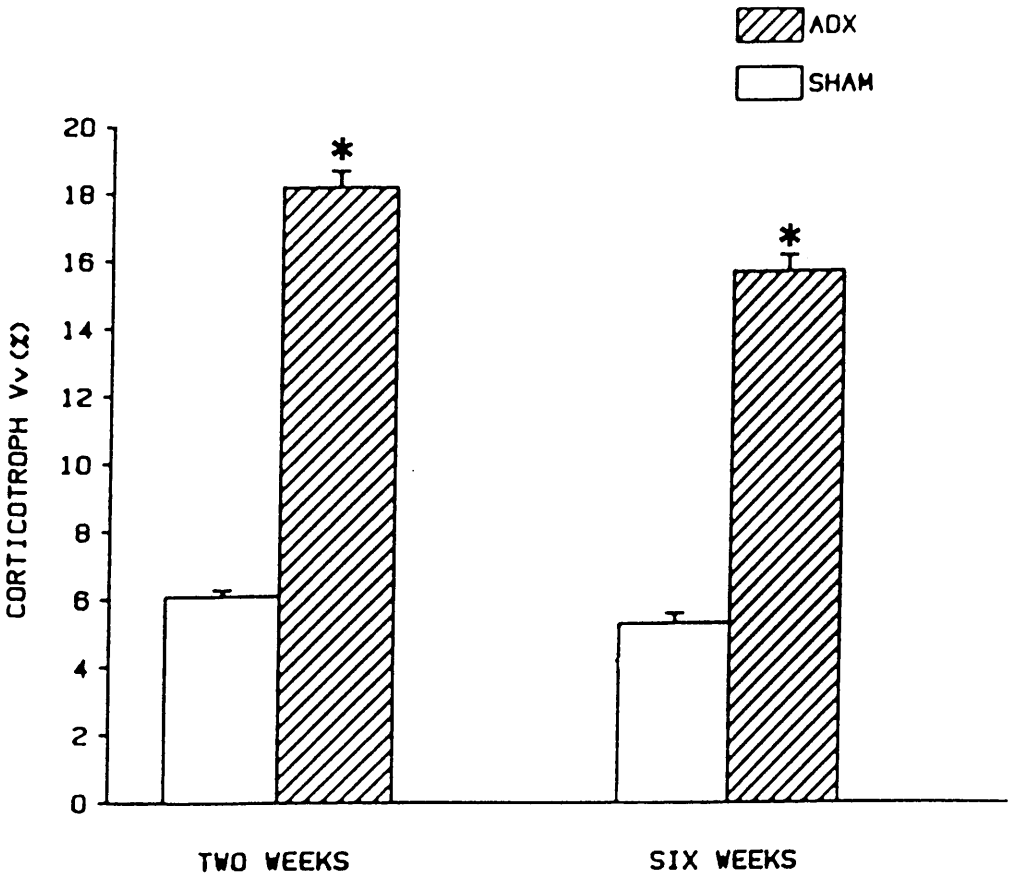
1976) and it is therefore possible that it might be an important factor in the control of corticotroph growth following adrenalectomy.

Obviously, the number of animals in the present study is small, and results must therefore be interpreted with some caution. However, the discrepancy between the effects of adding VP to the lower and higher dose of CRF is of interest. With the lower dose, it would appear that there is a further increase in corticotroph Vv, which would suggest either increased proliferation or increased ACTH content with more cells identifiable by immunohistochemical staining. A possible explanation for the fall in Vv at the higher dose would be increased secretion of ACTH with this combination and thus fewer immunopositive corticotrophs. This aspect is investigated further in Chapter 4 by examining the degree of granulation in the corticotrophs in these groups of animals. Further studies on the mitogenic effects of VP and VP/CRF combinations would obviously be of interest.

In none of the groups of CRF treated animals did corticotroph Vv reach the levels achieved after adrenalectomy. This may reflect the presence of negative feedback in the CRF treated animals, a failure to achieve maximal stimulation with the doses administered or the multifactorial nature of the stimulus following adrenalectomy.

Figure. 2:1

Effect of adrenalectomy on corticotroph Vv in female rats.



Results are shown as mean ± SEM

* $p < 0.001$ vs Control.

Figure. 2:2

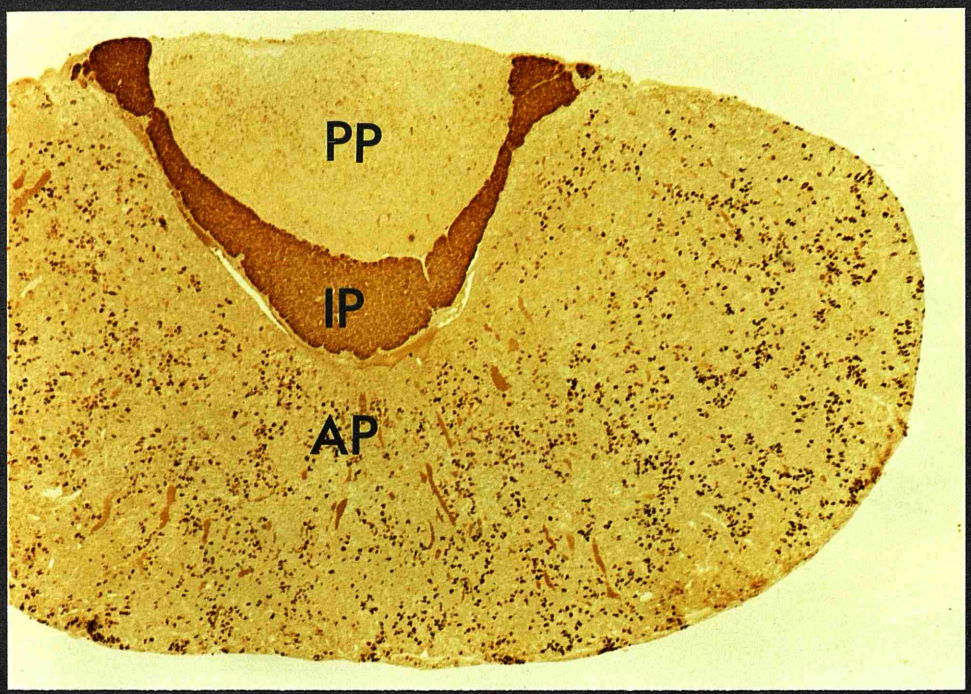
Normal rat pituitary. (PAP technique: anti $ACTH_{1-24}$: (x 36).

Corticotrophs are randomly distributed throughout the anterior lobe (AP). The corticotrophs of the intermediate lobe (IP) are also identified by this antibody.
PP = posterior lobe.

Figure. 2:3

Normal rat pituitary. (PAP technique: anti $ACTH_{1-24}$: (x 182).

Corticotrophs are seen as stellate cells with processes extending between neighbouring bearing cells.


PP
IP
AP

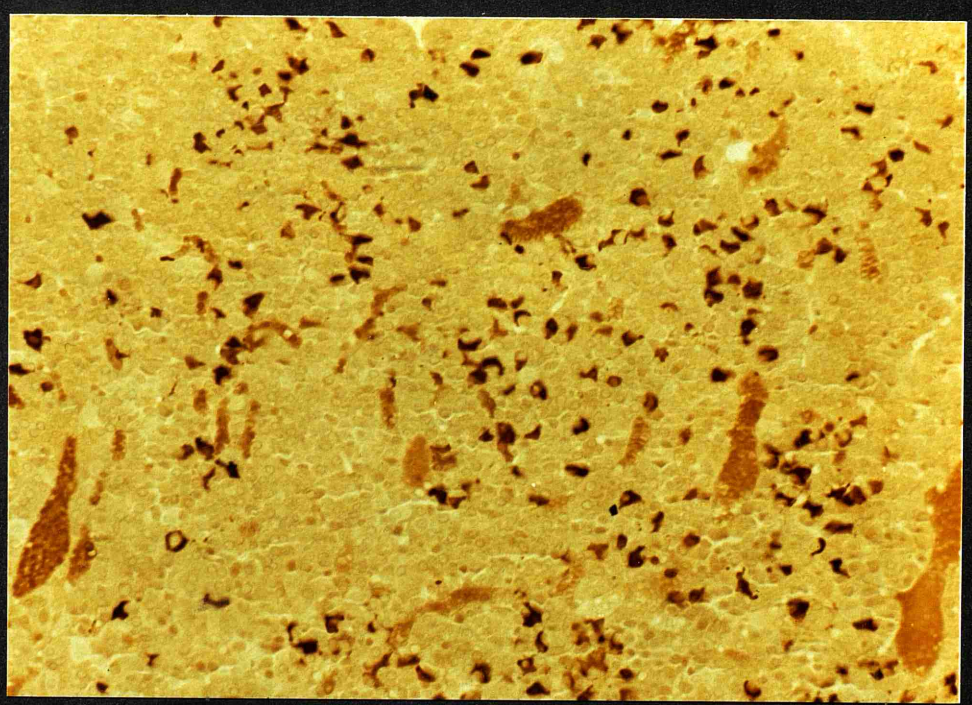


Figure. 2:4

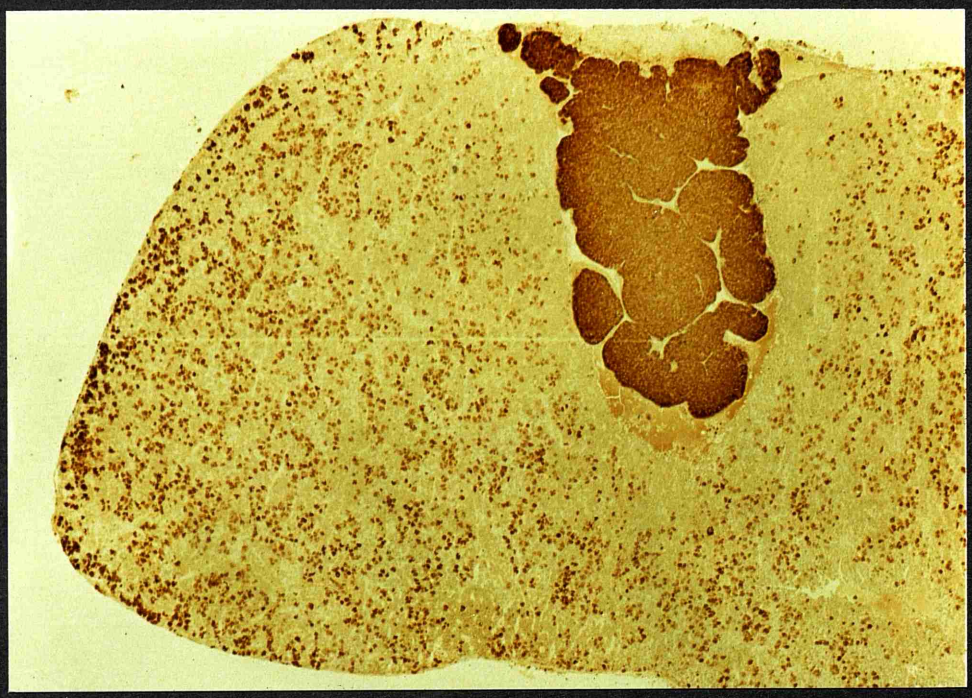
Female rat : 2 weeks post-adrenalectomy (x 36).

There is an obvious increase in the corticotroph population.

Figure. 2:5

Female rat : 2 weeks post-adrenalectomy (x 182).

Corticotrophs are subjectively increased in number and also size.



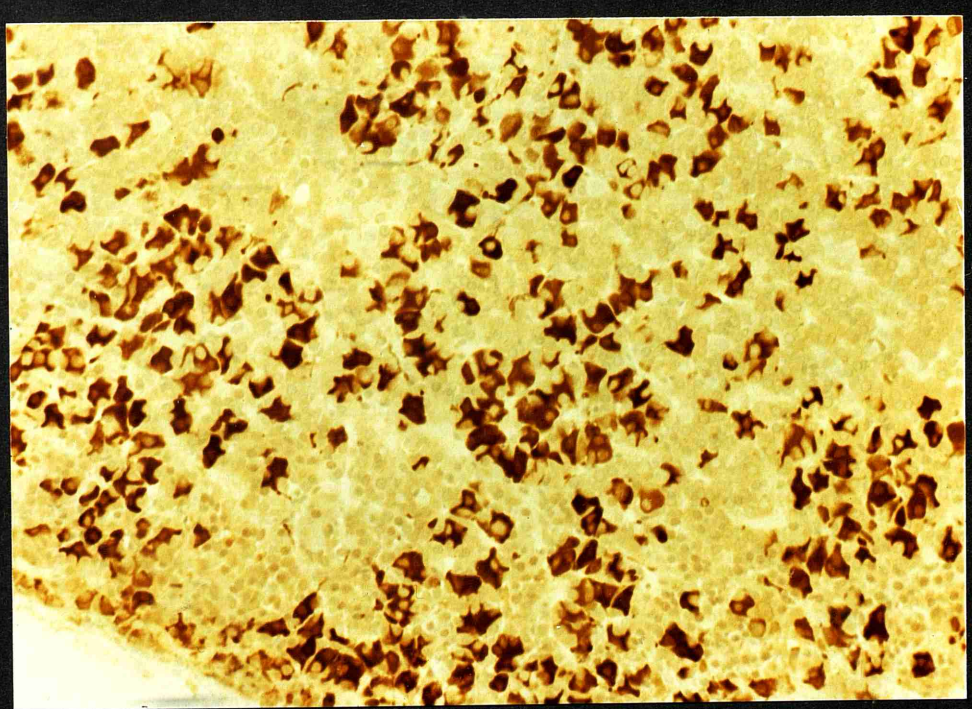
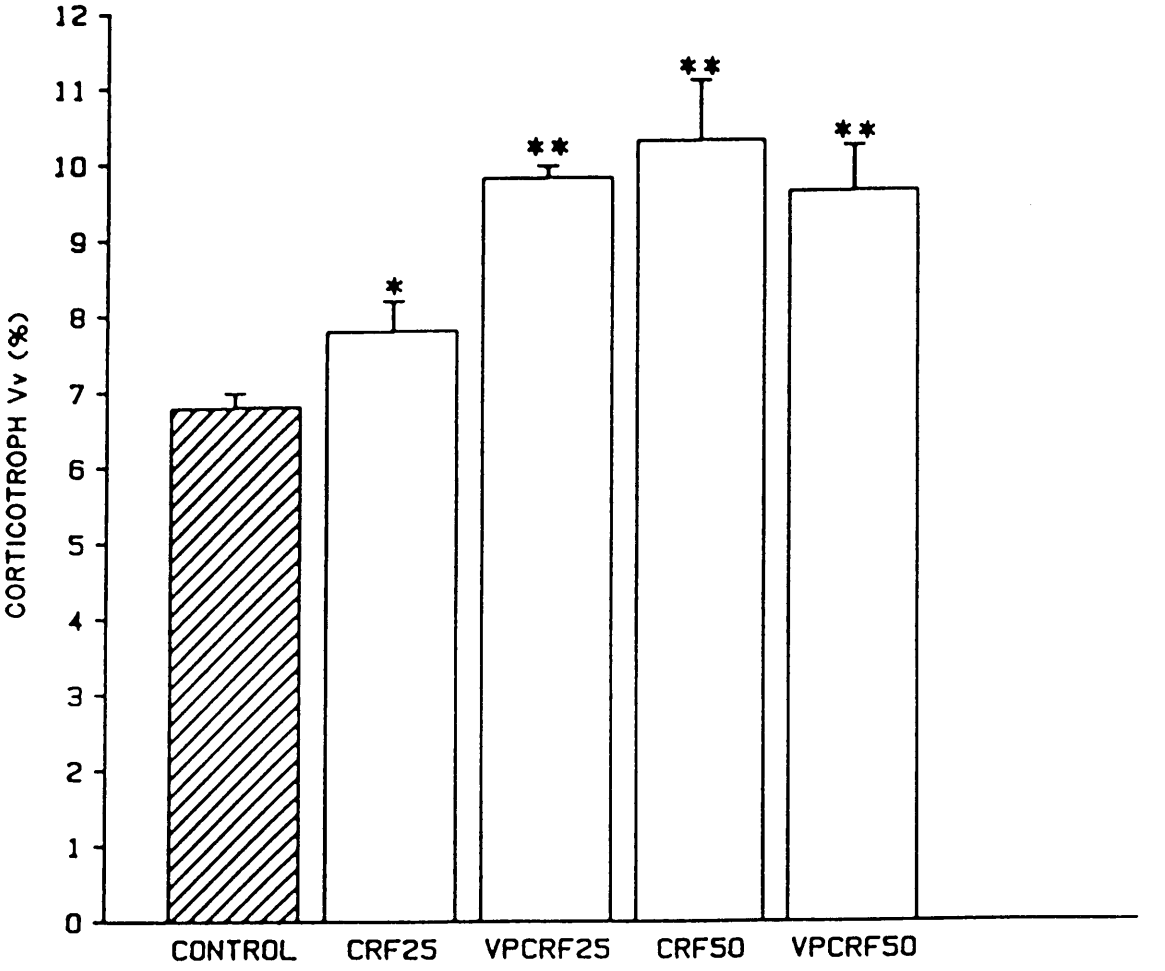


Figure. 2:6

Effect of CRF administration alone or with VP on corticotroph Vv in male rats.



Results are given as mean ± S.E.

* $p < 0.01$ vs Control.

** $p < 0.001$ vs Control.

CRF given as µg/Kg B.W.

VP given at a dose of 0.4 I.U./Kg B.W.

Figure. 2:7

Male rat. CRF 25µg/Kg B.W. : 2 weeks (PAP technique anti ACTH x 182).

Cells are moderately increased in size and number compared to controls but the change is less marked than following adrenalectomy.

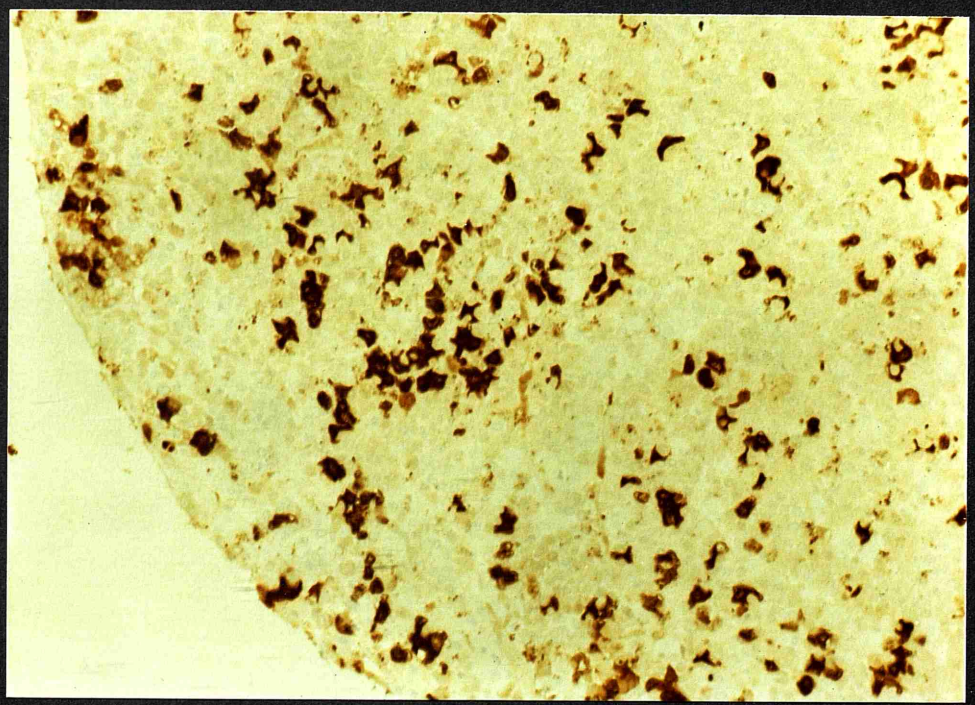


Table.2:1 **Effect of the administration of CRF ± VP on the weight of adrenal and pituitary glands in male rats.**

Treatment	n	Adrenal weight mg/100mg B.W.	Pituitary weight mg/100mg B.W.
Saline	9	14.9 ± 1.2	3.25 ± 0.6
CRF 25	9	16.9 ± 0.9*	3.21 ± 0.4
VP CRF 25	8	17.8 ± 0.8*	3.4 ± 0.3
CRF 50	9	18.5 ± 1.1**	3.4 ± 0.5
VP CRF 50	6	19.4 ± 0.8**	3.5 ± 0.26

Results are shown as mean ± S.E.

* $p < 0.01$ vs control
** $p < 0.001$ vs control

CRF doses are given µg/Kg body weight.
VP was administered at a dose of 0.4 I.U./Kg.

CHAPTER 3

INHIBITION OF CORTICOTROPH FUNCTION - QUANTITATIVE LIGHT MICROSCOPIC STUDIES

INTRODUCTION

Dexamethasone is a synthetic glucocorticoid which binds to a single cytosolic receptor (the classical glucocorticoid receptor) with high affinity (de Kloët and McEwen, 1976). It has much greater biological potency than corticosterone, approximately 25-30 times when measured by the anti-inflammatory effects (Brooks, 1979) and 70 times when assessed by its ability to deplete the cytosol of several tissues of free glucocorticoid receptor (Ichii, 1981).

Its site of negative feedback within the HPA axis has been a matter of some controversy. It can be shown to bind preferentially to anterior pituitary *in vivo* (de Kloët, Wallach and McEwen, 1975) and inhibits stimulated ACTH release in rats after hypothalamic lesions (Takebe et al, 1974) indicating a site of action in the pituitary gland itself. *In vitro* studies have shown that it can bind to cells of hippocampal nuclei (McEwen et al, 1975). In addition, reduced levels of hypothalamic CRF activity have been reported following dexamethasone administration (Chowers et al, 1967), more in keeping with a central or hypothalamic role. In contrast, increased levels of CRF activity have also been reported following dexamethasone (Krieger et al, 1977). A possible explanation for these discrepancies has been provided by the studies of Sakakura et al (1981) and Sakakura, Yoshioko and Kobayashi (1982) whose data suggest that the site of action is dose dependent, low doses acting at pituitary levels and high doses permitting significant quantities to gain access to more central sites.

There are published data that functional inhibition of corticotrophs by chronic glucocorticoid administration results in a reduction both in corticotroph size and numbers (Baker and Drummond, 1972; Caselitz and Saeger, 1979). However, a recent quantitative study (McNicol, 1985; McNicol et al, submitted for publication) showed no significant reduction in corticotroph Vv in the male rat after 2 or 6 weeks treatment with dexamethasone.

The aims of the present study were to extend the observations on the dexamethasone inhibited HPA axis. Firstly, in view of the sex differences, a quantitative analysis of corticotroph Vv was performed in the female rat as above. Secondly, the effects of longer-term (up to 6 months) administration of the steroid were monitored in both sexes. Finally, the early recovery phase following 6 weeks administration was examined in male rats. In all studies, an indirect measure of corticotroph function was made, based on adrenal weight and histology.

MATERIALS AND METHODS

Adult Sprague-Dawley rats were selcted and assigned to treatment groups as in Chapter 2. In all groups except 18 week dexamethasone administration, control animals received daily i.p. injections of physiological saline.

Experiment 1.

Female rats (7/group) received daily i.p. injections of dexamethasone (Sigma) suspended in physiological saline in doses of 100µg, 200µg and 500µg/Kg B.W. for 6 weeks.

Experiment 2.

Male and female rats received dexamethasone as above at a dose of 100µg/Kg B.W. for 6, 18 or 26 weeks. Numbers of animals available were insufficient for an 18 week control group.

Experiment 3.

Male rats received dexamethasone 100µg/Kg B.W. daily for 6 weeks and were then sacrificed 2, 5 or 14 days after the treatment was stopped. The 6 week male groups in Experiment 2 were the control population for this study. Materials were collected, processed and analysed as before.

RESULTS

Experiment 1.

Body and pituitary weights.

Control animals gained 12.5% of their initial body weight. Animals receiving 100µg and 200µg/Kg dexamethasone had a mean loss of 6.2% and those receiving 500µg/Kg lost approximately 15% of their starting weight. Pituitary weights were reduced in all treated animals, but the changes were not significant when related to body weight.

Adrenal weights and histology.

There was a significant decrease in adrenal weight (Fig.3:1) at all doses.

Adrenal weight is expressed as a percentage of mean control adrenal weight to permit some comparison of male/female data since adrenal glands in the female rat are consistently heavier than in the male, both in terms of absolute values and when related to body weight.

Adrenal histology showed a reduction in total cortical

width, with some zonation at the lower doses, but little evidence of zonation at the highest dose (Fig.3:2).

Corticotroph Vv

There was no significant reduction in corticotroph Vv with any dose. 100µg/Kg B.W. produced an increase (Table.3:1). Corticotrophs were easily identified (Fig.3:3).

Experiment 2.

Body and pituitary weights

By twenty six weeks, control male rats had gained 139% and females 80% of their initial body weight. Those receiving dexamethasone gained 55% (male) and 36% (female). There was no significant difference in absolute pituitary weights, but when related to body weight, there was a relative increase in pituitary weight in dexamethasone treated animals at 26 weeks (Table.3:2) compared to controls.

Adrenal weights and histology

Again, at treatment intervals up to 18 weeks, there was a reduction in adrenal weight in both male and female rats compared to control animals (Table.3:2). In these animals the adrenal glands showed a reduction in cortical width, with loss of reticularis.

In 26 week treated female animals, the adrenal weights were again lower than controls. In male rats, however, adrenal weights in dexamethasone treated animals were higher on a weight - related basis than in control animals, although absolute weights were lower in treated animals.

Adrenal histology was consistent with inhibition of function at all times.

Corticotroph Vv (Table.3:3).

At 6 weeks, both sexes showed an increase in Vv compared to controls. This fell at 18 weeks but no saline controls were available for comparison. At 26 weeks, there was a discrepancy between the two sexes, the males having a slight increase in Vv compared to controls, the females a slight fall.

Experiment 3.

Pituitary weights

No significant change occurred in pituitary weight at 2 and 14 days. (Data not shown). Those collected at 5 days were lost in processing.

Adrenal weights and histology

Adrenal weight decreased significantly after 6 weeks of dexamethasone. Absolute adrenal weight increased after withdrawal of steroid, but over the time studied, this paralleled the increase in body weight (Table.3:4). Adrenal histology showed an increase in total cortical width and re-establishment of zonation over the 14 days (Fig.3:4).

DISCUSSION

The lower body weights in dexamethasone treated animals compared to age-matched controls are a reflection of the non-specific catabolic effects of the steroid.

An initial attempt to measure plasma ACTH by radio-immunoassay to confirm inhibition of corticotroph function was unsuccessful because the antibody, which had been raised to human ACTH did not produce specific binding of the rat peptide.

Changes in adrenal weight and histology give a fair indication of corticotroph function, but the correlation is not absolute. It has certainly been shown that ACTH is important in the control of adrenal weight (Fiala, Sproull and Fiala, 1956) and that other peptides derived from POMC may act along with ACTH in this respect (Estivariz et al, 1982). In addition, however, growth hormone has been shown to play a role in adrenocortical proliferation (Cater and Stack - Dunne, 1955) and dexamethasone appears to have a direct inhibitory action on cell proliferation in the gland unrelated to its effects on ACTH release (Wright, 1971).

It is difficult to find an ideal way of expressing adrenal weight, particularly where treatments induce changes in total body weight. This may be the reason for the apparent discrepancies in the 26 week group. If only absolute values are quoted, non-specific reduction related to general loss in body weight will be included. However, if adrenal weights are body-weight related, the significant increase in body fat seen in aging rats, particularly males, will produce a lower "control value" in these animals. The prevention of this accumulation by dexamethasone treatment most probably accounts for the apparently heavier adrenals and pituitary glands in the 26 weeks dexamethasone treated animals. It might have been better to relate adrenal weight to that of a non-endocrine internal organ (e.g. liver, kidney).

