Glucocorticoids and hypothalamo-pituitary-adrenal function

Prevention of suppression?

Glucocorticoïden en de functie van de hypothalamus-hypofyse-bijnier as
Wordt suppressie voorkomen?

PROEFSCHRIFT

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List of abbreviations

ACH	: acetylcholine.
ACTH	: adrenocorticotropic hormone.
AVP	: arginine vasopressin.
CRF	: corticotropin releasing factor.
CSF	: cerebro-spinal fluid.
GABA	: γ-aminobutyric acid.
HPA-axis	: hypothalamo-pituitary-adrenal axis.
5HT	: serotonin (5-hydroxytryptamine).
5HTP	: 5-hydroxytryptophan.
i.p.	: intra peritoneal.
LHRH	: luteinizing hormone - releasing hormone.
NA	: norepinephrine.
n.s.	: not significant.
PRL	: prolactin.
s.c.	: subcutaneously.

Contents

Chapter I

Review of literature and scope of this thesis

In this first chapter the current insights are reviewed on what is known about the regulation of the hypothalamo-pituitary-adrenal axis (HPA-axis). This introductory chapter has been divided in three different sections.

The first section (A) reviews the literature concerning the effects of glucocorticoids on the function and the regulation of the HPA-axis. The second part (B) focuses on the nature and the regulation of corticotropin-releasing-factor (CRF). Finally, in the third section of this chapter (C) the questions are formulated which are aimed to be answered in this thesis.

I.A.: THE SUPPRESSIVE EFFECT OF GLUCOCORTICOIDS ON THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS

In 1952 Fraser et al. (1) reported a case of post-operative irreversible shock in a patient who had used glucocorticoids for a long time. From that moment on several reports appeared in which post-operative shock and subsequent death were attributed to adrenocortical insufficiency due to long-term glucocorticoid therapy (2-8, 19).

Some patients with circulatory failure appeared to respond to parenteral glucocorticoid administration, which made it probable that there was a direct relation between shock and plasma corticosteroid levels. In 1961 Sampson et al. (9) confirmed this probability by the finding of low plasma cortisol levels in a patient with adrenal insufficiency which developed after stopping long-term glucocorticoid therapy. The number of carefully documented patients reported in the literature is rather small (9, 10, 11). The clinical symptoms to make the diagnosis of adrenocortical failure certainly are unreliable. As evidence for adrenocortical insufficiency Salassa et al. (3) found a striking reduction in the mean adrenal weight in a post-mortem study of patients who had received corticosteroids until the day of their death. A group of patients who died of similar causes, but did not receive glucocorticoids, had no signs of adrenal atrophy. In this study the reduction of adrenal weight was already found after treatment with glucocorticoids for 5 days.

Later studies demonstrated that the return of the adrenal weight to normal

after stopping glucocorticoid therapy does not necessarily mean functional recovery of the HPA-axis (Axelrod, 19).

Because it is impossible to prove *in vivo* the presence of an anatomic adrenocortical atrophy, the functional integrity of the HPA-axis is studied by measuring HPA-responsiveness to a variety of provocative tests. The reaction to the stress of an insulin-induced hypoglycemia, to the administration of metyrapone, lysine vasopressin or ACTH are wellknown tests, to examine the reactivity of the HPA-axis (141). Each test has a different mode of action: insulin-induced hypoglycemia acts at hypothalamic or higher brain levels, thus stimulating the release of CRF and consequently of ACTH and cortisol (12). Metyrapone blocks the conversion of desoxycortisol to cortisol in the adrenal cortex by inhibition of the enzyme 11β-hydroxylase. The decrease of plasma cortisol levels results in a compensatory increase in ACTH secretion which stimulates steroid production (13, 18). Eventually increased adrenal secretion of desoxycortisol is accomplished. Lysine vasopressin will stimulate directly the release of ACTH from the anterior lobe of the pituitary, and thus will cause a sharp increase in plasma cortisol concentrations. So, its mode of action is different from that of the insulin-induced hypoglycemia. This stimulation test of ACTH release was described by Webb-Peploe et al. (142) to distinguish various causes of Cushing's syndrome, but can also be used to investigate the integrity of the HPA-axis after the use of glucocorticoids (141). Administration of synthetic $ACTH_{1-24}$ results in a maximal stimulation of cortisol secretion, but only adrenocortical reserve is tested without supplying information on the functional activity of the hypothalamo-pituitary part of the HPA-axis (14).

In the clinical setting of a patient taking glucocorticoids it is important to have information on the following points:

1) how long does it take for glucocorticoids to induce a suppression of the HPA-axis?
2) how long does it take before the HPA-axis is fully recovered after a period of exposure to glucocorticoids?
3) which mechanisms play a role in the recovery of the HPA-axis?

I.A.1. *Time interval of suppression of the HPA-axis*

It is difficult to determine exactly from the literature how fast glucocorticoids induce suppression of the HPA-axis. The main reasons are that various investigators have been using different methods in evaluating HPA-integrity, while a wide range in dosage and duration of treatment with glucocorticoids in different patient groups makes a comparison of the results difficult.

Christy et al. (15) showed a decreased adrenal response to ACTH infusion in six patients who had been treated for 5-13 days with prednisone (20-30 mg daily). Plager et al. (16) demonstrated an impaired reaction to the administration

of ACTH and metyrapone in four patients who had received cortisol 100 mg daily for only three days. Recently Streck et al. (17) have studied the effects on HPA function of high dose corticosteroid therapy (prednisone 30 mg/day) during 5 days. They used insulin-induced hypoglycemia and ACTH infusion as provocative tests. These authors found a significant suppression of HPA activity with both tests as measured for 2 days after prednisone administration, suggesting an impaired ACTH secretion and an insufficient adrenal reserve. Five days after concluding steroid therapy the cortisol response to hypoglycemia had returned to pre-treatment levels, but surprisingly the cortisol response to ACTH remained impaired. Others (18–21) found an abnormal response to hypoglycemia, metyrapone or lysine vasopressin, while the reaction of plasma cortisol to ACTH was already normal. They suggest that the three tests mentioned first are more sensitive in evaluating the integrity of the complete HPA-axis than ACTH stimulation tests.

The insufficiency of the HPA-axis is more outspoken when the duration of glucocorticoid administration has been longer. Moreover, a dose-dependent relationship exists, in which higher doses of glucocorticoids produce a more severe suppression, especially when the dose exceeds that of the normal cortisol production, which varies between 15–30 mg a day. Axelrod (19) mentioned that replacement doses of glucocorticoids probably exert no important suppressive effects on the HPA-axis, as long as the mode of administration mimicks the circadian rhythm of cortisol closely. Under these circumstances there is no need to fear for an insufficient reaction of the HPA-axis to the stress of general anesthesia and surgery. This concept was studied by Danowski et al. (20), who did not observe signs or symptoms of acute adrenal insufficiency in 117 patients, who had been treated with low doses of glucocorticoids (cortisol or its equivalent in a dose of less than 25 mg daily) and underwent in total 80 stressful diagnostic or therapeutic procedures, without supplemental coverage with glucocorticoids. These patients had been treated with low doses of glucocorticoids for hirsutism, irregular menses, or infertility.

Others reported on the effects of higher doses of glucocorticoids on the response to stimulation tests and during stressful periods. Jasani et al. (10, 21) reported on patients with rheumatoid arthritis, treated with doses of glucocorticoids equivalent to 20–60 mg cortisol/day (mean 32 mg) over a prolonged period (6 months–5 years). Suppression of the HPA-axis was most outspoken after the use of the highest doses of glucocorticoids (i.e. mean 60 mg cortisol). Furthermore, during a small "standard" operation (synovectomy of the knee), plasma cortisol levels were significantly more reduced in the patients who had received the highest doses of steroids, implicating a diminished HPA-response to stress. In parallel, blood pressure during operation was also statistically significantly *lower* after the highest doses of glucocorticoids. In this study a group of patients occurred with normal reaction to ACTH stimulation, but an

insufficient response to metyrapone, and to insulin-induced hypoglycemia. This seems to underline further that an increase of cortisol in response to high concentrations of ACTH represents a maximal reaction of the adrenal cortex, but not necessarily normal functioning of the entire HPA-axis. On the other hand, a normal reaction of cortisol to metyrapone and to insulin-induced hypoglycemia suggests intact dynamic functioning of the HPA-axis, and in this category of patients no hypotension was observed during surgery (10, 19, 21).

I.A.2. *Time course of the recovery of the HPA-axis*

When reviewing the literature, no certainty exists on the precise duration of the recovery of the HPA-system after various periods of administration of pharmacological doses of glucocorticoids. The potency of the HPA system to recover completely remains intact, regardless of the duration of the exposure to and the amount of glucocorticoids used, if the dosage of drugs is tapered down gradually and slowly enough. In general, one may say that the level of the dose and the duration of administration of steroids determine the speed of the recovery of the HPA-axis. In the studies of Streck and al. (17) mentioned already, recovery of the HPA-axis took 5 days after cessation of a ten day prednisone schedule, as assessed with an ACTH stimulationtest and the reaction to insulin-induced hypoglycemia. This is one of the first studies in which HPA suppression and its recovery after short-term steroid administration were properly documented.

The classical study by Graber et al. (22) may serve to demonstrate the sequence of events after prolonged HPA suppression due to endogenous and exogenous hypercortisolism.

They studied 8 patients with Cushing's syndrome due to an autonomous adrenocortical tumor, and 6 patients treated for 1-10 years with pharmacological doses of glucocorticoids. Thus, high circulating cortisol concentrations were present in both patient groups, and ACTH levels were suppressed. After correction of hypercortisolism (removal of the adrenal tumor or discontinuation of the administration of exogenous steroids) they serially measured plasma cortisol and ACTH at 06.00 hr repeatedly for 12 months (Fig. I-1). In the first month after the correction of hypercortisolism plasma cortisol and plasma ACTH levels were subnormal, indicating suppression of hypothalamo-pituitary activity and of adrenocortical hormone secretion. Thereafter, in the second till third month plasma ACTH levels began to rise, but cortisol levels remained subnormal. From the third till the fifth month plasma ACTH levels became actually elevated in most patients, although plasma cortisol levels remained persistently lowered. Only 6 months after correction of hypercortisolism plasma cortisol levels began to rise under the

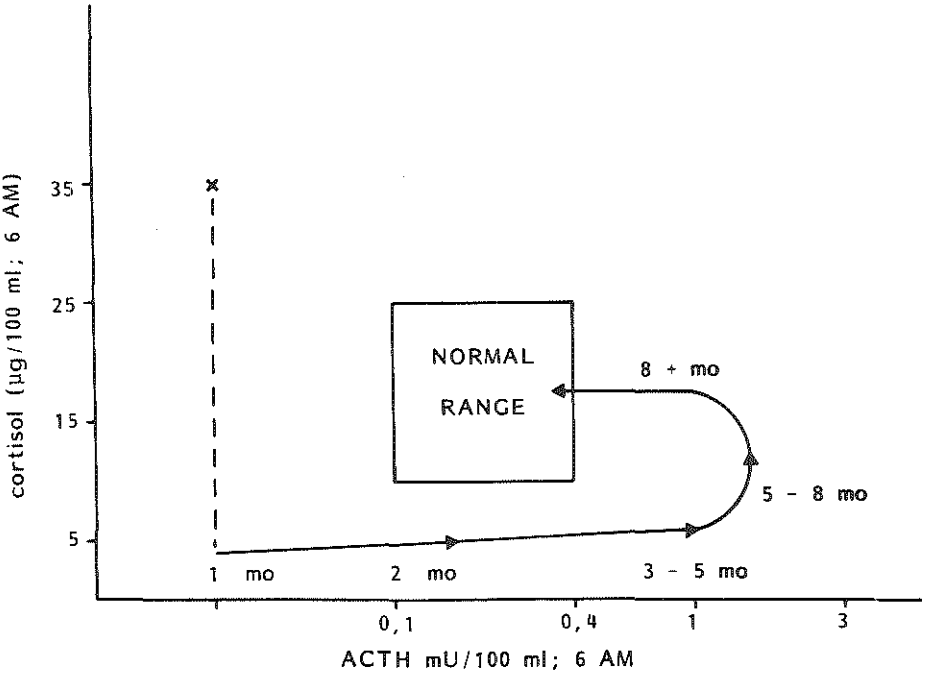

Fig. I-1. Recovery from prolonged pituitary-adrenal suppression (Graber 1967).

influence of the continuously elevated ACTH levels. Finally, after 8–9 months ACTH and cortisol levels returned within the normal range.

From this important study the following conclusions can be drawn:

1) during the recovery of prolonged suppression of the HPA-axis hypothalamo-pituitary function is restored before adrenocortical function
2) the recovery of pituitary function takes 1-2 months, while the recovery of the adrenocortical function takes much longer.
3) the recovery of basal hormone secretion by the complete HPA-axis after periods of suppression for longer than a year, requires at least 9-12 months.

Confirming these results Livanou et al. (23) found a gradual normalization of plasma cortisol levels stimulated by insulin-induced hypoglycemia over a time course of one year in patients after withdrawal of prolonged glucocorticoid treatment. Furthermore, another report showed an even more protracted recovery of the HPA-system taking up to two years after removal of adrenocortical tumors (24). Axelrod (19) concludes that the possibility of the occurrence of adrenal insufficiency should be kept in mind in patients undergoing any stressful procedure up to one year after withdrawal of glucocorticoids given for periods longer than 1 to 4 weeks.

I.A.3. *Mechanisms playing a role in the recovery of the HPA-axis*

Some controversy exists on the mode and site of action of glucocorticoids in suppressing ACTH release (25). The inhibitory effects of glucocorticoids on the functional activity of the HPA-system have been studied extensively in animal studies (26, 27, 27a, 28, 29, 30). In these studies the relationship between plasma and pituitary ACTH levels and the plasma concentrations of corticosterone were examined in non-stressed animals. An inverse relationship between ACTH and corticosterone levels has been reported in animals after exposure to corticosterone, and/or adrenalectomy. Thus, the concentration of plasma corticosterone seems to control the activity of the hypothalamo-pituitary system by regulating hypothalamic CRF activity and pituitary ACTH synthesis and release (31-34). There is evidence for the presence of glucocorticoid receptors in the anterior pituitary lobe, in the hypothalamus and in other higher brain centres via which glucocorticoids might exert their suppressive actions on the HPA-axis. Buckingham (28) commented on findings which possibly indicated that glucocorticoids exert a negative feedback effect via higher brain centres, c.q. extrahypothalamic sites, like areas of the amygdaloid bodies and the hippocampus (41).

Experiments *in vitro* have given ample evidence for the existence of functionally active glucocorticoid receptors in the hypothalamus (31, 37, 38, 39).

In other studies (35, 36) it appeared that the hypothalamic release of CRF and of CRF induced ACTH release was inhibited after glucocorticoid administration *in vivo*. The synthesis of ACTH itself seemed not to be inhibited however.

Stores of CRF in the median eminence of the hypothalamus are increased in adrenalectomized rats, but glucocorticoids prevent under those circumstances the release of CRF (40). Experiments on the effects of the administration of glucocorticoids on the regulation of ACTH under stressful conditions show a marked suppressing effect of high levels of circulating glucocorticoids on the stress responsiveness of the HPA-axis (41).

At present, the concept of a delayed or proportional feedback mechanism of glucocorticoid hormone controlling the release of ACTH in response to stressful stimuli is accepted. Sayers et al. (42) demonstrated the stimulatory effects of stressful stimuli on the HPA-system and subsequent inhibition of these effects induced by glucocorticoids. From this work and the work of Dallman et al. (43) it was concluded that there is a delay of about 2 hours, before there is a clear inhibition by glucocorticoids on stress responsiveness. However, Smelik (44) found no direct influence of single large doses of corticosterone on the responsiveness of the HPA-axis; only after normalization of circulating glucocorticoid levels there was evidence of inhibition of ACTH

release somewhat later. Others (Buckingham et al. (30); Hodges et al. (45)) have confirmed these negative observations.

There is also evidence for the existence of a rapid feedback system in the inhibition of stress-induced HPA activity. Dallman et al. (43) and Jones et al. (47) demonstrated that inhibition of ACTH secretion in response to stress occurred within a few minutes after the infusion of glucocorticoids. Jones et al. (47) have shown that this rapid feedback mechanism is dependent on the rate of increase of plasma corticosterone levels, but others have criticised the physiological significance of these findings (28). ACTH secretion was measured indirectly, anesthesia with barbiturates was used, and the rate of steroid-infusion was high (Buckingham; 28). In other studies the existence of a rapid feedback mechanism was confirmed and different receptors were demonstrated for rapid and delayed feedback actions of glucocorticoids (48).

Summarizing these results Gann et al. (46) suggest that the corticosterone-induced inhibition of CRF secretion is mediated mainly by the fast-rate-sensitive mechanism, but that the synthesis of CRF is controlled by the delayed level-sensitive system (49). However, modulation by glucocorticoids of CRF synthesis and secretion is evidently more complex. Some studies showed that both delayed- and rapid-rate-sensitive feedback mechanisms may inhibit CRF synthesis and secretion acting at sites remote from the CRF neuron (50). There is a distinct variety in the changes in responses to different stimuli induced by glucocorticoids. Some are inhibited by large doses of steroids, whereas the responses to other stimuli are suppressed at low stimulus intensity, but not at high intensity (46). The possibility of the existence of multiple pathways to the CRF neuron was raised: low threshold, steroid suppressible pathways as well as high threshold, non-steroid suppressible pathways to the hypothalamus would offer an explanation for these conflicting findings (5).

Having demonstrated the different sites at which glucocorticoids may exert their inhibitory actions under various conditions, it remains difficult to ascertain the relative importance of those different feedback sites under normal physiological conditions. From the available evidence, Buckingham (28) reasons that glucocorticoids exert their actions on stress-induced ACTH activity predominantly at hypothalamic centres, or even at higher brain centres, whereas ACTH secretion under basal non-stress conditions may be controlled via anterior pituitary lobe receptors. As shown by Hillhouse and Jones (52) and Yasuda and Greer (53), changes in the levels of circulating glucocorticoids influence pituitary ACTH secretion more readily than the CRF content of the hypothalamus. On the other hand, the suppressive action of corticosterone *per se* seems to be more pronounced at the hypothalamic level than at the level of the anterior pituitary gland (33, 39).

Influences of adrenocortical hormones, other than cortisol and corticosterone on HPA activity have been investigated by Jones and Hillhouse

(54). They suggested that due to differences in structure-activity relationships, both the 11β-OH and the 21β-OH groups of the adrenocortical steroids are essential for the fast-rate-sensitive feedback mechanism. Aldosterone does not seem to have an important inhibitory action on HPA-activity as may be concluded from observations on patients with primary hyperaldosteronism, although at high aldosterone concentrations inhibition of CRF secretion in rat hypothalami *in vitro* was observed (32).

I.B. CORTICOTROPIN RELEASING FACTOR

I.B.1. *Nature and regulation of CRF*

De Groot and Harris (55) were the first to demonstrate in 1950 an influence of the hypothalamus on the pituitary-adrenal system. In elegant experiments they implanted in rabbits an electrode in the hypothalamus, which was connected with a coil inserted between skull and scalp. The outer turn of the coil was connected to an indifferent electrode. By placing the rabbits head in an electromagnetic field they could induce a voltage in the coil and subsequently involve stimulation of the hypothalamus. Electrical stimulation of certain areas of the hypothalamus generated a remarkable increase in activity of the adrenal cortex. They postulated from these experiments that a humoral factor from nerve endings of the hypothalamus was released into the hypophyseal portal vessels, which appeared to stimulate ACTH release. Saffran et al. named this humoral substance in 1955: corticotropin releasing factor (CRF) (56). After successful experiments to extract substances with CRF activity from hypothalamic tissue in the years that followed many investigators attempted to elucidate the exact chemical identity of CRF, but it remained a mystery until very recently.

Porter et al. showed that extracts of blood from the capillaries of the hypophyseal portal vessels from hypophysectomized, stressed dogs caused an increased activity of the adrenal cortex in cortisol treated rats, thus providing further evidence of a hypothalamic CRF (57, 58). *In vivo* studies in rats and other animals demonstrated that crude extracts of hypothalami activated ACTH release also in the presence of sections through the hypothalamus or in corticosteroid treated animals (59, 60).

The exact chemical nature of CRF remained unknown until very recently. It became evident from extraction and purification studies that CRF is a polypeptide, since proteolytic enzymes like trypsin or pepsin were able to destroy CRF-like activity in hypothalamic extracts. Moreover, subsequent studies showed the existence of 2 fractions with CRF-like activity: α- and β- CRF, and a resemblance with vasopressin was noted (61, 62).

It was thought for some time that vasopressin might be identical with CRF. For instance in rats with diabetes insipidus induced by hypothalamic lesions the degree of inhibition of the HPA system appeared to have a proportional relationship with the degree of diabetes insipidus (63). Recent reports found a comparable elevation of the content of immunoreactive vasopressin *and* of CRF in adrenalectomized rats (64). After administration of glucocorticoids to adrenalectomized rats both the expected elevation of the CRF and the vasopressin content were prevented (64, 65).

In 1979 Gillies and Lowry (66) proposed that CRF might be a complex system, in which vasopressin is a major component which is modulated by other factors with weaker CRF activity. Using a CRF bioassay, chromatography and immunological techniques they found vasopressin-like material in the rat stalk median eminence to be identical to synthetic arginine vasopressin, but having only 30% of the biological activity of the extract. They suggested that this lack of full bioactivity of the major CRF peak, containing "arginine vasopressin-CRF" (AVP-CRF), was caused by removal of some synergistic factors. After stabilization of the chromatographic column with ascorbic acid, fractions from other areas in the chromatogram were recombined with the "AVP-CRF" complex, giving a synergistic effect. Other fractions did not have a significant bioactivity, measured by CRF bioassay. The combinations of added factors and "AVP-CRF", but also those factors with synthetic arginine vasopressin shared dose-response characteristics, identical to that of crude stalk median eminence. They concluded that the loss of bioactivity of this multifactorial system by separation techniques, which is caused by its instability under non-reducing conditions, is an important reason for the failure to characterize CRF.

Zimmerman et al. (67) found evidence in monkeys for the existence of a specific axonal pathway for vasopressin directly to the hypophyseal portal system. This pathway originated in the paraventricular nucleus of the median eminence of the hypothalamus. Glucocorticoids appeared to exert an inhibitory influence on this pathway. They also speculated that vasopressin might be either a CRF-like substance or an assistant of CRF.

On the other hand, there was as much, or even more convincing evidence that vasopressin is *not* identical to CRF in the rat. For instance: pentobarbitone/morphine was able to block the ACTH releasing effect of vasopressin (68). De Wied et al. (69) demonstrated the absence of pressor activity in hypothalami of hypophysectomized rats, in which CRF activity was present. In addition McDonald et al. (70) did not find a correlation between the pressor and CRF activity of hypothalami in response to stimuli as water deprivation, water loading or nicotine administration. It is important to mention that experiments with Brattleboro rats (rats with inherited diabetes insipidus) have shown a diminished activity of the hypothalamo-pituitary-adrenal system. But, median eminences or hypothalamic extracts from these rats still possess CRF bio-

activity, and these animals retain the ability to react to stress with a rise of circulating corticosterone concentrations (71, 72, 73, 74). In addition, in other observations Buckingham et al. made it clear that there had to be a difference in chemical structure between vasopressin and CRF from hypothalamic extracts (75). Those results are in contrast with the studies of Gillies et al. (66) mentioned above. Possibly, the methods used to demonstrate the CRF bioactivity, i.e. isolated pituitary cells vs. pituitary segments, varied in so many aspects, that important differences in the results were to be expected.

Only very recently, Vale et al. (76) reported the characterization of a polypeptide from ovine hypothalamic tissue, which was able to stimulate directly the secretion of ACTH. After ultrafiltration and chromatography, two zones with ACTH releasing activity were detected. The first zone had a higher intrinsic activity than the second, which on subsequent purification appeared to be arginine vasopressin. The zone with the highest ACTH releasing activity was further analysed by ion exchange chromatography and high pressure liquid chromatography. The primary structure of the major component was identified to be a polypeptide with 41 amino acids.