Nevertheless, the changes in adrenal weight and histology would be consistent with significant inhibition of corticotroph function and Lim et al (1982) have shown maximal inhibition of plasma ACTH levels by 20µg dexametha-

sone (equivalent to 100µg/Kg in the present study).

McNicol (1985) has previously demonstrated that corticotroph Vv in the male rat is not significantly reduced by the daily administration of dexamethasone in doses up to 200µg/Kg for 6 weeks and that at the 100µg/Kg dose, the Vv is increased. The present study has demonstrated that both of these are true also of the female rat. In addition, the administration of 500µg/Kg did not reduce Vv. These findings are in contrast to previous published data on the morphological effects of the long-term administration of glucocorticoids which suggested that a significant reduction occurs in the identifiable corticotroph population within the gland (Baker and Drummond, 1972; Kraicer et al, 1973; Caselitz and Saeger, 1979). This would be in keeping with the demonstration of reduced pituitary ACTH content (Fortier, 1959b). However, in those studies large doses of naturally-occurring glucocorticoids were administered, which have complex negative feedback effects.

While there is still some debate as to the site of action of dexamethasone, there is evidence that doses equivalent to those administered in the present experiments lower plasma ACTH, but do not reduce pituitary ACTH content (Sakakura et al, 1982; Lim et al, 1982). This would suggest a major effect on the inhibition of ACTH release rather than synthesis. These findings are consistent with the changes demonstrated in the present study. Maximal dissociation of these effects at 100µg/Kg dose could account for the increased Vv by allowing immunohistochemical identification of a group of corticotrophs which,

under physiological circumstances do not contain sufficient hormone to give positive staining.

The assessment of the changes in the 26 week treatment groups is difficult. The marked reduction in weight of treated groups obviously raises the possibility of non-specific alterations in the other cell populations in the gland. In addition, in normal animals, numbers of lactotrophs increase with age, eventually producing significant hyperplasia, particularly in females (Kovacs et al, 1980). We do not know whether this occurs in dexamethasone treated animals. For these reasons, it would be preferable to repeat these long-term studies looking at changes in total cell populations. This could be achieved more easily by disaggregation, calculation of total cells and differential counts of labelled hormone-producing populations.

In addition, the application of in situ hybridisation and dot blot or Northern blot analysis of POMC mRNA will be of interest, particularly in identifying differences between the mode of action of dexamethasone and natural steroids.

There were two main reasons for studying the recovery period following long-term dexamethasone administration. The first was to assess whether increased ACTH secretion following withdrawal of the steroid resulted in a reduction in the identifiable corticotroph population due to degranulation, similar to the changes seen after adrenalectomy (Siperstein and Miller, 1973). Nicholson et al (1984) studied the biochemical effects of withdrawal of dexamethasone after 14 days of administration. The dose of dexamethasone was similar to the present study but was

administered continuously in drinking water. They demonstrated reduced pituitary immunoreactive ACTH in inhibited rats, but only on the day of withdrawal was this significantly less than controls. In addition, they suggested that stimulation of ACTH release was impaired until day 5. The findings of our study would support a lack of sudden ACTH release. We cannot comment on ACTH content as quantitation is difficult in immunocytochemistry. It is possible that the 'slow feedback' induced by oral administration has had a more profound effect on ACTH synthesis than the 'intermediate feedback' produced by daily injections in the present study. Further comparative studies of these two modes of feedback would be of interest.

The second aim of this experiment was to monitor changes in adrenal weight. Nicholson et al (1984) suggested that by 5 days, although corticosterone output was normal, adrenal weight was still lower than control animals. In the present study we have demonstrated that zonation is reappearing by 5 days, consistent with corticosterone production. However, the increase in adrenal weight to 14 days parallels that of general body weight. This may reflect the inhibition of release not only of ACTH but of other POMC derived peptides involved in adrenal growth. Specific stimulated adrenal growth may therefore continue once normal function is re-established. A study of the cell kinetics of this response would help elucidate whether significant hypertrophy precedes any proliferative response.

The results demonstrated here would suggest that in

contrast to previous reports, it is possible to produce significant inhibition of corticotroph function without causing a major fall in the corticotroph population.

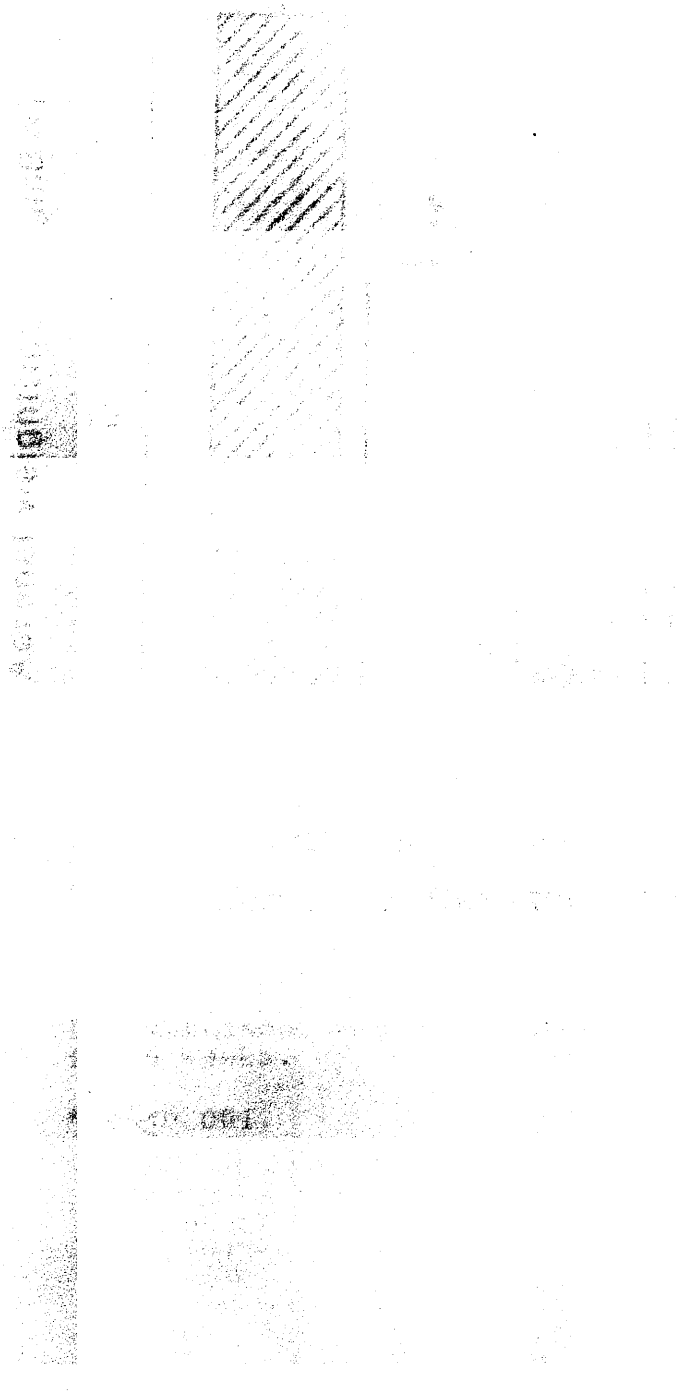
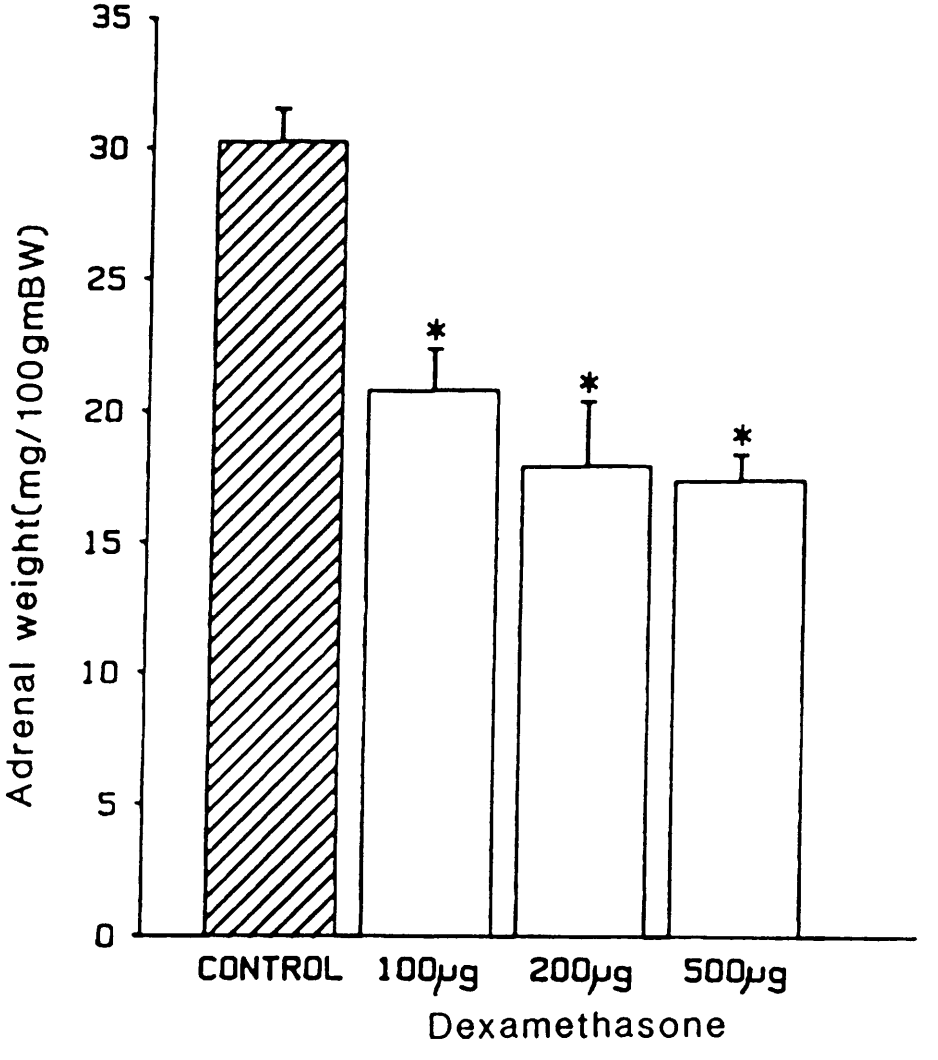


Figure 3:1

Changes in adrenal weight of female rats following dexamethasone administration.



Dexamethasone was administered daily for 6 weeks.

* $p < 0.001$.

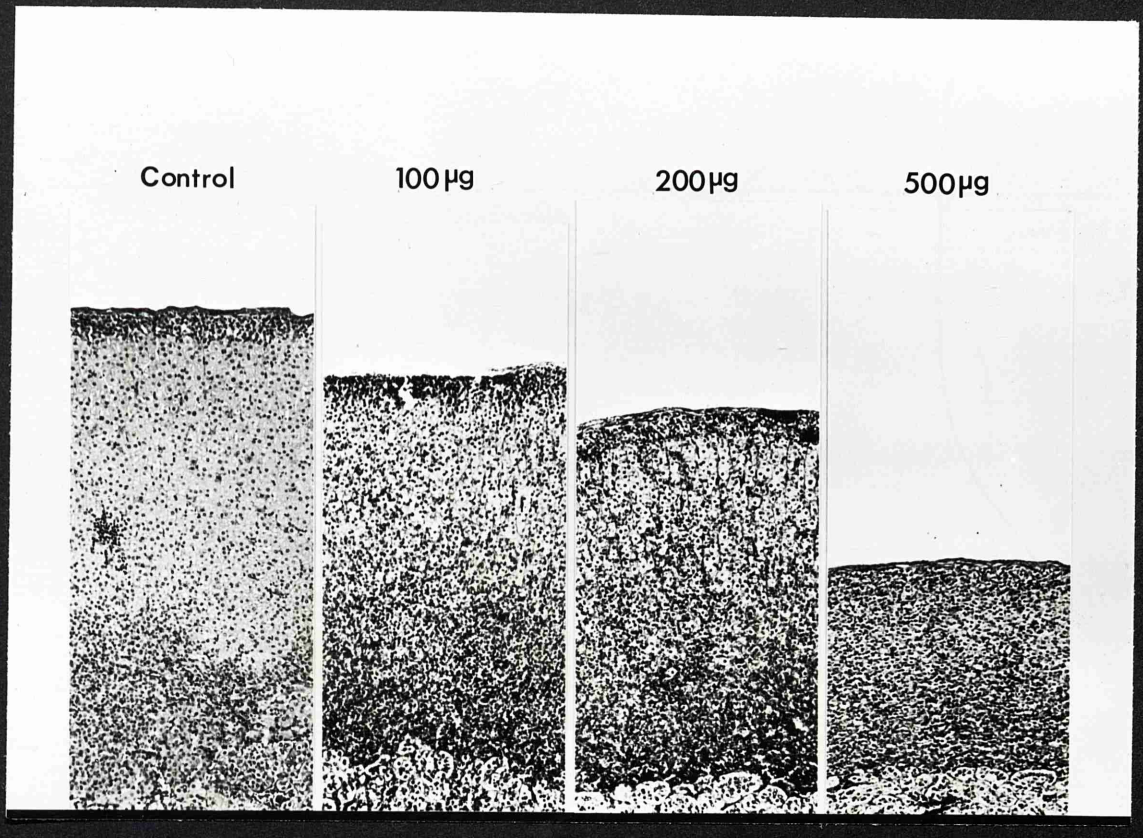
Figure. 3:2

Adrenal glands : female rats.
Dexamethasone administration for 6 weeks (H & E).

There is a dose dependent decrease in the width of the cortex with loss of zonation, more marked with the highest dose.

Figure. 3:3

Male rat : dexamethasone 100µg/Kg for 6 weeks (PAP : anti ACTH x 300).
Corticotrophs are easily identified. They do not appear reduced in numbers compared with controls.


Control
100 µg
200 µg
500 µg

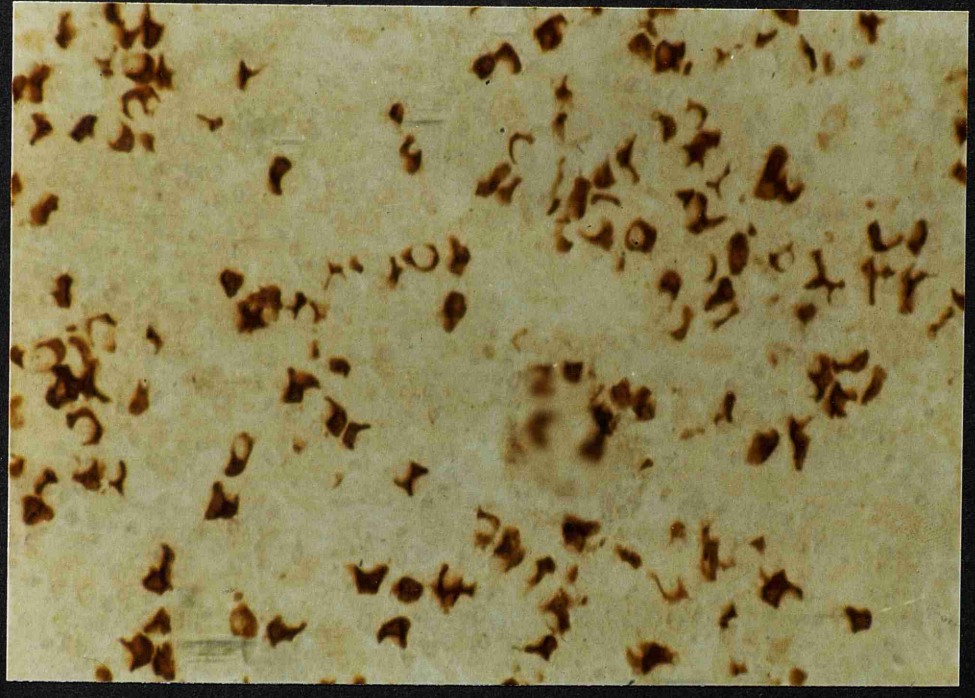


Figure. 3:4

Adrenal cortex : male rat.

6 weeks dexamethasone 100µg/Kg body weight;
recovery period up to 14 days after withdrawal.

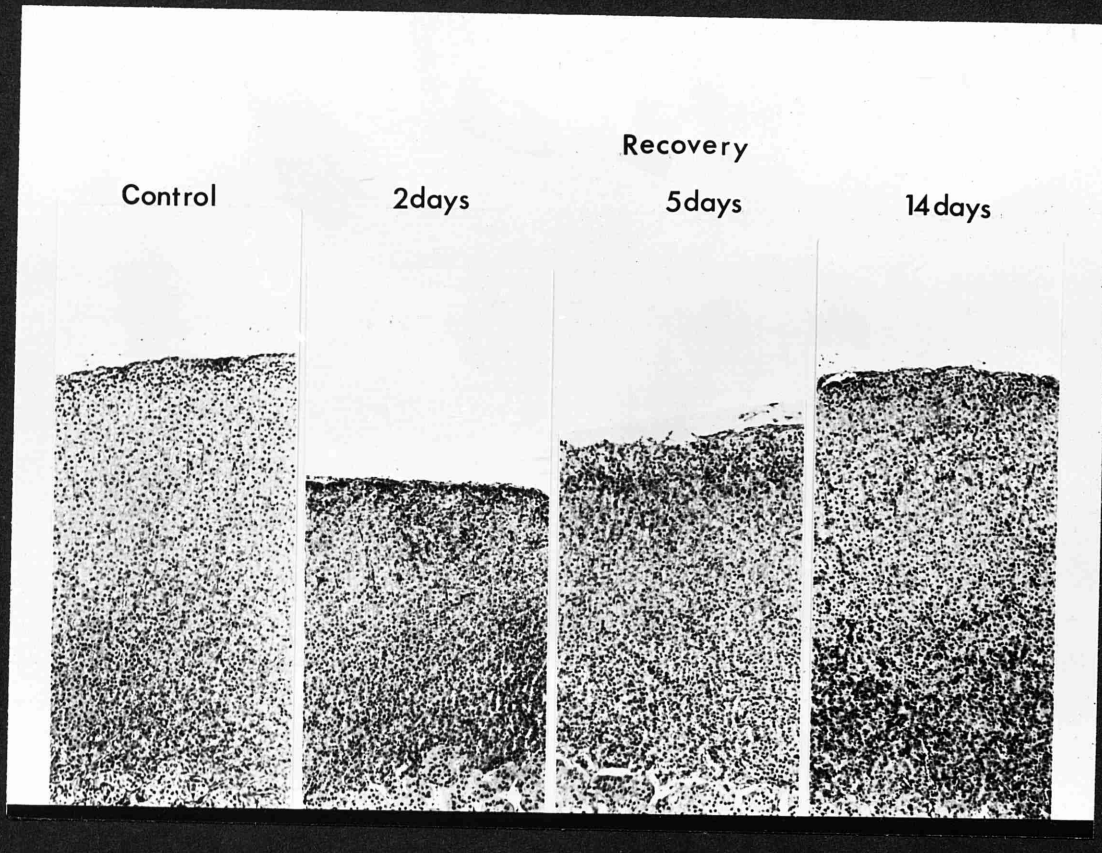

Recovery
Control
2days
5days
14days

Table.3:1 Effect of dexamethasone administration on corticotroph Vv in female rats.

Treatment	n	Corticotroph Vv%
Saline	8	5.28 ± 0.25
Dexamethasone		
100µg/Kg	8	6.45 ± 0.2*
200µg/kg	9	5.75 ± 0.25
500µg/Kg	9	5.73 ± 0.17

* $p < 0.01$ (two-tailed).

Table.3:2 **Effects of long-term dexamethasone administration on pituitary and adrenal weights in male and female rats.**

Treatment	Duration (weeks)	Pituitary weight*		Adrenal weight*	
		Male	Female	Male	Female
Saline	6	3.3 ± 0.3	5.1 ± 0.4	15.9 ± 0.4	28.8 ± 0.4
Saline	26	2.2 ± 0.1	3.6 ± 0.2	7.8 ± 0.9	22.6 ± 1.5
Dexamethasone 100µg	6	3.1 ± 0.2	5.8 ± 0.3	11.8 ± 0.6	18.4 ± 1.6
	18	3.1 ± 0.3	4.8 ± 0.4	10.5 ± 0.5	17.7 ± 1.4
	26	3.4 ± 0.7	5.3 + 0.3	9.8 ± 0.7	19.2 ± 1.3

* (mg/100gm B.W.).

Statistical analysis not performed.

Table.3:3 Effect of long-term dexamethasone administration on corticotroph Vv in male and female rats.

Treatment	Duration (weeks)	Corticotroph Vv (%)	
		Male (n)	Female (n)
Saline	6	6.2 ± 0.25(6)	5.28 ± 0.28(6)
Saline	26	6.4 ± 0.62(5)	5.04 ± 0.17(6)
Dexamethasone 100µg/kg	6	7.24 ± 0.58(5)	5.76 ± 0.2(5)
	18	5.87 ± 0.45(8)	4.47 ± 0.33(8)
	26	7.01 ± 0.19(5)	4.9 ± 0.4(8)

Statistical analysis was not performed on these data.

Table.3:4 Changes in corticotroph Vv, adrenal and body weight during recovery after dexamethasone administration.

Treatment	n	Corticotroph Vv%	Adrenal Weight mg/100gm B.W.	Body Weight (gm)
Saline	6	6.2 ± 0.25	14.9 ± 0.4	246
Dexamethasone 100µg/Kg	5	7.24 ± 0.58	11.8 ± 0.6*	300
Recovery				
2 days	8	6.9	10.4 ± 0.8	309
5 days	7	-	10.7 ± 0.8	353
14 days	8	6.8	10.6 ± 0.3	387

Results are shown as vs ± SEM.

* $p < 0.01$ vs Control.

CHAPTER 4

ULTRASTRUCTURAL STUDIES

A. CORTICOTROPHS: QUALITATIVE STUDIES

Introduction

(i) Stimulation of corticotroph function

The identity of the normal corticotroph in the rat anterior pituitary has been a matter of controversy, some groups suggesting that a single morphological type exists, while others claim that more than one cell type contains ACTH.

The classical and most numerous cell type was first recognised in a light microscopic-autoradiographic study by Siperstein (1963) as the cell showing morphological changes after adrenalectomy. Further ultrastructural (E.M.) investigation (Siperstein and Allison, 1965; Nakayama, Nickerson and Skelton, 1969; Siperstein and Miller, 1970) showed it to be stellate in shape with many processes engulfing other cells and extending to nearby vessels. The secretory granules are 200-300mμ in diameter and are arranged in a single row along the cell periphery. Rough endoplasmic reticulum is lamellar and scattered throughout the cytoplasm. Mitochondria are distinctly slender, sometimes have longitudinal cristae and are often bifurcated or twisted in unusual shapes. Direct immunohistochemical staining (Moriarty and Halmi, 1972) and indirect correlation of immunohistochemical staining of thick sections at light microscopic level with adjacent thin sections for electron microscopy (E.M.) (Bowie et al, 1973) have confirmed the "Siperstein Cell" as a source of ACTH synthesis.

A second type of cell has been implicated in ACTH

secretion (Kurosumi and Kobayashi, 1966; Kurosumi and Oata, 1966; Yamada and Yamashita, 1967; Rennels and Shiino, 1968; Costoff, 1973). This is described as a rather large, angular or polygonal cell with a few stout processes. The secretory granules are less than 200mμ in diameter; they are haloed and fill the entire cytoplasm rather than being limited to the cell periphery. It has been suggested that this "Kurosumi Cell" is a thyrotroph rather than a corticotroph (Siperstein and Miller, 1970; Moriarty and Halmi, 1972; Bowie et al, 1973). However, Kurosumi, Taniguchi and Inoue (1984) in a recent ultrastructural immunohistochemical study, have demonstrated ACTH in such cells. The existence of more than one type of ACTH secreting cell has also been suggested by Yoshimura and Nogami (1981) and Childs et al (1982) have reported that ACTH is also produced by a sub-population of gonadotrophs. Zak et al (1985) have recently proposed that these are all variants of a single cell type.

The ultrastructural changes occurring in corticotrophs in states of altered pituitary-adrenal function are incompletely documented, particularly with reference to the long-term effects. The changes following adrenalectomy have been studied in some detail, and initial hypertrophy and degranulation is followed by an increase in granules and a small decrease in cell area (Siperstein and Miller, 1973). The maximum time interval examined post-adrenalectomy is 3 weeks. There is also evidence that pharmacological adrenal blockade results in hypertrophy and alteration in ultrastructural features (Caselitz and Saeger, 1979; Zak et al, 1985). Such studies monitor the

effects of an overall increase in stimulation of the corticotroph, and the effects of specific stimulation by components of the corticotrophin-releasing complex (e.g. CRF, vasopressin) are even less well documented, although Caselitz and Saeger (1979) suggested that vasopressin administration produces hypertrophy and hyperplasia. The ultrastructural effects of CRF have not been documented, apart from a single report by Kurosumi et al (1984).

In the present study, the aims were to confirm and extend the observation in the post-adrenalectomy period by examining the ultrastructural changes in the Siperstein corticotroph at 2 and 6 weeks after adrenalectomy. In view of the known functional differences between the pituitary-adrenal axis of male and female rats, and of the quantitative differences in response discussed in Chapter 2, both male and female rats were included in this study. In addition, the effects of daily intraperitoneal injection of ovine CRF in doses which had been shown to produce a quantitative increase in the corticotroph Vv of male rats (McNicol, 1985) were studied, with and without the addition of vasopressin. These were compared with changes produced by adrenalectomy.

(ii) Inhibition of Corticotroph Function

The inhibitory action of glucocorticoids on the HPA axis is associated with certain ultrastructural changes in the corticotrophs which were characterized by Siperstein and Miller (1970) and were reported to be dose and time dependent. As with the other studies discussed in Chapter 1, their study was based on the administration of naturally

occurring glucocorticoids. The apparent discrepancy at light microscopic level between the reported effects of these steroids on the corticotroph population and the results obtained in our studies with dexamethasone is interesting. The aims of the present studies were to investigate these differences further by examining the ultrastructural effects of long term administration of corticosterone in doses which have been shown to reduce pituitary ACTH content (Fortier, 1959b) and comparing these changes with those seen after equivalent doses of dexamethasone.

MATERIALS AND METHODS

Animals and Treatments

Representative samples of anterior pituitary were taken from four animals in each of the following groups. In all cases, light microscopic examination confirmed that the corticotroph population in the glands sampled for E.M. was similar to the remainder of the group.

Adrenalectomy - 2 and 6 weeks, male and female.

Sham-adrenalectomy - 2 and 6 weeks, male and female.

CRF, 25µg/Kg and 50µg/Kg body weight/day for 2 weeks; male.

CRF 25µg/Kg and 50µg/Kg plus 0.4 IU/Kg vasopressin/day for 2 weeks; male.

Corticosterone 30 and 60mg/Kg body weight/day for 6 weeks; male and female.

Dexamethasone 500µg/kg body weight/day for 6 weeks; male and female.

Tissue Processing and Examination

Specimens were fixed immediately after removal in 4% glutaraldehyde, osmicated and embedded in epoxy resin. Golden sections (90nm) were cut on an LKB ultramicrotome, and mounted on copper grids. Sections were stained with lead citrate and uranyl acetate and examined in a Philips 301 electron microscope operated at 80KV.

Identification of Corticotrophs

Corticotrophs were identified using the criteria of Siperstein and Miller (1970) and Moriarty (1973).

RESULTS

(i)Controls

The features were similar in all control groups. Corticotrophs were similar to previous reports (Fig.4:1 and 4:2). No significant differences were identified between males and females.

In addition, cells similar to the "Kurosumi" cell were seen (Fig.4:3). They did not, however, contain "haloed" granules.

(ii)Corticotroph Stimulation

Adrenalectomy

In male and female rats, corticotrophs were easily identified and were subjectively increased in number at 2 weeks. While they were randomly distributed, they were seen in close association with blood vessels, and many had a bigger area of contact with these than in intact animals

(Figs.4:4 and 4:5). They were often cut in levels which did not pass through the nucleus (Fig.4:5). Increased cytoplasmic processes were identified which were often wider than those of control animals and exhibited an increased tendency to surround other cells.

Secretory granules were usually arranged in an interrupted row along the plasma membrane, but showed more tendency to aggregate than in controls (Fig.4:5). They were on average less electron dense than in controls. Some variation in shape and size was seen, particularly in those granules still in association with the Golgi complex (Fig.4:6).

Nuclei were enlarged, more eccentrically located. The chromatin was more condensed and more prominent nucleoli were present (Fig.4:7). Rough endoplasmic reticulum was more prominent, and was arranged in a parallel lamellar fashion occupying large areas of the cytoplasm (Figs:4:4 and 4:7). It was often seen in the wider cytoplasmic processes. There appeared to be an increase in the number of mitochondria. The Golgi complex showed variable distension and contained immature hormone granules.

Six weeks after adrenalectomy, corticotrophs appeared to have more and larger processes with an increased tendency to engulf other cells (Fig.4:8). All of these features were more prominent in female rats at both time intervals (Figs:4:9 and 4:10).

Corticotrophin-Releasing Factor (CRF) and Vasopressin (VP)

With CRF alone, corticotrophs showed increased numbers of larger processes, and this was more marked at the higher

dose, the overall stellate appearance often being difficult to recognise on a single section (Figs.4:11 and 4:12). An associated tendency to surround other cells was seen with the higher dose. Vasopressin augmented these changes slightly when given with the lower dose of CRF (Fig.4:13).

Secretory granules were subjectively increased in numbers with both doses of CRF. They were usually arranged in double or triple rows along the cell periphery. Granule aggregates were occasionally present towards the end of the larger processes (Fig.4:11). Granules showed variable electron density and appeared larger than in control animals. There was an increase in the numbers of granules in the central part of the cell.

There was no difference in the abundance of granules between animals treated with low dose CRF/VP and those with CRF alone (Fig.4:13). However, there was an obvious reduction in their numbers when vasopressin was given along with the higher dose (Fig.4:14).

(iii)Corticotroph Inhibition

Adrenal Weights

There was a significant reduction in adrenal weight with both doses of steroid. This was more marked in male than in female animals (Fig.4:15).

Corticosterone (30mg/Kg)

Female Rats (Fig.4:16)

The numbers of corticotrophs and the cell area were subjectively decreased. The characteristic stellate appearance was less frequent and the cytoplasmic processes

were less numerous, shorter and thinner than in corticotrophs of control rats and less often surrounded adjacent cells. The nuclei were round or oval with a smooth nuclear membrane and dispersed chromatin material. The nucleoli were single and less prominent. The secretory granules were reduced in numbers in a few corticotrophs but the majority exhibited a significant increase in their granule population. These were arranged in multiple interrupted rows along the plasma membrane and/or accumulated at the ends of the cytoplasmic processes. The granules were slightly variable in shape and size and, in some cells, were shown to be more abundant on one side of the cell than on the others.

Mitochondria were reduced in numbers in some cells, RER was identified in most cells. The Golgi complex was not prominent and contained few granules. Lysosomes were easily identified in some cells, but not in others.

Male Rats (Fig.4:17)

More prominent morphological changes were exhibited in the corticotrophs of the male rat after similar doses of corticosterone. In general, corticotroph outline was less complex than in control animals although the stellate appearance was still recognisable. The granule content, though increased in a few cells, was reduced in the majority of corticotrophs. There appeared to be dissociation of RER. The secretory granules again were unevenly distributed along the cell membrane and were variable in size and shape.

Corticosterone (60mg/Kg)

Female Rats

The appearances were more variable than with the lower dose of corticosterone. Many cells again showed an increase in granule content (Fig.4:18), while a few had reduced numbers of granules (Figs.4:19 and 4:20) often arranged asymmetrically along the cell membrane (Fig.4:19). Granule shape and size appeared more variable than in control animals (Fig.4:21). RER was more dissociated than at the lower dose (Fig.4:22).

Male Rats

Corticotrophs showed no significant differences from those occurring after treatment with the lower dose (Figs. 4:23 and 4:24).

Dexamethasone 500µg/Kg

The changes exhibited differed from those shown by the corticotrophs after an equivalent time and dose of corticosterone. In both sexes, corticotroph cell area was obviously reduced but much of the complexity in outline was retained. (Fig.4:25). The secretory granule population varied. A few cells showed a reduction in their granule population while the majority of corticotrophs exhibited a significant increase. (Fig.4.25). The granules were distributed unevenly along the plasma membrane and were accumulated at the capillary poles of the cells or in small aggregates through the cytoplasm and in association with Golgi complex, where their number was increased significantly. Again, the secretory granules in corticotrophs of both sexes displayed a considerable variation in

shape and size (Fig.4:26). The mitochondria were prominent and slightly increased in number. A lamellar arrangement of RER was present in most cells (Figs.4:25 and 4:27). The Golgi complex was more prominent with significantly higher granular content (Fig.4:25).

DISCUSSION

For several reasons, the cells chosen for detailed study in the present experiments were the classical Siperstein corticotrophs. Firstly, this cell is accepted by most workers in the field as the source of ACTH secretion. Secondly, unlike other cells, the morphological changes in the corticotroph after adrenalectomy and cortisol treatment are consistent with the changes in pituitary ACTH content (Gemzell et al, 1951; Fortier, 1959b). Thirdly, even if other ACTH secreting cells do exist, this is the predominant corticotroph, and finally, it is easily identified on morphological grounds. This last point has been of particular importance since, despite numerous attempts, we have been unable to achieve specific immunohistochemical staining of even Siperstein corticotrophs at electron microscopic level. It is not possible, therefore to comment on the nature of the cells resembling the "Kurosumi" corticotroph in the present study. The absence of "haloing" of granules in these cells in the present study might be explained on the basis of differences in fixation and/or staining since Siperstein and Miller (1970) have shown that the electron density of granules may alter under such conditions.

Most studies on the normal corticotroph have been

performed on male rats (Siperstein and Allison, 1965; Moriarty and Halmi, 1972), but the few which have examined female (Nakayama et al, 1969) or both sexes (Nakane, 1970; Zak et al, 1985) suggested that the overall morphology of the cells is similar in both, despite the functional differences. The findings of the present study would support this, as we were unable to demonstrate significant sex differences in animals of 10-12 and 16-18 weeks of age.

The fine structural changes in corticotrophs following adrenalectomy have been extensively studied in the male rat, and the changes demonstrated at 2 weeks are in keeping with previously published data (Moriarty and Halmi, 1972). The larger nuclear size and prominence of nucleoli are consistent with increased production of ribosomes, greater numbers of which will be required for translation of POMC mRNA, the level of which is raised following adrenalectomy (Jingami et al, 1985). The increased granulation of corticotrophs at 6 weeks compared with 2 weeks would support the finding that at 2 weeks after adrenalectomy, although pituitary ACTH content is increased, it is still rising, while by 6 weeks a plateau has been reached (Fortier, 1959a). The effect of adrenalectomy has not been previously documented in the female rat. It is interesting that at both time points the morphological changes in the female were more pronounced than in the male, in agreement with the overall quantitative light microsopic studies. This may be due to the higher circulating glucocorticoid levels in the normal female animal and the higher threshold of negative feed-back. This may result in a greater positive drive to the corticotroph following adrenalectomy.

Leroux and Pelletier (1984) have studied the internalisation and degradation of CRF by rat pituitary corticotrophs using combined electron microscopic/autoradiographic studies, but the only report of the structural changes following CRF stimulation is an abstract by Kurosumi et al (1984) who reported that "CRF administration results in increased exocytosis" without reference to the dose administered or to the duration of treatment. The mode of hormone secretion by the corticotroph is unknown, but exocytosis of granules is not documented as a feature of normal corticotrophs, and has in addition not been reported in the stimulated cells (Zak et al, 1985). The results of the present study are in agreement with the latter findings, since, although evidence of exocytosis was specifically looked for, none was found. The effects of CRF in the present study provided confirmation that the Siperstein cell is the main target for this peptide since the changes seen in these cells resembled those following adrenalectomy. The increase in granulation presumably reflects a direct effect of CRF on ACTH synthesis, which is in keeping with the known biochemical action of the peptide (Vale et al, 1983).

As with the quantitative light microscopic study on the effects of CRF (McNicol, 1985) the magnitude of the corticotroph response to CRF was less marked than that following adrenalectomy. This may reflect submaximal stimulation of the corticotroph by the dose of CRF administered, the intermittent nature of the stimulus as compared with that following adrenalectomy or the multi-factorial nature of corticotroph stimulation in the latter state. Alternatively, the action of exogenous CRF may be

modulated by glucocorticoid negative feedback which is absent in the adrenalectomised animal.

The further changes induced by the addition of vasopressin support its role as a component of the stimulating complex. The reduction in granulation produced by vasopressin in conjunction with the higher CRF dose probably reflect augmented ACTH release relative to POMC production. This could account for the lack of increase in corticotroph Vv seen in the light microscopic studies with this combination of CRF and VP.

The reduction in adrenal weight after corticosterone administration is consistent with inhibition of corticotroph function. The difference between male and female rats probably reflects the differences in threshold of feedback. The smaller corticotroph area and poorly developed cytoplasmic processes are consistent with the finding of Caselitz and Saeger (1979) after 3 weeks of methylprednisolone. A reduction in granule numbers, as seen in the male rats at both corticosterone doses could be due either to increased release of hormone, as in the early stages following adrenalectomy (Siperstein and Miller, 1970) or to reduced hormone synthesis. In the corticosterone-treated male rats, there was also significant dissociation of ribosomes from the endoplasmic reticulum. Ribosomes are specifically directed to the endoplasmic reticulum by the signal peptide of the molecule they are translating (Alberts et al, 1983) and pre-POMC has been shown to contain such a peptide (Nakanishi et al, 1979). The large numbers of free cytoplasmic ribosomes would be consistent with a reduction in POMC synthesis. This

would be in keeping with the suggested effects of such feedback with inhibition not only of ACTH release (Engeland et al, 1975) but also of synthesis (Roberts et al, 1979). The similar effects seen at both doses suggest that a maximal inhibitory effect was achieved at the lower dose. Although the corticotrophs appeared to be inhibited, the changes were less marked than in the study of Siperstein and Miller (1970) who reported complete elimination of corticotrophs after 7 days of a similar dose of cortisol. It is unlikely that these differences are due to the different steroids used as they have similar actions. They may be the result of different methods of treatment, their continuous administration in drinking water producing a more profound delayed feedback stimulus because of its relatively uninterrupted nature. The changes in the male rats in this study after corticosterone administration are in keeping with the reduction in pituitary ACTH content after the administration of a similar dose of cortisol for 32 and 55 days (Fortier, 1959b). The presence of small numbers of corticotrophs with an increase in granules suggests that in these, ACTH release was mainly affected. This would suggest heterogeneity among the corticotroph population.

In the female rat, the lower dose of corticosterone produced only an increase in granule content, with less obvious dissociation of ribosomes, suggesting a predominant effect on ACTH release. With the higher dose, the mixture of corticotrophs with increased and decreased granule content suggests that a threshold for the inhibition of ACTH synthesis may have been achieved at these doses.

Again, there is evidence of heterogeneity within the population. Even at this dose,the changes consistent with inhibition of synthesis are less pronounced than those induced in the male. These differences presumably reflect the higher threshold of negative feedback in the female.

The ultrastructural changes exhibited by corticotrophs after chronic dexamethasone administration have not been previously documented. In these animals, plasma corticosterone levels (data not shown) and reduced adrenal weight (Chapter 2) were consistent with inhibition of corticotroph function. The differences between the changes in dexamethasone-inhibited corticotrophs and those induced after an equivalent dose of corticosterone may reflect the reported differences in the mechanism of their actions on the HPA axis. The biochemical data of Sakakura et al (1982) suggested a primary inhibitory action of dexamethasone on ACTH release from corticotrophs, rather than on synthesis, particularly at low doses. There is evidence, however that dexamethasone can inhibit the production of POMC mRNA (Nakanishi et al, 1977; Jingami et al, 1985) but in these studies the dose of dexamethasone administered was much higher (up to 7 times the present dose). In addition, the animals had been adrenalectomised, so the administration of glucocorticoid was re-establishing negative feedback, whereas in the present study, the inhibition of basal function was being studied.

The abundance of the Golgi complex and rough endoplasmic reticulum along with the striking increase in the secretory granules in the present study would be consistent with the suggestion that ACTH release is inhibited to a

greater extent by dexamethasone than synthesis. The retention of the cellular outline after dexamethasone is not understood at present but may also indicate differences in the mechanism of inhibitory action of both steroids. It is interesting to speculate that a primary action of dexamethasone at the pituitary level (Sakakura et al, 1982) might still allow stimulation of the corticotrophs by hypothalamic factors, and thus allow them to maintain certain structural features. These differences however, require further investigation. The use of gene probes for POMC mRNA in in-situ hybridisation studies and in dot blot analysis of extracted mRNA would allow a more precise characterisation of the responses of these cells. The altered morphology of the secretory granules shown by the majority of inhibited cells was particularly obvious in corticotrophs of rats treated with dexamethasone and may reflect alteration in hormone synthesis and granule maturation. The mechanisms of this are unclear at present.

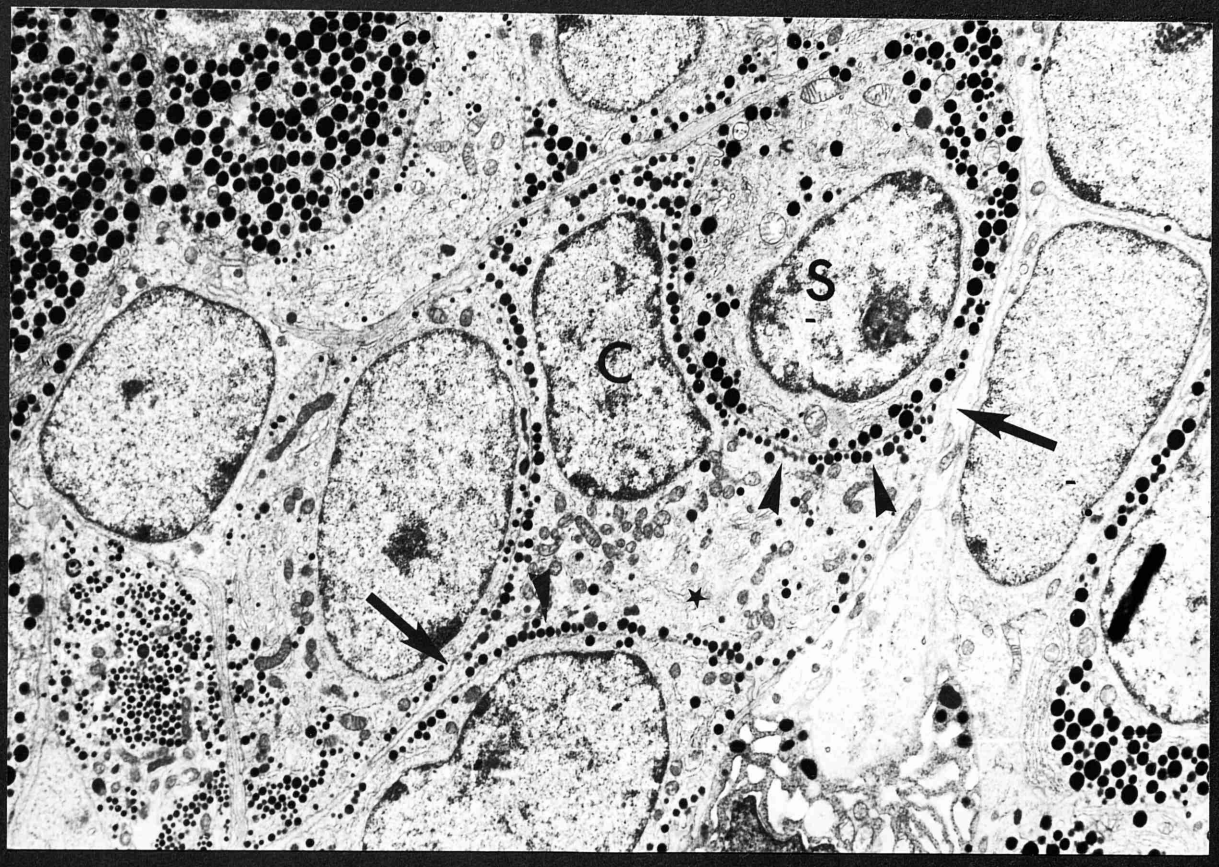
The increase in lysosomes and multivesicular bodies in the cytoplasm of suppressed cells is in keeping with intracellular breakdown of hormone in suppressed cells (Farquhar, 1971).

Figures. 4:1 and 4:2

Normal male rat pituitary. (x 6,600)

"Siperstein" corticotrophs are present (C) showing the typical stellate appearance with processes extending between other cells (➔). The nuclei contain one or two nucleoli, and dispersed chromatin. The secretory granules show the characteristic distribution along the cell membrane (▲▲). The mitochondria are slender, and often twisted. Rough endoplasmic reticulum (RER) (*) is usually arranged in short lamellae. The Golgi complex is prominent and may contain a few immature granules.

Somatotrophs (S) are also present.


S
C

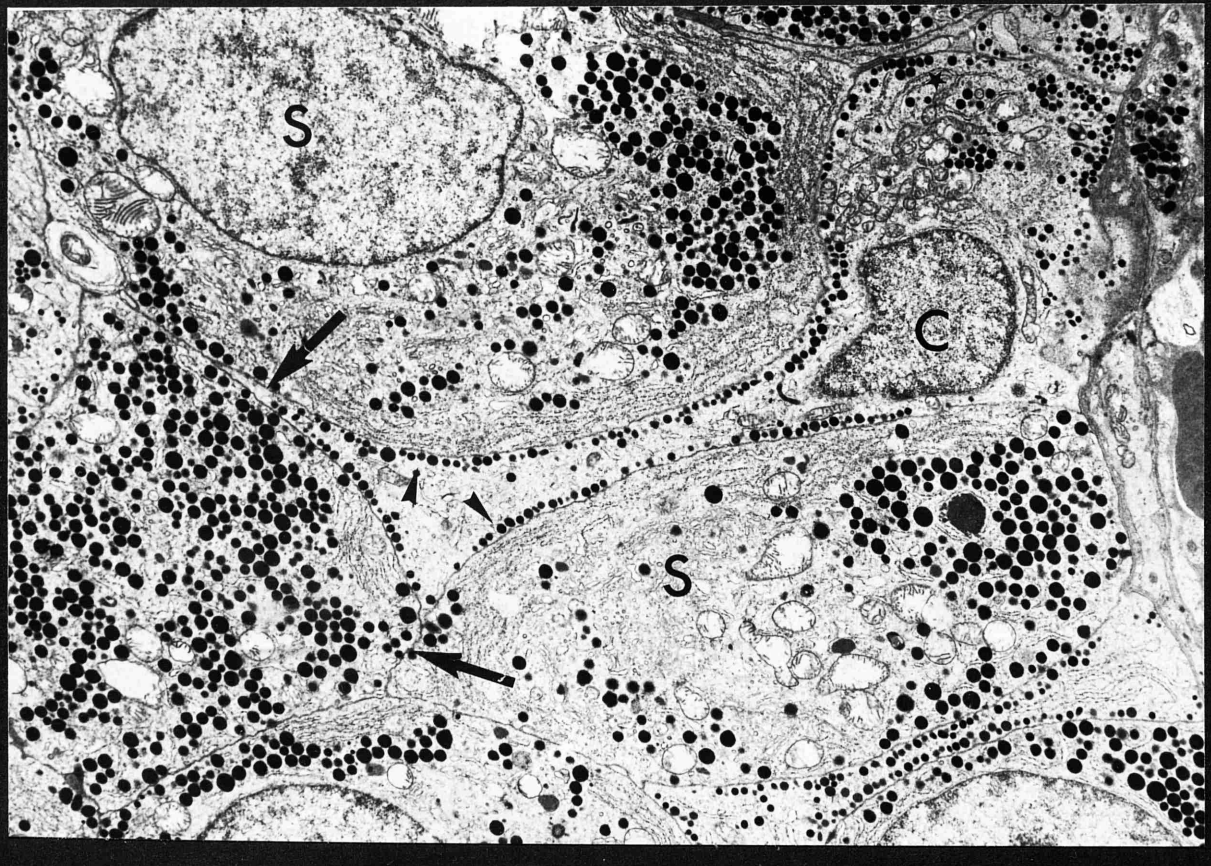
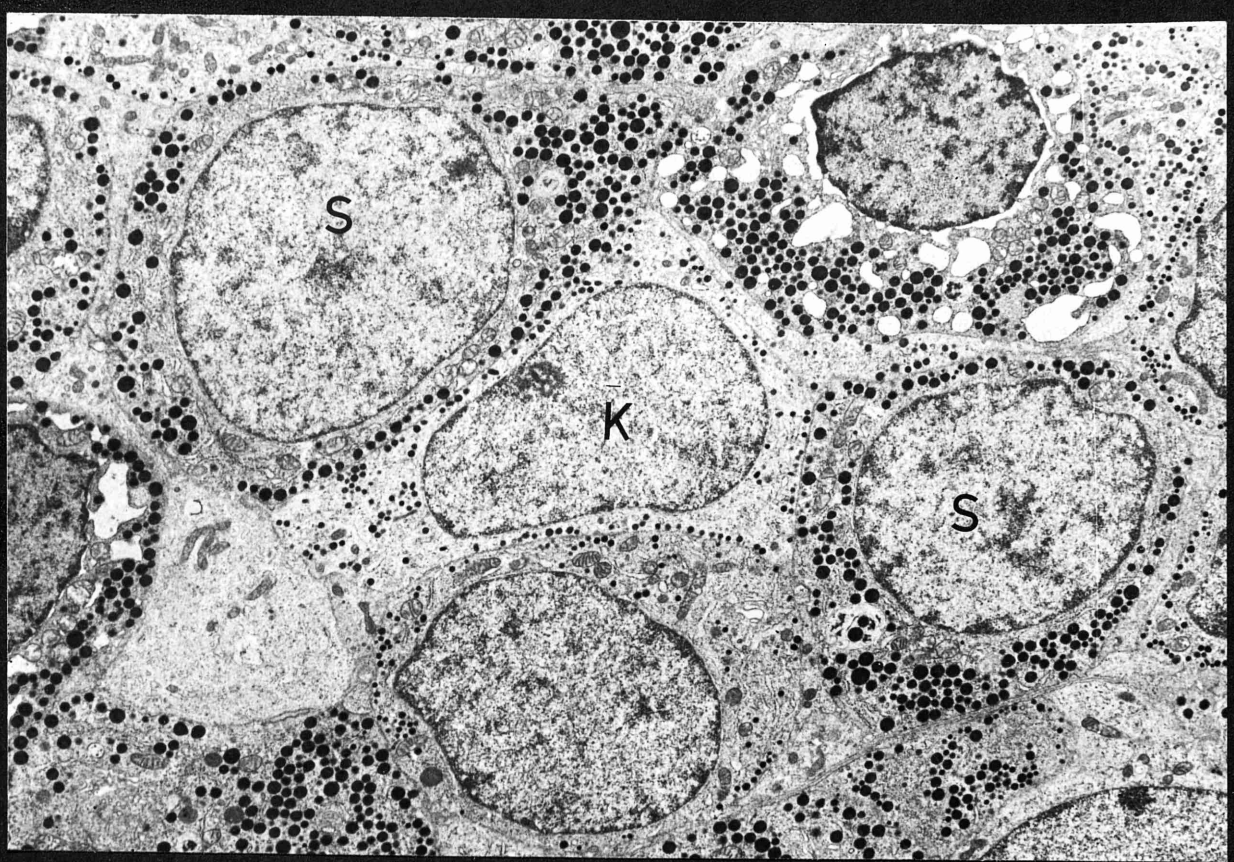

S
C
S

Figure. 4:3

Normal rat pituitary. (x 6,600).

A "Kurosumi" corticotroph (K) is present. The shape is stellate, but the secretory granules are smaller and more widely distributed within the cytoplasm.

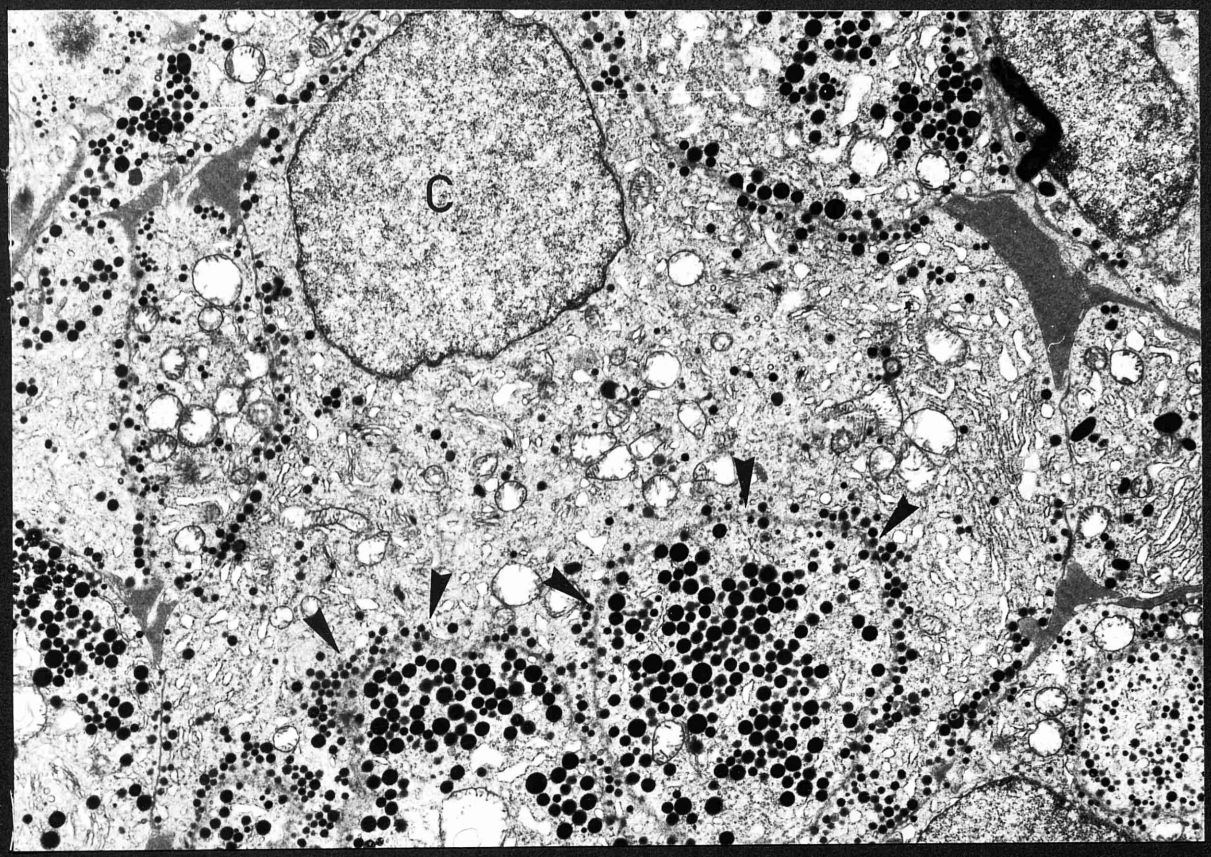
Somatotrophs (S) are also present.