After synthesizing CRF, these authors demonstrated that synthetic CRF_{1-41} was identical to the purified native CRF, and both were highly active in stimulating ACTH secretion *in vitro* and *in vivo*. Incubation of cultured pituitary cells with dexamethasone, prior to CRF administration, demonstrated a dose-dependent inhibition of ACTH release. They concluded that the high potency and intrinsic activity to stimulate ACTH secretion *in vitro* and *in vivo* and its presence in the hypothalamus indicated that this peptide actually was CRF.

I.B.2. *Regulation of the secretion of CRF*

In the last years many studies have been carried out on the control mechanisms, which regulate the secretion of CRF. Selye (77) was the first to find ACTH mediated effects on the adrenals produced after diverse stressful stimuli. Today, CRF is known to be one of the most important factors in regulating ACTH activity. The CRF content of the hypothalamus rises in accordance with, or just prior to increases in pituitary and plasma ACTH levels. Exposure of rats to ether stress results in an initial fall of the hypothalamic CRF content, followed by a sharp rise, which is directly followed by an increase of pituitary ACTH content and of ACTH release into the circulation and subsequently rising plasma corticosterone concentrations (31). Likewise, adrenalectomy causes an exaggerated response to stress, parallel to the increase of ACTH. On the other hand, administration of glucocorticoids reduced CRF and ACTH activity in a similar way (28, 31).

A prominent feature of the HPA-axis is the existence of a circadian rhythm. Probably, this is of great importance in maintaining vital metabolic processes

at adequate levels, with equally adequate concentrations of glucocorticoids, thus preventing overshoot (46, 79). The rhythm is conditioned by a light - dark cycle, and may be influenced by the time of food intake in some species, although probably not in man (80, 81, 82).

The pacemaker of the circadian rhythm lies in the neurons of the supra-chiasmatic nucleus (83): lesions in this particular area destroy the rhythmicity (84). It is also clear from anatomical studies that the supra-chiasmatic nucleus of the hypothalamus is stimulated by light stimuli mediated through the retino-hypothalamic tract. The ventromedial nucleus has to be intact, to entrain the circadian rhythmicity by feeding (85).

There are probably two mechanisms regulating the interaction between the circadian rhythmicity and the response to stressful stimuli. First, the efficacy of glucocorticoids to suppress ACTH in man and rat appears to be greatest when the concentrations of circulating glucocorticoids are the lowest (86). Secondly, serotonin (5-hydroxytryptamine) has a marked influence on circadian rhythmicity in adrenalectomized rats. It exerts a more profound stimulation of ACTH secretion when applied in the morning than in the evening. To explain these mechanisms, there has to be an independency from circulating glucocorticoid levels (87, 88). On the other hand the hypothalamic content of CRF is higher in the evening than in the morning, and there is clearly no direct correlation between the CRF content, and the tendency to secrete CRF in this situation (88). Krieger and Rizzo (89), and Vernikos-Danellis et al. (90) demonstrated a dependence of the circadian rhythmicity of corticosterone on serotonin. They found that blockade of serotonin synthesis prevented the circadian rhythmicity and a consequent rise in plasma glucocorticoids. However, the response to stress was not prevented, and even enhanced.

I.B.3. *Effects of ACTH on CRF: an internal "short" loop feedback?*

The negative feedback that glucocorticoids exert on the HPA-axis has been demonstrated conclusively, but some studies in the sixties and early seventies raised the possibility that circulating plasma ACTH levels might also directly influence CRF secretion and subsequent ACTH release from the anterior pituitary.

Gemzell and Heijkenskjöld (91) and Kitay et al. (92) found that the administration of ACTH reduced the anterior pituitary weight and the anterior pituitary ACTH content in adrenalectomized rats. They suggested that the secretion of ACTH is regulated not only by circulating glucocorticoid levels, but also by the circulating plasma ACTH levels: the existence of a "short" loop feedback mechanism was postulated. Hodges and Vernikos found a greater increase in plasma ACTH levels in adrenalectomized rats submitted to stress when the initial blood levels of ACTH were low, than when they were high (93).

Attempts to elucidate the mechanisms involved were made by Halasz and Szentagothai who implanted anterior pituitary tissue in the infundibular recess of the third ventricle of rats and found a suppression of adrenal function in the presence of such implants (94). Motta et al. (95) implanted cannulae bearing solid ACTH in the median eminence, frontal cerebral cortex and pituitary gland with stereotactic procedures. The rats were submitted to constant mild environmental stress. The results showed the ACTH implants in the median eminence to be effective in reducing significantly circulating corticosterone levels and anterior pituitary weight. They found no effects however of implants in the cerebral cortex or in the pituitary gland. Motta et al. explained these results as evidence for the existence of a "short" loop negative feedback mechanism of ACTH secretion, and supposed that the stalk median eminence contained receptors, responding to ACTH (96). It is noteworthy that the rats serving as controls in the experiments of Motta et al., had very high circulating corticosterone levels, probably due to the constant environmental stress. The same authors demonstrated that treatment of adrenalectomized-hypophysectomized rats with ACTH reduced the CRF content of the hypothalamus to the levels found in rats, which were only adrenalectomized, and postulated CRF activity to be regulated both by glucocorticoids and ACTH (97).

Using a pituitary incubation method to measure hypothalamic CRF bioactivity Seiden and Brodish (98) investigated the effects of adrenalectomy or hypophysectomy or both together on hypothalamic CRF activity. The results of their experiments indicated that there was in fact a "short" loop system in which ACTH influenced hypothalamic CRF activity: they found in all different conditions studied, an inverse relationship between the plasma level of ACTH and CRF activity. After injection of synthetic ACTH (2 U, s.c., 4 times daily for 1 week) adrenalectomized-hypophysectomized rats were shown to have a substantially lowered hypothalamic CRF activity when compared with adrenalectomized-hypophysectomized controls receiving saline injections. On the other hand they found no elevation of CRF activity shortly after adrenalectomy (5 or 7 days), as described by Motta et al. (97). The authors concluded that a role of plasma ACTH in regulating CRF activity might be important, but that the actions of adrenal glucocorticoids are probably of much more importance.

One of the few studies investigating a possible "short" loop feedback phenomenon in man was carried out by Upton et al. (99). In patients with lipoatrophic diabetes (Seip-Laurence syndrome), a rare genetically determined disease characterized by lipodystrophy, diabetes mellitus, hyperlipemia, hepatic insufficiency and chronic hypersecretion of CRF and LHRH (100), ACTH was administered and *plasma* CRF bioactivity was determined; the latter was shown to decrease in response to ACTH in 3 of 4 patients. Moreover, remarkably the cortisol levels throughout the day were high (24 to 35 μg/

100 ml), without an increase after the administration of high doses of ACTH.

The conclusions of Upton et al. on a possible direct "short" loop feedback effect of ACTH were based on these data in particular. No measurement of plasma ACTH levels in basal conditions were carried out, and the specificity of the method of plasma CRF assay is difficult to judge.

Takebe et al. (101) found that CRF bioactivity in the median eminence was suppressed after ACTH administration for 5 days to adrenalectomized-hypophysectomized rats under ether stress, but *not* under ether laparotomy stress with intestinal traction, indicating a possible hierarchy in different stress mechanisms.

Fehm et al. (102) found no evidence for the existence of an "ultra-short" feedback mechanism by the addition of ACTH to the medium of incubated pituitary cells in an attempt to inhibit ACTH release from the dispersed pituitary cells.

In summary, there are several well documented studies, presenting evidence for the existence of a "short" loop negative feedback mechanism in which plasma ACTH levels may inhibit CRF secretion at the hypothalamic level. On the other hand, almost all these data have been obtained in stressed adrenalectomized and/or hypophysectomized rats, and the importance of the reported phenomenon under physiological conditions has yet to be shown.

I.B.4. *Neurotransmitters and CRF secretion*

The mechanisms which control the secretion of CRF have been examined by many investigators with many different methods.

It has been demonstrated that excitatory and inhibitory pathways originating from specific areas in the hypothalamus, the amygdalic bodies, the hippocampus, but possibly also in the thalamus, the basal septal area, and parts of the reticular formation are involved in the regulation of CRF release (103, 104, 105). One of the problems in interpreting these results was that the use of *in vivo* techniques in which drugs are administered intraventricularly, or directly implanted into certain brain areas, does not necessarily result in specific data. For instance, drugs may diffuse to other areas, the stress of implantation may influence the outcome, interactions of the drugs with different receptors may occur after drug administration, with different results (28).

The use of an *in vitro* method in the study of CRF release, in which whole rat hypothalami are incubated with different putative neurotransmitters, made it more easy to demonstrate inhibitory or excitatory effects of different neurotransmitters, thus also determining the site of action of those substances. However, one should bear in mind that results of *in vitro* studies might not be representative for the *in vivo* situation.

I.B.4a. *Stimulatory influences on CRF release*

The importance of a cholinergic pathway in the control of CRF has been demonstrated convincingly by several authors. Endröczi et al. were the first to implicate acetylcholine as putative neurotransmitter: they showed that the activity of the hypothalamo-pituitary-adrenal system was stimulated by implantation of carbachol in different brain areas (106). Others confirmed this observation (107, 108, 109, 110, 111). Jones et al. (54) investigated *in vitro* the stimulation of the secretion of CRF from both hypothalamus and median eminence, after incubation with acetylcholine at different concentrations. A dose-dependent stimulation of the release of CRF from the hypothalamus was found with acetylcholine concentrations ranging from 1-5 pg/ml. Incubation of acetylcholine with tissue of the median eminence did not cause a release of CRF, even at high doses. In the presence of 10 ng norepinephrine, CRF release at concentrations of 3 pg/ml acetylcholine was significantly less, but again a dose dependent rise in CRF release was noted at higher concentrations. No effects of acetylcholine on CRF secretion were noted from hypothalamic tissue containing the ventromedial, dorsomedial and paraventricular nuclei (54). Both the synthesis and release of CRF are stimulated by acetylcholine (112, 113, 114, 122). The actions of acetylcholine on CRF secretion are probably mediated by nicotinic *and* muscarinic cholinergic receptors. Jones et al. stated that the mediation is predominantly nicotinic, because only hexamethonium, and not atropine, fully antagonizes the acetylcholine mediated release of CRF (54). In contrast with these results are those of Buckingham who found total inhibition of acetylcholine induced CRF release only by a combination of specific nicotinic and muscarinic receptor antagonists (112).

More controversy exists concerning the role of serotonin. Fuller (115) recently reviewed the evidence from *in vitro* and *in vivo* studies that serotonergic neurons stimulate the release of CRF from the hypothalamus. For some time there was considerable uncertainty whether the influence of serotonin on CRF and ACTH activity was excitatory or inhibitory. Implantation of serotonin in vivo either into the lateral ventricles, or into hypothalamic areas had no effect on basal CRF secretion, but inhibited the response to ether stress and surgical stress (116, 117). Others seemed to support these findings (90, 118).

Krieger and Rizzo (89) were the first to suggest that serotonin might have a stimulatory effect on CRF production and release. More recently it was shown that serotonin in vitro at concentrations ranging from 0.1 to 10 ng/ml stimulated CRF release in a dose dependent manner (54). Cyproheptadine, a relatively non-specific serotonin antagonist, and methysergide antagonized these effects (54).

Local application of serotonin to certain brain areas as the ventral hippocampus and the reticular formation resulted in an acute rise of plasma gluco-

corticoids in guinea pigs (119), whereas Krieger and Krieger observed an elevation of glucocorticoids after serotonin implantation directly in cat brain (109). In man and rhesus monkeys infusion of 5-hydroxytryptophan (5-HTP), a precursor of serotonin, significantly elevated the circulating glucocorticoid concentrations (120, 121). However, it became clear from the studies of Vermes and Telegdy (116, 117), that *in vivo* experiments in rats involving intracerebral administration of serotonin did not induce directly an excitatory response of the pituitary-adrenal system to this neurotransmitter. The existence of a cholinergic interneuron as mediator of serotonin-induced CRF release was suggested by Jones et al. (54), because they found an inhibition of the stimulating effect of serotonin by hexamethonium and atropine.

Pharmacological *in vivo* studies involving various agents further elucidated the enhancing effect of serotonin on HPA activity. Those agents consisted of several groups: 1) serotonin precursors like 5-HTP; 2) direct serotonin receptor agonists like quipazine and L-(m-chlorophenyl)-piperazine; 3) serotonin re-uptake inhibitors like fluoxetine, zimelidine, and certain tricyclic antidepressants (123); 4) serotonin releasers like p-chloroamfetamine, fenfluramine and norfenfluramine (115). The elevation of plasma corticosterone levels in rats by the serotonin precursor 5-HTP is stereospecific, and is potentiated by monoamine oxidase inhibitors. Additional evidence of serotonin as a particular mediator of CRF release, is greatly strengthened by the stimulation of this release by fluoxetine, a serotonin re-uptake inhibitor (124). The serotonin receptors involved in the 5-HTP-induced elevation of CRF may become supersensitive, as shown by Kawa et al. (125) and Clemens et al. (126). Kawa described augmentation of 5-HTP induced CRF release by pretreatment with 5,6-dihydroxytryptamine intraventricularly, while Clemens found an enhancement of 5-HTP-induced CRF release after feeding rats a tryptophan free diet.

Of the direct serotonin receptor agonists quipazine has been studied most extensively; it caused a dose-dependent increase in plasma corticosterone levels in rats, which was not influenced by fluoxetine pretreatment, indicating a direct serotonin receptor stimulation (127).

Fluoxetine has a maximal stimulating effect on CRF release within 1-4 hours (128).

As described by Lahti and Barshun (123) and Sigg et al. (129) certain tricyclic antidepressants may act as specific serotonin re-uptake inhibitors, and elevate plasma corticosterone levels. Especially imipramine and chlorimipramine cause such elevations.

Fenfluramine is a serotonin-releasing compound used clinically as an anorectic drug. It also elevates CRF-induced corticosterone levels (115). By depleting serotonin concentrations in the brain the excitatory effect of fenfluramine can be antagonized (115).

p-Chlorophenylalanine is a potent serotonin-depleting drug, which is able to

antagonize CRF release. It is therefore used in testing a possibly serotonergic pathway (130). p-Chlorophenylalanine is able to prevent the stimulating effects of p-chloroamphetamine, a serotonin releasing drug (130).

I.B.4b. *Inhibitory influences on CRF release*
The hypothalamic secretion of CRF is inhibited by catecholamines and by monoamines, in which group γ-aminobutyric acid (GABA) has a predominant place.

Ganong et al. performed many studies on the role of catecholamines in regulating the secretion of CRF (131). They found an inhibition of stress-induced ACTH secretion in dogs by the catecholamine precursor L-dopa and by drugs that release catecholamines (132). It became likely from their experiments that L-dopa had to be transformed in order to be able to inhibit ACTH secretion (133, 134). Dopamine does not seem to play an important role, since dopamine-receptor stimulating drugs like apomorphine do not have a clear effect on ACTH secretion (134), although initial studies reported an inhibitory effect of dopamine when implanted intraventricularly (135). Norepinephrine had a clear inhibitory influence on ACTH secretion after intraventricular administration (134). Hillhouse et al. (114) and Jones et al. (54) found a dose-dependent reduction of CRF release in response to acetylcholine, when norepinephrine was administered in doses of 3-10 ng/ml. This inhibition by norepinephrine was abolished by phentolamine, a specific α-adrenoceptor antagonist. Others confirmed this, and found propranolol, a β-adrenoceptor antagonist, to be effective to prevent a norepinephrine-induced inhibition of CRF release (136). Norepinephrine is able to inhibit acetylcholine- as well as serotonin-induced stimulation of CRF release (114). Epinephrine and α-adrenoceptor agonists like phenylephrine and methoxamine produced a similar inhibitory effect on CRF secretion *in vitro* as norepinephrine (137). In conclusion, all the available evidence points towards an α-adrenergic inhibitory pathway in the production and release of CRF.

The effects of GABA on CRF release were investigated by Makara and Stark (138), Marvin du Pan and Gomez (139), and Jones et al. (54). Infusion of GABA into the third ventricle of rats inhibited the response of the HPA-axis to surgical stress. GABA caused inhibition of CRF release in response to acetylcholine and serotonin *in vitro* in a dose-dependent way. GABA is present in high concentrations in the lateral preoptic region of the rat hypothalamus (54, 138, 139). An antagonist of GABA, picrotoxin, produced a significant rise in plasma corticosterone in rats, who were conscious at the time of the experiments. Also, under anesthesia, picrotoxin caused a rise in circulating corticosterone concentrations (138). Picrotoxin prevented *in vitro* the inhibition of GABA (10 ng/ml) on the release of CRF in response to serotonin significantly (54). Intraventricular injection of bicuculline, another GABA antagonist,

caused *in vivo* a rise in ACTH release. This was completely prevented by the simultaneous administration of GABA (54). In addition, Jones et al. (54) did not find interactions between the inhibitory effects of GABA and norepinephrine. The possibility of an auto-feedback effect of CRF on its own release, mediated by a GABA inhibitory neuron, was raised by the experiments of Yagi and Sawaki (140), who found that collaterals from parvicellular cells in the tuberoinfundibular region in the hypothalamus synapse with GABA inhibitory neurons. From all the studies mentioned above, it is clear that GABA exerts an inhibitory effect on CRF release. However, some doubt still exists on the presence of an auto-feedback of CRF, as mediated by the inhibitory GABA neuron. It is possible that there is a direct inhibition of CRF release by the GABA neuron, without a collateral axon of the CRF neuron.

In summary, Jones et al. proposed a model of the neurotransmitters, involved in the release of CRF from the rat hypothalamus (54). The existence of a cholinergic interneuron, mediating the serotonin-induced CRF release is disputed by others (28, 12). Nevertheless, evidence accumulates that serotonin, possibly by influencing circadian rhythmicity, has a stimulating capacity on CRF release, as does acetylcholine. GABA-ergic and adrenergic pathways inhibit the production and release of CRF (Fig. I-2).

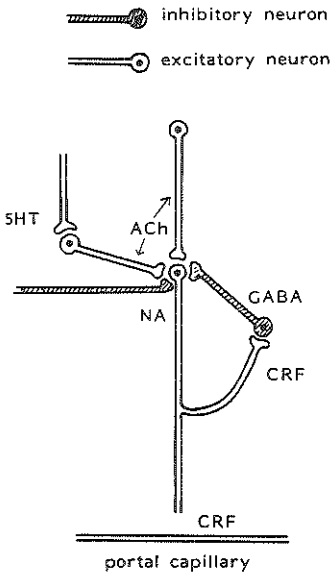

Fig. I-2. Proposed model of neurotransmitter regulation in the release of CRF from hypothalamus (Jones; 1976).

I.C. QUESTIONS TO BE ANSWERED IN THIS STUDY

1. How important is the hypothesized direct "short" loop negative feedback of ACTH on its own synthesis and release? Can the model of an ACTH secreting tumor-bearing rat be used to study the relationships between inhibitory and stimulatory effects on the hypothalamo-pituitary axis?
2. Is it possible to stimulate the hypothalamo-pituitary axis by alterations in the putative hypothalamic neurotransmitter levels, even in the presence of high circulating glucocorticoid levels in the rat?
3. Does suppression of the HPA-axis occur in man, even after short-term glucocorticoid administration? If so, how long takes its recovery? What effects can be observed on cortisol secretion during the stress of anesthesia and operation?
4. Does simultaneous administration of glucocorticoids in combination with a substance with possible CRF stimulating properties, prevent the suppression of the hypothalamo-pituitary-adrenal axis in man?

The results, which are presented in this thesis to answer the questions formulated, have been obtained in two different ways.

In chapter II the use of the model of the ACTH/PRL secreting transplantable tumor 7315a is described. In addition the possible direct "short" loop feedback effect of ACTH is studied in this model.

In chapter III it is investigated whether the systemic administration of CRF-stimulating drugs will be able to counteract the suppressive effects of glucocorticoids.

The data, concerning questions 3 and 4, whether short-term glucocorticoid administration causes suppression of the HPA-axis in man and whether this suppression can be prevented, will be discussed in detail in chapter IV and V.

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Chapter II

The model of the transplantable ACTH-secreting pituitary tumor 7315a. No evidence for a direct "short" loop feedback of ACTH

In this chapter we will describe the effects of a transplantable ACTH-secreting pituitary tumor on the HPA-axis.

II.A. METHODS

II.A.1. *The transplantable ACTH and Prolactin secreting pituitary tumor 7315a*

The transplantable ACTH and prolactin (PRL) secreting pituitary tumor 7315a used in our experiments was obtained from Dr. R.M. MacLeod, Charlottesville, Virginia, USA. Female Buffalo rats with a body weight varying between 150–250 g were inoculated subcutaneously on the back between the scapulae with a suspension of the tumor cells, as described earlier by MacLeod et al. (1). The rats were housed five to six per cage under artificial light from 06.00 to 21.00 h, and given free access to water and rat chow. After three to four weeks, when the tumors measured approximately $1.5 \times 1.5 - 2 \times 2$ cm^2, the experiments were performed. The measurement of the tumor in centimeters squared has been shown to correlate significantly with tumor weight (2). The animals were killed by decapitation between 08.30 - 09.30 h.

After decapitation the pituitary was quickly removed, and the anterior lobes were separated from the intermediate lobe. The anterior lobes were incubated in 2 ml medium 199 (GibcoBio-cult) $NaHCO_3$ 1.25 g/l. The flasks were incubated in a Dubnoff shaker at 37°C, in an atmosphere of 95% O_2/ 5% CO_2, for 4 hours. After incubation the medium was removed, and the pituitary glands rinsed and homogenized in 2 ml distilled water, and acidified with 1 ml HCL (0.1 N). The ACTH content of the medium and the content of anterior lobe were measured using the bioassay as described below. All data are expressed as means ± S.E.M. Statistical analysis was done with analysis of variance.

II.A.2. *Bioassay of ACTH*

We used a bioassay for the determination of the ACTH concentration, kindly

provided by Dr. G.H. Mulder (Amsterdam). The methodology and the characteristics of the bioassay have been described in detail in his thesis (3).

In short the ACTH bioassay is performed as follows: twelve female Wistar rats, with a body weight of approximately 150-200 g are sacrificed by decapitation between 08.30 and 09.30 hour. The adrenals are removed, cleaned of adhering adipose tissue, and subsequently cut in pieces, until a suspension is obtained. This suspension is placed in a flask containing collagenase type I (Sigma) 30 mg in 10 ml Krebs-Ringer-bicarbonate-glucose buffer (KRBG buffer). The composition of 100 ml of this buffer is as follows: 80 ml distilled water, 10 ml Stock A (NaCl 119 mM, KCL 4.7 mM, $MgSO_4 . 7H_2O$ 1.2 mM, $CaCl_2 . 2H_2O$ 2.5 mM, KH_2PO_4 1.2 mM) and 10 ml Stock B ($NaHCO_3$ 24.9 mM), and 0.209 g d-glucose (11.6 mM).

All the glass materials used have been siliconized. The suspension of adrenal tissue in the collagenase-KRBG buffer was incubated in a Dubnoff shaker for fifty minutes, under an atmosphere of 95% O_2/5% CO_2, at 37°C. The dispersion, initiated by incubation in the collagenase containing buffer was completed by gently sucking up adrenal tissue pieces with a Pasteur pipet, and blowing them out. Usually about 50 pipet excursions sufficed to get almost total disruption of adrenal tissue parts. The cloudy suspension was centrifuged at 100 × g, during 10 minutes, at a temperature of 20°C; the speed of the centrifuge was accelerated very slowly, while at the end of the procedure no brakes were used. The pellet was resuspended in 10 ml KRBG with 0.5% bovine albumin. This suspension was filtered through a nylon cloth, with micropores of 60 μM, and was centrifuged again for 10 minutes at 100 × g. This pellet was resuspended in 1 ml KRBG buffer with 0.5% albumin and then added, very carefully to 10 ml KRBG buffer with 2% albumin. The tube was centrifuged 5 minutes at 10 × g. The final pellet was resuspended with 60-70 ml KRBG buffer, containing 0.5% albumin. Furthermore, 10 μl $CaCl_2 . 2H_2O$ 8.08% and 100 E Trasylol^R (Bayer) per ml KRBG buffer were added to stabilize the suspension and antagonize the potentially deleterious effects of the collagenase. The cell suspension was pipetted into teflon incubation flasks, each flask already containing 0.1 ml sample and receiving 1 ml of prepared adrenal cell suspension. During the whole procedure, the cell suspension was kept in constant, gentle movement by mechanical stirring to ensure equal cell samples.