S
K
S

Figures. 4:4 and 4:5

Male rat pituitary: 2 weeks post-adrenalectomy (x 7,920).

There is an obvious increase in cell area of corticotrophs (C) with greater contact with blood vessels. Many sections show little nucleus (Fig.4:5). The cytoplasmic processes are thicker and often surround other cells (▲▲). The RER is increased, and most obviously lamellar in arrangement (*). Granules are increased in number and more randomly distributed.


C

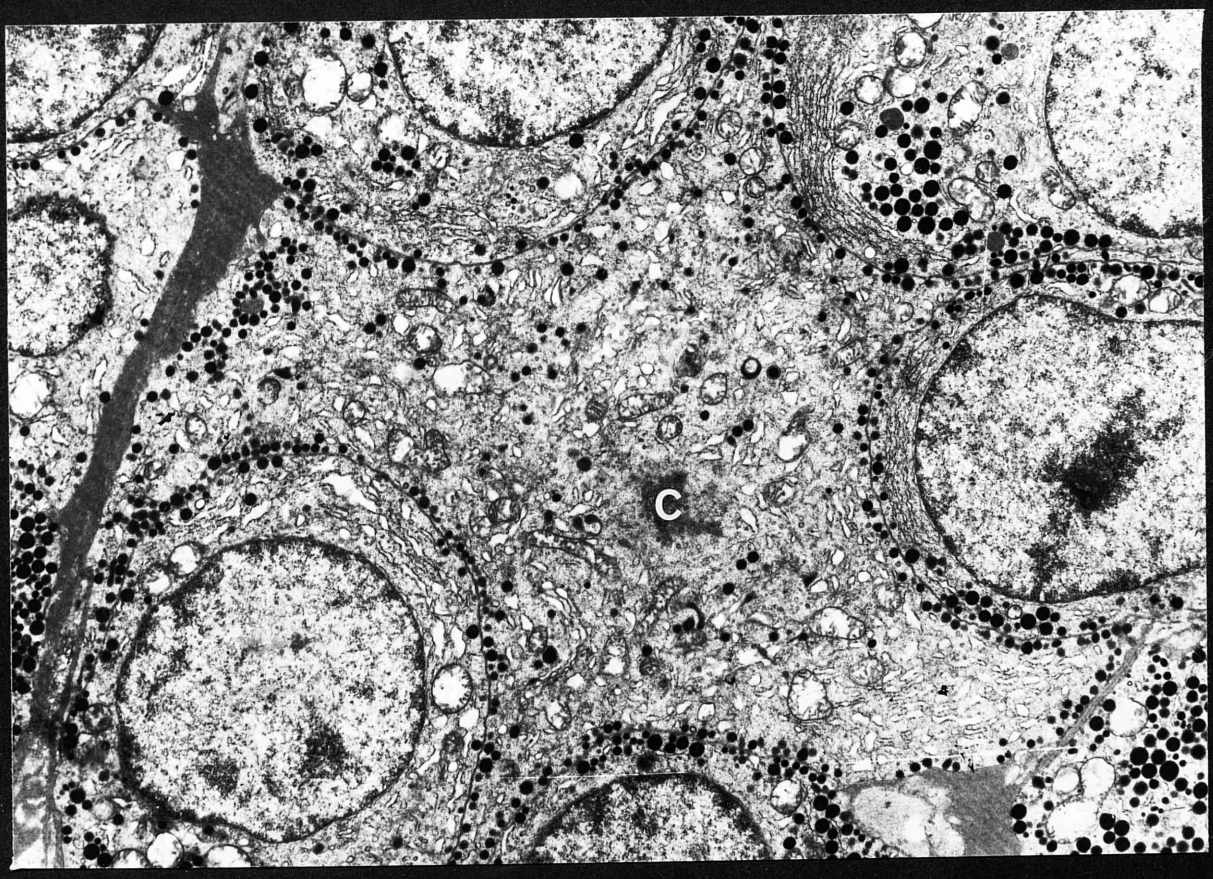

C

Figure. 4:6

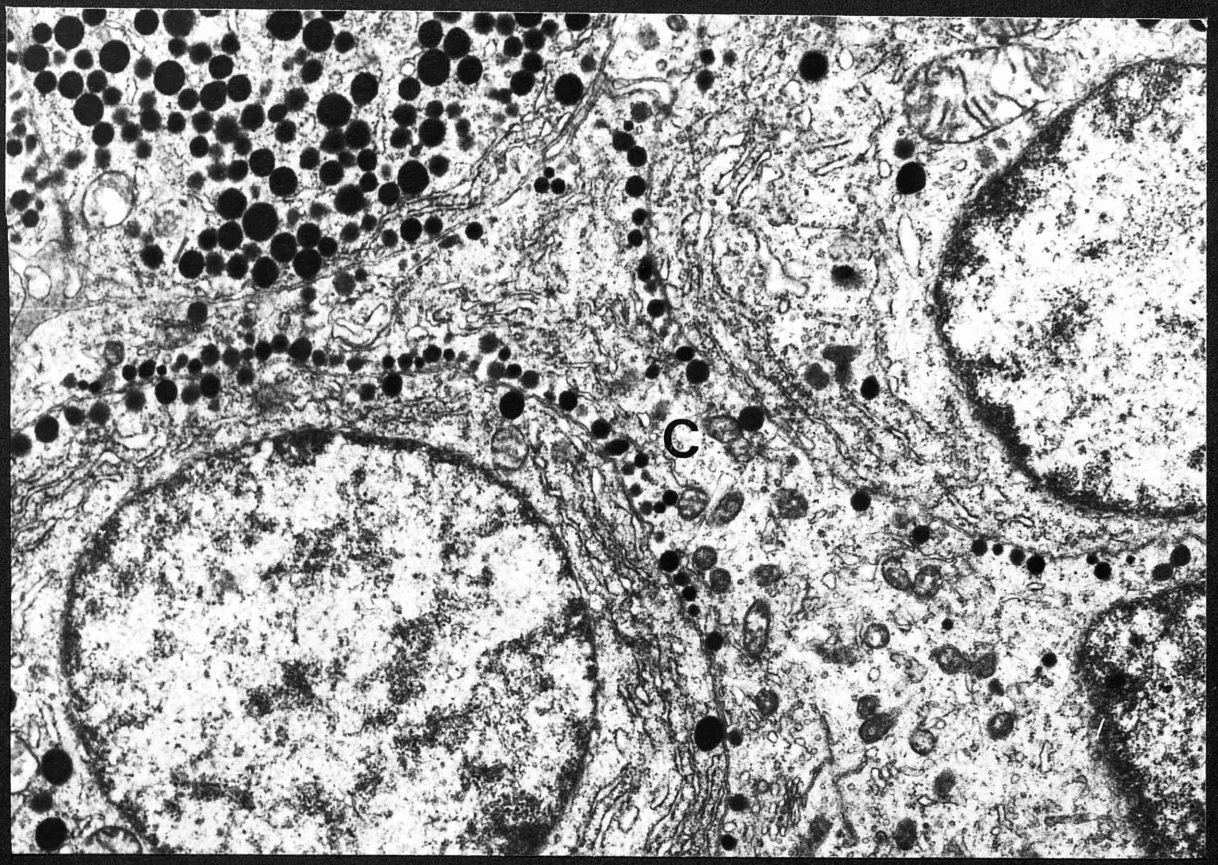
Male rat pituitary: 2 weeks post adrenalectomy (x 9,460).

Section of a corticotroph (C) with the secretory granules showing variable degrees of electron density.

Figure. 4:7

Female rat pituitary: 2 weeks post-adrenalectomy. (x 4,840).

This illustrates the more prominent changes occurring in the female gland. Nucleoli are prominent. RER is markedly increased (*). There is a reduction in the granule content of the cells.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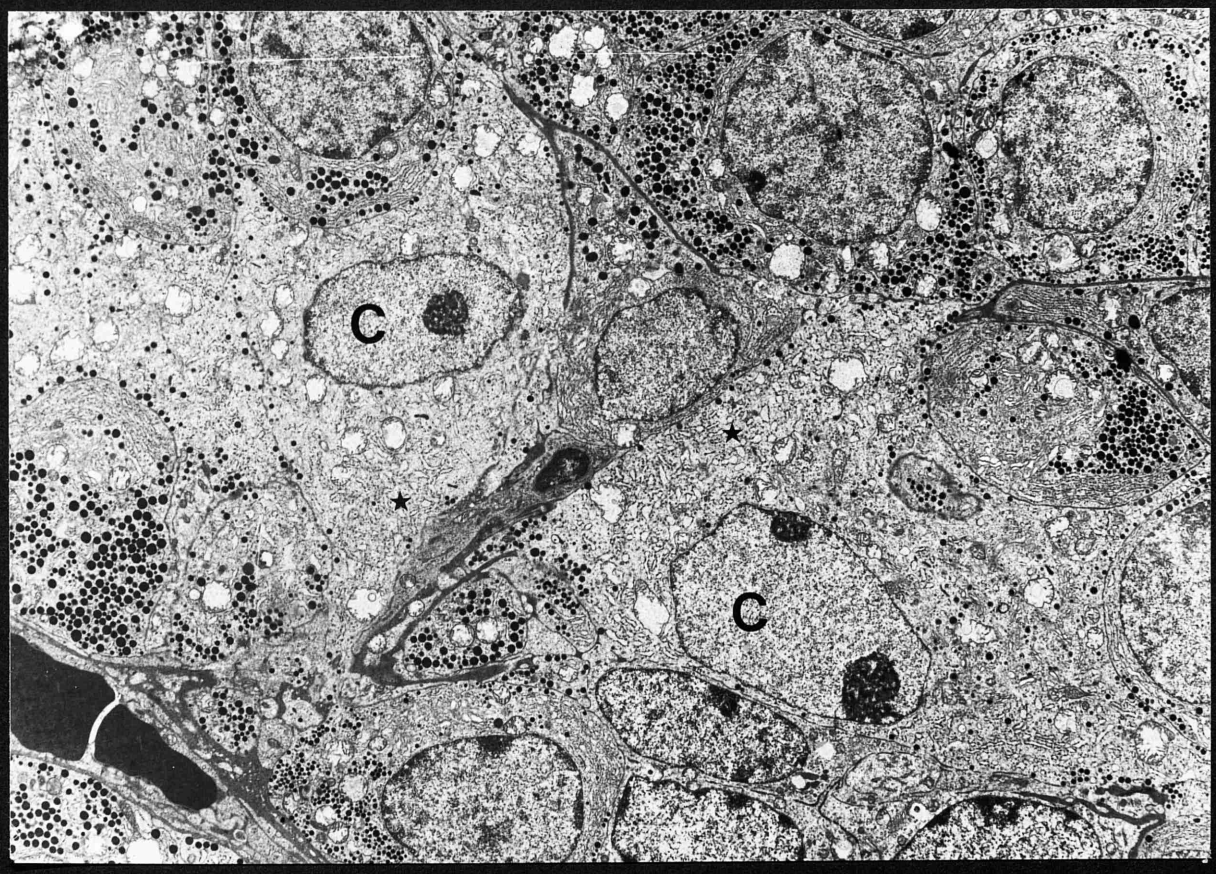

C
C

Figure. 4:8

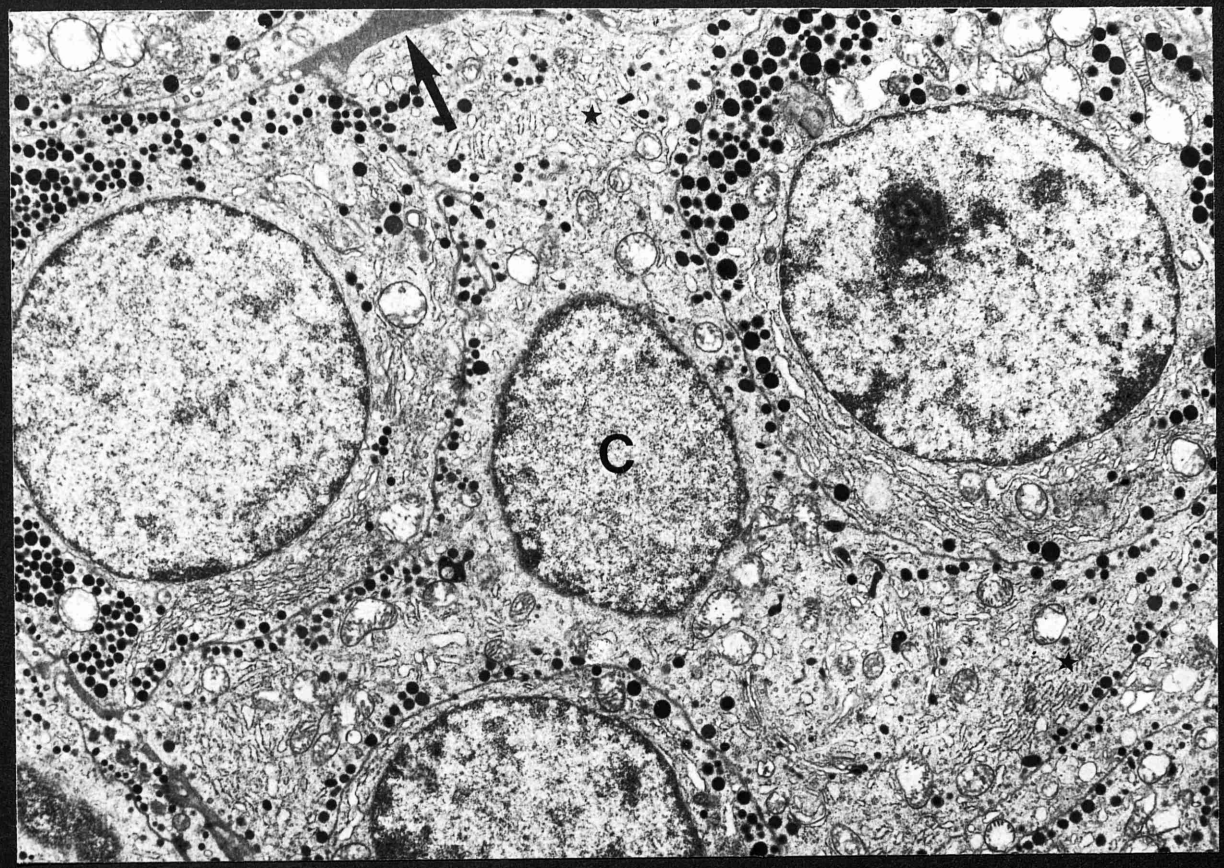
Male rat pituitary: 6 weeks post-adrenalectomy (x 6,600).

Changes are similar to those seen at 2 weeks. (↓ indicates vessel : * RER).

Figure. 4:9

Female rat pituitary: 6 weeks post-adrenalectomy (x 6,600).

Changes are similar to those seen at 2 weeks.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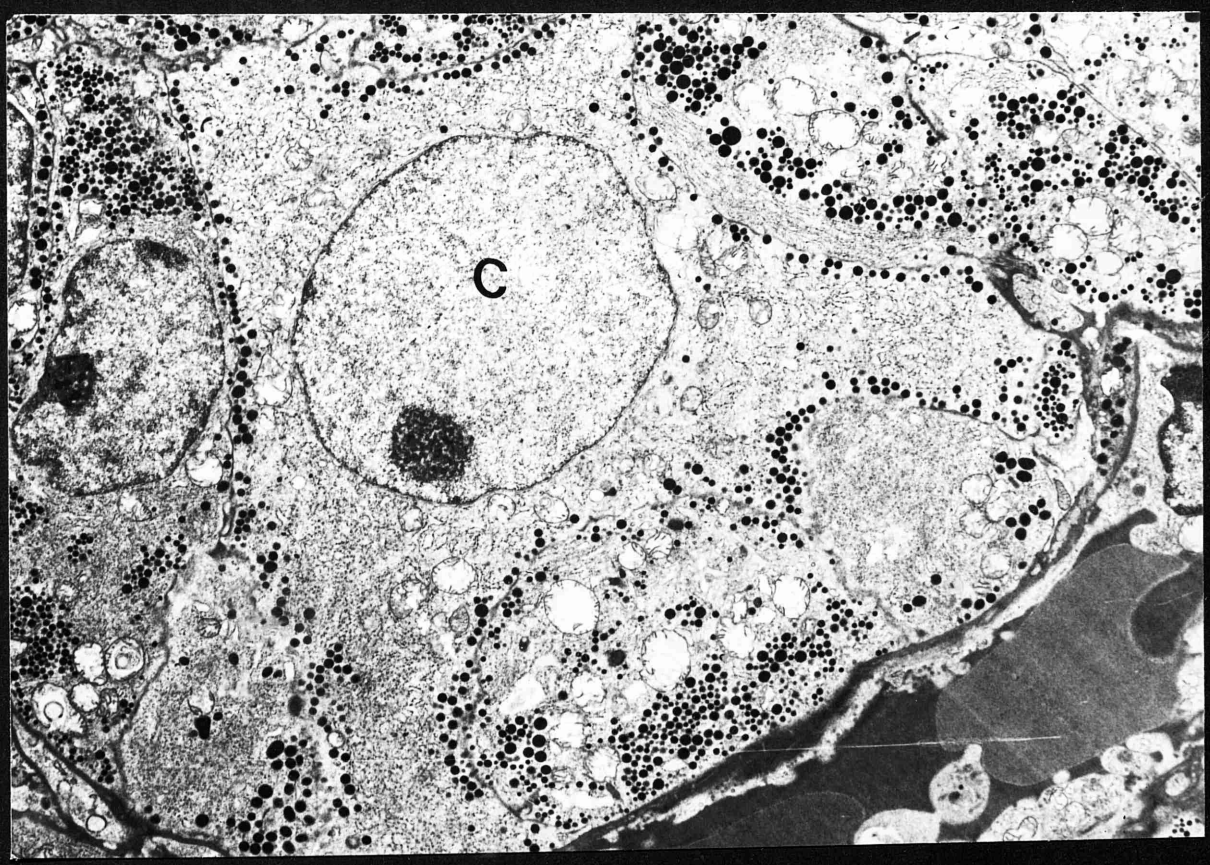
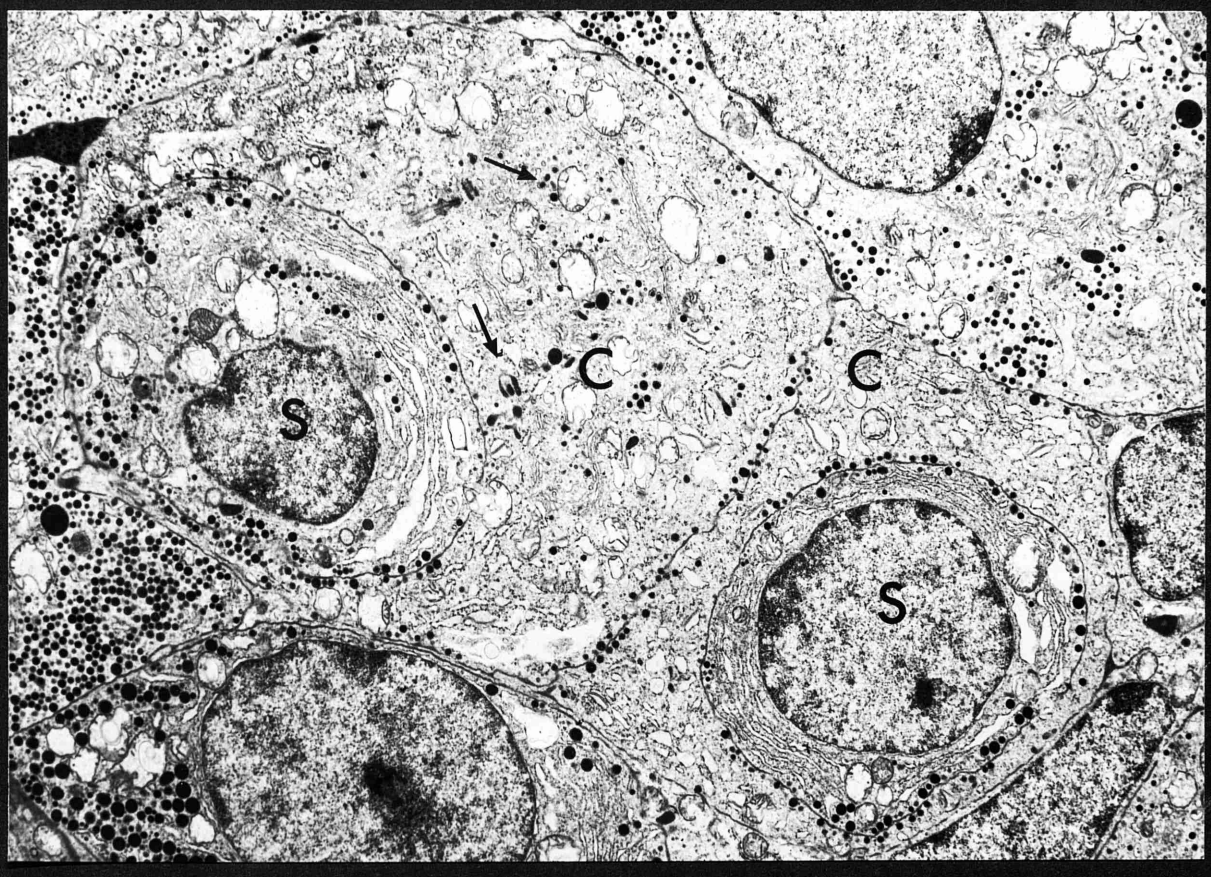

C

Figure. 4:10

Female rat pituitary: 6 weeks post-adrenalectomy (x 6,600).

Sections of two corticotrophs (C) are present. These are completely engulfing somatotrophs (S). The secretory granules in association with the Golgi complex (→) exhibit variable shapes and sizes.

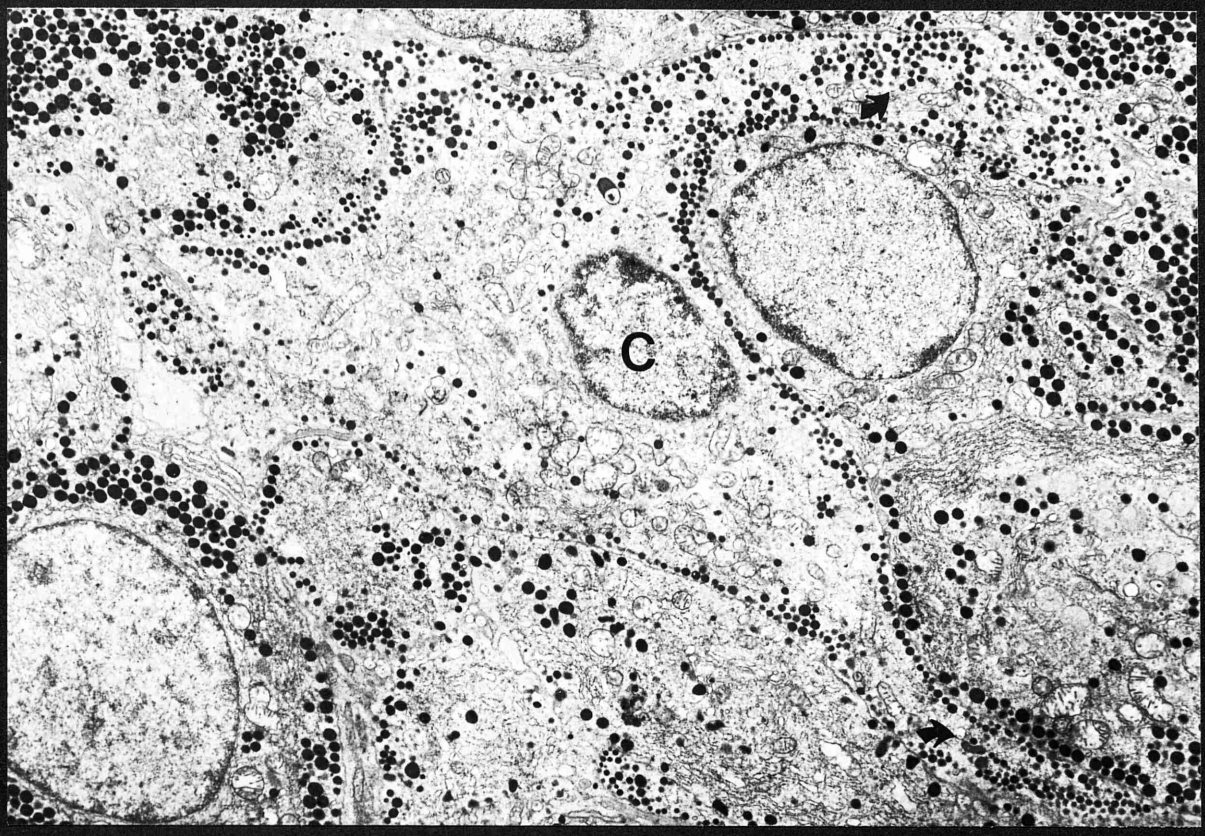

S
C
C
S

Figures. 4:11 and 4:12

Male rat pituitary: CRF (50µg/Kg/day) for 2 weeks (x 6,600).

4:11 Corticotroph (C) showing remarkable irregularity of outline with a few wide cytoplasmic processes. The secretory granules are increased in number, accumulating mainly in the cytoplasmic processes (↗).