The standard aliquots of synthetic $ACTH_{1-24}$ (Organon) were added at the same time as the samples to be measured. When using the third International Standard, 10 pg $ACTH_{1-24}$ equals 1 μU. The standard series of synthetic $ACTH_{1-24}$ consisted always of the same range concentration:

standard 1 = 615.38 μU/ml
standard 2 = 307.69 μU/ml
standard 3 = 153.85 μU/ml

standard 4 = 61.54 μU/ml
standard 5 = 30.77 μU/ml
standard 6 = 15.38 μU/ml
standard 7 = 6.15 μU/ml
standard 8 = 3.07 μU/ml

This standard series was prepared in teflon tubes, containing saline 0.9% and albumin 0.5% at pH 3.5. Then, 0.1 ml of these concentrations was added to the incubation tubes. A sample containing only saline 0.9% and albumin was always included in the bioassay as a blank. All samples were measured in duplicate, and incubated for two hours and 15 min. in a Dubnoff shaker, at 37°C, under an atmosphere of 95% O_2/5% CO_2. At the end of the incubation the content of the tubes was added to siliconized glass tubes with round bottoms. Each teflon tube was rinsed with 0.5 ml distilled water, and this was added to the glass tubes. Then 5 ml dichloromethane (Merck) was added and the glass tubes were shaken by hand for 30 seconds, to extract the corticosterone to be measured. The aqueous layer containing cells and proteins was separated from the dichloromethane layer by centrifuging at 1200 X g for 2 min. The upper, aqueous layer was removed using a water operated suction pump. Then, 4 ml dichloromethane containing the corticosterone, was pipetted into clean glass tubes with a flat bottom. A fluorescence reagent, consisting of 30% absolute alcohol and 70% concentrated sulphuric acid, was then added to the glass tubes, each tube receiving 2 ml. The glass tubes were again shaken by hand for 30 sec. After 30 min. the upper layer consisting of dichloromethane was removed.

The fluorescence was measured 60 min. after addition of the fluorescence reagent, using a spectrofluorimeter (Fluorimeter 1000 M, Perkin-Elmer) with an excitation wavelength of 473 nm, and an emission wavelength of 526 nm. This measurement of corticosterone is a modification of the method described by Silber et al. (4). For equilibration of the fluorescence curve we used samples of 0, 0.2, 0.6 and 1.0 μg corticosterone in 1 ml KRBG buffer plus 0.5 ml distilled water. The fluorescence readings of corticosterone were plotted against the standard ACTH series, using a logarithmic scale for the standard ACTH concentrations.

The total number of adrenal cells obtained in each assay, and many variables in the incubation method, have been carefully evaluated by Mulder in his thesis (3). We were able to confirm the high correlation coefficient between the ACTH standard concentrations and the fluorescence readings found in this assay, ranging from 0.985 to 0.997 for the linear log dose response from ACTH standard 7 (6.15 μU/ml) to standard 3 (153.85 μU/ml). The interassay variation in 4 bioassays of ACTH amounted to 6%. The intraassay variation was 10%.

II.A.3. *Radioimmunoassay of ACTH and corticosterone*

The plasma ACTH concentration and the ACTH content and release of the anterior lobe of the rat pituitary were occasionally also measured using a radioimmunoassay of plasma ACTH, as provided by the Radiochemical Centre Amersham (Buckinghamshire) (5). Preliminary extraction of plasma samples onto glass beads was carried out, as described by Ratcliffe and Edwards (6), while the pituitary media and contents were measured without extraction. The interassay variation was 7%. The intraassay variation amounted to 12%. Plasma corticosterone was measured with a competitive protein binding assay (7).

II.B. RESULTS

The results obtained in order to study the value of the use of the transplantable ACTH/PRL secreting pituitary tumor 7315a, have been divided in the following sections:

1. The relation between the weight of the tumor and the weight of the adrenals.
2. The relationship between the weight of the tumor and the circulating concentrations of ACTH and corticosterone.
3. The effect of the tumor on:
   a) anterior pituitary lobe weight.
   b) ACTH content of the anterior lobe
   c) ACTH release by the anterior lobe.
4. The effect of adrenalectomy on ACTH content and pituitary weight.
5. A comparison between the biological and radioimmunological activity of tumor ACTH.

II.B.1. *The relation between the weight of the tumor and the weight of the adrenals*

A linear relationship existed between the weight of the 7315a tumor and the total weight of the two corresponding adrenal glands (Fig. II-1). The correlation coefficient was $r=0.826$ ($p<0.001$). The data of three different experiments were pooled, the number of tumor-bearing rats being in total 59. The mean adrenal weight in the control non-tumor bearing animals of these three experiments was 51.5 ± 2.1 mg (mean ± S.E.M.; n=18).

II.B.2. *The relation between the weight of the tumor and the circulating concentrations of ACTH and corticosterone*

The plasma ACTH concentrations of 10 tumor-bearing animals amounted to

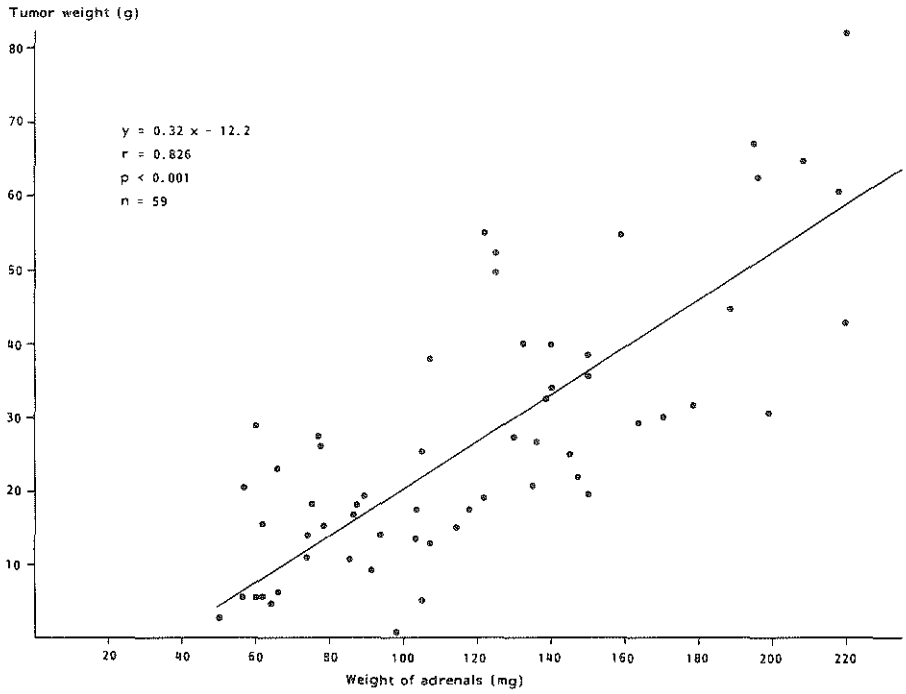

Fig. II-1. The correlation between tumor weight and adrenal weight in female rats carrying the ACTH/PRL secreting tumor 7315a.

7668 ± 1190 pg/ml (mean ± S.E.M.). There was a highly significant correlation between the weight of the tumor of these animals and the circulating ACTH levels (Fig. II-2; $r=0.823$; $p<0.001$). In five control non-tumor-bearing rats plasma ACTH levels amounted to 241 ± 43 pg/ml.

Plasma corticosterone levels of the 10 tumor-bearing rats mentioned above amounted to 46.2 ± 3.8 μg/100 ml, while the levels in 5 non-tumor-bearing controls were 19.6 ± 6.2 μg/100 ml (mean ± SEM) ($p<0.01$ vs. tumor-bearing control rats). There was no correlation between the total adrenal weight and circulating corticosterone levels, nor between the weight of the tumor and plasma corticosterone concentrations. The ACTH content of the anterior pituitary lobe was significantly depressed in tumor-bearing rats when compared with controls (see below), but this did not correlate with the circulating corticosterone levels. The plasma ACTH concentration did not show a correlation with the amount of ACTH present in the anterior pituitary gland.

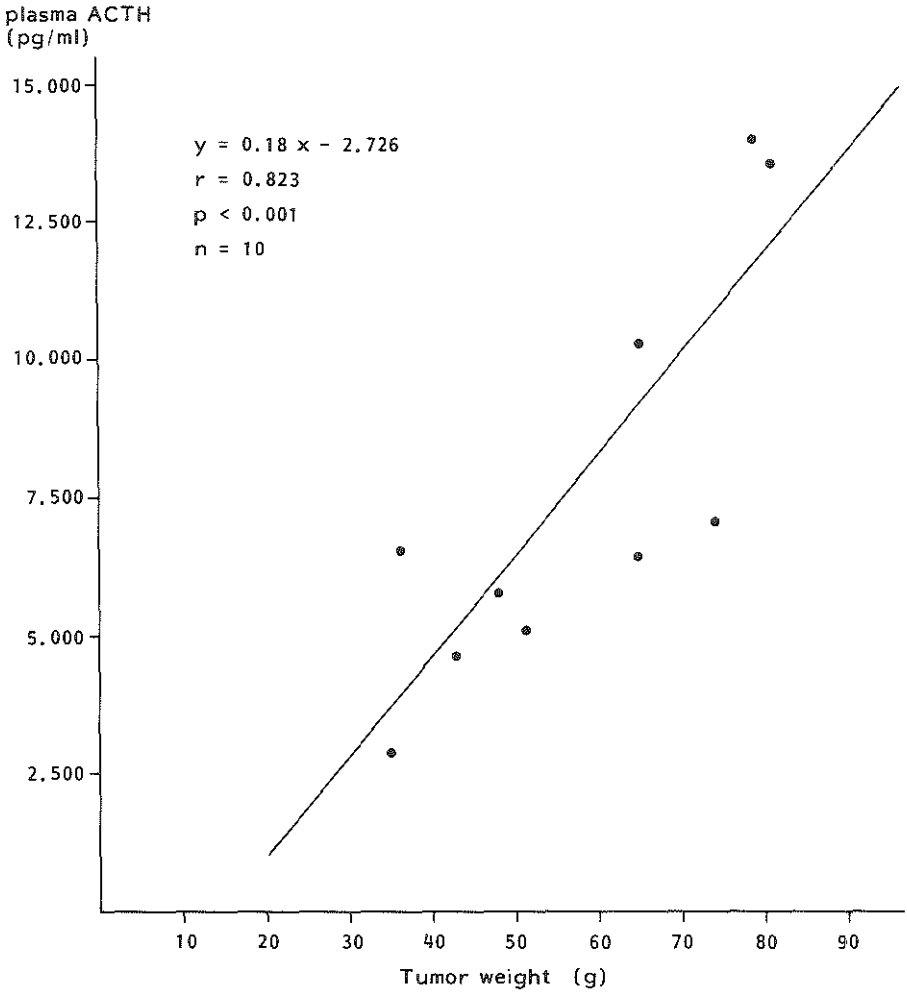

Fig. II-2. The correlation between plasma ACTH and tumor weight in female rats carrying the ACTH/PRL secreting tumor 7315a.

II.B.3. *The effect of tumor transplantation on the ACTH content and release of the anterior pituitary lobe*

A statistically significant reduction of the ACTH content of the anterior pituitary gland was consistently found in tumor-bearing rats. In Fig. II-3 the effect of tumor transplantation on the ACTH content per total pituitary gland and per mg pituitary are shown as an example of the results obtained in three different experiments. (ACTH measured by bioassay and expressed in μU/total gland or μU/mg pituitary). In Table II-I the results of ten different experiments

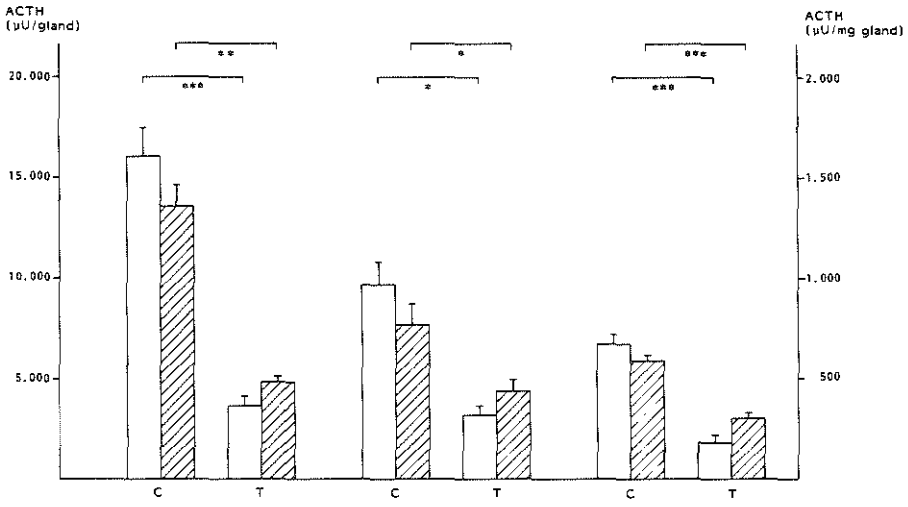

Fig. II-3. The effect of tumor transplantation on the ACTH content of the anterior pituitary gland expressed in μU/total gland, and μU/mg gland (hatched bars), in female rats in 3 different experiments (8 rats per group; mean ± S.E.M; C = controls; T = tumor-bearing rats).

* $p<0.02$
** $p<0.005$
*** $p<0.001$

are summarized, in which the significant reduction is also evident. The mean ACTH content per total pituitary is 10687 μU/gland, whereas the mean content in rats with a tumor is only 3395 μU/gland ($p<0.001$), ranging from 6977 to 16002 μU/gland and from 1821 to 5219 μU/total gland for controls and tumor rats respectively. When expressed per mg pituitary gland, the same reduction of ACTH content is observed, though in percentages a slightly smaller reduction is present.

The release of ACTH from the anterior pituitary lobes incubated *in vitro* for 4 hours of the same groups of rats, is presented in Table II-II. The release of ACTH *in vitro* expressed per gland shows great variations, but a reduction in release is noted in tumor rats versus controls in all but two of the experiments ($p<0.02$). However, no significant reduction is present, when data are expressed in μU ACTH per mg pituitary gland. The percentages of reduction of ACTH content in tumor rats versus controls ranged from 47.9% to 81.4% with a mean of 60.2% when measured per total gland. The percentage of change per mg pituitary ranged from 27.6% to 70.0% (mean 51.7%). There was no correlation between these reductions in anterior pituitary ACTH content and tumor weight nor with absolute content and tumor weight (Table II-III).

The mean weight of the anterior lobes of the pituitary gland of control rats

Table II-I. The ACTH content per total anterior pituitary gland, and per mg anterior pituitary gland, in female control rats and tumor-bearing rats (6-8 per group) in 10 consecutive experiments

	ACTH content (μU/total gland)		ACTH content (μU/mg gland)	
	Controls	Tumor	Controls	Tumor
1	10023 ± 2016	5219 ± 654	938 ± 139	679 ± 81
2	11617 ± 852	4087 ± 264	1044 ± 61	517 ± 20
3	10124 ± 930	4401 ± 811	1190 ± 78	610 ± 112
4	6977 ± 268	1981 ± 312	576 ± 34	309 ± 34
5	10833 ± 637	2043 ± 346	936 ± 81	281 ± 48
6	16002 ± 1572	3641 ± 503	1344 ± 138	477 ± 45
7	10682 ± 399	2492 ± 274	860 ± 38	343 ± 35
8	9767 ± 1250	3249 ± 505	760 ± 103	445 ± 59
9	9808 ± 566	1821 ± 231	927 ± 77	306 ± 38
10	11034 ± 883	5017 ± 566	1113 ± 68	715 ± 53
$\bar{x}$	10687 ± 710	3395 ± 403	969 ± 69	468 ± 51
	a		a	

All values expressed as mean ± S.E.M. in μU ACTH as measured by ACTH bioassay.
$\bar{x}$ represents mean values of these 10 experiments.
[a] $p < 0.001$ vs. controls.

was 11.56 ± 0.36 mg, while the mean pituitary weight of the tumor-animals was significantly reduced: 7.29 ± 0.18 mg ($n = 25$; $p<0.001$), a reduction in weight by 36.9%. There was no correlation between tumor weight and the reduced pituitary weight in 25 tumor-bearing rats.

II.B.4. *The effect of adrenalectomy on the pituitary ACTH content and weight*

The effects of bilateral adrenalectomy (4 weeks before) were studied in control rats and in animals with a transplanted tumor (Table II-IV). In adrenalectomized rats a marked rise in ACTH content was evident when compared with normal rats, the mean ACTH content per total gland rising from 18 800 ± 1400 μU in controls to 52 500 ± 12 700 μU in the adrenalectomized rats ($p<0.02$).

As was to be expected, a significant fall in mean ACTH content of the anterior pituitary gland was found in tumor controls when compared with normal rats (4600 ± 1100 vs. 18 800 ± 1400; $p<0.001$). But, adrenalectomized rats with the transplanted ACTH/PRL secreting tumor demonstrated a likewise

Table II-II. ACTH release by anterior pituitary glands of control and tumor-bearing rats, incubated *in vitro* for 4 h

	ACTH release in medium (μU/total gland)		ACTH release in medium (μU/mg gland)	
	Controls	Tumor	Controls	Tumor
1	161.8 ± 37.7	86.4 ± 6.7	13.5 ± 3.0	10.5 ± 1.1
2	41.3 ± 5.1	17.7 ± 3.4	3.3 ± 0.6	2.2 ± 0.4
3	38.9 ± 2.7	27.6 ± 1.1	3.5 ± 0.3	3.6 ± 0.3
4	30.5 ± 2.8	31.5 ± 5.5	2.5 ± 0.3	5.6 ± 1.6
5	55.5 ± 8.0	24.9 ± 6.9	4.9 ± 0.9	3.3 ± 0.9
6	86.7 ± 4.3	44.4 ± 13.7	8.4 ± 1.2	7.7 ± 1.9
7	39.6 ± 4.9	11.1 ± 3.9	3.2 ± 0.4	1.6 ± 0.6
8	16.9 ± 2.1	9.2 ± 2.5	1.3 ± 0.2	1.3 ± 0.3
9	64.9 ± 6.4	69.7 ± 10.1	7.1 ± 1.4	10.0 ± 1.4
$\bar{x}$	59.6 ± 14.5	35.8 ± 8.8	5.3 ± 1.3	5.1 ± 1.2
	a		b	

All values expressed as mean ± S.E.M. in μU ACTH as measured by ACTH bioassay.
$\bar{x}$ represents mean values of these 9 experiments.
a $p < 0.02$ vs. control.
b n.s.

Table II-III. The percentage of reduction (% change) of ACTH content in anterior lobe pituitary per gland and mg gland resp., in tumor-bearing rats versus controls, and the absence of a relationship with mean tumor weight (g)

	% Change of ACTH content		Mean Tumor weight
	Gland	mg Gland	
1	-64.8	-50.5	49.6 ± 10.8
2	-56.5	-48.7	17.4 ± 4.6
3	-71.6	-46.6	22.1 ± 2.2
4	-81.3	-70.0	26.7 ± 3.5
5	-77.3	-64.6	43.8 ± 11.0
6	-76.7	-60.1	38.7 ± 7.1
7	-66.8	-41.5	41.4 ± 13.3
8	-81.4	-67.1	38.7 ± 8.3
9	-54.5	-35.9	27.3 ± 5.2
10	-47.9	-27.6	27.0 ± 6.3
$\bar{x}$	-60.2	-51.7	33.3 ± 3.3
	a		
		a	

a n.s.

increase in the ACTH content of the anterior pituitary lobes, which was highly significant in comparison with rats with a transplanted tumor *and* intact adrenals (39 500 ± 9 100 μU vs. 4 600 ± 1100 μU; $p<0.001$) (Table II-IV). The same results were found, when ACTH content was expressed per mg pituitary. Moreover, there was no significant difference in ACTH content of the anterior pituitary gland of adrenalectomized rats versus adrenalectomized tumor-bearing rats (52 500 ± 12 700 μU vs. 39 500 ± 9100 μU resp.; n.s.). The weight of the tumor of the adrenalectomized animals did not differ from that in the control tumor-bearing rats.

In Table II-V data regarding the ACTH content per pituitary and the release of ACTH *in vitro* are summarized. The changes in the pituitary ACTH content in adrenalectomized rats and in tumor-bearing rats, who underwent also bilateral adrenalectomy, are essentially the same as described in Table II-IV. The changes are reflected in a similar way in the ACTH release from the anterior pituitary lobe *in vitro*: a significant rise of ACTH release occurred after bilateral adrenalectomy in both control and tumor-bearing groups (adrenalectomy vs. controls: 156.6 ± 26.8 vs 41.5 ± 5.1 μU/total gland resp.; $p<0.05$; tumor + adrenalectomy vs. tumor controls: 128.6 ± 16.0 vs. 17.7 ± 3.4 μU/total gland resp.; $p<0.01$). Again, the release of ACTH in vitro amounted to comparable levels in both adrenalectomy and tumor + adrenalecomy groups (156.6 ± 26.8 vs. 128.6 ± 16.0 μU/total gland; n.s.) (Table II-V). The percentages of changes in ACTH release by the anterior glands *in vitro*, and in pituitary ACTH content, plotted against the normal control group, amounted to -57% and -49% resp. in rats with a tumor only, to +278% and 331% resp. in

Table II-IV. The effects of bilateral adrenalectomy on the ACTH content of the anterior pituitary of control and tumor-bearing rats, expressed per total gland and per mg pituitary

	ACTH content (μU/gland)	ACTH content (μU/mg gland)
Controls (n = 5)	18 000 ± 1 400	1644 ± 181
Tumor controls (n = 5)	4 600 ± 1 100[a]	592 ± 121[a]
Adrenalectomy (n = 5)	52 500 ± 12 700[c]	4277 ± 111[c]
Tumor + adrenalectomy (n = 5)	39 500 ± 9 100[b,c]	4074 ± 713[b,c]

ACTH in μU/gland and μU/mg pituitary; 5 rats per group; mean ± S.E.M.

[a] $p < 0.001$ vs. controls.

[b] $p < 0.001$ vs. tumor controls.

[c] $p < 0.02$ vs. controls.

Table II-V. Effects of bilateral adrenalectomy on ACTH release by the anterior pituitary gland *in vitro* (in μU/gland, and μU/mg gland) and its ACTH content (in μU/gland, and μU/mg gland) of control rats and rats with a transplanted tumor (5 per group ± S.E.M.)

	ACTH release *in vitro*		ACTH content	
	(μU/total gland)	(μU/mg gland)	(μU/total gland)	(μU/mg gland)
Controls (n = 5)	41.5 ± 5.1	3.3 ± 0.6	17040 ± 2770	1340 ± 24
Tumor controls (n = 5)	17.7 ± 3.4[a]	2.2 ± 0.4	8680 ± 310[a]	105 ± 6[a]
Adrenalectomy (n = 5)	156.6 ± 26.8[a]	12.9 ± 2.2[a]	73600 ± 16880[a]	600 ± 137[a]
Tumor + adrenalectomy (n = 5)	128.6 ± 16.0[a,b]	15.1 ± 3.4[a,b]	81800 ± 11780[a,b]	931 ± 203[a,b]

[a] $p < 0.05$ vs. controls.
[b] $p < 0.01$ vs. tumor controls.

adrenalectomized control rats, and to +211% and 381% resp. in rats with a tumor and adrenalectomy.

The effects of adrenalectomy of control animals and tumor-bearing rats on the anterior pituitary weight showed no difference in adrenalectomized control rats vs. intact controls (12.6 ± 0.8 vs. 11.7 ± 0.8 mg; n=5; n.s.) and a significant increase in adrenalectomized tumor-bearing rats vs. tumor controls (9.36 ± 0.75 vs. 7.48 ± 0.39 mg; $p < 0.05$) (Fig. II-4).

II.B.5. *A comparison between the biological and radioimmunological activity of tumor ACTH*

The 7315a tumor turned out to secrete an ACTH-like polypeptide with a relatively low biological activity. The bio-activity of the plasma samples of the 10 tumor-bearing rats mentioned under II.B.2. amounted to only 5% of the concentration measured with the specific radioimmunoassay.

When the total content of ACTH in the anterior lobe of the pituitary was determined by RIA and bioassay, however a linear relationship was found, with a high correlation coefficient ($r=0.95$). So there is a highly significant correlation between both methods ($p<0.001$) (Fig. II-5). When the release of ACTH from the pituitary was measured, after incubation of the anterior lobes during four hours, again a significant correlation was observed ($r=0.94$; $p<0.001$).

II.C. DISCUSSION

The finding of a linear relationship between the plasma ACTH concentration and the weight of the pituitary tumor and between the weight of the tumor and the adrenal weight demonstrated the profound influence of this tumor on the adrenal gland: it caused a tumor weight dependent hyperplasia of the adrenals. Whereas the normal adrenal weight of these rats amounts to around 50 mg, tumors of 30-40 grams may produce a four- to five-fold increase in adrenal weight. It is evident that this increase is mainly caused by the large amounts of ACTH secreted by this tumor, a situation comparable with the existence of Cushing's syndrome in man, caused by an ectopic ACTH production.

The comparison of the plasma ACTH levels obtained by radioimmunoassay and bioassay respectively, suggests that this tumor produces mainly inactive parts or incomplete aminoacid residues of the ACTH polypeptide and that only 5% of the circulating "ACTH-molecules" is biologically active. Still, this amount seems to be high enough to induce the marked changes in adrenal weight and the doubling of circulating plasma corticosterone levels.

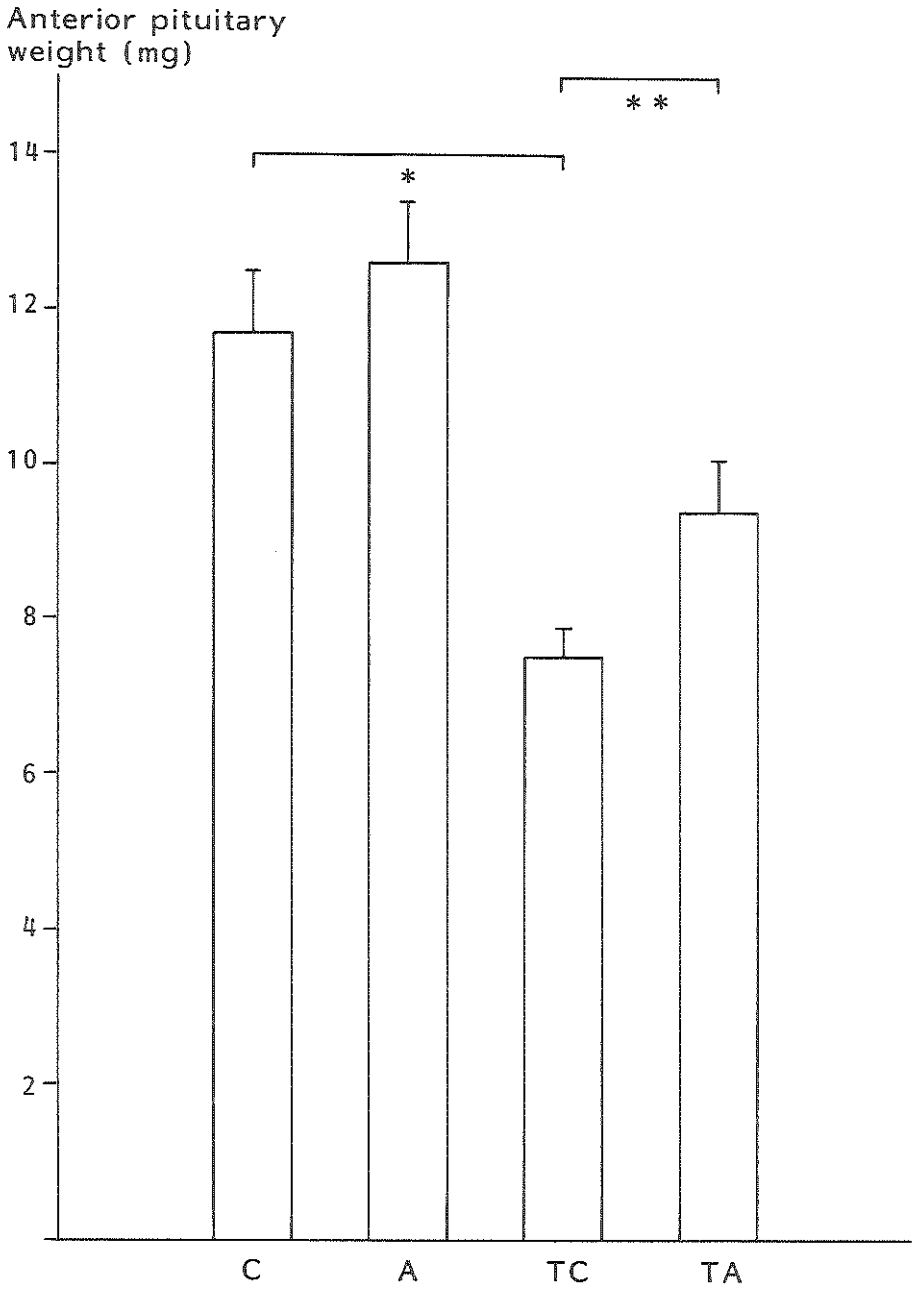

Fig. II-4. The mean anterior pituitary weight (in mg ± S.E.M.) in control rats (C); adrenalectomized rats (A); tumor controls (TC); and adrenalectomized tumor rats (TA) (6 female rats per group).

* $p<0.001$

** $p<0.05$.

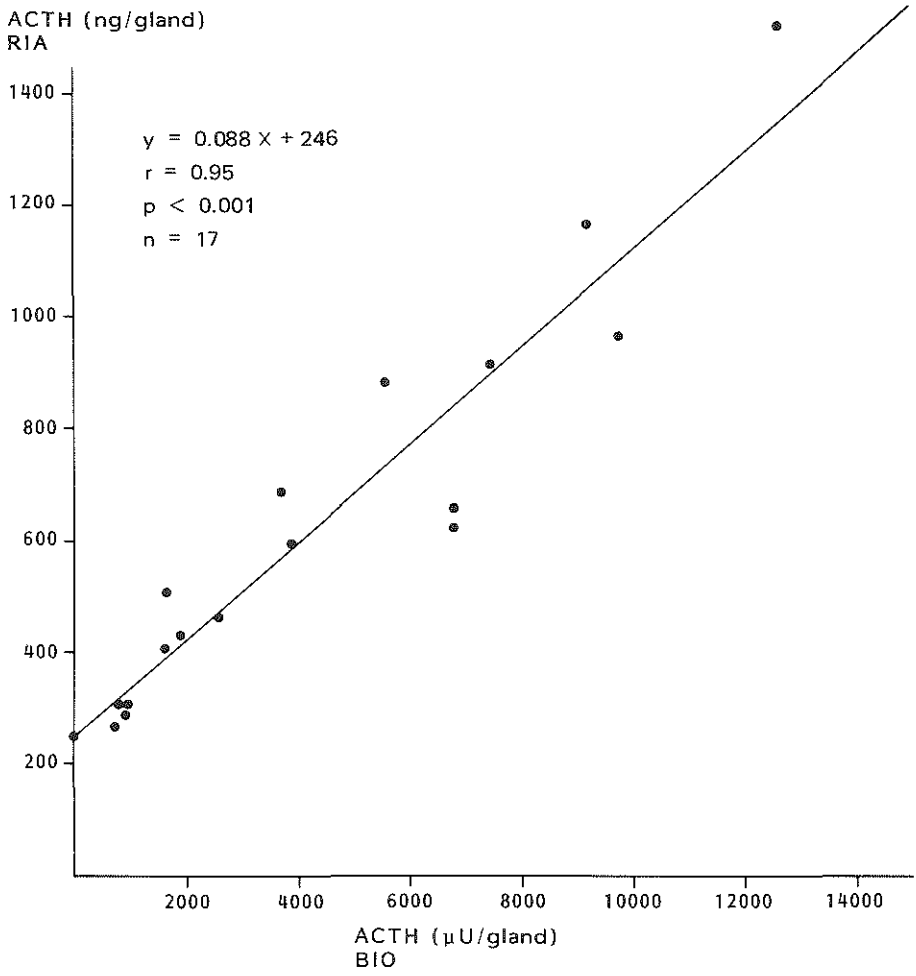

Fig. II-5. The correlation of ACTH content per anterior pituitary gland as measured by radioimmunoassay (in ng/gland) and by bioassay (in μU/gland).

One may speculate on the contribution of hyperprolactinemia to the enormous increment in the size of the adrenal gland. Recent experiments with a pure PRL-secreting tumor derived from the 7315a tumor, however, showed a normal weight of the adrenal gland in the presence of plasma PRL levels above 10 000 ng/ml (unpublished data). The absolute corticosterone levels of the plasma in 7315a tumor-bearing rats were only doubled in comparison with those of control rats. However in man the actual levels of plasma glucocorticoids have been reported to show a poor correlation with the cortisol secretion rate measured over 24 hrs in patients with severe Cushing's syndrome.

These more extensive peripheral effects of marginally elevated plasma cortisol levels may be ascribed to the disappearance of the circadian rhythm of ACTH and cortisol secretion resulting in persistingly (slightly) elevated plasma cortisol levels over 24 hrs.

The marked reduction of the anterior pituitary weight in rats with a transplanted tumor is not entirely due to the conspicuous changes in the pituitary-adrenal system. An important contribution is made by the excessive production and secretion of prolactin by the transplanted tumor, which has a concomitant suppressive effect on prolactin producing pituitary cells, resulting in an additional reduction in pituitary weight. The quantitative importance of prolactin may be stressed by the fact that the prolactin content of the pituitary is about 250 μg in control rats and 80-100 μg/total pituitary in tumor-bearing rats, while the ACTH content when measured with radioimmunoassay has to be expressed in *ng*/pituitary, a difference of a factor 1000 (8).

A constant reduction in the pituitary ACTH content was found in all rats with the transplanted ACTH/PRL secreting tumor, indicating an impaired synthesis. A similar reduction in the release of ACTH per total anterior pituitary *in vitro* is noted, which might well be considered as an impaired secretory potency. One may speculate on the mechanism of this persistent reduction. There is some evidence, though scattered, that high levels of ACTH, administered into the median eminence of the hypothalamus, might influence the secretion of ACTH from the pituitary through a "short" loop feedback system on its own secretion (9, 13) (see section I.B.3.). Jones et al. found that increasing concentrations of ACTH were able to inhibit CRF release by hypothalami incubated *in vitro* (10). On the other hand there is abundant evidence of a strong negative feedback system, in which glucocorticoids exert an inhibition of CRF and ACTH synthesis and release (see section I.B.2.).

In our opinion this mechanism is mainly involved in the suppression of pituitary ACTH synthesis, and in the inhibition of pituitary ACTH secretion in the 7315a tumor-bearing rats. The synthesis of biologically active ACTH by the ACTH/PRL tumor, and subsequent secretion into the systemic circulation, produces bilateral adrenal hyperplasia. Adrenal corticosterone production is stimulated and the secretion from these hyperplastic adrenals is enhanced. The high circulating corticosterone concentrations exert, via a negative feedback mechanism, a suppressive influence on CRF release, and ACTH synthesis and release. Important evidence for the existence of this sequence of events is presented by the data obtained in the adrenalectomy experiments. Bilateral adrenalectomy of control rats *without* a tumor produced an enormous rise in the ACTH content, and release from the anterior pituitary, as was shown in many previous similar experiments (10, 11). In adrenalectomized rats carrying the ACTH/PRL secreting tumor a similar significant rise in pituitary ACTH content was found when compared with rats with the same trans-

planted tumor, and *without* bilateral adrenalectomy. It was demonstrated that the release of ACTH from the anterior pituitary was also significantly stimulated. Moreover, no significant differences were found in these experiments between adrenalectomized control rats and adrenalectomized tumor-bearing rats.

These data can be explained in only one way: apparently bilateral adrenalectomy is able to abolish completely the suppression of the ACTH synthesis and release by the anterior pituitary gland found in tumor-bearing rats. This implies that the continuous elevation of circulating corticosterone concentrations is the most important factor causing this pituitary suppression. The negative feedback mechanism by plasma corticosterone regulates the activity of the hypothalamo-pituitary-adrenal axis. In consequence, it is concluded that the large amounts of ACTH released into the systemic circulation from this ACTH/PRL secreting tumor do not exert a direct effect on the synthesis and release of ACTH by the pituitary. This implies that the importance of a "short" loop feedback mechanism of ACTH on its own secretion (by inhibiting CRF release?) is in this model not a very dominant one. One should bear in mind that most experiments, that have described this phenomenon in the literature were done either *in vitro* or with the use of hypothalamic implants, which does not necessarily implicate physiologic conditions (see section I.B.3.).

It may be clear from the considerations mentioned above, that the model of the ACTH/PRL secreting tumor offers many advantages in studying the suppression of the hypothalamo-pituitary system *in vivo*. As mentioned earlier, this tumor mimicks hypercortisolism in man caused by an ectopic ACTH producing tumor. Furthermore, this model might be applied to the investigation of mechanisms involved in the suppression of the HPA-axis, induced by exogenous glucocorticoids and the recovery of part of this axis after withdrawal of steroids. The common denominator is in both circumstances the presence of continuously high circulating glucocorticoid concentrations, which are responsible for the development of Cushing's syndrome. A diagram may further exemplify this (Table II-VI). We wanted to use the model of this transplantable tumor, instead of long-term administration to rats of high doses of glucocorticoids parenterally or via the drinking water, in order to be sure of persistently high levels of circulating corticosterone with low variability during three or four weeks in large groups of rats. This aim turned out to be difficult to achieve when using exogenous glucocorticoids in studies with rats (12).

II.D. CONCLUSIONS

1) The subcutaneous implantation of the ACTH/PRL secreting tumor 7315a in rats causes a suppression of the synthesis and release of ACTH in the anterior pituitary lobe.

Table II-VI. Sequence of events in the presence of ectopic ACTH production by the ACTH secreting tumor in rats, and of administration of high doses of glucocorticoids in man

RAT	MAN
Ectopic production of ACTH by the ACTH secreting tumor	Exogenous administration of glucocorticoids
↓	↓
Bilateral adrenal hyperplasia	High circulating glucocorticoid levels in the systemic circulation
↓	↓
High circulating corticosterone concentrations	Suppression of synthesis and secretion of ACTH in/from the anterior pituitary
↓	↓
Suppression of synthesis and secretion of ACTH in/from the anterior pituitary	Suppression of production and secretion of cortisol from the adrenals
	↓
	Bilateral atrophy of the adrenals

2) This suppression is mediated by a negative feedback mechanism, in which the high circulating corticosterone concentrations from hyperplastic adrenal glands are the main determining factor.
3) The high circulating levels of plasma ACTH, secreted by the transplanted tumor, probably do not directly exert a significant suppression on the synthesis of ACTH by the anterior pituitary. A "short" loop negative feedback mechanism seems not to play an important role in this tumor model.
4) This tumor model is therefore comparable with the situation, in which high concentrations of circulating glucocorticoids cause suppression of CRF and ACTH release.

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Chapter III

Effects of putative neurotransmitter enhancing drugs on the hypothalamo-pituitary axis of tumor bearing rats

III.A. INTRODUCTION

In the previous chapter we have described the effects of the transplantable ACTH/PRL producing tumor 7315a on pituitary ACTH synthesis and release. We also discussed the feasability of this experimental model to study the effects of high circulating glucocorticoid levels on pituitary ACTH synthesis and release. As stressed previously, the conditions of the hypothalamo-pituitary axis in this model are comparable with those of man during exogenous glucocorticoid administration.

As chronic suppression of the ACTH content of the anterior pituitary of tumor-bearing rats in the presence of high circulating corticosterone levels was a constant feature, we were interested to know whether prevention or abolishment of this suppression was possible. If ACTH synthesis could be stimulated by enhancing CRF release, even in the simultaneous presence of high circulating corticosterone concentrations, potentially a way might be opened to prevent suppression of the hypothalamo-pituitary axis in man during the administration of pharmacologic doses of glucocorticoids.

We have tried to influence pituitary ACTH synthesis and release in tumor-bearing rats with pharmacologic agents which are assumed to exert a stimulatory effect on CRF release, through modulation of excitatory neurons. We have thus concentrated mainly on the serotonergic and cholinergic pathways. Furthermore, we looked for pharmacologic agents able to penetrate the blood-brain barrier. Finally, all drugs had either to be available as drugs registered and approved by the Netherlands drug administration or to be known as having a low toxicity without important side-effects. Applying these criteria, a drug found to be able to prevent the suppression of pituitary ACTH synthesis and release in the tumor-bearing rat, might subsequently be shown to be able to do the same in man.

It has been mentioned in chapter I that the effects of many serotonergic and cholinergic drugs on the activity of the HPA-axis have already been studied in detail. However, most of these experiments evaluated the effects of the various substances on the HPA-axis under basal or stress conditions. Reports on the actions of these drugs in the simultaneous presence of high circulating glucocorticoid concentrations are very few (1).

In this chapter we describe the effects of the following pharmacologic agents on the pituitary ACTH content and release in tumor-bearing rats. The drugs can be categorized according to their presumed specific pathway:

A. serotonin - enhancing:
   1- chlorimipramine
   2- fenfluramine
B. acetylcholine - enhancing:
   1- piperidine
   2- carbachol (2-carbamoyl)-oxy-ethyl-trimethylammoniumchloride)
   3- CDP-choline (cytidine-diphosphatocholine)
   4- pyridostigmine

The potential serotonin enhancing effects of antidepressants have been demonstrated by Sigg et al. (2) and Lahti and Barsuhn (3). Chlorimipramine appears to be a strong serotonin re-uptake blocker, which results in rising ACTH-levels and thus causes a rise in circulating corticosterone levels in rats. The enhancement of serotonergic neurotransmission by fenfluramine was recently reviewed by Fuller (4, 5). Fenfluramine, as a serotonin releasing drug was able to stimulate plasma corticosterone concentrations in rat. Carbachol, CDP-choline and pyridostigmine are nicotinergic cholinergic agents, while piperidine is a specific nicotinic cholinergic receptor agonist (6).

III.B. METHODS

To evaluate the effects of the various pharmacologic agents we used Buffalo rats, weighing 200-220 g, inoculated with the transplanted ACTH/PRL secreting pituitary tumor 7315a. The agents to be injected were administered, when the size of the transplanted tumor was about 1.5 X 1.5 - 2 X 2 cm^2. We used either subcutaneous or intraperitoneal injections.

Chlorimipramine (Anafranil^R, Geigy) was obtained in ampoules with 12.5 mg/ml. The drug concentration was diluted with saline 0.9%, to get suitable injection volumes of 0.25 ml, 0.3 ml, or 0.5 ml. In total, rats received chlorimipramine in doses of 0.5, 1.0, 1.5, 2.0 and 3.0 mg/day/rat (2, 3). The drug was injected twice daily for five days, or once a day for 12 days, s.c.

Fenfluramine (Ponderal^R, fenfluraminechloride, Servier) tablets of 20 mg were dissolved in saline 0.9%, and 0.25 ml or 0.5 ml of this solution was injected intraperitoneally once a day to total doses of resp. 0.8, 1.6, 2.0, 3.2 mg/day/rat. The period of injections was 5, or 12 days.

Piperidinechloride crystals (Merck) were dissolved in saline 0.9% and doses of resp. 0.375 and 0.5 mg/day/rat were injected i.p. once a day, for 5, 13 or 14 days.

Carbachol (Doryl^R, Merck) in ampoules of 0.25 mg/ml was diluted with

saline 0.9%, and rats were given doses of resp. 0.062 and 0.10 mg/day/rat for 5 days, divided over two doses s.c.

Pyridostigmine (Mestinon^R, Roche) in ampoules of 5 mg/ml was diluted with saline 0.9%, rats received 60 μg/day for 5 days i.p.

Cytidine-diphosphatocholine (CDP-choline^R, Sigma) was dissolved in saline and rats received 1 mg/day/rat for 5 days, once daily i.p. In all experiments injection volumes ranged between 0.2 - 0.5 ml.

The number of rats varied per experiment from 5-8 per group, which will be specified for each experiment separately. All tumor-bearing controls received saline 0.9% injections i.p. The last injection was given one hour before sacrificing the rats. Rats were decapitated without preceding ether anesthesia. The anterior lobe of the pituitary was removed, and incubated for 4 hours, when studies of the release of ACTH in the medium were planned. The tumor, and sometimes the adrenals were removed, cleaned of surrounding adipose tissue and weighed. The ACTH content of the anterior pituitary and its release into the medium was measured with the ACTH bioassay. For further, detailed information on the procedures we may refer to the previous chapter (II.A.2.).

III.C. RESULTS

III.C.1. *Chlorimipramine*

After 5 days of injection s.c. of chlorimipramine (resp. 0.5, 1.5, 3.0 mg/day/rat divided over two doses) in tumor-bearing rats, the ACTH content of the anterior pituitary increased significantly compared with tumor-bearing controls, when expressed per total gland, as well as per mg pituitary (tumor + chlorimipramine 1.5 mg and 3.0 mg vs. tumor controls $p<0.01$ and $p<0.005$, resp. (Fig. III-1)). No significant increase was noted when the rats received chlorimipramine 0.5 mg/day. The suppression of pituitary ACTH content as seen in tumor-bearing controls, was completely overcome, and the levels of the ACTH content in the groups with the higher chlorimipramine doses were fairly equal to the ACTH content of control rats *without* an ACTH secreting tumor. The mean anterior pituitary weight in tumor-bearing rats injected with chlorimipramine was not different from that in tumor controls (resp. 7.77 ± 0.13 mg; 7.60 ± 0.28 mg; 7.86 ± 0.40 vs. 7.66 ± 0.17 mg; n.s.). Likewise there were no significant differences between the tumor weight in the various groups with a tumor (21.0 ± 6.9 g; 12.6 ± 11.4 g; 17.9 ± 6.1 vs. 27.0 ± 6.3 g) and also not of the weight of the adrenal glands. In another experiment, tumor-bearing rats received chlorimipramine 2.0 mg/day/rat, for 5 days divided over two doses: the pituitary ACTH content in the chlorimipramine treated group rose to 11646 ± 1474 μU/total gland (mean ± S.E.M.) compared with 5778 ±

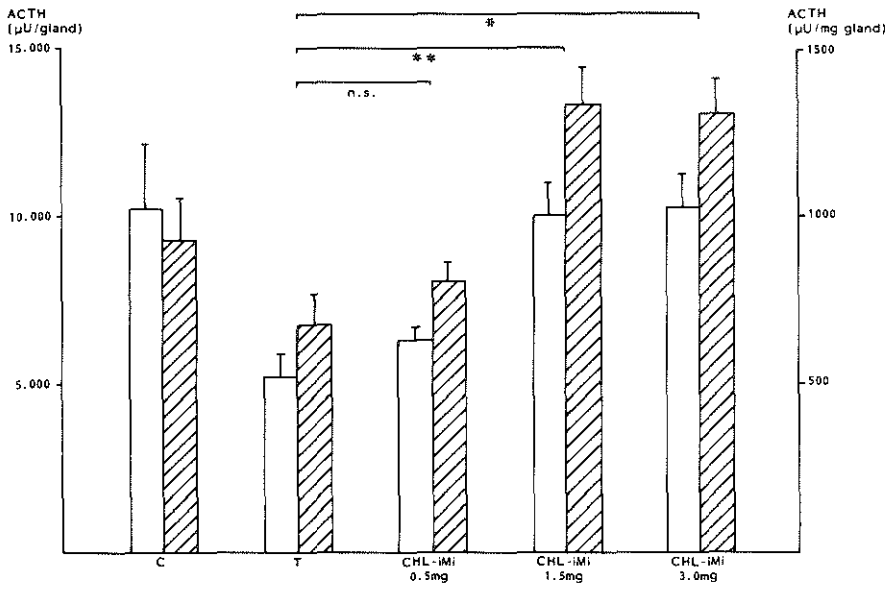

Fig. III-1. The effect of chlorimipramine on the ACTH content of the pituitary gland in μU/gland and μU/mg gland (hatched bars), in female rats. (5 rats per group; mean ± S.E.M.; C = controls; T = tumor-bearing rats; CHL-IMI = chlorimipramine 0.5 mg/day, 1.5 mg/day; 3.0 mg/day; 5 days; twice daily).
* $p<0.005$
** $p<0.01$

1478 μU/total gland in the tumor controls ($p<0.002$; n=5). The pituitary ACTH content of non-tumorbearing control rats was 18694 ± 1347 μU/total gland and thus considerable higher than in the experiment mentioned before. Other similar experiments with chlorimipramine in the same doses confirmed this rise in pituitary ACTH content.