4:12 There is an obvious increase in cell area of the corticotroph (C) which is engulfing a somatotroph (S).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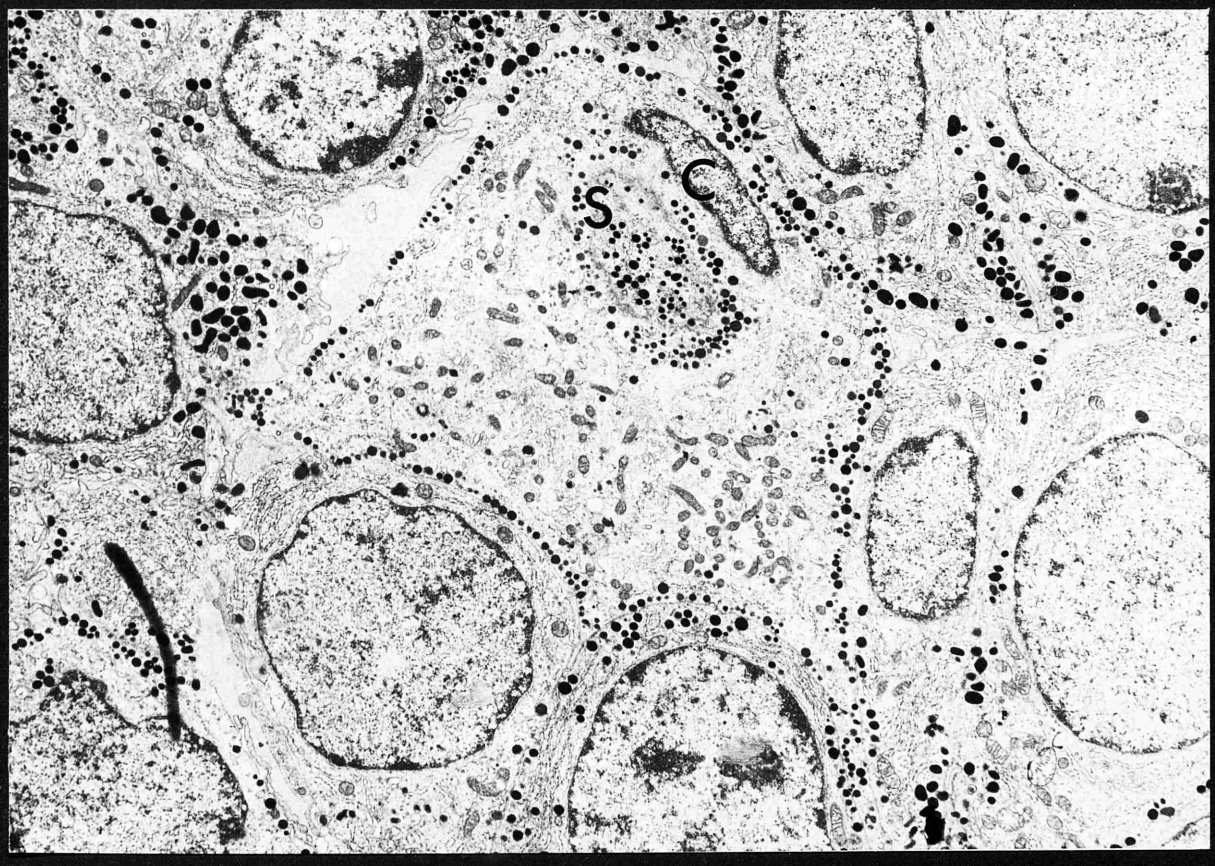

S
C

Figure. 4:13

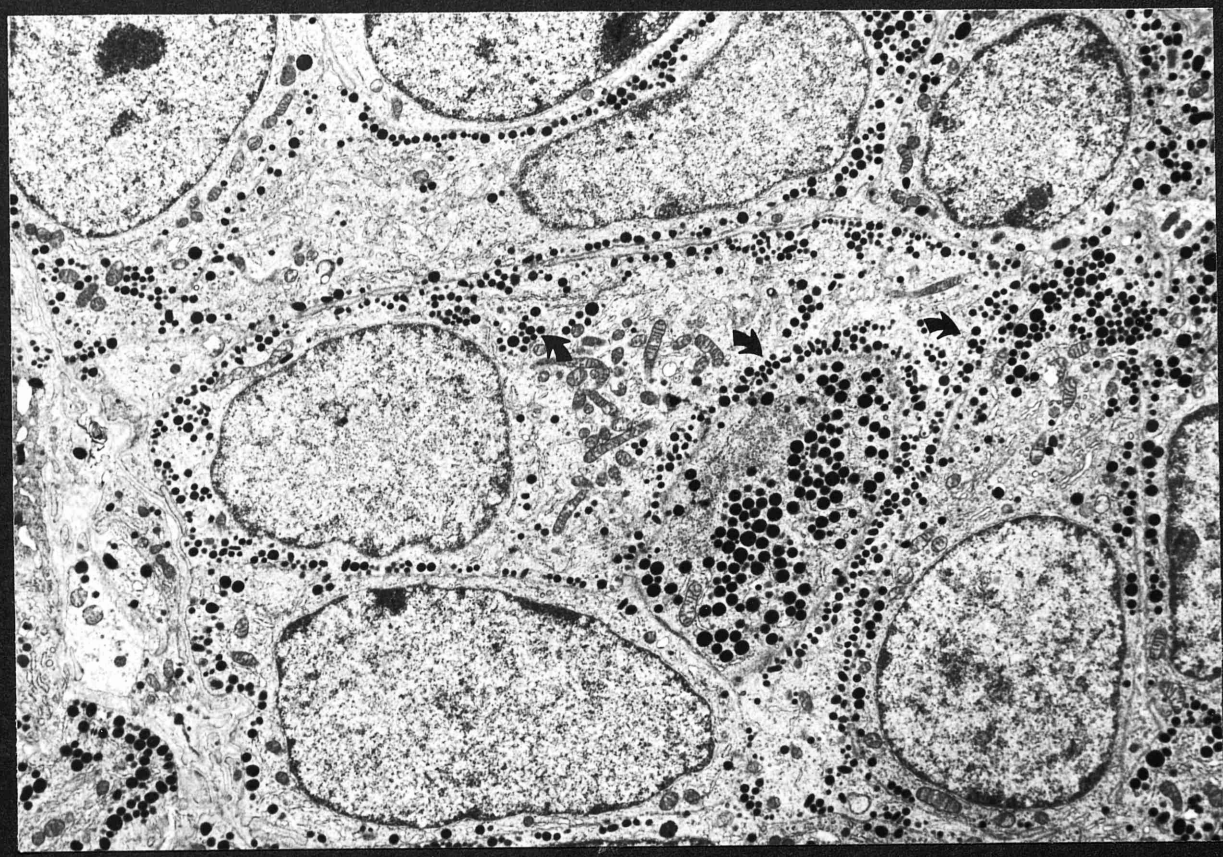
Male rat pituitary: CRF (25µg/Kg B.W.) plus vasopressin (VP) (0.4 IU/Kg) for 2 weeks. (x 6,600).

There is an increase in cell area of the corticotroph (C). Secretory granules are prominent (↗).

Figure. 4:14

Male rat pituitary: CRF (50µg/Kg) plus VP (x 6,600) daily for 2 weeks.

There is a significant reduction in the number of secretory granules present (→) in this corticotroph (C).



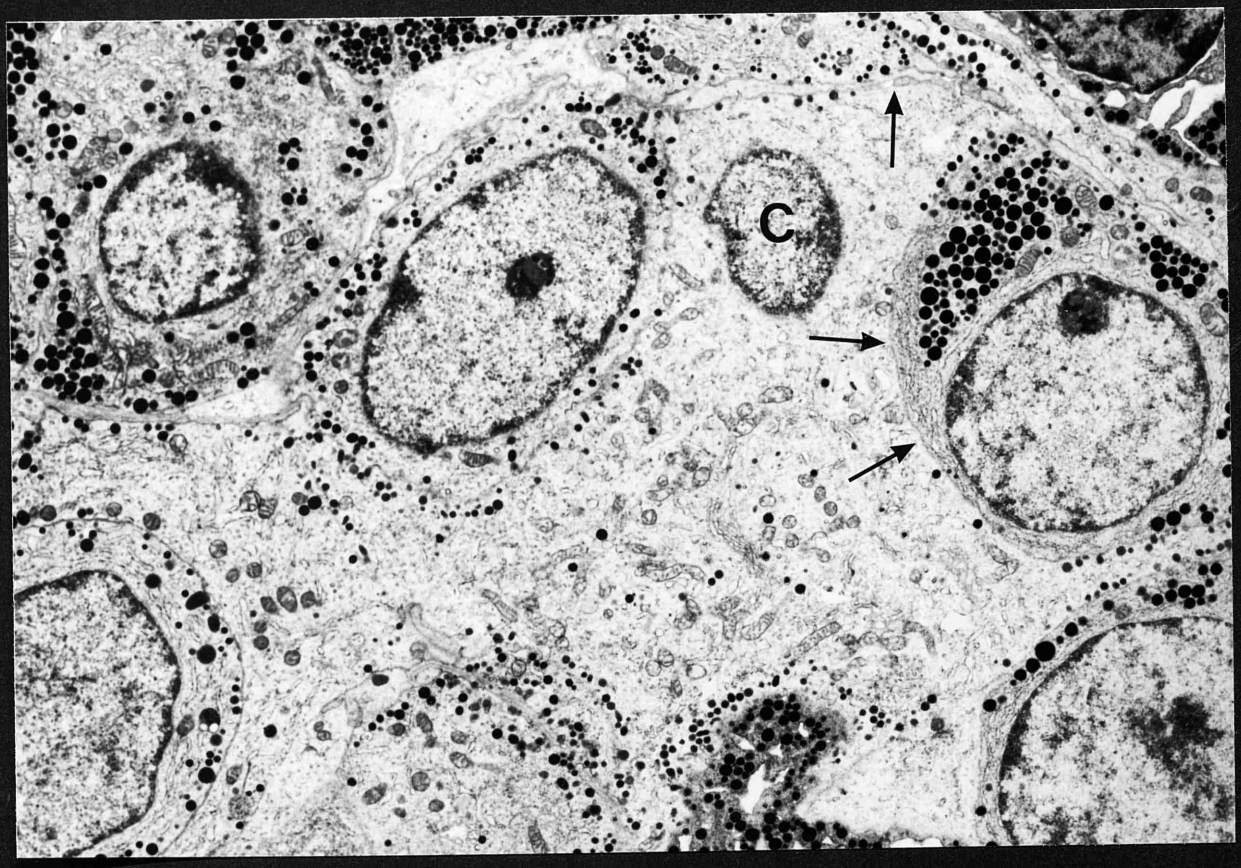
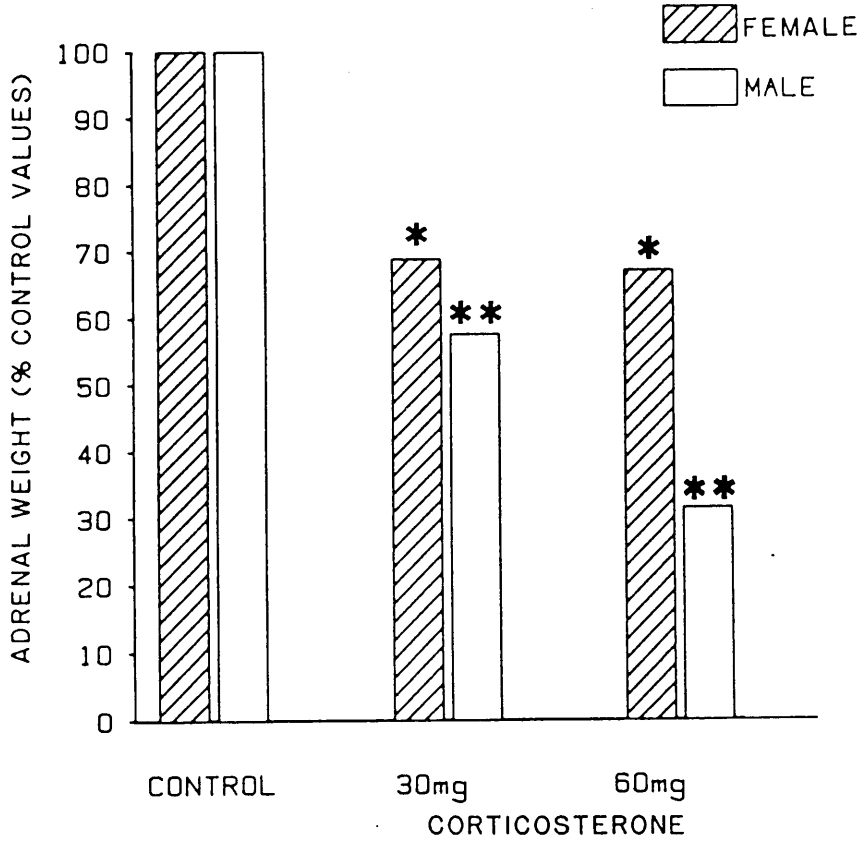

C

Figure. 4:15

Effect of corticosterone administration on adrenal weight in male and female rats.



Steroid/Kg body weight

* $p < 0.01$ vs Control.

** $p < 0.001$ vs Control.

Adrenal weights are shown as a percentage of mean control value because of the consistently heavier adrenals in normal female rats.

Figure. 4:16

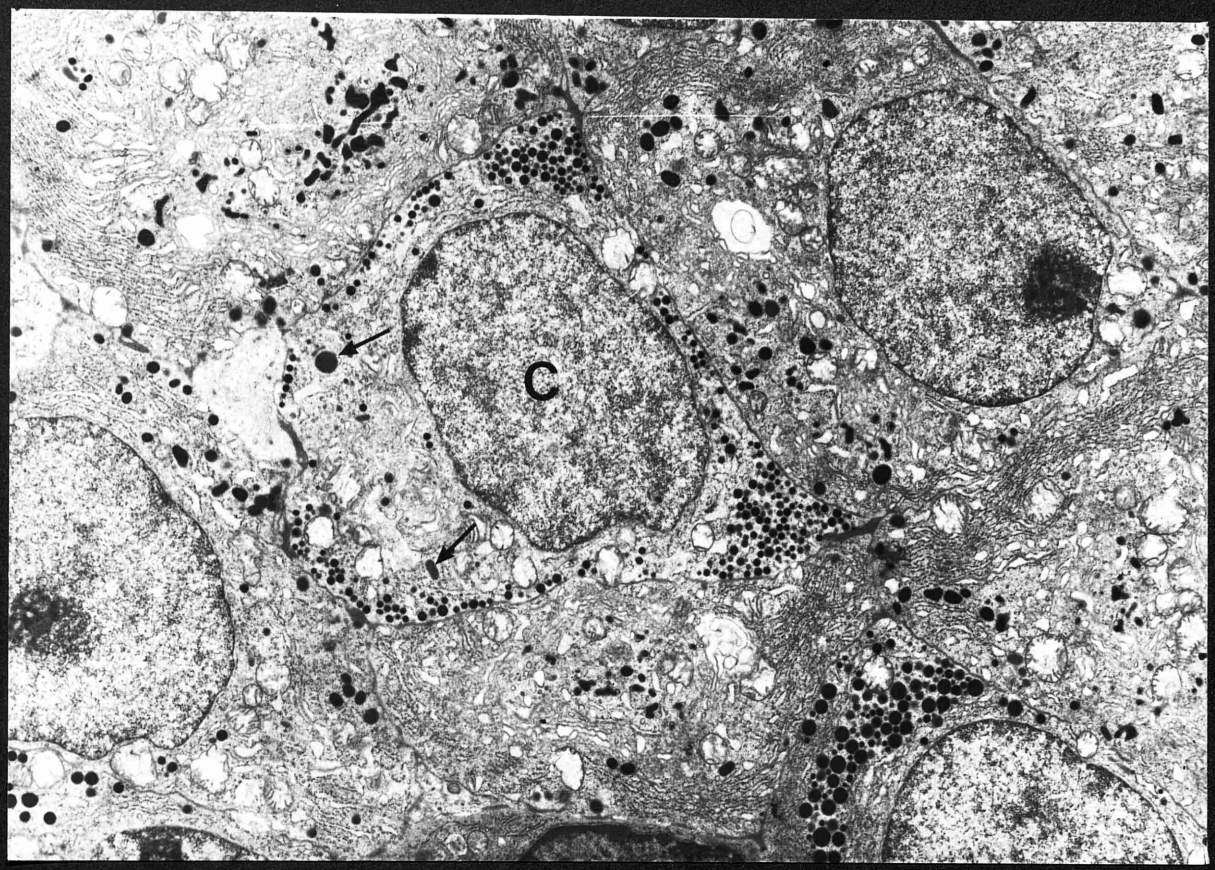
Female rat pituitary: corticosterone 30mg/Kg: 6 weeks (x 7,920).

The corticotroph (C) shows a less pronounced stellate shape. There is accumulation of granules in the processes and these are more variable in shape and size than controls. Mitochondria appear reduced in number. The Golgi complex is not prominent. Lysosomes are easily identified (➔).

Figure.4:17

Male rat pituitary: corticosterone 30mg/Kg: 6 weeks (x 13,760).

Corticotroph (C) with few secretory granules accumulating at one end of the cell. Other changes are same as above.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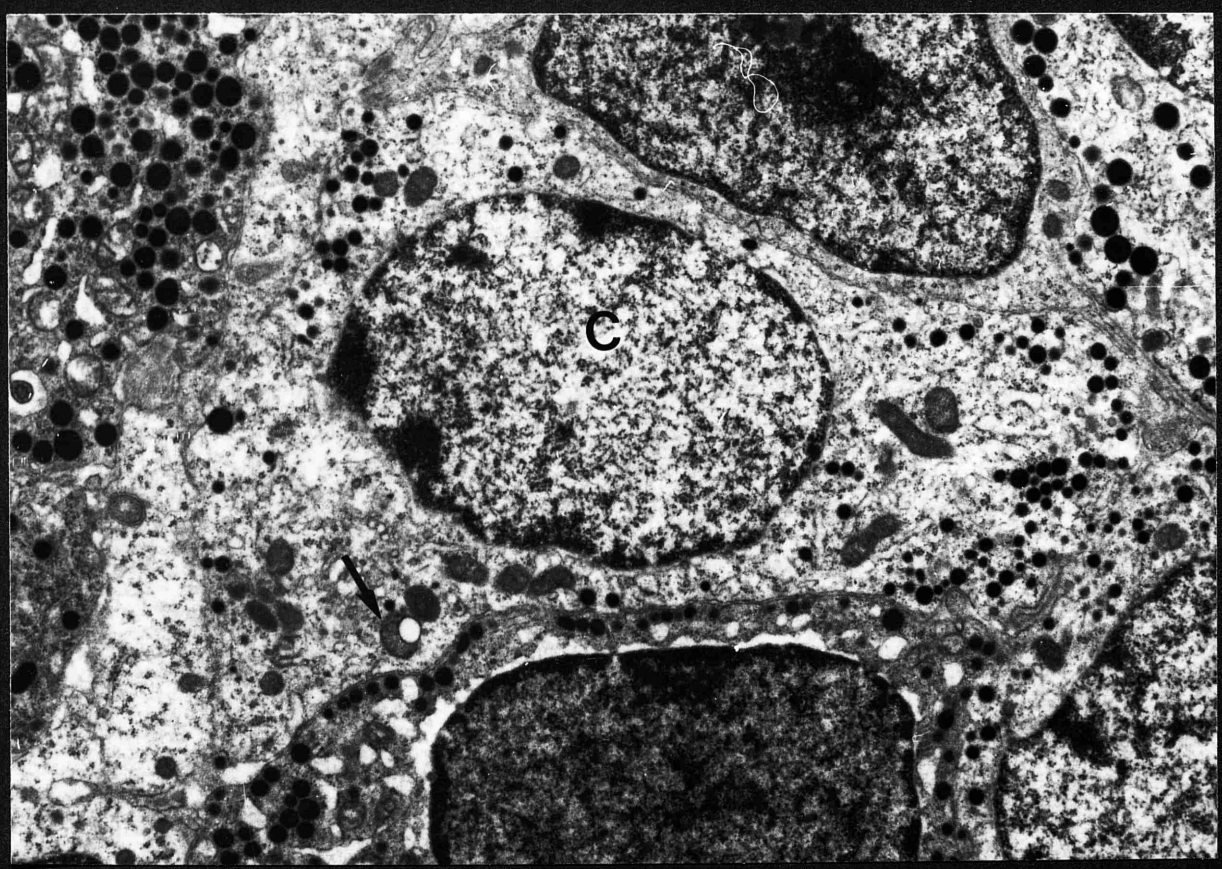
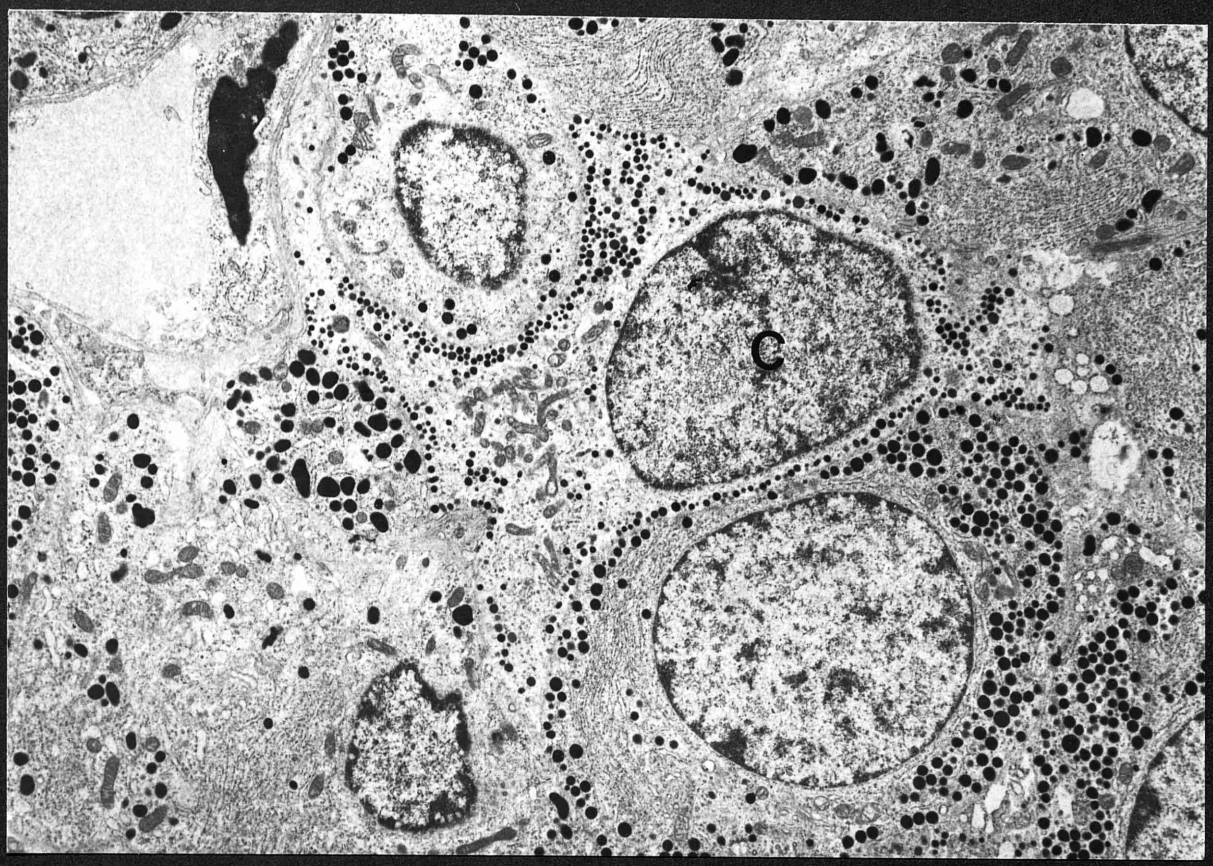

C

Figures. 4:18 and 4:19

Female rat pituitary. Corticosterone 60mg/Kg: 6 weeks.

4:18 (x 6,600) Corticotroph (C) showing stellate shape with an increase in secretory granules. Mitochondria are abundant. There is evidence of dissociation of RER.

4:19 (x 6,600) There is asymmetry of distribution of granules and variability in shape and size.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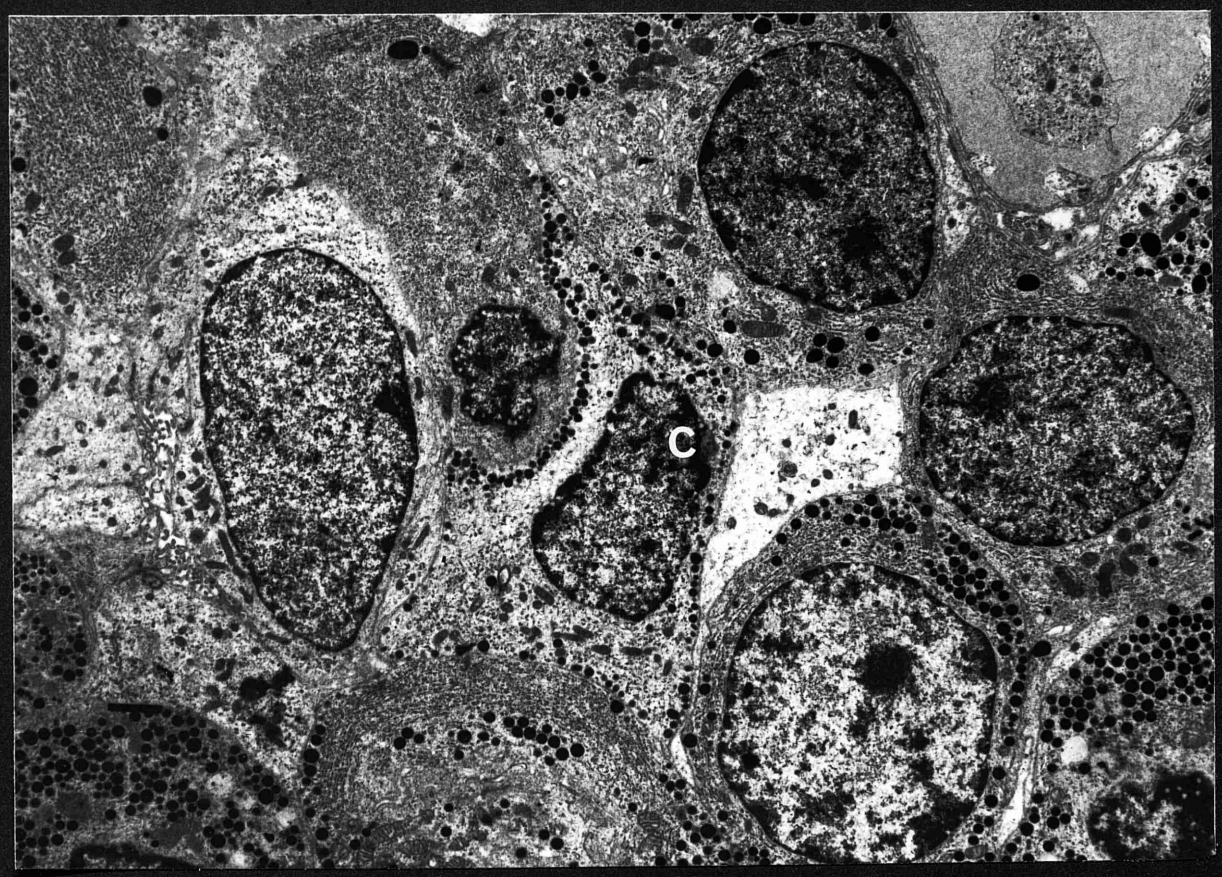
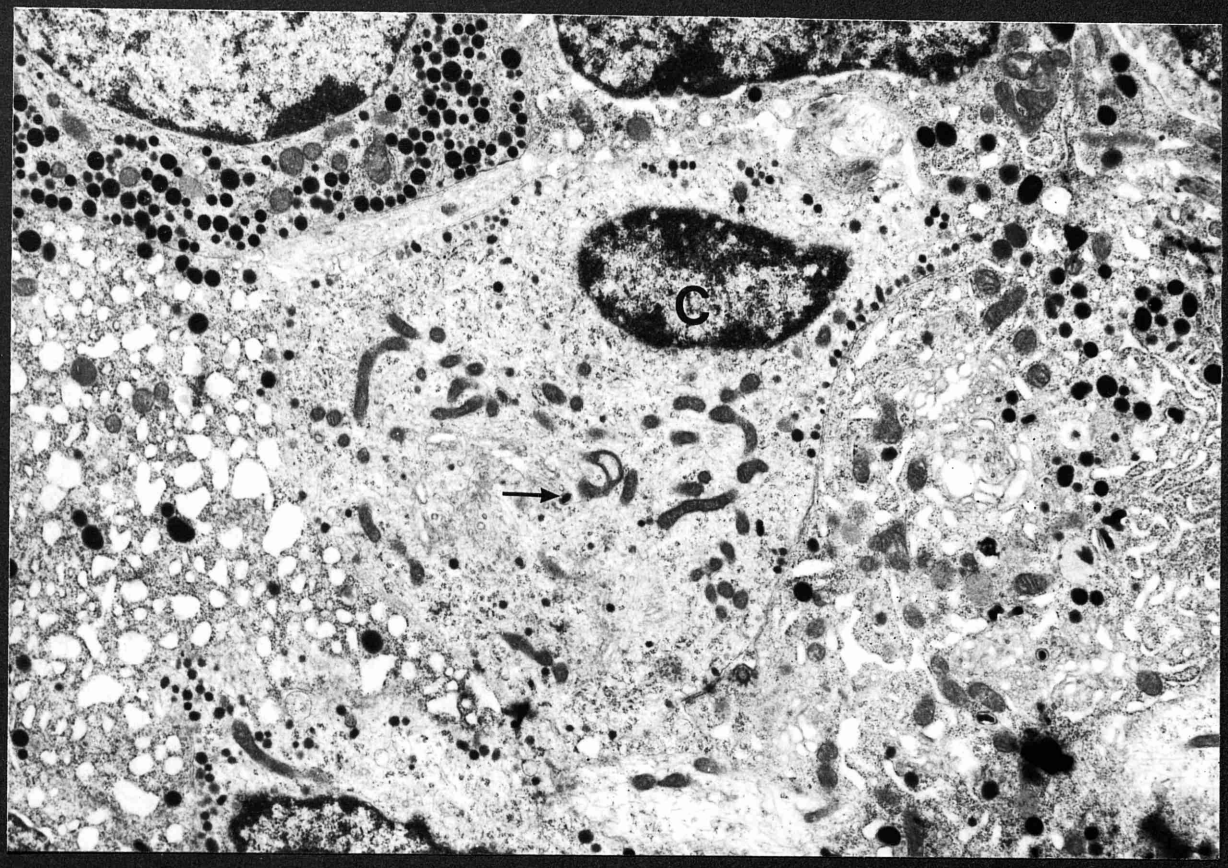

C

Figures. 4:20 and 4:21

Female rat pituitary: corticosterone 60mg/Kg: 6 weeks.