We measured ACTH release into the medium from the anterior pituitary gland of chlorimipramine treated tumor-bearing rats. During incubation of the pituitary for 4 hours, the ACTH release into the medium from a group of 8 rats, who received chlorimipramine 2 mg/day/rat (twice daily) for 12 days, was significantly higher, when compared with control rats with *and* without a tumor (Table III-I; $p<0.05$ and $p<0.05$ resp.). The same results were found when data were expressed per mg pituitary, and thus taking into account the diminished pituitary weight in tumor-bearing animals (Table III-I).

In two of three other experiments, in which the pituitaries were incubated for 4 hours, the chlorimipramine treated groups (2 mg/day for 12 days once daily, or 3 mg/day for 5 days, twice daily) also showed a rise in the release of ACTH into the medium in comparison with the tumor control

Table III-I. The effect of chlorimipramine on the ACTH content and the release of ACTH in medium per total pituitary gland, and per mg pituitary gland in female rats

	ACTH (μU/total gland)		ACTH (μU/mg gland)	
	Content	Medium	Content	Medium
Controls (n = 7)	11034 ± 853	64.9 ± 6.4	1113 ± 68	7.1 ± 1.4
Tumor controls (n = 6)	5018 ± 567	69.7 ± 10.1	715 ± 53	10.0 ± 1.4
Tumor + chlorimipramine (n = 8)	7348 ± 711[b]	92.0 ± 13.8[a,b]	1074 ± 108[b]	13.4 ± 2.0[a,c]

ACTH in μU/gland and μU/mg gland; 6-8 rats per group; mean ± S.E.M. Chlorimipramine: 2 mg/day, 12 days, twice daily.

[a] $p < 0.05$ vs. controls.

[b] $p < 0.05$ vs. tumor controls.

[c] n.s. vs. tumor controls.

groups (Table III-II). Again, there was a similar increase in pituitary ACTH content in chlorimipramine treated rats, and no significant difference in mean pituitary, tumor or adrenal weight. Adrenal weight for example in one experiment: tumor controls vs. chlorimipramine: 191.5 ± 3.6 vs. 212.3 ± 26.8 mg resp.; n.s.

III.C.2. *Fenfluramine*

The effects were investigated of administration of two doses of fenfluramine (0.8 and 1.6 mg/day/rat for 5 days) on pituitary ACTH content in the tumor-bearing rats. Fenfluramine 0.8 mg/day, twice daily caused a significant increase in ACTH content (4455 ± 783 μU/total gland vs. 2469 ± 208 μU/total gland in tumor controls; $p<0.05$). The higher dose of fenfluramine 1.6 mg/day, twice daily did not significantly increase the ACTH content of the pituitary, (3692 ± 719 vs. 2469 ± 208 μU/total gland; n.s.) (Fig. III-2). In another experiment using a higher dose of fenfluramine (3.2 mg/day for 5 days) pituitary ACTH content was 3332 ± 451 vs. 1981 ± 312 μU/total gland in tumor-control rats ($p<0.02$).

In yet another experiment, in which tumor-bearing rats were given fenfluramine 2 mg/day/rat (one injection, for 12 days) there was again a rise in pituitary ACTH content and also in the release of ACTH after incubation during 4 hours (Table III-III; $p<0.05$), when compared with tumor controls.

However, the effects of fenfluramine in causing an increase in ACTH release into the medium, were less consistent than the effects of chlorimipramine. In two other experiments using doses of fenfluramine of less than 3 mg/day there was no detectable difference in the ACTH release in the drug treated and tumor control group.

Table III-II. The effect of different doses of chlorimipramine, with different duration of administration, on the release of ACTH in medium per total pituitary gland, in female rats

	ACTH (μU/total gland)		
Controls	55.5 ± 8.0	16.9 ± 2.1	30.5 ± 2.8
Tumor controls	24.9 ± 6.9	9.2 ± 2.5	31.5 ± 5.5
Tumor + chlorimipramine	44.4 ± 11.6[a]	13.1 ± 1.6[a]	35.1 ± 7.7[b]
	2 mg/12 days	2 mg/12 days	3 mg/5 days

ACTH in μU/gland; 5-6 rats per group; mean ± S.E.M. Chlorimipramine 2 mg/day, 12 days, once daily; chlorimipramine 3 mg/day, 5 days, twice daily.

[a] $p < 0.05$ vs. tumor controls.

[b] n.s. vs. tumor controls.

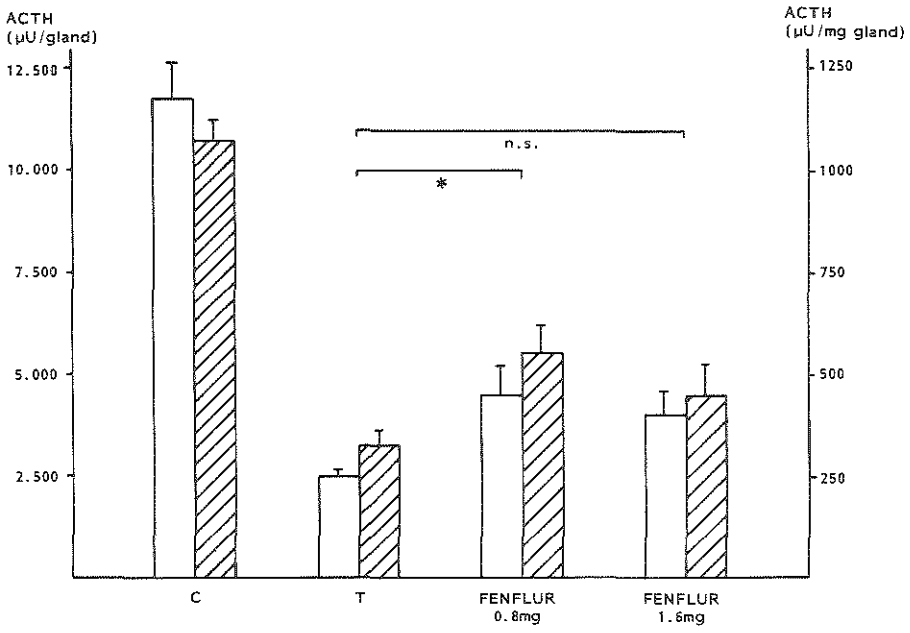

Fig. III-2. The effect of fenfluramine on the ACTH content of the pituitary gland in μU/gland and μU/mg gland (hatched bars), in female rats. (5 rats per group; mean ± S.E.M.; C = controls; T = tumor-bearing rats; FENFLUR = fenfluramine 0.8 mg/day; 1.6 mg/day; 5 days; twice daily).
* $p<0.05$

III.C.3. *Piperidine*

Piperidinechloride, used in two different concentrations (0.375 and 0.5 mg/day/rat; two injections i.p. for 5 days), caused in three experiments a marked rise in the ACTH content of the anterior pituitary glands; piperidine 0.375 mg: 7528 ± 1212 vs. 4401 ± 811 μU/total gland in tumor controls; $p=0.05$; piperidine 0.5 mg: 3742 ± 435 vs. 2326 ± 181 μU/total gland in tumor controls; $p<0.025$. In the third experiment (Table III-IV) the pituitary content and release of ACTH were simultaneously studied: both the release and the content increased after piperidine administration (0.5 mg/day, 5 days, twice daily) significantly ($p<0.05$), when compared with tumor-bearing control animals.

III.C.4. *Other cholinergic drugs*

The effect of the other cholinergic drugs on the pituitary ACTH content, as used in this tumor model were less clear. In Table III-V the effects of two

Table III-III. The effect of fenfluramine on the ACTH content and the release of ACTH into the medium per total pituitary gland, and per mg pituitary gland

	ACTH (μU/total gland)		ACTH (μU/mg gland)	
	Content	Medium	Content	Medium
Controls (n = 7)	11034 ± 833	64.9 ± 6.4	1113 ± 68	7.1 ± 1.4
Tumor controls (n = 6)	5018 ± 567[a]	69.7 ± 10.1	715 ± 53[a]	10.0 ± 1.4
Tumor + fenfluramine (n = 8)	7140 ± 679[b]	102.2 ± 16.4[a,b]	922 ± 72[b]	15.1 ± 2.9[a,b]

ACTH in μU/gland and μU/mg gland; 6-8 rats per group; mean ± S.E.M. Fenfluramine 2 mg/day, 12 days, once daily.

[a] $p < 0.05$ vs. controls.

[b] $p < 0.05$ vs. tumor controls.

Table III-IV. The effect of piperidine on the ACTH content and the release of ACTH into the medium per total pituitary gland and per mg pituitary gland in female rats

	ACTH (μU/total gland)		ACTH (μU/mg gland)	
	Content	Medium	Content	Medium
Controls (n = 5)	6977 ± 267	30.5 ± 2.8	573 ± 34	2.5 ± 0.3
Tumor controls (n = 6)	1981 ± 311	31.5 ± 5.5	298 ± 39	5.6 ± 1.5
Tumor + piperidine (n = 6)	3093 ± 434[b]	58.3 ± 18.1[a,b]	438 ± 50[b]	9.1 ± 3.3[a,b]

ACTH in μU/gland and μU/mg gland; 5-6 rats per group; mean ± S.E.M. Piperidine 0.5 mg/day, 5 days, twice daily.

[a] $p < 0.05$ vs. controls.

[b] $p < 0.05$ vs. tumor controls.

Table III-V. The effect of carbachol on the ACTH content per total pituitary gland and per mg pituitary gland in female rats

	ACTH content		ACTH content	
	Total gland	mg Gland	Total gland	mg Gland
Controls (n = 6)	22990 ± 2240	2206 ± 110	23210 ± 3510	2179 ± 250
Tumor controls (n = 5)	10020 ± 1580	1218 ± 191	4460 ± 610	569 ± 36
Tumor + carbachol (n = 5)	10830 ± 1800[a]	1550 ± 214[a]	3610 ± 340[a]	495 ± 45[a]
	0.062 mg/5 days		0.1 mg/5 days	

ACTH in μU/gland and μU/mg gland; 5-6 rats per group; mean ± S.E.M. Carbachol 0.062 mg/day, 5 days, twice daily; carbachol 0.1 mg/day, 5 days, twice daily.

[a] n.s. vs. tumor controls.

doses of carbachol on the total pituitary content of tumor-bearing animals are summarized: it is clear that carbachol has no obvious effect in augmenting the ACTH content.

Efforts to induce an increase in the pituitary ACTH content with CDP-choline or pyridostigmine remained fruitless. In experiments with CDP-choline (1 mg for 5 days) and pyridostigmine (0.06 mg/day i.p. for 5 days) the total ACTH content of the pituitary was not significantly different from the pituitary content in tumor-bearing control rats.

III.D. DISCUSSION

It is clear from the results obtained in this chapter, that some of the pharmacologic agents used, are able to prevent the suppression of the pituitary ACTH release and content of rats bearing the ACTH/PRL secreting tumor 7315a. We have demonstrated in chapter II that the secretion of ACTH from the transplanted tumor has no profound depressing influence on the pituitary ACTH content, which stresses the importance of the high circulating corticosterone concentrations in inducing this suppression. Thus, the glucocorticoid induced suppression of CRF release from the hypothalamus and the subsequent inhibition of pituitary ACTH synthesis and release can be overcome by the administration of pharmacologic agents, known from the literature to stimulate CRF release.

Chlorimipramine (Anafranil^{R}, Geigy) is a tricyclic antidepressant, frequently used in the psychiatric field in dosages ranging from 100 to 150 mg per day (15). Chlorimipramine induced consistently an increase in the ACTH content of the anterior pituitary gland, to levels comparable with the pituitary ACTH content of control rats *without* a tumor. Thus, chlorimipramine is able to prevent or reverse completely the suppression of the ACTH synthesis and release, even in the presence of the high circulating corticosterone concentrations characteristic for this experimental model. We found no significant differences in tumor weight or in the weight of the hyperplastic adrenals in tumor-bearing animals, whether or not treated with chlorimipramine. Thus it might be concluded that this compound influenced pituitary ACTH synthesis and release at the hypothalamic or pituitary level, and *not* at the adrenal level. Moreover, a dose-reponse relationship appears to be present, considering the progressive increase in ACTH content with higher doses of chlorimipramine (Fig. III-1). In the highest dose used (3.0 mg/day) the pituitary ACTH content had normalized (compared with controls) and no further increase was noted. The release of ACTH from the anterior pituitary was also stimulated by chlorimipramine given for 5 or 12 days. In each experiment ACTH release increased to levels similar to that found in normal controls, or rose even above levels

found in the medium of the pituitary glands from normal control animals. Again, as mentioned in chapter II, the release of ACTH into the incubation medium from the pituitary of tumor-bearing controls was not always less than that of normal controls. After all, only 1-2% of the total pituitary ACTH content is released during the 4 hr incubation period into the medium. As both the total pituitary ACTH content, and the release of ACTH increased after chlorimipramine administration, this indicates probably a stimulation of ACTH synthesis and secretion.

As mentioned before, Sigg et al. and Lahti and Barsuhn (2, 3) reported on the potentiating effect of tricyclic antidepressants on serotonin. They found that chlorimipramine (10 mg/kg i.p.) is a strong serotonin re-uptake blocker which enhances the effect of pretreatment with 5-hydroxytryptophan on plasma glucocorticoids. They concluded that chlorimipramine stimulated ACTH secretion through serotonergic pathways. Fuller explained the mechanism of the stimulating actions of serotonin re-uptake blockers (4, 5). After re-uptake inhibition, the serotonergic neuron decreases the synthesis and turnover of serotonin. The concentration of already released serotonin in the synaptic cleft is increased, and enhances the stimulative serotonergic action on the CRF neuron, due to blockage of re-uptake of serotonin (7, 8). Re-uptake is assumed to be the major means by which serotonin is inactivated at the neural synapse, so this type of compounds should facilitate neurotransmission by serotonergic pathways (8, 22).

Further evidence that serotonin re-uptake inhibition induces an increase in serotonergic functions, includes a) the ability of other selective inhibitors of serotonin like fluoxetine, zimelidine, paroxetine, p-bromo-EXP 56, and Org 6582 to raise corticosterone concentrations in plasma (4); and b) the ineffectivity of a structural isomer of fluoxetine to enhance steroidogenesis when certain terminal groups are not in the same specific structural position as in fluoxetine itself (4, 11, 12).

Chlorimipramine is metabolized into the secondary amine desipramine, which was suggested in the past years to be the active drug (13). It is now clear that desipramine is just as active as imipramine (and chlorimipramine), and not *more* active (14). The half-life is 13 ± 3 hours, and 89 to 94% of the drug is present, bound to plasma proteins (13). The systemic availability varies considerably, due to an extensive first-pass metabolism in man (14), which occurs in the liver after absorption of the drug in the gut. The active drug (or its active metabolite desipramine) penetrates the blood-brain barrier, but there is a mean ratio of plasma vs. cerebro-spinal fluid concentration of 30 : 1 (15). In this last study the authors demonstrated that there is a positive correlation between the level of the cerebro-spinal fluid chlorimipramine concentrations and those patients with endogenous depression, who responded well to the drug. In the non-responders cerebro-spinal fluid concentrations of the

drug were significantly lower, with a much higher plasma-CSF ratio. They speculated that this might be explained by significant variation in the unbound fraction of chlorimipramine in plasma, as described by Glassman et al. (16).

Fenfluramine is a serotonin-releaser, enhancing CRF and ACTH release, as described by Fuller et al. and Schettini (5, 17, 18). This drug depletes brain serotonin stores, and does probably the same in the hypothalamus. The half-life of the drug is 11-20 hours, and it is for 34% bound to plasma proteins. The drug is metabolized into nor-fenfluramine, which is pharmacologically active. The biological availability is equal when administered orally or systemically (19, 20, 21). It is used in man as an inhibitor of appetite in dosages between 60 and 120 mg/day.

It is clear from our experiments with fenfluramine that an increase in pituitary ACTH content could be induced, when compared with tumor controls, but to a lower level than that found in normal controls. Fenfluramine could not completely overcome the suppression of pituitary ACTH content in tumor-bearing animals, not even at higher dosages. The increase of ACTH release from the pituitary gland was also less pronounced than the one observed after chlorimipramine. Moreover, some experiments, comparable as to the dosage used and time period of administration of fenfluramine showed inconsistent results. So, in this tumor model fenfluramine was not as effective as chlorimipramine in overcoming the effects of high circulating corticosterone levels on pituitary ACTH content.

Piperidine chloride was the third pharmacological agent able to raise the pituitary ACTH content and to stimulate ACTH release *in vitro*. This compound is a nicotinic cholinergic receptor stimulator, which was recently reported to enhance sleep-related and insulin-induced growth hormone secretion (6), thus establishing a cholinergic mechanism on the control of growth hormone secretion. We used piperidine in our experiments considering the stimulative role of cholinergic neurons on the release of CRF. A complete normalization of the pituitary ACTH content, as found in chlorimipramine-treated tumor-bearing rats, was not observed after piperidine treatment. This might indicate that in the dosages used, piperidine is less potent in stimulating CRF release than chlorimipramine.

Piperidine is normally present in the brain and cerebro-spinal fluid (23). It can be produced by a decarboxylase in the brain from pipecolic acid, an intermediate of lysine metabolism (24, 25). Piperidine concentrations increase in sleeping mice (26), while sleep-inducing effects of piperidine have been noted in the cat, but not in man (27, 28). The endogenous nicotinic cholinergic receptor stimulation of piperidine was first noted by Euler (29) and Abood (30). The compound passes easily the blood-brain barrier, especially in the mesencephalon, where concentrations of the compound are found which are even higher than in the blood, indicating a local synthesis (31).

Carbachol, CDP-choline and pyridostigmine had no apparent influence on the pituitary ACTH content of tumor-bearing rats. These compounds are all cholinergic agents, the latter an anticholine esterase agent. It is not quite clear why these drugs failed to exert a stimulating influence on ACTH synthesis. Carbachol and CDP-choline pass the blood-brain barrier in man (32, 33, 34) and have strong muscarinic agonist properties (35). We used doses of drugs comparable with those used in studies mentioned above. The half-life of these compounds is relatively short. Perhaps administration should have been done more frequently and divided over the day.

III.E. CONCLUSIONS

In summary, we were able to demonstrate that two pharmacologic agents with distinct serotonin stimulating actions, chlorimipramine and fenfluramine, can stimulate the synthesis *in vivo*, and the release *in vitro* of ACTH from the anterior pituitary. Even more important is the observation that these drugs do so in the simultaneous presence of high circulating glucocorticoid concentrations, induced by the ACTH secreting tumor 7315a. Chlorimipramine is even capable of preventing the suppression of ACTH synthesis completely. Chlorimipramine is thought to act as a "neurotransmitter", stimulating serotonergic actions on the CRF neuron, and thus stimulating ACTH synthesis and secretion. Fenfluramine is less potent than chlorimipramine in producing this effect. Of the four cholinergic stimulating agents tested, only piperidine was capable to produce an increase in pituitary ACTH synthesis and release *in vivo*. Our results confirm the importance of serotonergic pathways in the control and stimulation of CRF and ACTH release, as stressed by Fuller et al. (4) and Buckingham et al. (36). One might speculate on the mutual balance of the serotonergic and cholinergic pathways in regulating the activity of the hypothalamo-pituitary-adrenal axis: in this tumor model the serotonin dependent pathway can be manipulated much more effectively and the cholinergic pathway seems to be of minor importance.

The results of the administration of chlorimipramine and fenfluramine in rats with high circulating corticosterone levels made us wonder whether similar actions may be observed in man, receiving high doses of glucocorticoids. Data on this question will be presented in the fifth chapter of this thesis.

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Chapter IV

Effects of short-term administration of glucocorticoids on hypothalamo-pituitary-adrenal function in man

IV.A. INTRODUCTION

The suppressive effect of the administration of glucocorticoids for prolonged periods on the activity of the HPA-axis in man has been demonstrated convincingly (see chapter I). There is, however, little information regarding the effect of the administration of glucocorticoids for a relatively short period, for example 7-15 days. In one of the few well-documented studies, Streck and Lockwood (1) demonstrated a suppression of the HPA-axis in man after the administration of 30 mg prednisone per day for 10 days.

We wanted to investigate whether this impairment in the activity of the HPA-axis occurs in all individuals, after the administration of glucocorticoids in pharmacological doses for a short period. Moreover, such a suppression of the reactivity of the HPA-axis could have implications for patients, receiving glucocorticoids for a short period because of diagnostic tests or for therapeutic reasons. At present it is generally advised to supplement only those patients, who have been treated with glucocorticoids for periods of several weeks or more, with high doses of cortisol, in case of planned stressful diagnostic or therapeutic procedures. This is done in order to prevent adrenocortical insufficiency (2, 3). Thus, the demonstration of a suppression of the HPA-axis in patients after short-term glucocorticoid administration might have similar implications.

Clinically there are very few circumstances in which this question can be investigated properly. Patients with established hypercalcemia have to go through many diagnostic procedures to elucidate the etiology of their elevated serum calcium levels (4, 6). Primary hyperparathyroidism is a frequent cause of hypercalcemia in man. To discriminate between primary hyperparathyroidism and other causes of hypercalcemia, like sarcoidosis and malignancies, pharmacological doses of glucocorticoids may be used (prednisone 30 mg/day, during 10 days) (5). In primary hyperparathyroidism serum calcium remains constant during the course of glucocorticoid administration, whereas the other causes of hypercalcemia are characterized by a marked decrease or normalization of serum calcium level during that period. Thus, we used the administration of glucocorticoids in patients with suspected primary hyperpara-

thyroidism to investigate the effects on the HPA-axis, and on cortisol levels during the stress of a subsequent neck exploration for removal of one or more enlarged parathyroid glands.

IV.B. METHODS AND PATIENTS

We studied 6 patients with hypercalcemia, who were thought to have primary hyperparathyroidism in view of an elevated serum calcium, and a lowered serum phosphate level, decreased renal tubular transport of phosphate and typical abnormalities in the bone biopsy (6). The age of the patients (2 males, 4 females) ranged from 47 to 69 years. The HPA-axis was studied using ACTH-stimulation and metyrapone administration.

In the case of the ACTH stimulation test, an indwelling needle was placed in a suitable vein of the forearm, while patients were in bed to avoid stress. 45 Minutes after insertion of the needle blood samples for cortisol determination were drawn at 30, 15 and 0 min. before administration of ACTH. Synthetic $ACTH_{1-24}$ (Cortrosyn^R, Organon, Oss), 0.25 mg in 2 ml was then injected intravenously, and samples were drawn after 30, 60 and 90 min. The mean maximal increment of cortisol after ACTH amounted to 494 ± 31 in 10 normal individuals with a range between 405 and 597 nmol/l. Normal basal plasma cortisol levels at 15.00 hr amounted to 213 ± 28 (129-359 nmol/l) in 10 controls. A maximal increment of cortisol after ACTH of more than 195 nmol/l is considered to be evidence of normal adrenocortical function. All ACTH stimulation tests were performed at 15.00 hour.

Metyrapone (Metopiron^R, Ciba) was given orally in a dose of 750 mg every 4 hours for 24 hours, starting at 08.00 hour. After 24 hours, at 08.00 hour the following day a blood sample was drawn by venous puncture for determination of the plasma desoxycortisol concentration.

Plasma desoxycortisol concentrations after the administration of metyrapone were measured in 43 normal individuals. The levels were higher than 350 nmol/l in 36 normal individuals, and between 230 and 350 nmol/l in 7 normal individuals and never less than 230 nmol/l. Therefore values between 230 and 350 nmol/l, are considered to be borderline normal. The ACTH stimulation test and the administration of metyrapone preceded the administration of glucocorticoids with 2 resp. 1 day. Prednisone (3 × 10 mg/day) in tablets of 5 mg was given during 10 days. At day 1,2,3,5,7,9 and 10 blood samples were drawn for measurement of serum calcium, phosphate, and cortisol at 08.00 hour. One day after stopping prednisone, at 15.00 hour, the ACTH stimulation test was repeated. The next day, again a metyrapone test was performed.

The following day (3 days after termination of prednisone administration)

all patients whose tests pointed to the diagnosis of hyperparathyroidism underwent an operation to explore the size and aspect of the parathyroid glands. All operations were scheduled to start at 08.00 hour. Anesthesia was uniformly initiated in all patients by the administration of penthothal, fentanyl, and pavulon. NO_2/O_2 gas mixtures, and ethrane were used during anesthesia. During operation blood pressure was recorded at 15 min. intervals. Before induction of anesthesia, a separate indwelling needle was placed in a suitable vein to obtain blood samples during the operation and thereafter. Samples for cortisol determination were drawn at 08.00, 08.30, 09.00, 09.30, 10.00, 10.30 and 11.00 hour. Finally, a third ACTH stimulation test was performed 1 day post-operatively at 15.00 hour. A schematic diagram of the whole protocol is shown in figure IV-1.

As a control group, patients were studied in whom a single, non-cystic, "cold" nodule in the thyroid gland was diagnosed. To exclude malignancy, these patients underwent exploration of the thyroid, an operation comparable with the one in the former group. 7 Patients (7 females) in age ranging from 33 tot 67 years, were studied. Anesthesia was carried out in the same manner as in the group of patients with primary hyperparathyroidism. Blood pressure was measured at 15 min. intervals. During the operation blood samples were drawn at the same time intervals as in the first group of patients.

Cortisol was measured using a radioimmunoassay using the kit supplied by Clinical Assays (Cambridge, MA.) 11-Desoxycortisol was measured by radioimmunoassay after extraction, using the procedure, described earlier for the estimation of oestradiol (7, 8) but without chromatography. All patients gave informed consent to perform the various procedures. All data are expressed as mean ± S.E.M. Statistical analysis of data was performed with Student's paired t-test, or analysis of variance.

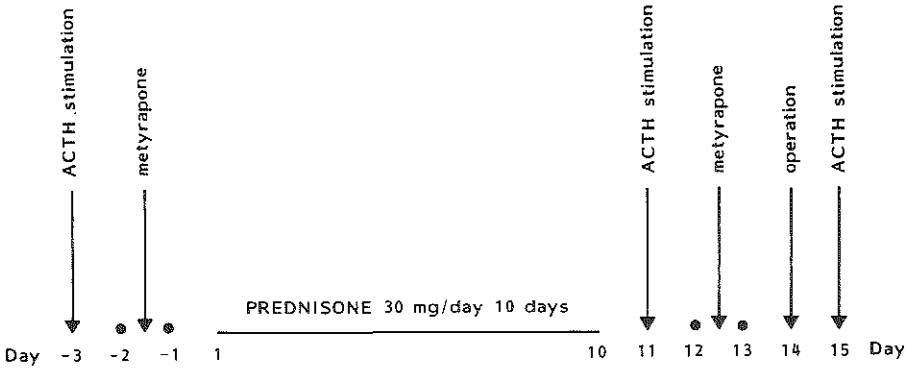

Fig. IV-1. A schematic diagram of investigation of the HPA-axis in six patients with suspected primary hyperparathyroidism.

IV.C. RESULTS

IV.C.1. *ACTH stimulation tests and metyrapone tests*

The results of three consecutive ACTH stimulation tests showed an unexpected pattern. In Fig. IV-2 plasma cortisol levels are shown at 0 min. (15.00 h) in the six glucocorticoid treated patients, before, immediately after the 10 day-course of prednisone, and one day after the parathyroid exploration. Before prednisone administration the cortisol levels showed a marked variation; the values were found to range from 52 to 510 nmol/l (mean 245 ± 70 nmol/l). Immediately after conclusion of the prednisone course variations in plasma cortisol concentrations were much less pronounced (mean 176 ± 17 nmol/l). In three patients the basal plasma cortisol levels before ACTH administration were decreased after the prednisone administration, but in contrast, in three others a slight increase was noted at the same time. Overall there was no significant change in basal plasma cortisol values before and after prednisone administration. However, in 5 patients, pre-ACTH plasma cortisol levels, 1 day after operation, were significantly higher than those immediately after the prednisone course (mean 280 ± 29 vs. 176 ± 17 nmol/l; $p<0.05$).

Thereafter the maximal increment of plasma cortisol (= Δcortisol = highest level of cortisol minus basal level) after ACTH stimulation was investigated. After the administration of prednisone, a sharp fall in the maximal increment of cortisol after ACTH was demonstrated in all patients, when compared with Δcortisol levels before prednisone (mean 184 ± 25 vs. 511 ± 137 nmol/l resp.; $p<0.05$; Fig. IV-3). In the six patients Δcortisol levels before prednisone ranged from 268 - 1152 nmol/l; after prednisone administration the Δcortisol level ranged from 94 - 272 nmol/l. In four of these patients it did not exceed the 195 nmol/l (Δcortisol 94, 153, 165 and 185 nmol/l), which is considered to represent the lower limit of normal. The mean fall in Δcortisol was 64.0% in comparison with the values before prednisone. One day after operation Δcortisol levels were significantly elevated, in comparison with the maximal increment of plasma cortisol after ACTH immediately after the prednisone course (mean 574 ± 77 vs. 184 ± 25 nmol/l resp.; $p<0.02$; Fig. IV-3). Thus, both basal cortisol levels and the maximal increment of cortisol after ACTH were significantly higher one day post-operatively than immediately after the glucocorticoid period. Furthermore, the Δcortisol level after operation was not significantly different in comparison with the pre-prednisone Δcortisol levels (mean: 574 ± 77 vs. 511 ± 137 nmol/l; n.s.; Fig. IV-3 and 4). No alteration was found in the basal cortisol levels before prednisone and one day after operation (Fig. IV-4).

The results of metyrapone administration in the 6 patients with primary hyperparathyroidism are shown in Fig. IV-5. After administration of mety-

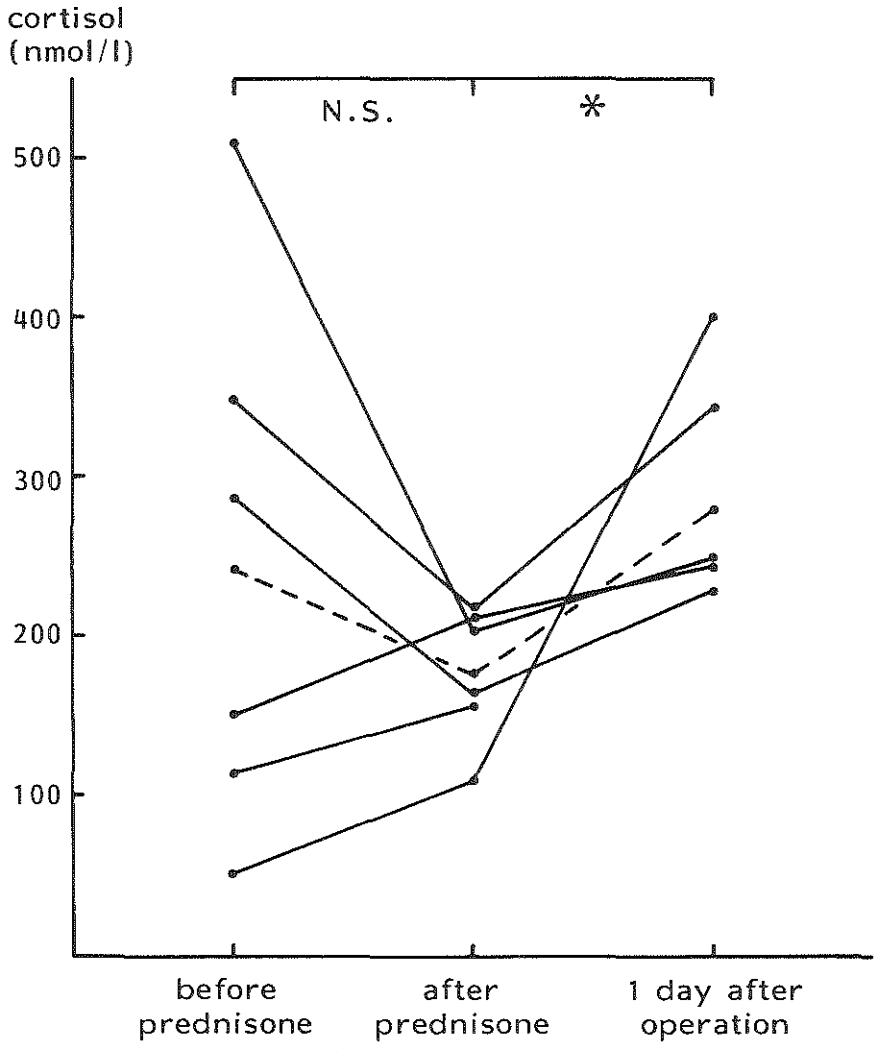

Fig. IV-2. The effect of the administration of prednisone (30 mg/day for 10 days) on basal plasma cortisol levels at 15.00 hour before ACTH stimulation (0 min.), before prednisone, 1 day after withdrawal of prednisone, and one day after operation. (- - - -) represent mean values.

* $p<0.05$.

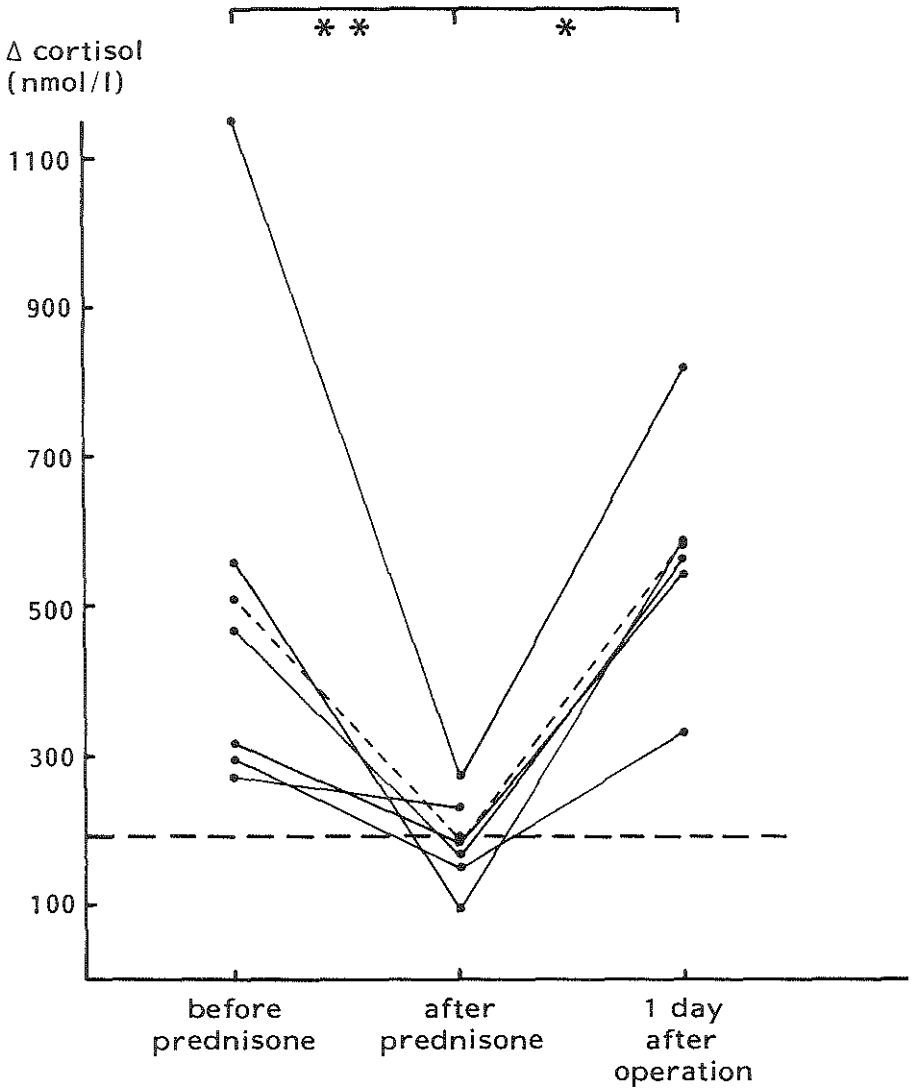

Fig. IV-3. The maximal increment of plasma cortisol levels in response to ACTH (Δcortisol) before prednisone, 1 day after withdrawal of prednisone, and one day after operation. (- - - -) represent mean values.

* $p<0.02$.

** $p<0.05$.

— — — represents lowest normal limit of Δ cortisol (195 nmol/l).

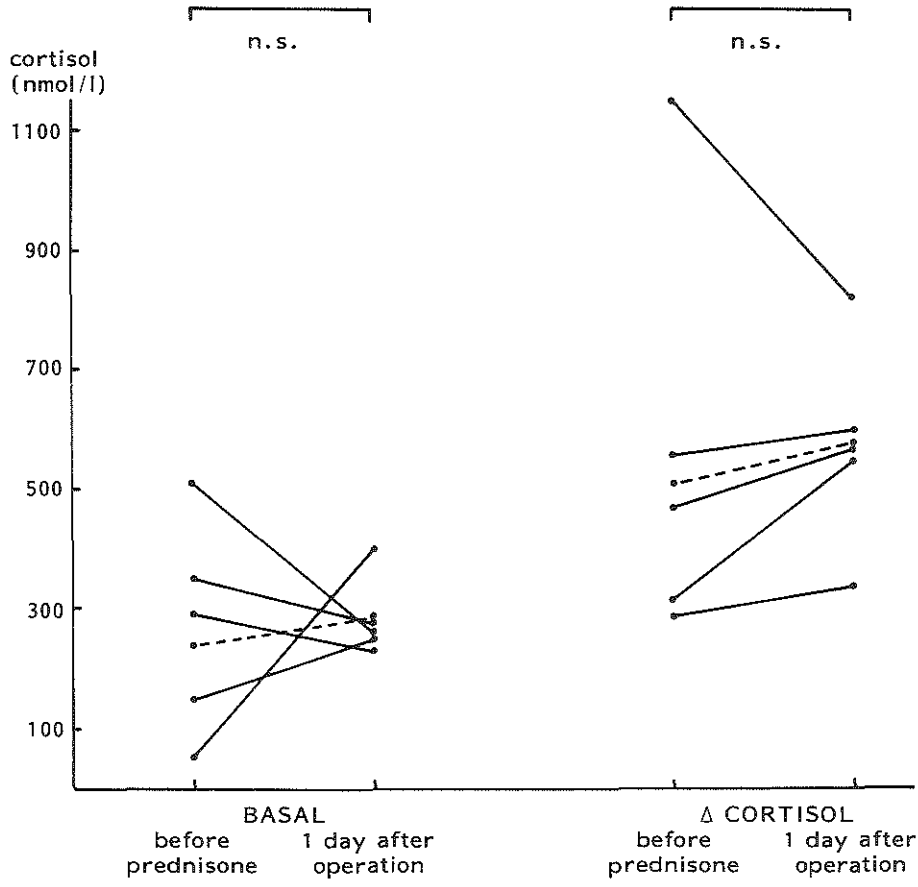

Fig. IV-4. A comparison of plasma cortisol levels at 15.00 hour (0 min. and Δcortisol) before prednisone treatment and one day after operation. (- - - -) represent mean values.

rapone 6 X 750 mg/24 hours for the first time, a value of desoxycortisol of more than 350 nmol/l was reached in all 6 patients. In all 6 patients the plasma desoxycortisol concentration after metyrapone was reduced markedly after the period of glucocorticoid administration. The mean plasma desoxycortisol value after metyrapone was 672 ± 99 nmol/l before, and 389 ± 68 nmol/l ($p<0.02$) after prednisone.

The cortisol values, at 08.00 hour, during 10 days of administration of prednisone 3 X 10 mg/day in the six patients are plotted in Fig. IV-6. The mean cortisol level at day 1 was 270 ± 51 nmol/l, but it was 135 ± 29 nmol/l at day 10 ($p<0.05$). Thus a decrease in mean cortisol levels of 135 nmol/l was found after 10 days of glucocorticoid administration. The mean cortisol levels did not exceed 175 nmol/l from day 3 on to reach a nadir at day 10.

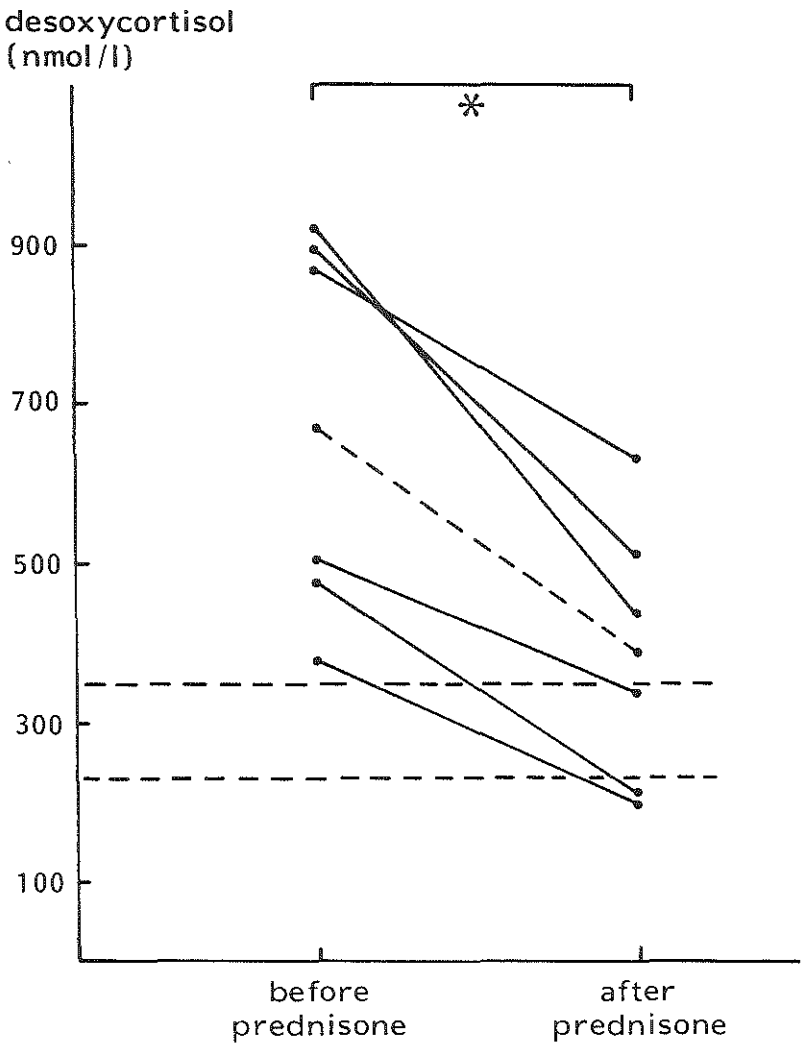

Fig. IV-5. The effect of administration of prednisone (30 mg/day for 10 days) on desoxycortisol levels after 4.5 g of metyrapone before and after prednisone. (- - - -) represent mean values.

* $p<0.02$.

— — — represents borderline area (230–350 nmol/l).

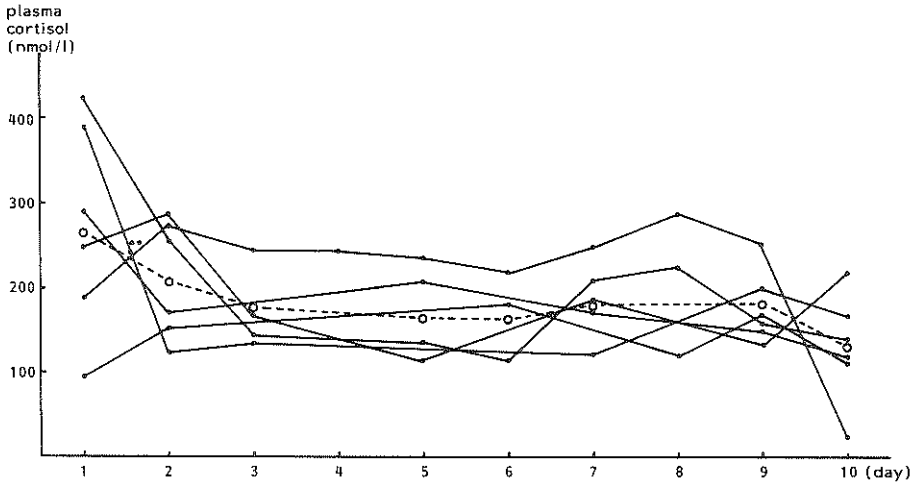

Fig. IV-6. Plasma cortisol levels at 08.00 hour during prednisone treatment (30 mg/day, for 10 days). O- - - - -O represent mean plasma cortisol levels. Mean plasma cortisol levels at day 10 vs. mean plasma cortisol level at day 1: $p<0.05$.

IV.C.2. *Plasma cortisol levels during operation*

The cortisol values of the prednisone-treated patients during the period of induction of anesthesia, operation, and recovery, from 08.00 till 11.00 hour, are summarized in Table IV-I. The mean cortisol levels showed a tendency to fall from 405 ± 39 at 08.00 to a nadir of 284 ± 56 nmol/l at 09.30 hour (n.s.); thereafter a gradual increase was found to 586 ± 125 nmol/l at 11.00 hour. In patient 1,2,3,4 a parathyroid adenoma was found between 09.00 - 10.00 hour. Anesthesia was ended in these patients at about 10.00 hour. The stress of extubation and the recovery from anesthesia caused the plasma cortisol to rise markedly. During the time, that was spent in the recovery room till 11.00 hr., the cortisol levels rose even further in patients 2,3,4. In patient 5 a parathyroid adenoma was eventually found in thymic tissue, just above the aortic arch, at 11.00 hour. From 09.30 to 10.30 hour hypotension was induced by ethrane to prevent capillary bleeding in the operation field. With induction of this hypotension the plasma cortisol rose from 210 to 495 nmol/l, maintaining a plateau till the end of this procedure (Fig. IV-7).

Patient 6 underwent a second exploration of the parathyroid region, after an unsuccessful operation in another hospital. It was anticipated that the adenoma was located in the anterior mediastinum, as angiography of this area showed a blush of contrast dye, suggesting a tumor. In this patient the same pattern in plasma cortisol levels was observed as in patients 2, 3 and 4: after

Table IV-I. Plasma cortisol levels of six patients with primary hyperparathyroidism during operation

	Plasma cortisol (nmol/l)						
Time (hours)	08.00	08.30	09.00	09.30	10.00	10.30	11.00
Patient 1	370	360	430	243	450	243	300
2	483	451	-	-	-	604	686
3	523	307	353	375	499	1057	1027
4	425	246	348	250	368	389	538
5	250	218	210	495	400	263	198
6	378	244	216	159	153	516	767
$\bar{x}$	405	304	311	284	374	512	586
S.E.M.	39	36	43	56	60	123	125

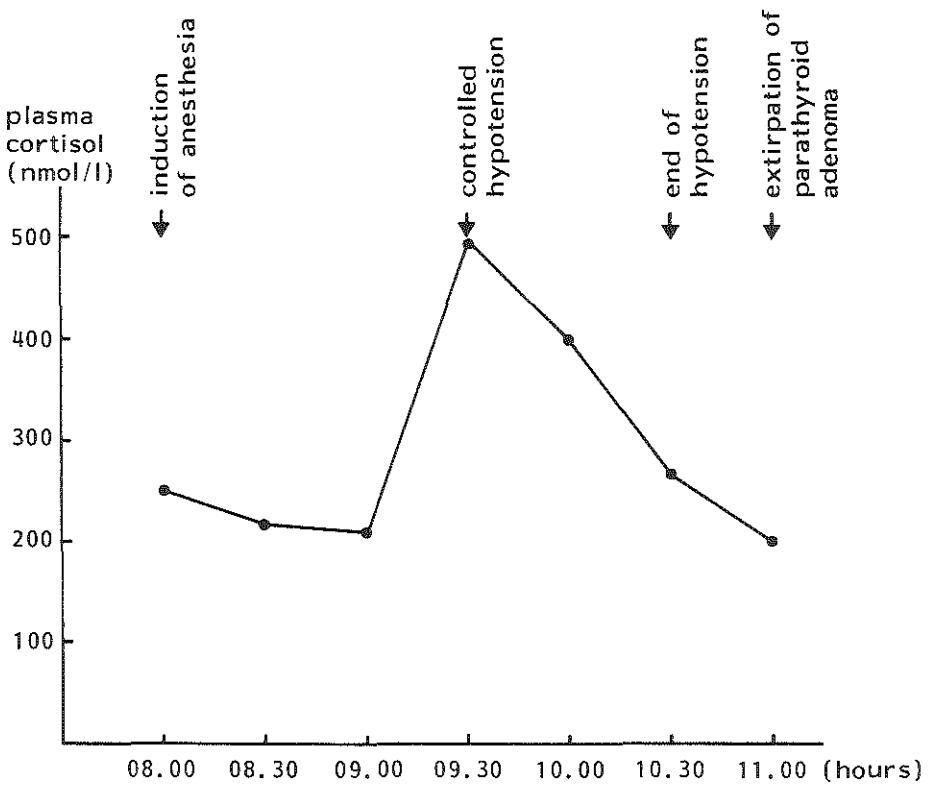

Fig. IV-7. Plasma cortisol levels in a patient with hyperparathyroidism during neck exploration.

an initial normal level of cortisol at the induction of anesthesia, plasma cortisol fell to lower levels ranging from 250 - 150 nmol/l. From the start of the procedure of sternotomy, plasma cortisol rose sharply, reaching a value of 516 nmol/l at 10.30 hour, at which time the severed sternum was closed with traction. The cortisol level rose even further to 767 nmol/l during the recovery from anesthesia, at 11.00 hour (Fig. IV-8). In all six patients a parathyroid adenoma was found and proven histologically.