4:20 (x 6,600) Reduced numbers of secretory granules are present, of variable shape, size and electron density. A few granules are seen in Golgi complex. (→). There is dissociation of RER.

4:21 (x 39,000) This illustrates the variability in secretory granules in a corticotroph process (C).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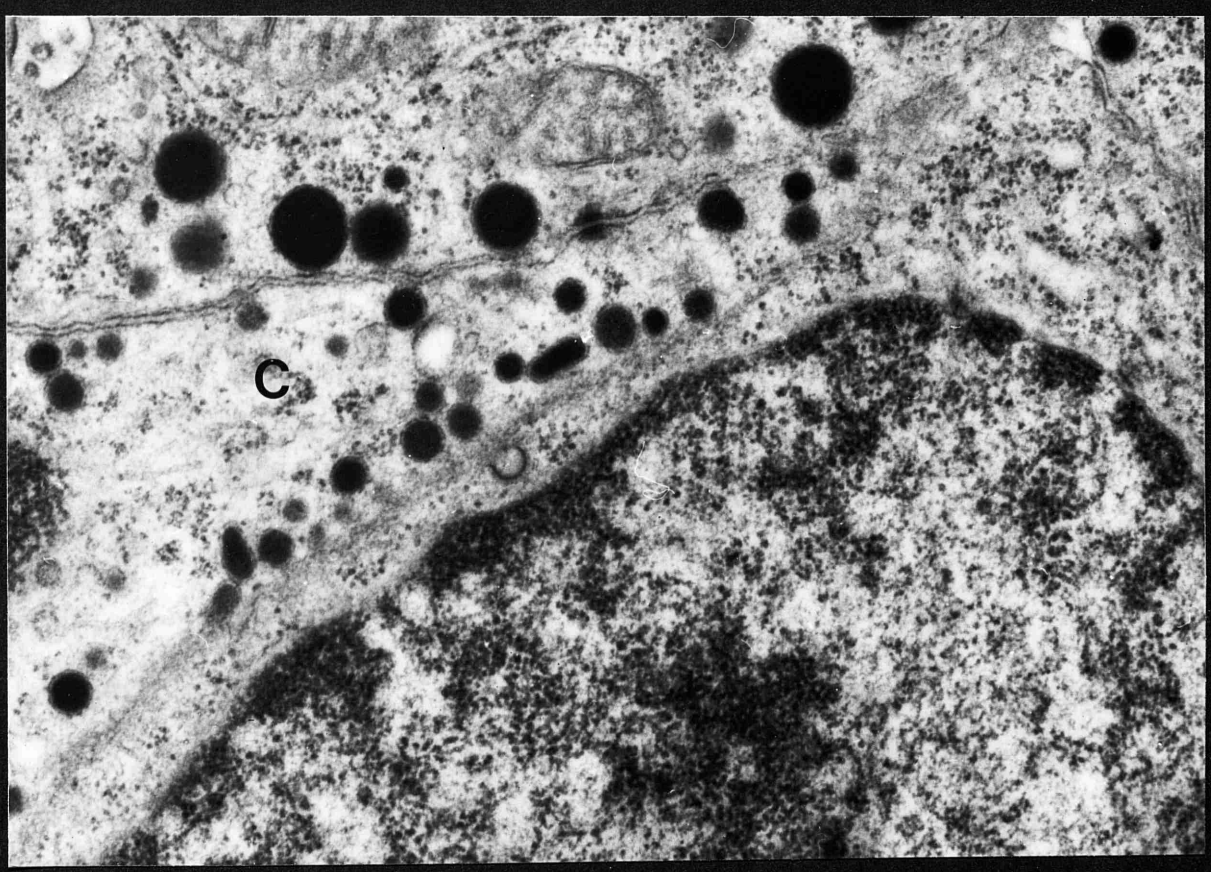
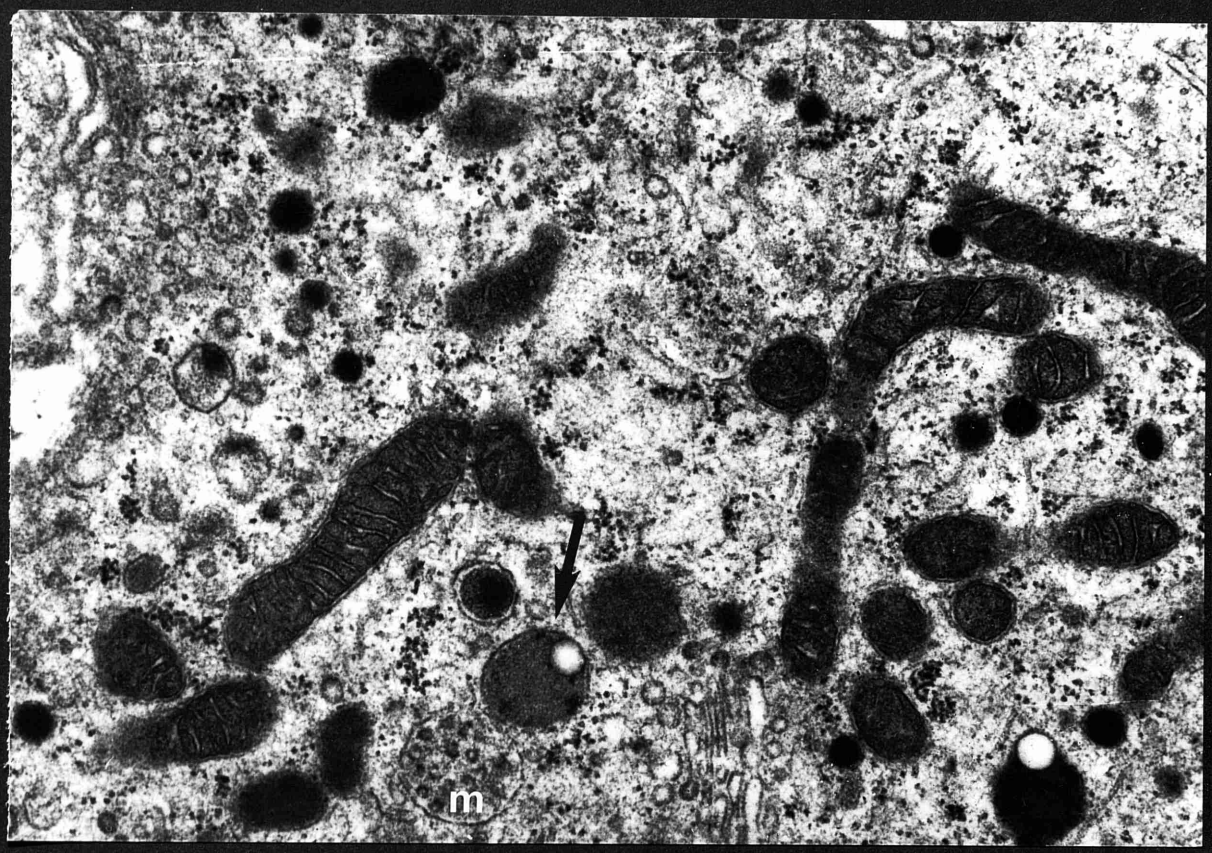

C

Figure. 4:22

Female rat pituitary: corticosterone 60mg/Kg: 6 weeks. (x 39,600).

Marked dissociation of ribosomes from RER is seen (▲▲). Lysosomes are present (⟶)and a multivesicular body (m) is present.


m

Figures. 4:23 and 4:24

Male rat pituitary: corticosterone 60mg/Kg: 6 weeks.

4:23 (x 10,750) Secretory granules are again variable in size and shape in this corticotroph (C) which has lost its characteristic stellate shape.

4:24 (x 9,460) Corticotroph (C) showing stellate shape but reduced secretory granules of variable shape and size.

Figures. 4:23 and 4:24

Male rat pituitary: corticosterone 60mg/Kg: 6 weeks.

4:23 (x 10,750) Secretory granules are again variable in size and shape in this corticotroph (C) which has lost its characteristic stellate shape.

4:24 (x 9,460) Corticotroph (C) showing stellate shape but reduced secretory granules of variable shape and size.

Figure. 4:25

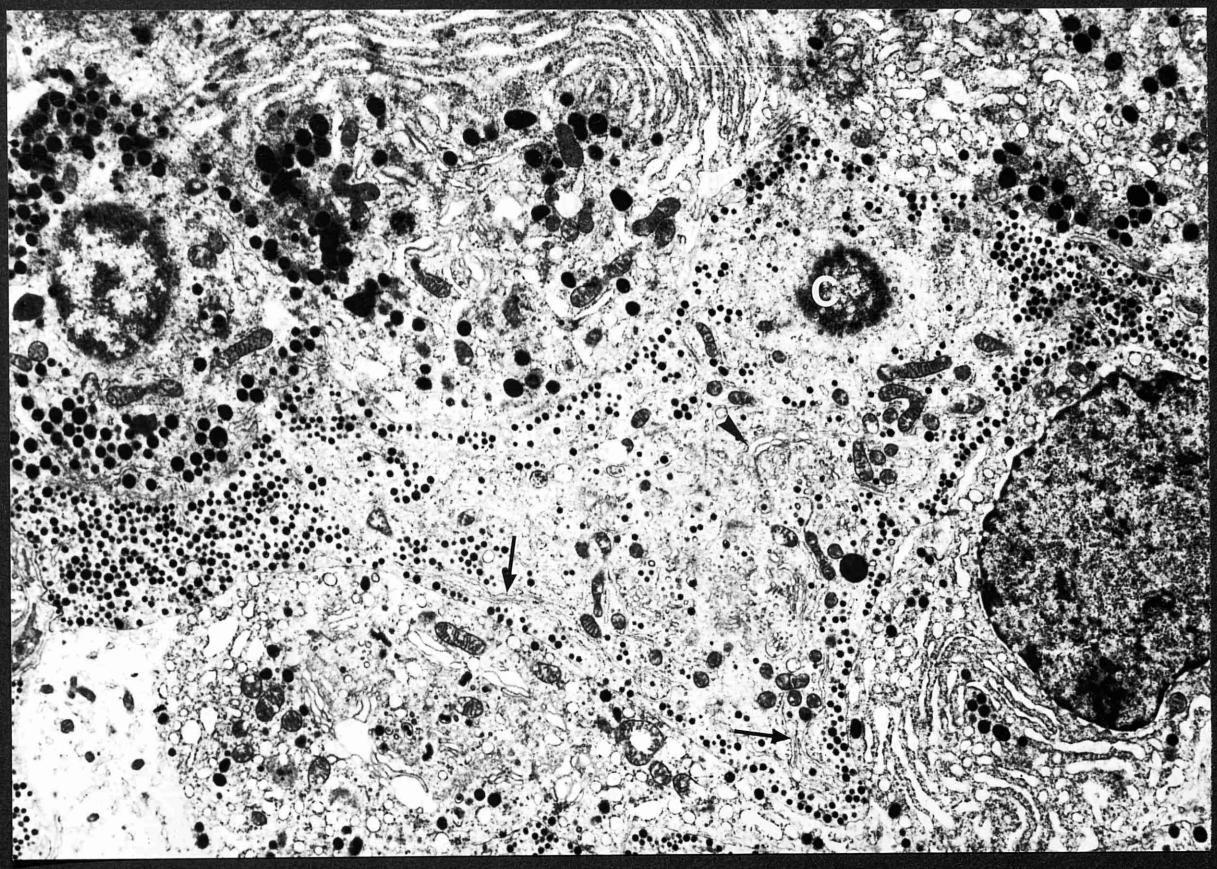
Female rat pituitary: dexamethasone 500µg/Kg: 6 weeks. (x 7,920).

Corticotroph (C) showing considerable complexity of outline. There is an obvious increase in secretory granules with massive aggregation in cell processes. RER is easily identified (➔). The Golgi complex is prominent (▲▲) and contains secretory granules.

Figure. 4:26

Female rat pituitary: dexamethasone 500µg/Kg: 6 weeks. (x 12,980).

Numerous mitochondria are seen. Secretory granules show variation in shape and size.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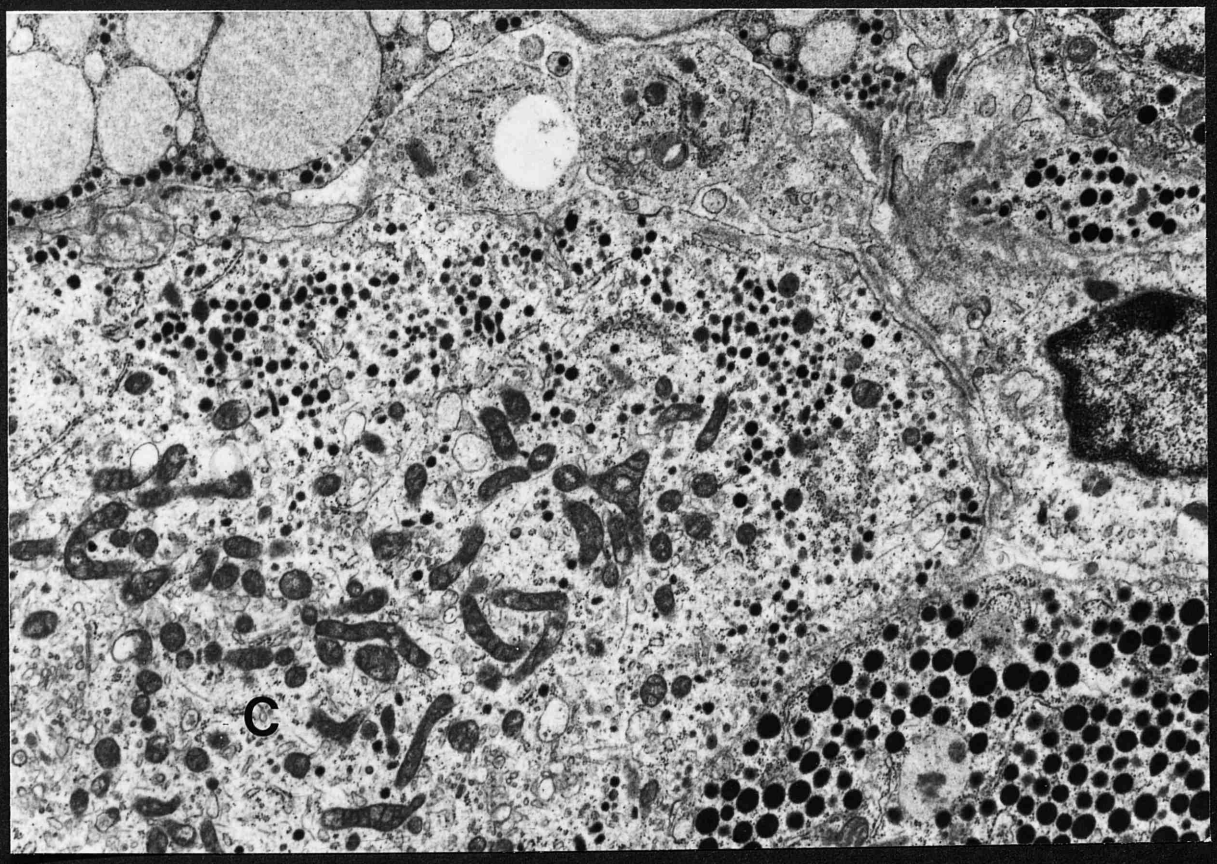
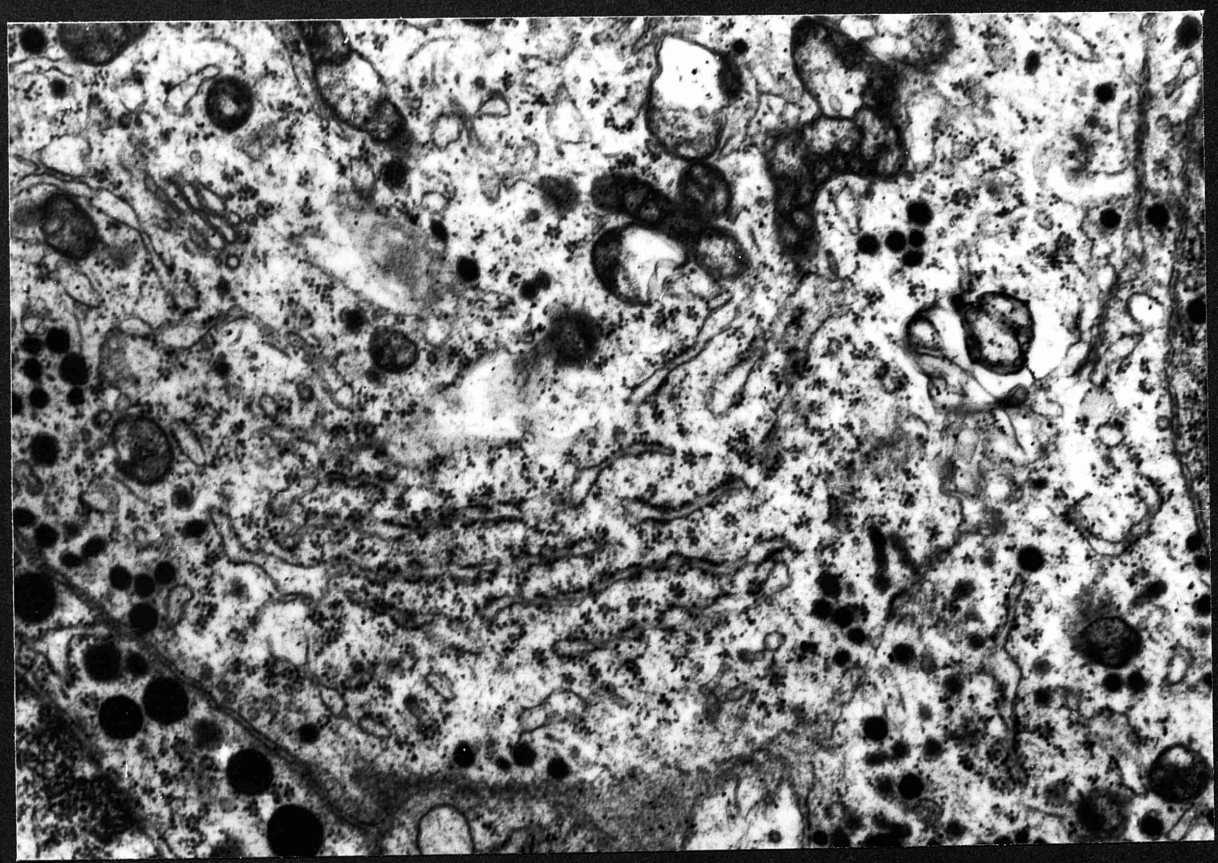

C

Figure. 4:27

Rat pituitary: dexamethasone 500µg/Kg: 6 weeks. (x 31,200).

Section of corticotroph showing abundant RER.



B. CORTICOTROPHS: QUANTITATIVE STUDIES

Introduction

The measurement of corticotroph cell area as an estimate of cell size has been reported only twice in the intact male rat (Siperstein and Miller, 1973; Westlund et al, 1985) the former study based on planimetry of electron micrographs, the latter on combined cell/point-counting at light microscopic level over a grid of known area. However, the values reported varied markedly in the two studies, the latter reporting a mean cell area of approximately twice that of the former. In both of these studies, the effects of adrenalectomy were also documented, in the former for up to 3 weeks, and in the latter over the first 48 hours. In addition, the short-term effects of CRF were monitored in the second study.

In the present study, corticotroph cell area was estimated in control and post-adrenalectomy male and female rats and in male rats following the administration of CRF with or without VP.

Subjective changes in granule distribution and size were discussed in section A of this chapter, and a recent study (Zak et al, 1985) suggested that corticotroph stimulation produced different granule sizes in male and female rats. In view of these findings, granule size was also estimated.

Measurement of the Secretory Granule Diameter

Corticotrophs were selected at random from a series of prints at a final magnification of x2880. Measurement of granule diameter was carried out by using a light microscope with an attached magnifying drawing tube. The image of the granules was viewed and measured by means of a calibrated linear graticule eyepiece. The final results represented a mean of a minimum of 20 readings per cell and an average of 20-30 cells per group.

RESULTS

All results are shown as mean ± SEM

(i) Cell Area

Sham-Adrenalectomy. Results are shown in Figs.4:28 (male) and 29 (female).

Corticotroph cell area in female rats 2 and 6 weeks after sham-adrenalectomy was slightly higher than in males, but this did not achieve statistical significance.

Adrenalectomy. Results are shown in Figs.4:28 (male) and 29 (female).

An increase of 140% and 130% was noted in the mean corticotroph cell area of male and female rats respectively 2 weeks after adrenalectomy. At 6 weeks, corticotrophs in female rats showed an even higher value for mean cell area than at 2 weeks. The increase in the cell area during the period from the 2nd to the 6th week was less than that achieved in the first 2 weeks after adrenalectomy. Corticotrophs in male rats, on the other hand, exhibited a clear reduction in cell area at 6 weeks after adrenalectomy

compared to the 2 week test animals. These were, however, greater than in control animals.

These overall changes in cell area were largely due to changes in the cytoplasmic area, but there was a small increase in nuclear area.

CRF. Results are shown in Fig.4:30.

Low (25µg) and high (50µg/Kg body weight) dose CRF administration increased the mean corticotroph cell area by 26% and 32% respectively of that in control animals. This, again, was mainly due to the increase in cytoplasmic area. The administration of vasopressin in a dose of 0.4 IU/Kg along with CRF induced a larger increase in cell area than CRF alone. This was clearly noticeable in the group which received high dose CRF where the overall increase in mean cell area was 45%.

(ii)Granule Size

Sham-Adrenalectomy. Results are shown in Table.4:1.

Mean granule diameter in both male and female rats were not significantly different. Maximum granule diameter, however, was greater in male rats.

Adrenalectomy. Results shown in Table.4:1.

Two weeks after adrenalectomy, there was a significant increase in the mean granule diameter which was similar in both sexes. At six weeks, mean values in male rats were not changed significantly but the value was reduced in female rats.

CRF/Vasopressin. Results are shown in Table.4:2.

The mean granule diameter was greater than the controls after treatment with the low CRF dose (25µg/Kg)

either alone or with VP but this did not achieve significance. The values were significantly lower after high dose CRF (50µg/Kg) administration. The addition of VP resulted in an even lower mean value than CRF alone.

Glucocorticoid Treatment. Results are shown in Table.4:3.

The secretory granules in the corticotrophs of male rats showed a significant reduction in their diameter after low (30mg/Kg) and high (60mg/Kg) dose corticosterone. No change was demonstrated in female rats. Dexamethasone treatment in a dose of 500µg/Kg induced a significant reduction in the granular diameter of both sexes.

DISCUSSION

As discussed earlier, in the two previous reports corticotroph cell area in intact rats was estimated by different methods from that used in the present study. Siperstein and Miller (1973) who used a similar method to ours reported a mean total cell area in rats of 48μm^2 while Westlund et al (1985) obtained a value of 97μm^2. Our values in intact rats (which range between 43 and 53μm^2 in the various control groups) correlate well with those of the former study. The higher values established by the latter investigation are thought to be due to the different method of cellular assessment, since a similar type of analysis carried out on paraffin embedded sections from some of our control animals yielded similar values to those established by Westlund's group. This may be due in part to the differences in section thickness and to distortions produced in the tissue by the electron microscope. It has been shown for the first time in the present study that the corticotroph cell area in female rats is similar to that in males since previous investigations were carried out only on male animals.

The increase in the cell area following adrenalectomy was monitored in previous studies but again, however, the results were not in agreement. An increase of 30% (Siperstein and Miller, 1973) and 347% (Westlund et al, 1985) were reported 24 hours after adrenalectomy. Changes in the cell area were followed up by the first study and found to reach a maximum of 92μ^2 by day 5 then plateau until day 21 after adrenalectomy when the cell area measured 82μ^2. Our results at 2 weeks are slightly higher

($121\mu^2$) than that reported by Siperstein ($90\mu^2$) at the same time interval. This may reflect differences in fixation.

The significant reduction in mean cell area in male rats at 6 weeks compared with 2 weeks established in this study and the decreasing values at the end of a 2 week plateau reported by Siperstein and Miller (1973) suggest that the hypertrophic response of the corticotroph declines sometime between the 3rd and 6th week after adrenalectomy. Since a day-by-day record of changes is not available, it is not possible at present to determine the exact time at which this stage is reached. The comparatively higher values for corticotroph cell area in female rats at 6 weeks suggests a greater magnitude of response to adrenalectomy. This is in keeping with the changes in the overall volume density observed at light microscopic level as well as the subjective ultrastructural changes at the same time interval. This sex difference in response requires more detailed investigation.

Changes in corticotroph cell area were studied after CRF stimulation by Westlund et al (1985) using the same light microscopic method as in their post-adrenalectomy studies. After 48 hours of continuous intraperitoneal infusion of 10 and 50ng/minute of CRF (14.4 and 72.0µg/day), they noted an increase of 162% and 185% control values respectively. These values were obviously lower than those reported for adrenalectomy at the same time interval. They did not report longterm studies. Although the absolute values are different, the relative magnitude of the increase in cell area after CRF administration compared with adrenalectomy is similar in their study and

the present one. This would suggest that CRF has a significant hypertrophic effect on the corticotroph. The generally higher values established by the previous study after CRF administration could be attributed to the difference in the dose, duration and method of administration as well as to the method used for the calculation of cell area. The consistently higher values reported for cell area after adrenalectomy compared with that following CRF treatment might again reflect the differences in stimulation discussed in section A.

Previously reported measurements of corticotroph secretory granules are variable. Some caution must be exercised in the interpretation of studies such as this, due to the changes which may be produced by the various fixation and staining procedures and to the possibility of sampling errors. The range of 60-246nm established in this study for male rats correlate well with the reported maximum diameter of 200-250nm (Siperstein and Miller, 1970) and the range of 60-200nm (Zak et al, 1985). The higher values of 200-300nm reported by Moriarty and Halmi (1972) could be attributed to the use of immunohistochemically stained sections in which the granules appear larger due to the precipitation of PAP molecules. In our study, the maximum granule diameter in female rats appears slightly lower than in the male, although Zak et al (1985) made no comment on this in their study. Whether the higher mean granule diameter seen in the CRF control group compared with other control groups reflects differences in fixation is unclear. However, all the CRF study specimens were processed at the same time, and should presumably have

undergone similar structural changes and can therefore be compared with one another. This does, however, highlight the problem of attempting to compare the effects of one treatment with another.

The studies presented here confirm and extend the observations of Moriarty and Halmi (1972) on the post-adrenalectomy corticotroph and Zak et al (1985) on the aminoglutethimide treated rat that an increase in granule size occurs in states of increased corticotroph stimulation. The exact mechanism of these changes is uncertain at present. It is possible that it reflects increased hormone content of granules. However, one might then assume that CRF would produce a similar effect. The changes demonstrated with this peptide however are almost impossible to interpret at present, but may reflect the preferential release of larger granules from corticotrophs following CRF stimulation. Neurosecretory granules contain not only peptide hormones, but a variety of structural proteins, (e.g. chromogranins). Perhaps the changes reflect alterations in these components rather than in hormonal content.

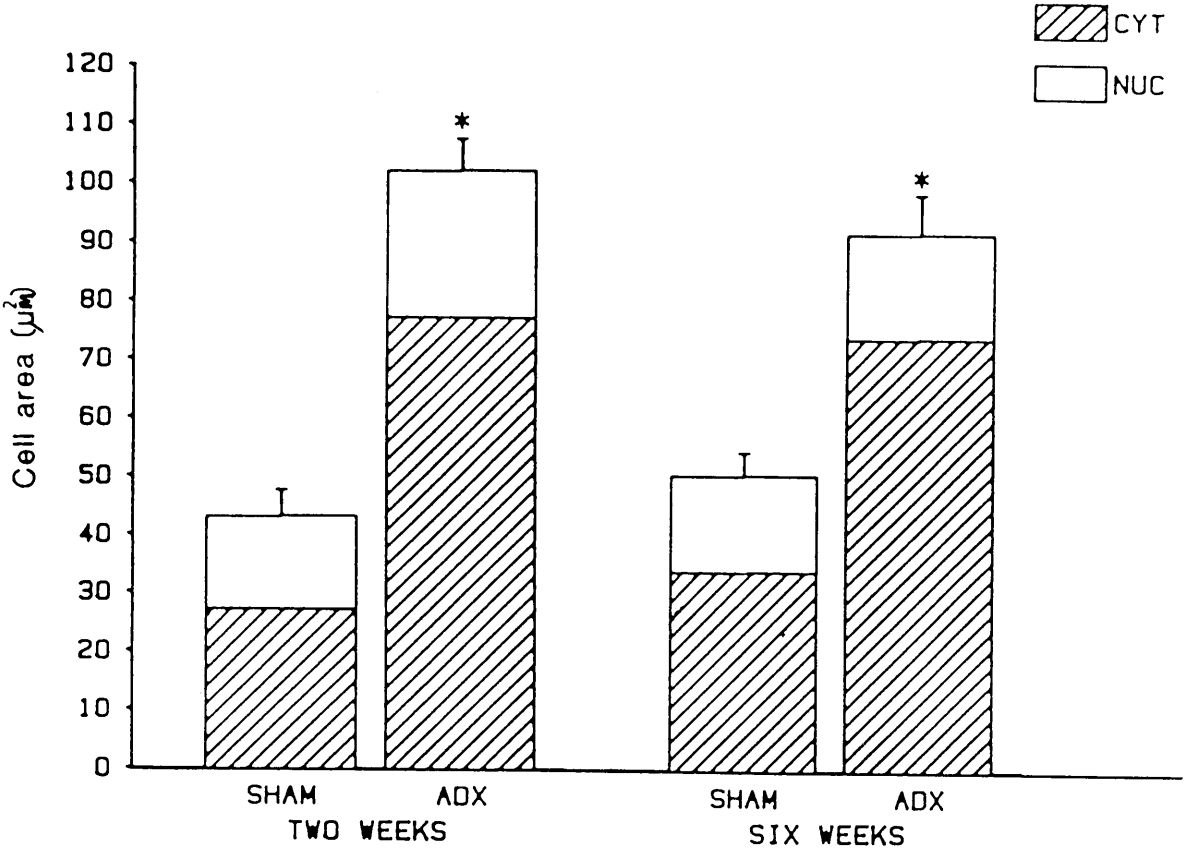
These studies also confirm the subjective impression of reduction in granule size in dexamethasone treated animals and in corticosterone treated males, but again the reason for the alterations are unclear.

While one must interpret the absolute values obtained in a study such as this with some caution, the results have highlighted several interesting features. Further ultrastructural immunocytochemical studies localising ACTH and related peptides, other novel peptides (e.g. neuromedin

U)(Steel et al, 1987) and structural proteins may help elucidate further the alterations demonstrated in granule size and morphology.

Figure. 4:28

Effect of adrenalectomy on the cell area of corticotrophs in male rats.

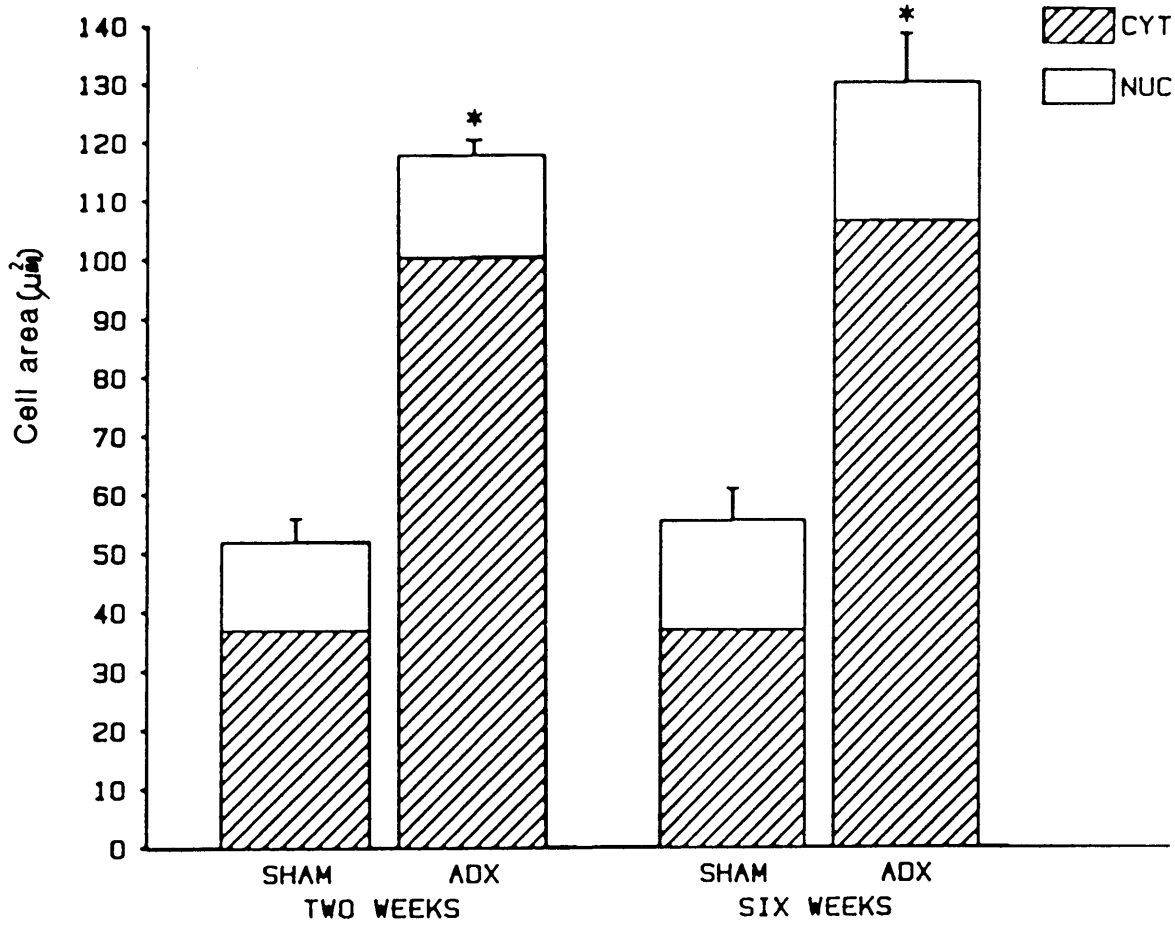


Results are shown as mean ± S.E.M.

* $p < 0.001$ vs Control.

Figure. 4:29

Effect of adrenalectomy on the cell area of corticotrophs in female rats.

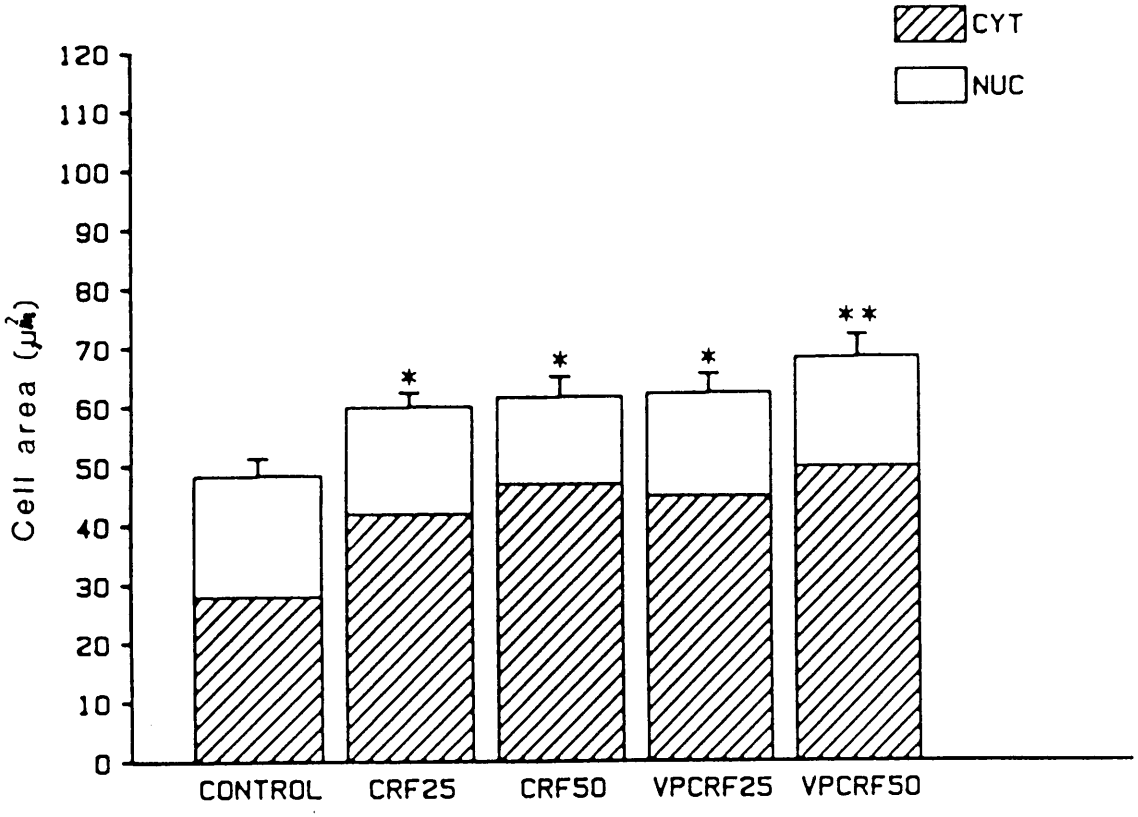


Results are shown as mean ± S.E.M.

* $p < 0.001$ vs Control.

Figure. 4:30

Effect of the administration of CRF with or without VP on the cell area of corticotrophs of the anterior pituitary.



Results are shown as mean ± S.E.M.

* $p < 0.01$ vs Control.

** $p < 0.001$ vs Control.

Table. 4:1 Effect of adrenalectomy on the granule diameter of corticotrophs.

Treatment	Weeks	Sex	Granule Diameter (nm)
Sham		MALES	145 ± 3.4
Adrenalectomy	2		179 ± 4.3*
	6		178.2 ± 3.0*
Sham		FEMALES	135 ± 3.8
Adrenalectomy	2		172 ± 3.0*
	6		160 ± 2.7*

Results are shown as mean ± S.E.M.

* $p < 0.001$ vs sham adrenalectomised rats.

There was no significant difference in granule diameter of sham groups in both sexes.

Table.4:2 Effect of the administration of CRF with or without VP on the granule diameter of corticotrophs.

Treatment	Granule Diameter (nm)
Saline	163.9 ± 3.5
CRF 25	174.0 ± 3.2
CRF 50	150.0 ± 2.6*
VP CRF 25	166.9 ± 3.3
VP CRF 50	132.6 ± 2.5*

Values are shown as mean ± S.E.M.

* $p<0.002$ vs control (two-tailed).

CRF doses are given as µg/Kg.

VP is given at a dose of 0.4IU/Kg.

Table.4:3 Effects of corticosterone and dexamethasone administration on the granule diameter of corticotrophs in the anterior pituitary.

Treatment	Dose	GRANULE DIAMETER	
		Male	Female
Saline		140.2 ± 4.5	132.6 ± 4.5
Corticosterone	30mg/Kg	116.6 ± 2.0*	136.4 ± 2.4
	60mg/Kg	116.3 ± 2.08*	130.4 ± 2.6
Dexamethasone	500µg/Kg	115.9 ± 2.1*	100.7 ± 23.1*

Values are shown as mean ± S.E.M.

* $p < 0.001$ vs Control.

C. FOLLICULOSTELLATE CELLS

In addition to the hormone producing cells in the anterior pituitary, a further population of cells - the folliculostellate cells (FS) - has been recognised. They contain the protein originally identified in brain, S-100 (Nakajima, Yanaguchi and Takahashi, 1980) and the glial structural protein, glial fibrillary acid protein (GFAP) (Velasco, Roesmann and Gambetti, 1982). The origin of these cells and their functions are unknown, but several hypotheses have been put forward, including roles as supporting cells, phagocytes, ion transport cells or a stem cell population (reviewed in Shirasawa et al, 1983). There have been suggestions that their numbers may increase in the rat following some alterations in pituitary function (Shirasawa et al, 1983) and Shiotani (1980) reported hypertrophy of these cells in the rabbit pituitary following adrenalectomy. The features and distribution of these cells and their relationship to corticotrophs were studied by immunohistochemical methods by light microscopy and at a morphological level by electron microscopy in normal and post-adrenalectomy rats.

MATERIALS AND METHODS

Immunohistochemistry

Folliculostellate cells were identified using the PAP immunoperoxidase technique with a primary antibody to S-100 protein raised in rabbit (Dako). Their relationship to corticotrophs was demonstrated using a double-staining

technique, identifying S-100 as above using diaminobenzidine as a substrate and ACTH by the alkaline-phosphatase (APAAP) technique (Appendix 1, p100) developing the colour reaction with Fast Red.

Ultrastructural analysis was made on the previously described material.

RESULTS

Folliculostellate cells were scattered diffusely in large numbers throughout the gland (Fig.4:31) usually lying singly or in small groups (Fig.4:32). They appeared smaller than corticotrophs and showed no specific association with them, either in control glands, or following adrenalectomy (Fig.4:32). There was no subjective increase in numbers following adrenalectomy.

Ultrastructurally, they were easily identified and showed the characteristic features (Figs.4:33 and 34). The cells contained no neurosecretory granules. There were numerous polyribosomes. Where they lay adjacent to each other, junctional complexes were present (Fig.4:33) but these did not appear to occur where contact was with a hormone secreting cell. They had numerous microvilli projecting into pseudolumina (Fig.4:33) or interdigitating in gaps between cells (Fig.4:34). Cilia were also seen (Fig.4:33). The cells were not obviously hypertrophied in adrenalectomized rats.

DISCUSSION

No specific association of FS cells with corticotrophs has been demonstrated and no obvious alteration in either morphology or numbers following adrenalectomy, although Shiotani (1980) reported hypertrophy in the rabbit. This may reflect species variation or may be related to the fact that his study was carried out between 3 and 11 days following surgery, whereas the present one was at 2 or 6 weeks. The only functional alteration to date which has been reported to increase this cell population is castration, thyroidectomy having no effect (Shirasawa et al, 1983). The function of F-S cells remains obscure.

F-S cells can be recognised specifically by the microvilli, which are not seen on hormone - producing cells in the pituitary. The presence of cilia however, is not specific as these were seen in various cell types. Presumably these are not related to motility of cells at this site. They may have a role in clearing of secretory products. No granulated F-S cells were identified in the present study in contrast to a study on the goat pituitary (Shirasawa, Yamaguchi and Yoshimura, 1984). This may again reflect species variation, but would suggest that they are not hormone-producing precursor cells in the rat. Their function remains obscure.

Figure. 4:31

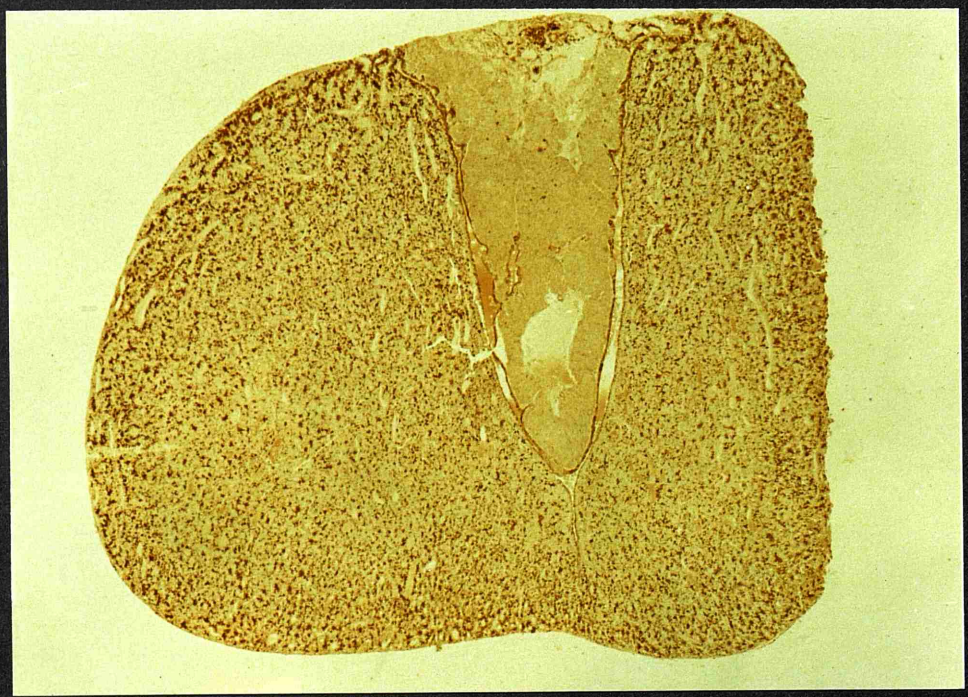
Normal rat pituitary (PAP : anti-S100 x 29).

Folliculostellate (FS) cells are distributed uniformly throughout the gland.

Figure. 4:32

Male rat pituitary : 2 weeks post-adrenalectomy. (Double immunostaining technique, anti-S100 (brown), anti-ACTH (red) x 182).

There is no obvious association between corticotrophs and FS cells. There is no apparent increase in numbers of FS cells.



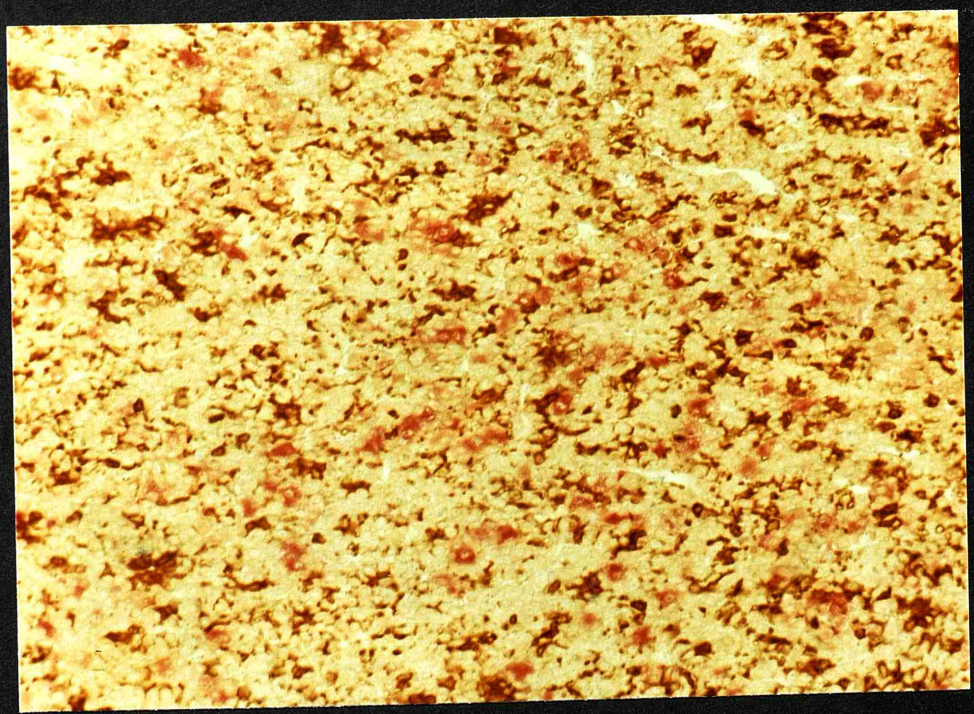


Figure. 4:33

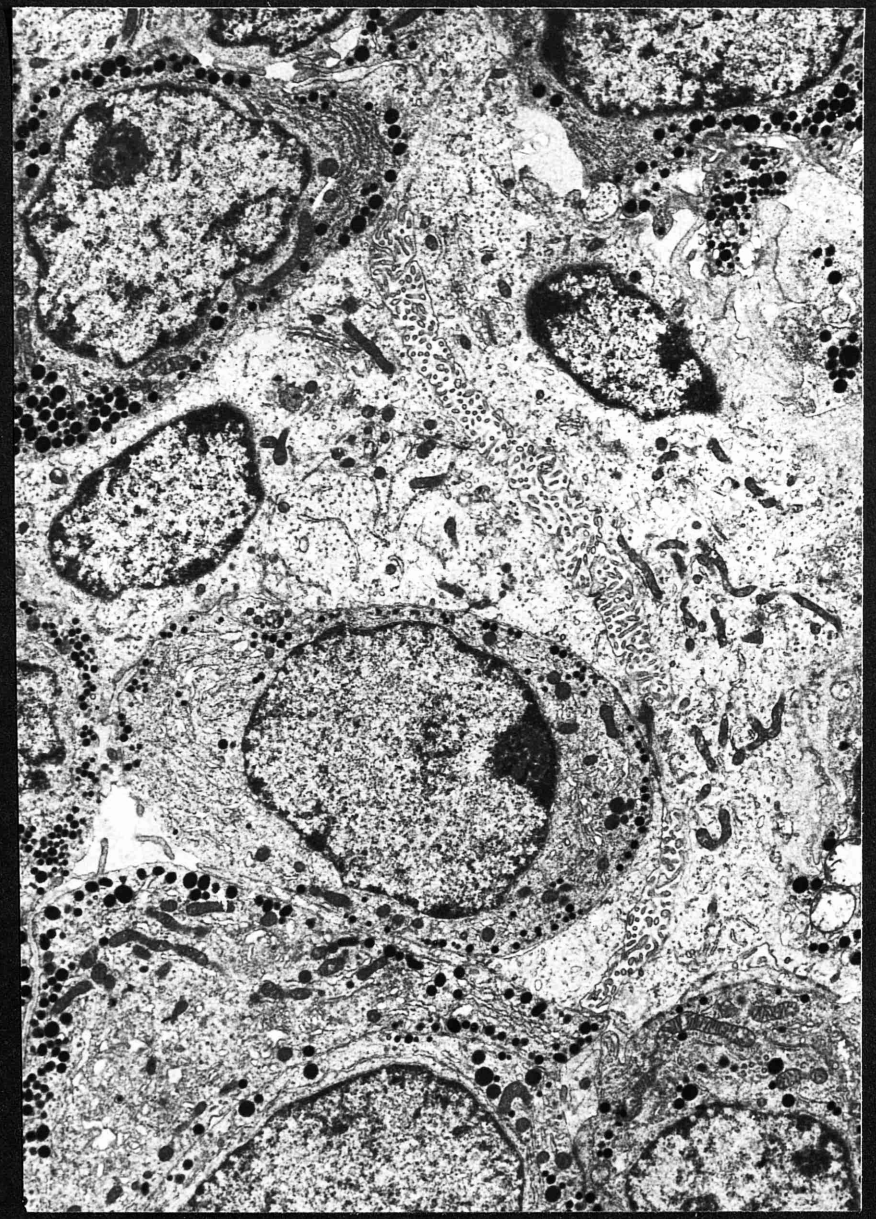
Normal rat pituitary : folliculostellate cell (FS) (x 9,460).

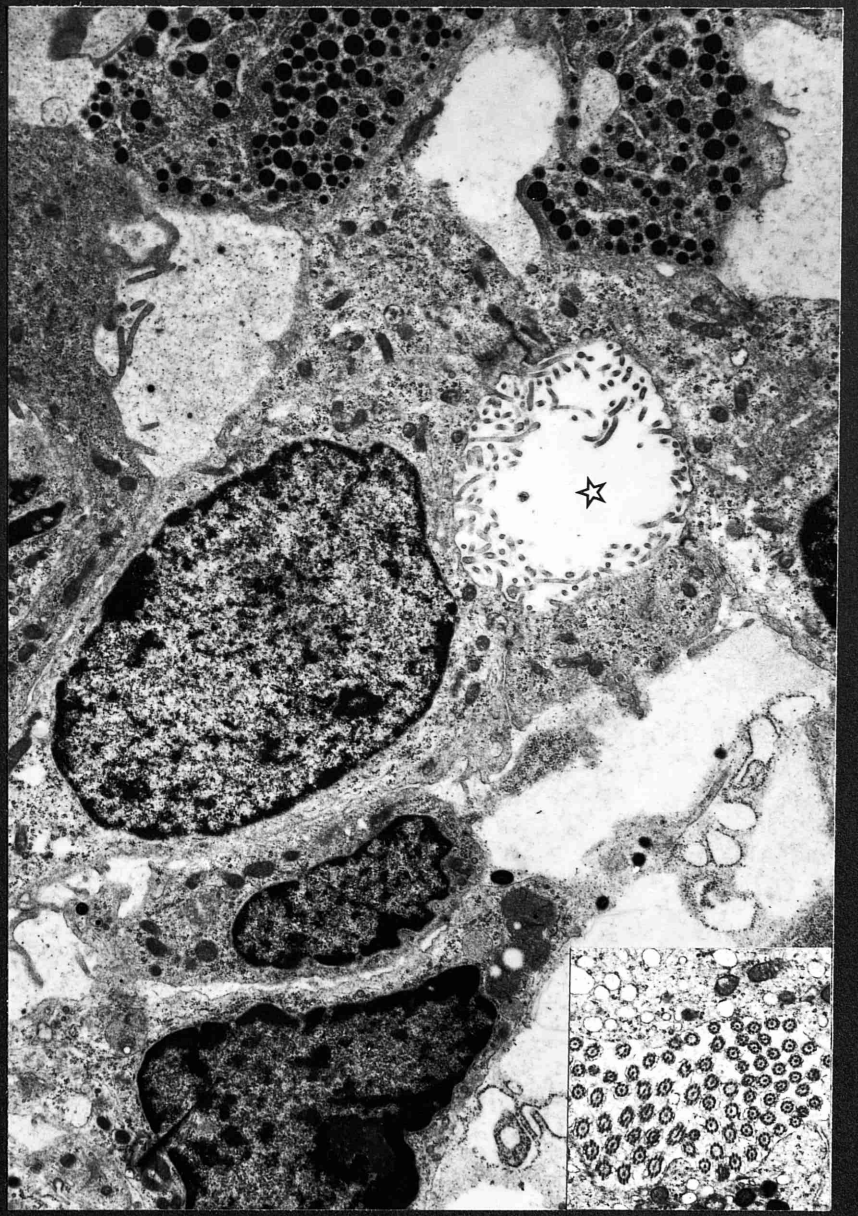
These cells are characterized by an irregular outline with agranular cytoplasm. They usually form aggregates and surround a pseudolumen (☆). Long microvilli project into this. In several of these cells, cilia were identified, with a characteristic arrangement of microtubules. The inset (upper right) shows an extreme example of the presence of cilia on a FS cell.