The plasma cortisol concentrations of seven patients with a single cold nodule in the thyroid, undergoing a thyroid exploration, are shown in Table IV-II.

The control patients, who underwent exploration of the thyroid region showed a similar pattern of plasma cortisol levels, as the one observed in the prednisone treated patients. The total operation time of the thyroid exploration ranged from 90 min. to 3 hours, and was thus comparable with the duration

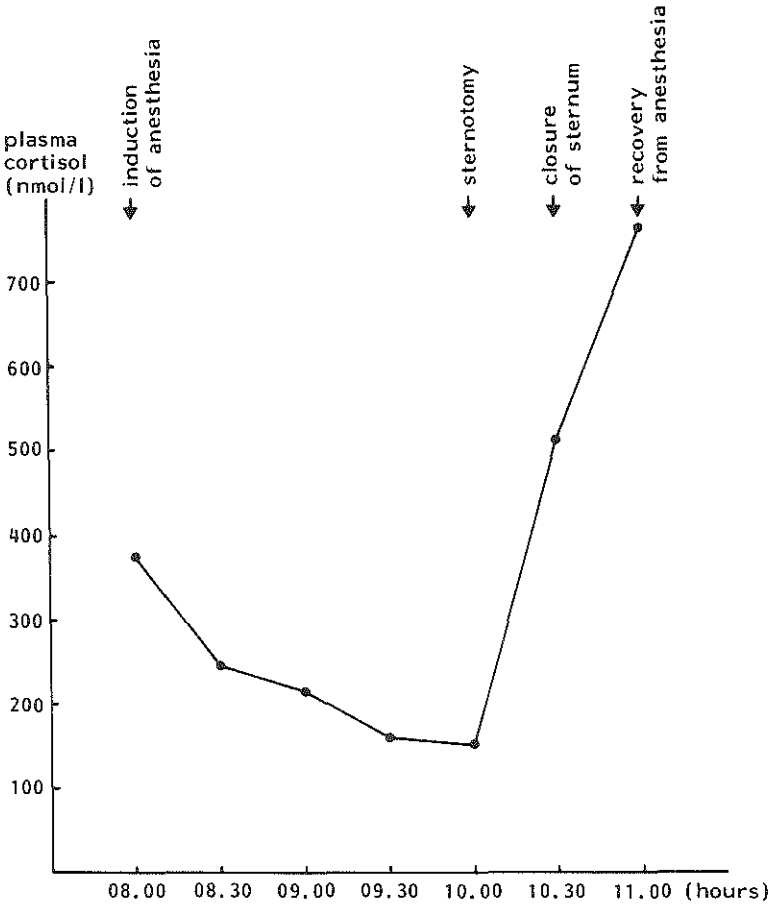

Fig. IV-8. Plasma cortisol levels in a patient with hyperparathyroidism during neck operation.

of parathyroid exploration. In the control group a similar and sharp increase in the plasma cortisol concentration was seen after 09.00 hour, which rose to even higher levels at 10.00 hour and later, representing the recovery of anesthesia and the period spent in the recovery room. The mean cortisol levels rose from 488 nmol/l at 09.00 hour to a maximum of 931 nmol/l at 11.00 hour. Between the induction of anesthesia at 08.00 hour and 09.00 hour the mean levels fluctuated between 468 and 534 nmol/l. Patient F, a female of 66 years old, presented very high plasma cortisol values throughout the whole duration of the thyroid exploration, which took 2½ hours (levels fluctuating between 954-1353 nmol/l). She did not have signs of hypercortisolism at clinical or biochemical examination.

Table IV-II. Plasma cortisol levels of seven patients with a "cold" thyroid nodule, serving as controls, during operation

	Plasma cortisol (nmol/l)						
Time (hours)	08.00	08.30	09.00	09.30	10.00	10.30	11.00
Patient A	630	425	347	239	215	297	681
B	213	255	289	336	369	472	472
C	667	667	543	811	505	822	862
D	304	278	300	1053	1384	1080	1079
E	308	791	899	1126	1203	1044	1177
F	1041	1231	954	1209	1109	1240	1353
G	111	93	81	834	1418	1200	894
$\overline{X}$	468	534	488	801	886	879	931
S.E.M.	123	147	124	143	191	139	113

Patient G on the other hand, showed remarkably low cortisol levels at the beginning of the operation, ranging from 111 nmol/l at 08.00 hour to 81 nmol/l at 09.00 hour. At 09.30 hour, the moment of removal of a part of the thyroid gland, containing the cold nodule, cortisol levels rose sharply to 834 nmol/l, and even higher to 1418 and 1200 nmol/l at the period of recovery from anesthesia. A comparison of the mean cortisol values found in the patients who had received prednisone before operation, and those of the control group is made in Fig. IV-9. All mean values in the prednisone treated group were below those of the controls, at all times of study. Especially from 09.30 hour on the two lines diverged significantly ($p<0.05$). When the areas under both curves are compared, the area under the curve representing the plasma cortisol values of glucocorticoid treated patients is significantly smaller than that of the controls ($p<0.05$). The mean maximal increment of plasma cortisol from 09.00 - 09.30 till 11.00 hour amounted to 251 ± 127 nmol/l in the group of hypercalcemia patients and to 443 ± 94 in the thyroid patients. This difference is significant ($p<0.05$).

The clinical events during and after operation were not different between the 6 prednisone treated patients and the 7 control patients. No complications or signs and symptoms which could be related to (relative) adrenocortical insufficiency were noted. Blood pressure in the period before, during and in the first 24 hrs after operation were also not different between both groups of patients.

IV.D. DISCUSSION

From this study it is clear that the short-term effect of glucocorticoids on the HPA-axis is pronounced. The results of the administration of metyrapone, which assesses the integrity of responsiveness of the HPA-axis, demonstrates an impairment of the reactivity of the HPA system after 10 days of prednisone administration. A similarly lowered responsiveness of the adrenal cortex to ACTH was shown in these patients.

Abnormalities in the response of the HPA-axis to metyrapone and ACTH testing were reported by Plager (9) to occur after 3 days of glucocorticoid treatment (cortisol 100 mg/day). Christy found an impairment of the axis after similar short treatment periods (10). On the other hand, Treadwell et al. (11), using metyrapone, could only find an abnormal response if patients were treated for more than 15 months. It must be noted, however, that most of their patients took lower doses of prednisone (15 mg or less) than in our study and the other reports quoted.

When we interpret the data obtained in the three consecutive ACTH stimulation tests, it is clear that the administration of glucocorticoids for 10 days,

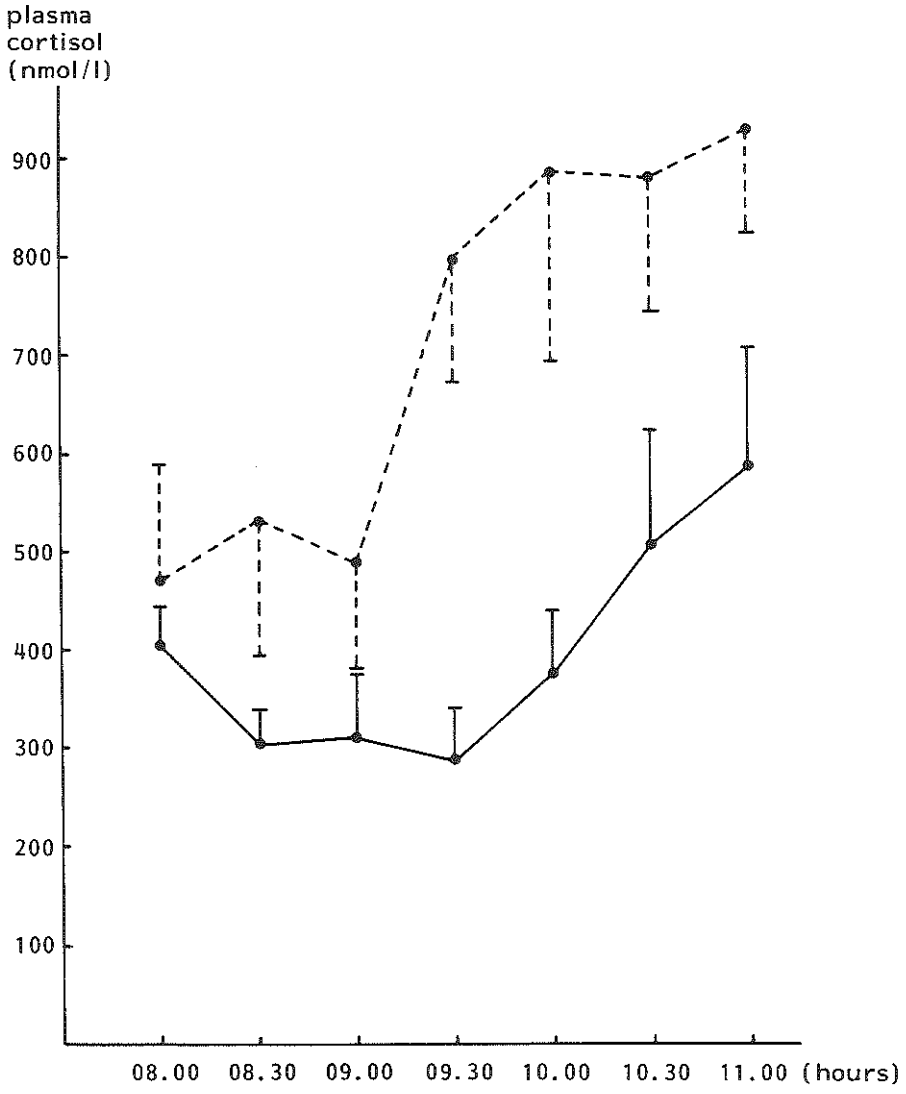

Fig. IV-9. Plasma cortisol levels (mean ± S.E.M.) of 6 patients with primary hyperparathyroidism (pretreated with prednisone) (————) and of 7 patients with a "cold" thyroid nodule, serving as controls, who underwent a similar neck exploration (- - - - -).

impairs the adrenocortical response to ACTH significantly. Especially the maximal secretory capacity of the adrenals (represented by Δcortisol) was decreased after 10 days of prednisone in all patients. Basal plasma cortisol levels at 15.00 hour (immediately before ACTH administration) were not lowered after 10 days of prednisone, although plasma cortisol levels at 08.00 hr. were statistically significantly suppressed (Fig. IV-6). This could be caused partly by the fact that the diurnal rhythm of plasma cortisol before prednisone resulted in relatively low levels at 15.00 hour, while it is well known that differences in "internal" stress, as anxiousness and tensions are able to cause marked variations in the plasma cortisol concentration. This wide variation in basal plasma cortisol levels makes clear, oncemore, how little information with regard to the integrity and responsiveness of the HPA-axis can be deducted from single plasma cortisol determinations.

It is possible to determine from our data the duration of suppression of the HPA-axis. The basal plasma cortisol levels, and the maximal response to ACTH were completely normalized one day after the operation, that is, five days after the end of the course of glucocorticoid administration. However, one should bear in mind, that in these tests a supraphysiological concentration of ACTH is used, forcing the adrenals to secrete cortisol to the maximum of their capacity. It is uncertain whether under these circumstances physiological concentrations of ACTH, secreted by the anterior pituitary gland, are able to meet the needs for cortisol of the bodies homoiostasis. As will be discussed later, the levels of plasma cortisol concentrations obtained during the operation indicate that they are not. Combining the results of metyrapone and ACTH stimulation, we demonstrated that the response of the HPA-axis was still impaired three days after cessation of the glucocorticoids, but was normal again after five days. Thus, the recovery was completed between the third and fifth day. As demonstrated by Graber (12, see chapter I, Fig. I-1), ACTH secretion recovers before the cortisol release. This does not necessarily mean that the HPA-system is completely normal when there is a normal respons on ACTH stimulation, taking into account the high dosage of ACTH used. The metyrapone stimulation gives more accurate information on the integrity of the hypothalamo-pituitary, and pituitary-adrenal responses (13, 14, 15, 16, 19). Furthermore, we found a significant rise both in the basal and maximally stimulated plasma cortisol levels, one day postoperatively when compared with those immediately after cessation of the prednisone, indicating a recovery of adrenal function (17, 20, 21, 22).

It is interesting to see that, although responses of plasma cortisol to ACTH testing were normal one day later, the cortisol levels one day earlier (on the day of operation) were significantly lower in the glucocorticoid treated patients, when compared with the controls. At all times measured, the plasma cortisol levels were lower in the first group. In both groups the patterns of the cortisol

levels were similar during the total period of observation. At 08.00 hour, when the general anesthesia was induced, the cortisol levels were normal (400 - 475 nmol/l), indicating a stress factor to the patient. During the actual surgery plasma cortisol levels fell in the glucocorticoid treated patients until 09.30 hour. This indicates that this period was relatively little stressful (18).

In patient 5, whose blood pressure had to be controlled with induced hypotension, an immediate rise of the cortisol level was found, indicating the response of a well known control mechanism (23). The patient, undergoing a reexploration of the cervical area with sternotomy, showed a very impressive rise of plasma cortisol levels, in particular when the sternum wound was closed with forceful traction on the steel sutures. Although both groups of patients demonstrated a sharp rise in cortisol levels after the end of the operation, during termination of the general anesthesia, and during the period they spent in the recovery room, it is clear from the divergence of both lines in Fig. IV-9 that in the control group the adrenals were able to produce much more cortisol than those in the glucocorticoid treated group. Thus, from this figure it can be concluded that prednisone administration impaired the responsiveness of the adrenals in their maximal secretory capacity. The suppression, which was found immediately after withdrawal of the glucocorticoids was still to some extent present at the fourth day, after stopping. On the fifth day, however, a normal respons of cortisol to ACTH stimulation was found.

Several authors (Jasani et al. (23), Kehlet et al. (24), Marks et al. (18) and Sampson et al. (25)) have reported on possible correlations between responses of the HPA system and plasma cortisol levels during induction of general anesthesia and operations, in patients previously or simultaneously treated with pharmacologic doses of glucocorticoids. Investigating the HPA responsiveness with a short-term ACTH stimulation test (plasma cortisol levels 30 min. after the intravenous injection of 0.25 mg ACTH), Jasani et al. found a correlation between the maximal response of plasma cortisol levels after ACTH and the maximal cortisol levels during operations. When the HPA system was tested, using prolonged infusion of ACTH during 6 hours, a similar correlation between the maximal rise in cortisol levels and the levels of cortisol during anesthesia and surgery was found (18, 25). In other words, one might expect normal reactions of the HPA system, at stressful periods, in glucocorticoid treated patients, when a previous ACTH testing was normal. In our results the responsiveness of the HPA-axis was less than that of the normal control group, as might be expected by the previous abnormal responses on ACTH and metyrapone testing.

Although we did not observe signs of circulatory collapse in any of the glucocorticoid treated patients, these results indicate that even after such a short period of glucocorticoid administration (relative) adrenocortical insufficiency might occur. Thus, anesthesiologists and physicians should be

aware of this potential hazard, and consider appropriate actions, like the supplemental administration of glucocorticoids (13).

Jasani et al. (14, 26) demonstrated that a normal response of cortisol to ACTH stimulation does not imply normal reactivity to metyrapone, insulin-induced hypoglycemia or lysine-vasopressin. To assess the HPA integrity more accurately the use of both ACTH stimulation and metyrapone testing may be advocated.

In summary, we demonstrated a suppression of the hypothalamo-pituitary-adrenal system after a short period (10 days) of glucocorticoid administration in pharmacologic dosages. Immediately after the withdrawal of the glucocorticoids, both ACTH stimulation and metyrapone testing showed an impaired response, indicating a substantial influence on the HPA system.

This impairment in the response was present for 3-4 days; thereafter a normal responsiveness of the adrenal part of the HPA system was found again.

During a period of prolonged stress (induction of general anesthesia, surgery, reversal of anesthesia, and the post-operative period) the secretory capacity of the adrenals was impaired in the glucocorticoid treated patients, when compared with a control group, undergoing a similar stressful procedure.

These data suggest that even after short courses of glucocorticoids the occurrence of adrenocortical insufficiency is a real possibility and that the deleterious effects of these drugs can be serious.

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Chapter V

Effects of chlorimipramine: a drug with possible serotonin enhancing effects on the glucocorticoid mediated suppression of the HPA-axis, in man

V.A. INTRODUCTION

The aim of this study was to investigate whether it is possible to prevent the negative effect of glucocorticoids on the HPA-axis *in man*. We wanted to know whether drugs, which are known to stimulate ACTH secretion are able to prevent partially the suppressive effects of glucocorticoids on pituitary-adrenal function. In the previous chapter (chapter IV) we demonstrated the profound suppressive effects of short-term glucocorticoid administration on the HPA-axis. In chapter III it was shown that chlorimipramine, a pharmacological agent with serotonin enhancing effects on CRF release was capable to prevent in rats the suppression of the pituitary ACTH release and content which was induced by a transplantable ACTH secreting pituitary tumor. In this chapter the effects of the chronic administration of chlorimipramine are described on the prednisone-induced suppression of HPA-function in patients with suspected hyperparathyroidism.

V.B. METHODS AND PATIENTS

Six patients with hypercalcemia, who were suspected to have primary hyperparathyroidism were asked to participate in the study. The same criteria (as mentioned in chapter IV) were applied to make the diagnosis of hyperparathyroidism probable. The ACTH stimulation test and the metyrapone stimulation test were performed as described in the previous chapter. Exactly the same protocol was followed as described (Fig. IV-1) for the study of the effect of prednisone on the HPA-axis in the hypercalcemic patients. However, after the completion of the first ACTH and metyrapone stimulation tests, chlorimipramine was administered together with prednisone. Prednisone 10 mg (in tablets of 5 mg) was given three times a day during 10 days. Chlorimipramine (Anafranil^{R}, Geigy) was given orally in tablets of 25 mg. On the first and second day of the prednisone administration chlorimipramine was given in a dose of 2 X 25 mg; on the third day in a dosis of 3 X 25 mg; from the fourth until the tenth day in a dose of 4 X 25 mg a day. Thereafter the study was

completed in accordance with the protocol described earlier in detail. Again, all patients, in whom the diagnosis of primary hyperparathyroidism was made, underwent exploration of the parathyroids, three days after completing the combined drug regimen. One patient was not operated upon because the serum calcium level normalized after prednisone administration. The operation and anesthesia were performed as described earlier (chapter IV). All six patients (1 male, 5 females) in age ranging between 45 and 50 years, gave written informed consent. Data are expressed as mean ± S.E.M. Statistical analysis of the data was performed with Student's paired t-test, or analysis of variance.

V.C. RESULTS

V.C.1. *Effects of chlorimipramine on ACTH-and metyrapone stimulation tests*

The results of the consecutive ACTH stimulation tests are shown in Fig. V-1. In one patient an ACTH stimulation test could not be repeated on the day after the operation. The plasma cortisol levels at 0 min., before ACTH administration, did show a significant decrease when values before prednisone/chlorimipramine administration were compared with values after conclusion of the drug regimen. The cortisol levels before ACTH varied from 167 to 315 nmol/l (mean 249 ± 25 nmol/l) in the ACTH stimulation test, which was performed before the administration of the drugs. In the second test, after stopping the two drugs, plasma cortisol levels at 0 min. varied from 50 to 156 nmol/l (mean 104 ± 14 nmol/l). There was a significant fall in mean values at 0 min. before and after the administration of prednisone and chlorimipramine. (Fig. V-1; $p<0.01$). Again a significant increase could be demonstrated when mean basal cortisol levels after operation were compared with those of the ACTH stimulation test, performed after discontinuation of the drugs (mean 225 ± 32 vs. 104 ± 14 nmol/l; $p<0.05$; ranges 139 - 328; resp. 50 - 156 nmol/l; Fig. V-1).

In Fig. V-2 the maximal increment of plasma cortisol (Δcortisol) after ACTH administration is shown before and after the administration of prednisone and chlorimipramine and one day after operation. After the combined drug regimen the mean value of Δcortisol levels was significantly lower in comparison with the one found before drug treatment (mean 273 ± 22 vs. 660 ± 48 nmol/l; $p<0.005$). The Δcortisol values after drug treatment ranged in the six patients from 205 to 368 nmol/l; those found before the start of the drug administration ranged from 501 to 791 nmol/l. Thus, all Δcortisol values exceeded the lowest normal limit of 195 nmol/l. When the maximal increment of cortisol levels after ACTH one day after operation was compared with the Δcortisol levels after drug treatment a significant increase was noted. The

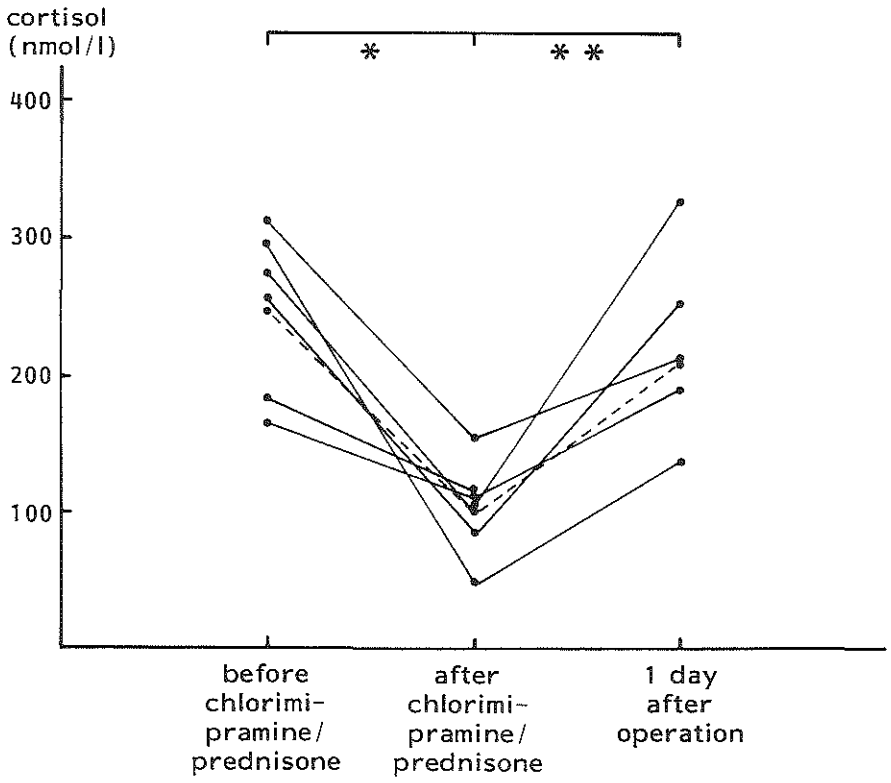

Fig. V-1. Basal plasma cortisol (nmol/l) before administration of ACTH, prior to treatment with chlorimipramine (2 X 25 mg → 4 X 25 mg/day) and prednisone 3 X 10 mg/day, after combined drug treatment and 1 day after operation resp. in 6 patients with suspected primary hyperparathyroidism. (●- - - -●) represent mean values.
* $p<0.01$
** $p<0.05$.

values ranged from 396 to 828 (mean 652 ± 72) nmol/l after operation, vs. 205 to 368 (mean 273 ± 22) nmol/l after drug treatment ($p<0.01$; Fig. V-2). The basal plasma cortisol levels and the maximal increment of plasma cortisol after ACTH did not show significant changes when a comparison was made between values before the prednisone/chlorimipramine treatment and values one day after operation (basal levels: mean 249 ± 25 vs. 225 ± 32 n.s.; Δcortisol: 660 ± 48 vs. 652 ± 72 nmol/l n.s.; Fig. V-3).