Figure. 4:34

Normal rat pituitary : folliculostellate cell (FS) (x 7,920).

Two adjacent FS cells showing interdigitating microvilli in an apparent gap between cell membranes.





CHAPTER 5

CELL PROLIFERATION STUDIES

INTRODUCTION

As discussed in Chapter 1, the post-natal growth of the anterior pituitary gland is the result of both hypertrophy and hyperplasia of the various cell populations. Maximal cell proliferation occurs in immature animals (Hunt, 1942; Crane and Loomes, 1967; Nakai et al, 1969) with low levels of mitotic activity in adult rats. The factors controlling cell turnover in the mature gland are unclear, but there is evidence of a circadian variation in mitotic activity (Nouët and Kujas, 1975) and of oestrus-related changes in the female (Crane and Loomes, 1967).

Changes in adenohypophyseal cell growth have been reported in association with altered HPA function, H^3-thymidine uptake falling after corticosterone administration and increasing after adrenalectomy (Crane and Loomes, 1967; Nakane et al, 1977; Stepien, Karasek and Pawlikowski, 1981). The time course of these changes has not been fully investigated, however, nor has the role of specific hypothalamic stimulating factors in producing them.

The aims of the present study were to examine the circadian variation in mitoses in the anterior pituitary gland of normal adult male Sprague-Dawley rats and to monitor the changes occurring in the early stages following adrenalectomy and as a result of CRF administration. A metaphase arrest technique was used to assess mitotic activity. Only male animals were included in the study to avoid the problem of possible cyclical changes in females.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats weighing 200-250gms were assigned on a random basis to the following treatment groups.

1. Intact animals sacrificed at 1200, 1800, 2400 and 0600.
2. Bilaterally adrenalectomized or sham-adrenalectomized and sacrificed 2, 7 or 14 days following surgery.
3. Daily i.p. injection of synthetic ovine CRF (Sigma) (50µg/Kg BW) or physiological saline for 2 or 7 days before sacrifice.

All animals received a single intraperitoneal injection of vincristine sulphate (Oncovin, Eli Lilly and Co.,) (1mg/Kg BW) 4 hours before sacrifice, and were sacrificed in the same order as the injection was given. All treatment groups were sacrified around 1400.

Pituitaries were fixed in buffered formalin and embedded horizontally in paraffin wax. 3µm sections were cut through the gland and sets of 3 sections from levels through each gland, were stained with Meyer's haematoxylin (for general mitotic count) or using the PAP immuno-peroxidase method with an antibody to ACTH 1-24 (for the assessment of mitotic corticotrophs). Total metaphases were counted manually. The total area of each anterior lobe section was measured using a computerised image analyser, (as described for measurement of cell area, p 72) and a "mitotic index" calculated as metaphases

per mm^2. In order to take into consideration the cellular hypertrophy following adrenalectomy and CRF administration, and differences in tissue shrinkage of various blocks during processing, this was converted to metaphases per 1000 cells. For this, representative sections from each group were photographed to a final magnification of x300 and nuclei in two randomly chosen areas of known dimension were counted for each print, allowing the total number of cells per mm^2 to be calculated.

For the assessment of corticotroph mitotic activity, manual counting of ACTH - positive mitotic cells was performed. The remainder of the analysis was performed as before. This part of the study did not include the circadian rhythm groups.

Analysis of circadian rhythm was performed using a computerised programme ('single cosinor system').

RESULTS

Circadian Rhythm Study

Metaphases were easily identified (Fig.5:1). There was little evidence of degeneration of metaphases or of significant numbers of cells in anaphase. Numerical data are shown in Fig.5:2. There was a significant ($p < 0.002$) circadian rhythm of mitotic activity with a peak (5.49/1000 cells) at 1100 and a trough (3.32/1000 cells) at 2300.

Post Adrenalectomy

Numerical results are shown in Table.5:1. No differences were identified between saline injected and sham-adrenalectomized animals and only one set of control values is therefore shown.

There was an obvious subjective increase in mitotic activity of the gland at 7 days post-adrenalectomy, with a marked increase in the numbers of mitotic corticotrophs (Fig.5:3). These were rounded up and showed diffuse cytoplasmic staining.

A significant increase ($p<0.05$) in total adeno-hypophyseal mitotic index was seen only in rats sacrificed one week after adrenalectomy. However, mitotic cortico-trophs were increased significantly ($p<0.001$) at both times.

CRF Administration

Again, there was an obvious subjective increase in mitotic activity.

Results are shown in Table.5:1. The mitotic index was significantly increased in CRF treated animals at both 2 ($p<0.001$) and 7 ($p<0.02$) days, but mitotic corticotrophs were significantly increased only at 2 days ($p<0.02$).

DISCUSSION

Various methods exist for the assessment of proliferative activity *in vivo*. In the present study, the mitotic activity of the anterior pituitary was estimated using a stathmokinetic technique, in which an anti-microtubular agent (e.g. vincristine or colchichine) is injected at a fixed interval prior to sacrifice to block dividing cells at the metaphase stage, thus allowing greater numbers of mitotic figures to be counted. This was thought to be particularly suitable for the pituitary gland of the adult rat in view of the previously reported low basal levels of proliferative activity (Nouët and Kujas, 1975). In addition, the combination of the technique with immunohistochemical staining allowed the assessment of specific changes in proliferation in corticotrophs. In theory, the selection of an optimal dose of a stathmo-kinetic agent should be made for each tissue on the basis of a dose-response curve and analysis of linearity of metaphase accumulation (Wright and Appleton, 1980). Because of limitations on time and numbers of animals available, these studies could not be performed in the present study and the dose selected was based on previous studies on rat tissues (Wynford-Thomas, Stringer and Williams, 1982a and b; McNicol, personal communication). The lack of obvious degenerating metaphases and of anaphase escape would suggest that it lay within the acceptable range.

Previous reported levels of basal mitotic activity in the anterior pituitary have been based on the analysis of "natural" mitoses (Nouët and Kujas, 1975) or on a stathmo-

kinetic technique (Sakuma, Shirasawa and Yoshimura, 1984). The former study reported a mean value over a 24 hour period of 0.97/1000 cells in adult male Sprague-Dawley rats. If it is assumed that there was linear accumulation of mitoses in the present study, this is of the same order of magnitude as our result. However, the study of Sakuma et al (1984) demonstrated a single count of 31.7/mm^2 at 0900, 6 hours after colchicine administration to Wistar-Imamichi rats. Whether this reflects true strain differences between Wistar and Sprague-Dawley (6.48/mm^2 in the present study) is unclear. Nouët and Kujas (1975) also suggested that factors such as method of fixation may influence the number of mitoses identified in tissue sections although this should apply only to studies in which cells can complete their cycle, and not to metaphase arrest studies.

It is generally accepted that circadian rhythms of proliferative activity occur not only in tissues with a known functional rhythm (e.g. thyroid) (Wynford-Thomas et al, 1982a) but also where no such obvious functional variation occurs (e.g. skin) (Scheving and Pauly, 1967). Comparison of reported rhythms depends on the method of analysis of proliferation, the lighting schedule and possibly the day of the week and the time of year at which the study is performed, since circaseptan and circannual rhythms have also been reported in metabolic parameters.

In the present study, the application of the widely accepted technique of cosinor analysis has demonstrated a significant circadian rhythm of mitotic activity in the anterior pituitary gland with a peak at 1100. Since this

mitotic index reflects accumulation of mitoses, the actual peak is probably prior to 1100. The only previous study we have been able to identify on rhythmicity of pituitary mitotic activity was based on the analysis of natural mitoses on an hourly basis, and demonstrated two sharp peaks at 0600 and 1100 (Nouët and Kujas, 1975). The present study was not designed to identify such ultradian variation. Direct comparison of the rhythms cannot be made therefore because of these differences, and also because the lighting schedule was slightly different in the two studies. However, it is of interest that both studies suggest that maximum cell division occurs in the early lights-on period. The correlation of growth and functional rhythms in the pituitary would be extremely complex, and would require detailed differential analysis of immuno-histochemically defined mitotic cells, and the identification of a possible stem cell population.

Previous investigations have shown that ^{3}H-thymidine uptake increases in the post-adrenalectomy phase. Crane and Loomes (1967) demonstrated a significant increase in adult male albino rats at both one and two weeks, although levels were lower at 2 weeks than at one. Nakane, Setalo and Mazurkiewicz (1977) reported maximum uptake at 6 days of ^{3}H-thymidine administered in vivo. They suggested on the basis of immunohistochemical staining that cell division was occurring in a non-corticotroph population at this time point and that only later (12 days post-adrenalectomy) did significant numbers of corticotrophs divide. This peak of uptake around 6 days has also been demonstrated by in vitro labelling (Mastro, Shelton and Hymer, 1969).

In the present study, mitotic activity was assessed at 2, 7 and 14 days after adrenalectomy, and a significant increase in mitotic index was identified only at 7 days. This would correlate with the peak labelling at 6 days described above. Our results however, do not suggest that a significant increase in cell proliferation continues until 2 weeks, and are thus not in keeping with Crane and Loomes' (1967) study. They are, however, consistent with quantitative analysis of the changes in the overall corticotroph population of both male (McNicol, 1985) and female (Chapter 2) rat which showed that the major expansion of this cell population has been achieved by 2 weeks after adrenalectomy. This suggests that if hypothalamic factors are involved in the stimulation of the growth response, their functional and growth promoting effects eventually become dissociated, as has been demonstrated for the goitrogen-treated thyroid (Wynford-Thomas et al, 1982b). This may reflect changes in direct receptor mediated growth responses, or alterations in the interaction of these peptides with other growth factors. In contrast to the study of Nakane et al (1977) we have demonstrated significant proliferative activity in corticotrophs at both 2 and 7 days. These, however, account for only 20-25% of the mitotic cells. Further immunohistochemical and ultrastructural studies will be required to identify whether this reflects cell division in other hormone-producing cell types or in a stem cell population.

The absence of an overall increase in mitotic activity at 2 days may indicate that corticotrophs are stimulated to

divide before other cell populations after adrenalectomy. However, in other experimental growth models including the regeneration of liver following partial hepatectomy (Bucher, 1967; Zieve, Anderson and Lindblad, 1985) and the growth of the contralateral adrenal gland following unilateral adrenalectomy (McNicol and Penman, 1987) peaks of proliferation may be demonstrated at 24 and 72 hours following the growth stimulus, with a fall at 48 hours. Further studies are underway at present to assess whether such early peaks occur in the pituitary following bilateral adrenalectomy.

To date, although there was experimental evidence that CRF could induce hypertrophy of corticotrophs (Westlund et al, 1985) the evidence for an effect on cell proliferation was circumstantial, based on the occurrence of corticotroph hyperplasia in a patient with a CRF secreting tumour of prostate (Carey et al, 1984). The present study has demonstrated a mitogenic effect of CRF on the anterior pituitary. Whether this is a direct effect of the peptide or a permissive action for other growth factors cannot be assessed from these in vivo studies. The results, which have been confirmed in a further small group of rats, raise several interesting points. The level of mitotic activity at 2 days is higher than at any time point following adrenalectomy. This may, reflect our sampling times in the post-adrenalectomy phase and the studies outlined above should elucidate this further. However, it would appear that the majority of cells in mitosis are not corticotrophs. Further immunohistochemical studies are required to assess whether these are other hormone producing cells

or a stem cell population.

In vitro analysis of the growth-promoting effects of the peptide on pituitary cells and of its interaction with other hypothalamic peptides and growth factors will be of importance in the assessment of its role in controlling the size of the anterior pituitary corticotroph population.

Figure. 5:1

Rat anterior pituitary. (Haematoxylin x 728).

Metaphases are easily identified after vincristine administration.

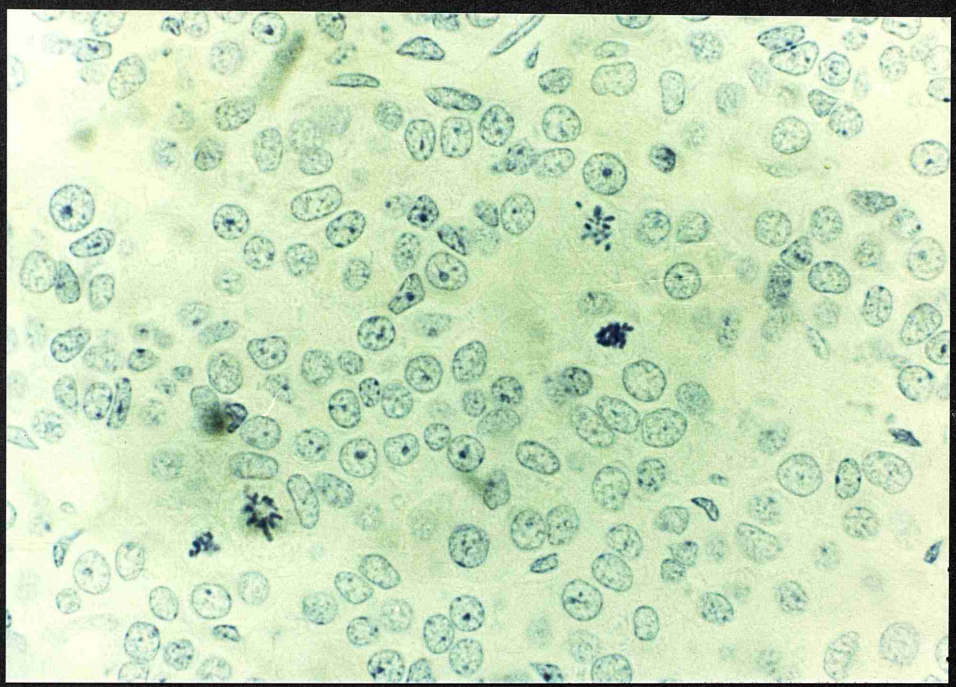
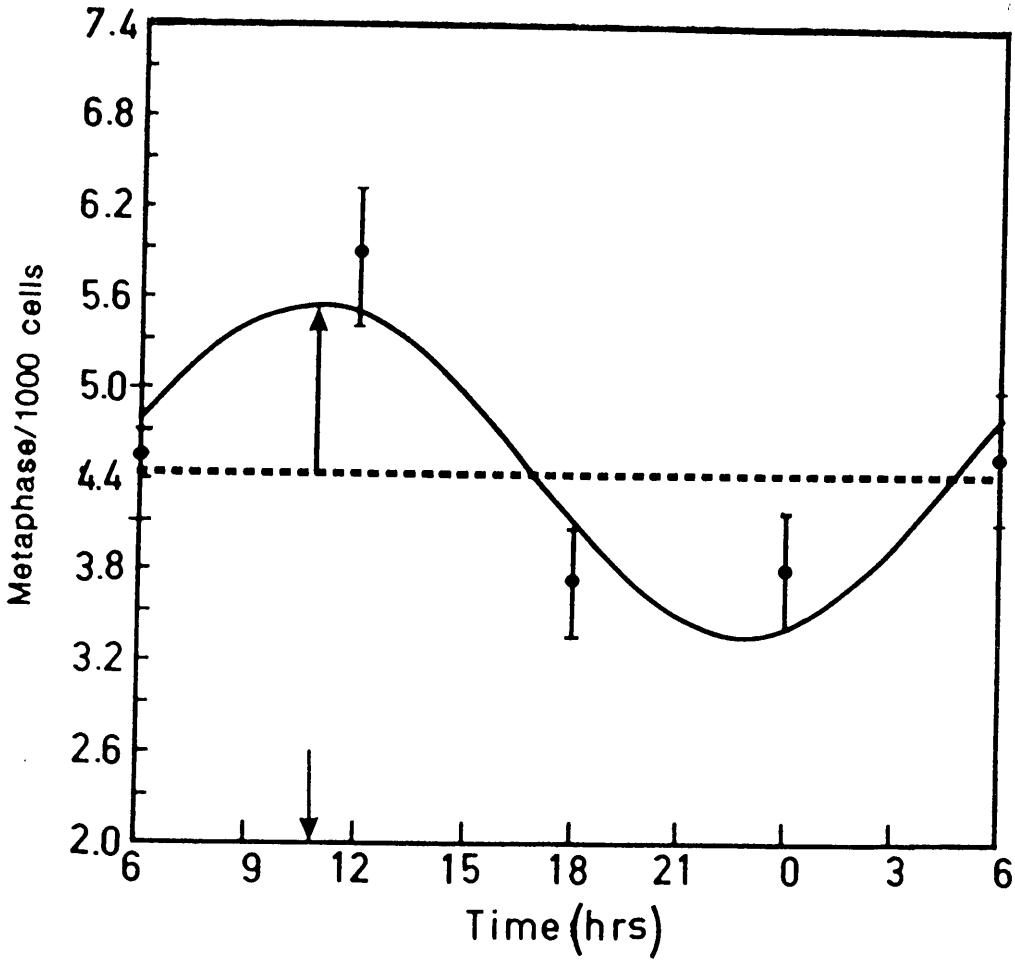


Figure. 5:2

Circadian rhythmicity of adenohypophyseal mitosis in normal rats.



Curve is obtained from the best fit of sine wave through the mitotic indices calculated for each time point using a stathmokinetic technique.

Results are shown as mean ± SEM.

Peak mitotic activity occurred around 1100 (↓).

Figure. 5.3

Rat anterior pituitary gland : 7 days post-adrenalectomy : vincristine treated. (PAP : anti-ACTH : x 728).

Significant numbers of mitotic corticotrophs are present. The cells are rounded up with diffuse cytoplasmic staining.

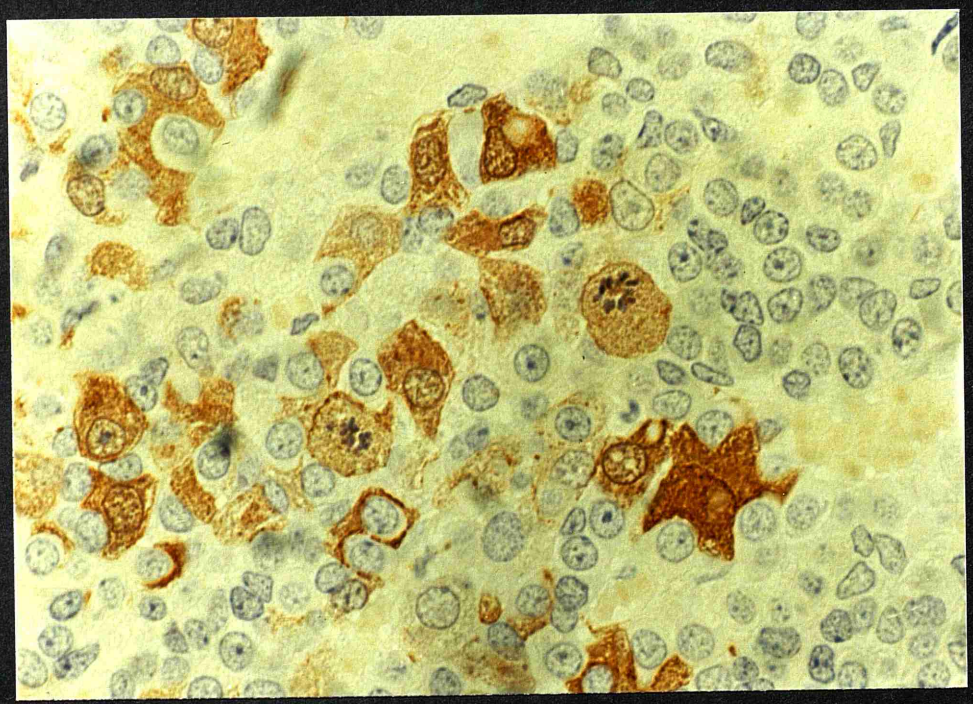


Table.5:1 Effect of adrenalectomy and CRF administration on the mitotic activity of the anterior pituitary gland.

Treatment	Duration	n	Mitoses/ 1000 cells.	Mitotic Corticotrophs/ 1000 cells.
Control		7	4.49 ± 0.38	0.34 ± 0.06
Adrenalectomy	2 days	7	4.18 ± 0.52	0.9 ± 0.17***
	1 week	7	8.24 ± 1.8*	2.26 ± 0.29***
	2 weeks	7	4.28 ± 0.59	0.19 ± 0.03
CRF	2 days	7	12.03 ± 0.79***	0.66 ± 0.11**
	1 week	7	7.45 ± 0.67**	0.4 ± 0.06

CRF was given at a dose of 50µg/Kg body weight.

Results are shown as mean ± S.E.M.

* $p<0.05$
** $p<0.02$
*** $p<0.001$

CHAPTER 6

FINAL DISCUSSION

The aims of this thesis were to extend the observations on the morphological changes induced in the anterior pituitary corticotroph population, and to some extent in the adrenal gland, by specific alterations in pituitary-adrenal function. Examination of such changes should make it easier to understand the pathology of the human pituitary gland in diseases of the HPA axis.

The general morphological changes were examined at both light microscopic and ultrastructural level using a combination of qualitative and quantitative assessment. The use of Vv as a measurement of changes in the size of the corticotroph population has the advantage of being easier to perform than total cell counts, but the disadvantages have already been discussed. Particularly in a complex organ such as the pituitary gland, the possibility of producing non-specific alterations in Vv of a small population such as the corticotrophs must be considered. The possible paracrine inter-relationships of pituitary cells are poorly understood and this might also lead to indirect changes in cell populations. More detailed studies would probably be better performed using total cell counts, particularly where long-term observations are involved. The automated analysis of disaggregated glands with immunocytochemical labelling of the different cell populations would be easier than total cell counts on tissue sections. Vv will be useful, however, in pilot studies to screen for possible effect of new hormones or growth factors, particularly in dose-response studies.

This method of analysis consistently demonstrated a greater response to bilateral adrenalectomy in the female rat than in the male. This was confirmed by the quantitative ultrastructural studies showing parallel changes in corticotroph cell area. Whether this means that the difference is due only to differences in the degree of hypertrophy or to a greater increase in cell numbers in the female rat is unclear at present. Analysis of cell numbers and a comparative study of the cell kinetics of the response in the two sexes would be of interest in investigating this further. The time course of the growth response in the male pituitary following adrenalectomy requires further characterization. However, it would appear that proliferation is an early response to the increased stimulation.

The studies on the effects of CRF would support a role for this peptide not only in the control of corticotrph function but also in the regulation of the size of the corticotroph population, both by stimulating hypertrophy and cell proliferation. This increase is dose dependent, but whether cell proliferation is also has not been studied. As discussed in Chapter 5, the role of CRF in cell proliferation can only be examined further by a combination of in vivo and in vitro studies examining its effects alone and in combination with other hormones and growth factors.

The ultrastructural changes following CRF and CRF/VP administration need further examination. It will require a combination of biochemical and molecular biological studies on levels of ACTH and POMC mRNA within the gland to assess

whether changes in granule content are associated with changes in ACTH synthesis and/or release.

The changes in granule morphology associated with alterations in corticotroph function are of interest. Ultrastructural immunocytochemistry must be used to investigate these further. The inability to label corticotrophs specifically at EM level was a problem in these studies. The fact that staining could be achieved on semi-thin resin-embedded sections suggests that it was a technical problem related to grid staining. Further studies are underway at present to establish the technique.

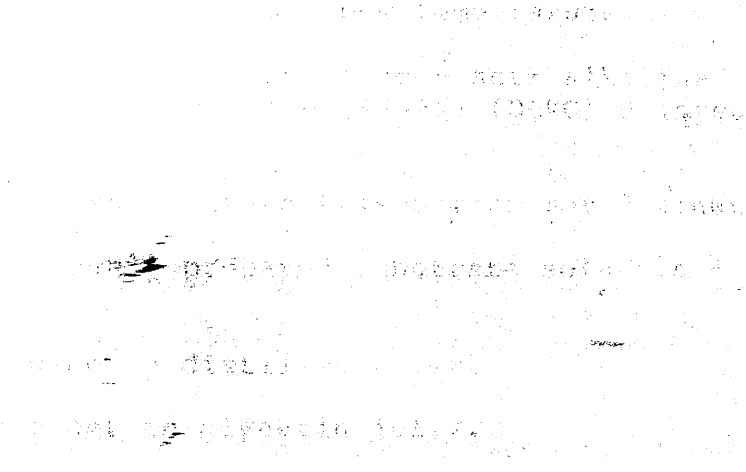
Quantitative studies at ultrastructural level are more difficult than at light microscopic level because of sampling errors due to the small size of the blocks. In the present studies, the samples taken for EM analysis were representative of the general changes as judged by light microscopic immunohistochemical staining and sampling was wider than in other reported studies. However, one must consider distortion caused by processing, by the beam, and by relative differences in fixation. Perfusion fixation might be considered as an alternative to immersion, although removal of the pituitary and immersion in fixative was complete within about a minute of death.

The differences in the effects of corticosterone and dexamethasone are extremely interesting and provide the first morphological evidence that these glucocorticoids act differently in negative feedback. These must obviously be examined further, comparing continuous as opposed to intermittent administration of the steroids. Again, a combination of EM immunohistochemistry and molecular

biological techniques are required.

The role of the folliculostellate cell remains unclear, but the lack of granulation and subjective changes in cell numbers suggests that it is not a stem cell, at least in the rat pituitary.

The studies outlined here have extended knowledge of the morphological changes in the pituitary corticotroph in states of altered function. They support the concept that increased stimulation results in a larger population, and suggest that this may be caused directly by CRF. While caution must be used in extrapolating the findings in the rat to man, this would be in favour of a hypothalamic or central origin for those cases of Cushing's disease with corticotroph hyperplasia. The changes seen with corticotroph inhibition raise the possibility that the degree of or type of inhibition of the HPA axis may depend on the particular glucocorticoid administered in clinical situations. This aspect of the studies however, requires more detailed analysis.



APPENDIX

Double Immunostaining Technique.

1. Dewax and bring to water.

2. Wash in 95% methanol containing 0.5% H_2O_2 for 30 minutes.

3. Rinse in water, then in tris-saline (0.05m tris buffer, pH 7.6 plus 0.9% sodium chloride).

4. Treat with normal swine serum (NSS) diluted 1:5 with do 0.05m tris buffer, pH 7.6 for 15 minutes. Drain off but do not wash.

5. Treat with specific antibody (anti-S100) (DAKO) diluted 1:100 with 5% NSS and applied for one hour at room temperature.

6. Wash in tris-saline for 15 minutes.

7. Treat with swine anti-rabbit IgG (DAKO) diluted 1:100 in 50% tris-buffer, 50% normal human serum for one hour.

8. Treat with peroxidase - antiperoxidase conjugate (PAP) (DAKO) diluted 1:100 in tris buffer with 5% NSS for 15 minutes.

9. Wash in tris-saline for 5-10 minutes.

10. Treat with 30mg 3,3, diaminobenzidine tetrahydrochloride (DAB) (Sigma) dissolved in 100ml tris buffer (0.03%) with 2 drops of hydrogen peroxide (0.01%) for 15 minutes.

11. Wash in water.

To stain the corticotrophs, repeat steps 4-7 using specific antibody to ACTH (DAKO) diluted 1:800 and applied overnight at room temperature.

9a. Apply alkaline phosphatase - anti alkaline phosphatase conjugate (APAAP) (DAKO) diluted 1:4 for 30 minutes.

10a. Wash slides in fresh tris-saline for 2 minutes.

11a. Apply freshly prepared substrate solution * for 15 minutes.

12a. Wash twice in distilled water.

13a. Water mount in glycerin jelly.

Results

Folliculostellate cells - Brown.

Corticotrophs - Red.

*Substrate solution (A-AP substrate).

1. Naphthol AS Bl Phosphoric Acid - 5.0mg.
2. Dimethyl Formamide - 2 drops.
3. Fast Red TR Salt - 5.0mg.
4. Veronal Acetate Buffer - 10ml.
5. lmM Levamisole. - 200µl.

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ERRATUM

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