The results of metyrapone administration in the patients studied are shown in Fig. V-4. In all patients the plasma desoxycortisol concentration after metyrapone was decreased when levels after drug treatment were compared with levels before treatment: range 323 - 459 (mean 372 ± 25) nmol/l after

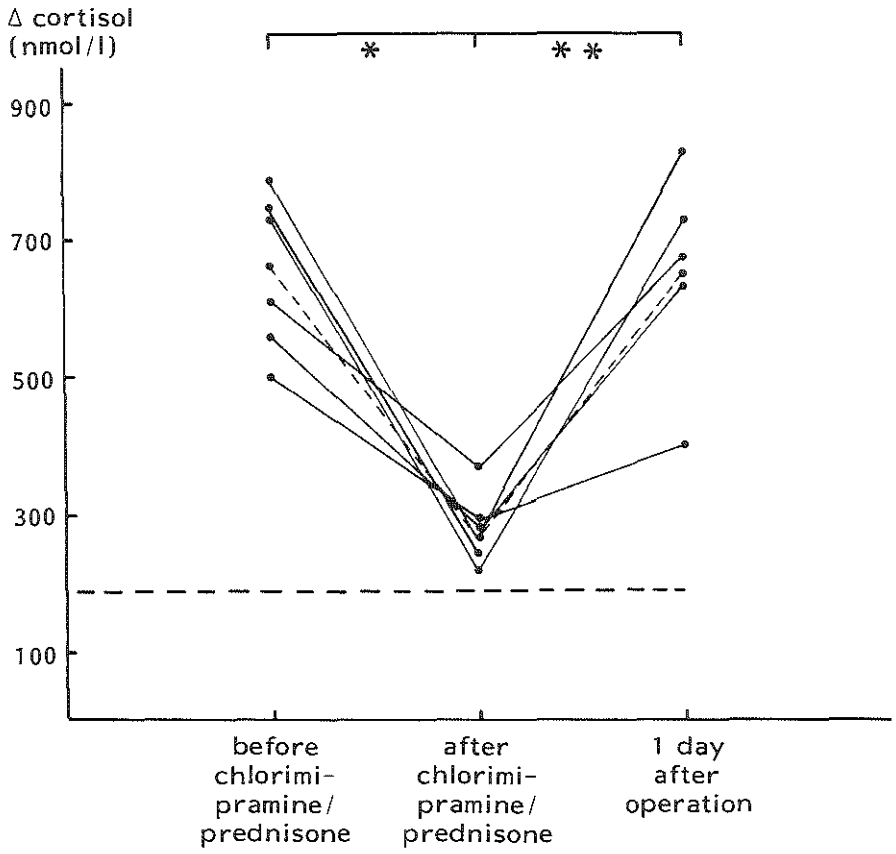

Fig. V-2. Maximal increment of plasma cortisol (nmol/l) (Δcortisol) after ACTH stimulation before and after treatment with chlorimipramine (2 X 25 mg → 4 X 25 mg/day) and prednisone (3 X 10 mg/day); and 1 day after operation resp., in 6 patients with suspected primary hyperparathyroidism (●- - - -●) represent mean values.
* $p<0.005$
** $p< 0.01$
- - - - - - represent lower limit of normal Δcortisol (195 nmol/l).

drug treatment vs. 491 - 630 (mean 548 ± 9) nmol/l; $p<0.05$; Fig. V-4. All metyrapone stimulation tests showed desoxycortisol values over 350 nmol/l after metyrapone in the pre-treatment test. After prednisone/chlorimipramine administration desoxycortisol concentrations after metyrapone were over 350 nmol/l in 3 patients, and between 230 - 350 nmol/l in the other 3 patients (323, 323 and 329 resp.).

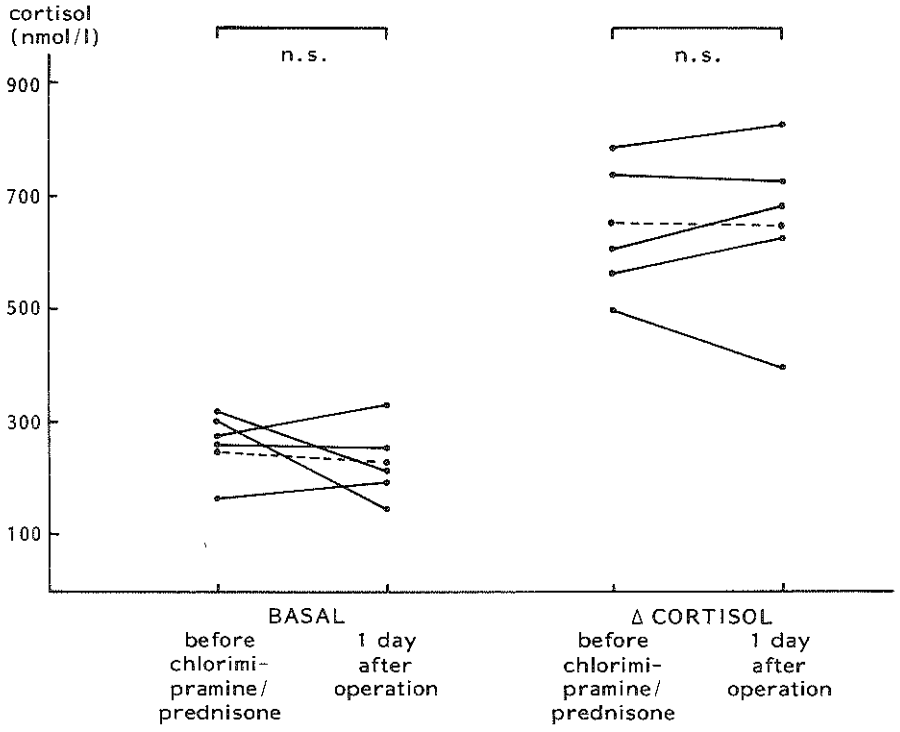

Fig. V-3. A comparison of basal plasma cortisol level and Δcortisol after ACTH stimulation before chlorimipramine/prednisone treatment and 1 day after operation, in 5 patients with definite primary hyperparathyreoidism. (●- - - -●) represent mean values.

V.C.2. *Effects of prednisone/chlorimipramine compared with prednisone only on ACTH- and metyrapone stimulation tests*

We compared the results of the ACTH stimulation test in the patients, who underwent the prednisone/chlorimipramine regimen with the same test in those who were treated with prednisone only, after completing the respective drug regimen. In Table V-I the plasma cortisol levels at 0, 30, 60 and 90 min., mean values, and mean Δcortisol levels of the ACTH stimulation tests in the two groups of patients are shown together. The mean basal plasma cortisol levels in all three ACTH stimulation tests did not differ significantly in either group. The mean values before drug treatment amounted to 245 ± 70 nmol/l in the group treated with prednisone only versus 249 ± 25 nmol/l in the group submitted to a prednisone/chlorimipramine regimen (n.s.). After the drug treatment those figures were 176 ± 17 nmol/l vs. 104 ± 14 nmol/l resp. (n.s.). One day after operation a mean basal cortisol value of 280 ± 29 nmol/l vs. 225 ± 32 nmol/l before treatment was found (n.s.).

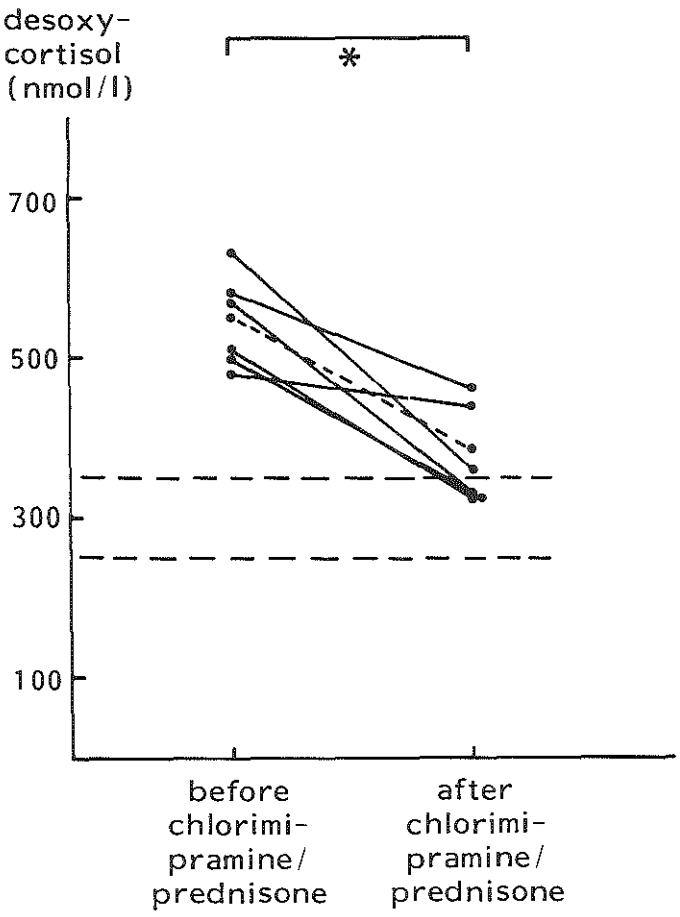

Fig. V-4. Plasma desoxycortisol (nmol/l) before and after chlorimipramine/prednisone treatment, following metyrapone administration, in 6 patients with suspected primary hyperparathyroidism, (● - - - ●) represent mean values.
— — — represents borderline area (230 - 350 nmol/l)
* $p<0.05$.

The maximal increment of plasma cortisol (Δcortisol) after ACTH administration in the patients treated with prednisone and chlorimipramine was significantly greater than the Δcortisol value in the patients treated with prednisone only; mean 273 ± 22 vs. 184 ± 25 nmol/l ($p<0.05$; Table V-I). Four of the six patients who were treated with prednisone only showed a subnormal response of Δcortisol levels directly after prednisone: 94, 153, 165, and 185 nmol/l. In the patients, who received prednisone/chlorimipramine treatment, Δcortisol levels ranged from 205 - 368 nmol/l; mean: 273 ± 22

Table V-I. ACTH stimulation tests in 12 patients with primary hyperparathyroidism, after treatment either with prednisone (30 mg/day for 10 days) or with chlorimipramine (2 × 25 mg → 4 × 25 mg/day and prednisone 30 mg/day for 10 days)

	AFTER PREDNISONE				AFTER CHLORIMIPRAMINE/PREDNISONE				
	Cortisol (nmol/l)								
	0	30	60	90	0	30	60	90	(min.)
1	217	370	342	279	112	311	376	571	A
2	156	-	380	388	156	301	344	361	B
3	203	288	380	388	84	312	346	362	C
4	214	182	307	308	110	296	339	391	D
5	160	230	253	325	50	265	365	418	E
6	108	267	255	380	113	295	351	361	F
$\bar{X}$	176	267	320	345	104	297	354	410	
SEM	17	31	24	19	14	8	6	37	
$\bar{\Delta}$		184 ± 25				273 ± 22			
				a					

$\bar{X}$ mean cortisol level ± S.E.M.
$\bar{\Delta}$ mean maximal increment of cortisol.
a $p<0.05$.

nmol/l, thus in all exceeding the level of 195 nmol/l, which is considered to be the lower limit of normal.

The mean maximal increment of cortisol after ACTH treatment with either drug regimen amounted to 660 ± 48 nmol/l in the group to be treated with prednisone/chlorimipramine, as to 511 ± 137 in patients treated with prednisone only (n.s.).

One day after operation a comparison of the two drug regimes did not show a significant change in mean Δcortisol levels (652 ± 72 nmol/l with prednisone/chlorimipramine vs. 574 ± 77 nmol/l with prednisone only; n.s.). The decrement of Δcortisol levels was not significantly different when both drug regimes were compared.

Mean desoxycortisol levels before the drug administration were 548 ± 9 nmol/l in the "chlorimipramine" group, as to 672 ± 99 nmol/l in the "prednisone" group (n.s.). Mean desoxycortisol concentration after prednisone/chlorimipramine was 372 ± 25 nmol/l, while this concentration amounted to 389 ± 68 nmol/l after prednisone treatment only (n.s.). Two patients in the latter group did have desoxycortisol concentrations after metyrapone administration which were abnormal (both 211 nmol/l). In one patient this value was borderline (344 nmol/l); the others showed normal values. Those patients who underwent prednisone/chlorimipramine treatment had a normal value in three cases, while the others were at the lower limits of normal (323 to 329 nmol/l).

However, when the decrement of desoxycortisol (i.e. desoxycortisol before drug treatment minus desoxycortisol after drug treatment) was determined, a different pattern emerged: the mean decrement of desoxycortisol in the patients treated with prednisone only was 384 ± 52 nmol/l vs. 176 ± 34 nmol/l in the patients treated with both prednisone and chlorimipramine ($p<0.01$; Fig. V-5).

The cortisol levels at 08.00 hour during 10 days of administration of prednisone and chlorimipramine are shown in Fig. V-6. Again a decrease in mean cortisol levels was noted from 324 ± 69 on day 1 to 170 ± 34 at day 10 ($p<0.05$). There was no significant difference when the figures found in patients treated with prednisone/chlorimipramine were compared with the corresponding ones in those treated with prednisone only, neither on day 1 nor on subsequent days till day 10.

V.C.3. *Plasma cortisol levels during operation*

The plasma cortisol levels in the prednisone/chlorimipramine treated patients demonstrated from the start of anesthesia till the recovery period a pattern similar to the one found in patients treated with prednisone only. After initial high levels (mean 392 ± 70 nmol/l at 08.00 hour) the plasma cortisol levels

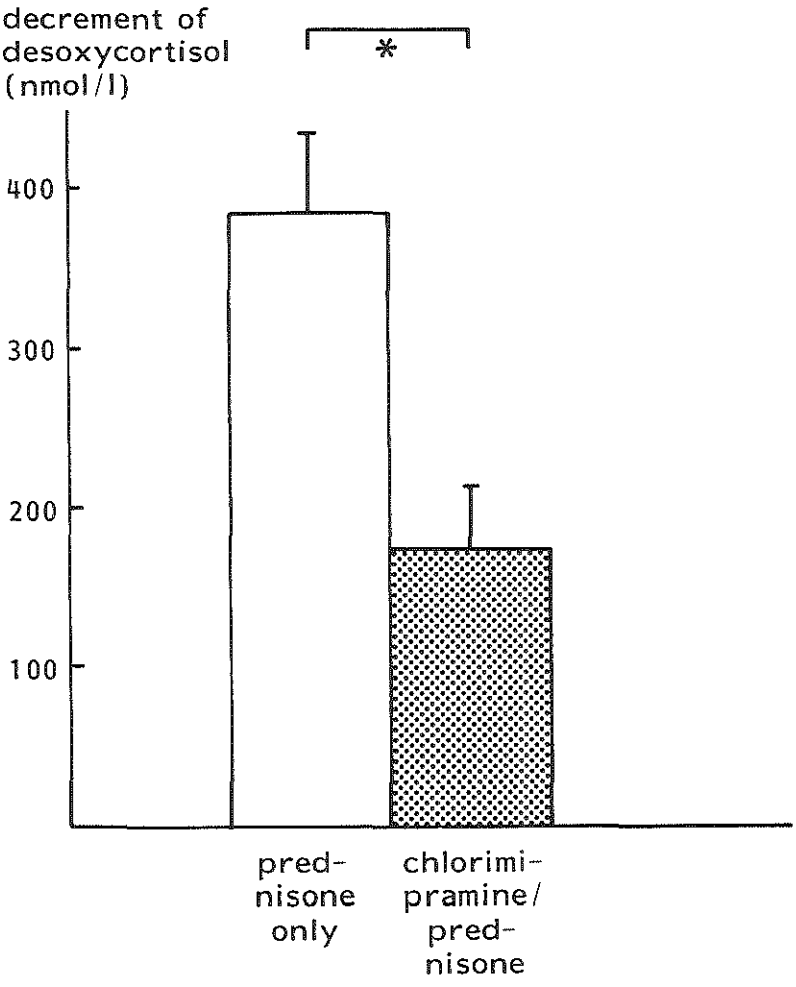

Fig. V-5. Mean decrement of desoxycortisol (nmol/l) after metyrapone administration, after treatment with prednisone (open bars) and after treatment with chlorimipramine and prednisone (hatched bars).
* $p<0.01$.

decreased to a lowest level of 234 ± 59 at 09.30 hour. Thereafter mean cortisol levels increased again to a maximum of 407 ± 117 nmol/l at 11.00 hour. There were no significant differences in mean plasma cortisol levels between both patient groups at any time (Fig. V-7). The results of 5 patients who underwent surgery of the parathyroid region are summarized in Table V-II. As mentioned before, in the sixth patient surgery had to be postponed because the plasma calcium level normalized on the day of the scheduled operation.

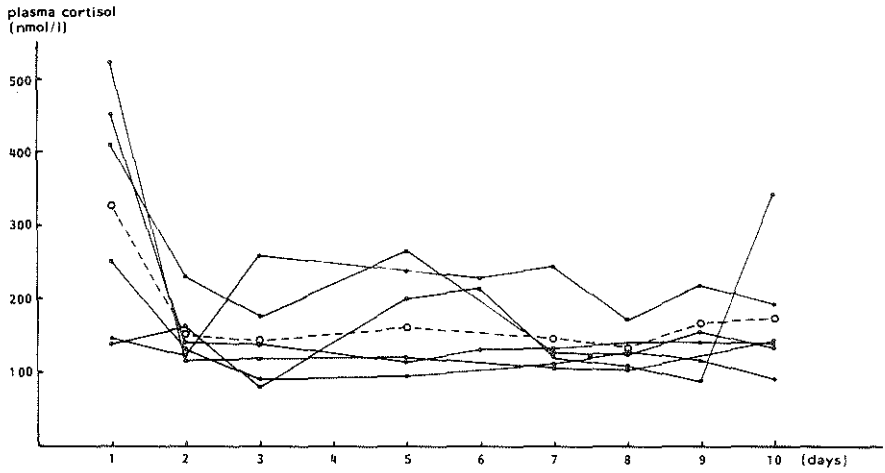

Fig. V-6. Plasma cortisol levels (nmol/l) at 08.00 hours during 10 days of treatment with chlorimipramine/prednisone in 6 patients with suspected primary hyperparathyroidism. (●- - -●) represent mean plasma cortisol.

During and after operation there were no obvious signs which might point to (relative) adrenocortical insufficiency: there were no important changes in blood pressure or heart rate. All patients recovered uneventful from operation and anesthesia. In this series of patients it was not necessary to induce hypotension during the operation at any time.

V.D. DISCUSSION

From the data reported certain conclusions can be drawn. It is clear that after administration of prednisone 30 mg/day combined with an increasing dose of chlorimipramine (50 → 100 mg/day) a significant impairment in responsiveness of the HPA-axis is still found. Addition of chlorimipramine in the doses used could not prevent this impairment. However, there are certain positive effects. Four of six patients showed an insufficient reaction of plasma cortisol to ACTH stimulation after administration of prednisone 30 mg/day, when related to the lower limit of normal of 195 nmol/l. In contrast all six patients, to whom chlorimipramine and prednisone had been given, had a normal response to ACTH stimulation. This suggests that the responsiveness of the adrenal cortex to ACTH stimulation was improved in the latter group. Moreover, the mean maximal increment of cortisol levels immediately after treatment with chlorimipramine and prednisone, following an ACTH stimulation test was significantly

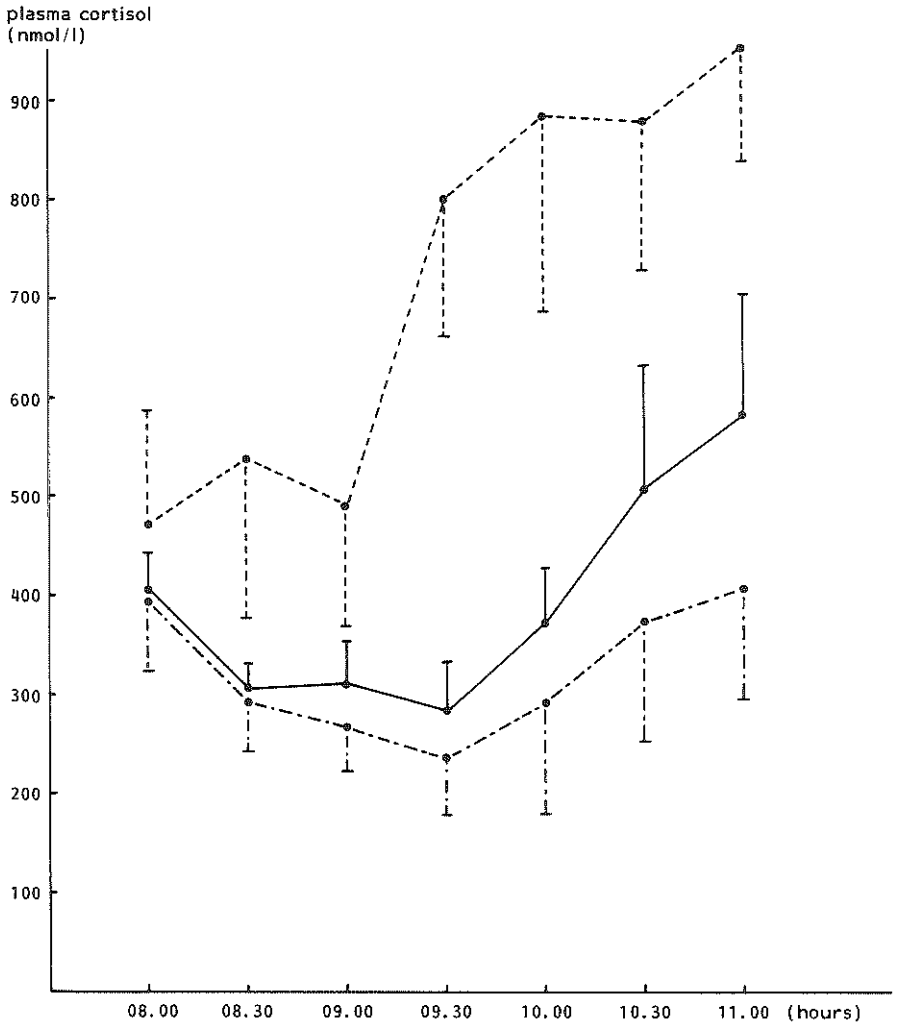

Fig. V-7. Comparison of mean plasma cortisol levels (nmol/l) ± S.E.M. in 6 patients with primary hyperparathyroidism after previous treatment with prednisone (30 mg/day, for 10 days) (———); and in 5 patients with primary hyperparathyroidism after previous treatment with chlorimipramine (50 → 100 mg/day, for 10 days with prednisone 30 mg/day, for 10 days) (– · – · – · –), during parathyroid exploration; 7 patients with a single thyroid nodule during neck exploration (- - - - -) served as a control group.

Tabel V-II. Plasma cortisol levels in 5 patients with primary hyperparathyroidism, during surgery, after previous treatment with chlorimipramine and prednisone for 10 days.

Cortisol (nmol/l)

	08.00	08.30	09.00	09.30	10.00	10.30	11.00	(hours)
1	547	344	324	278	240	278	235	
2	533	462	288	287	335	300	385	
3	165	123	131	82	75	87	83	
4	258	188	156	119	102	406	614	
5	457	342	405	402	695	800	717	
$\bar{x}$	392	292	263	234	289	374	407	
S.E.M.	70	56	52	59	112	118	117	

higher than the one found after treatment with prednisone only. This finding confirms the suggestion of an improved responsiveness of the "adrenal" part of the HPA-axis.

Desoxycortisol levels after metyrapone stimulation decreased less after treatment with prednisone and chlorimipramine than after treatment with prednisone alone. There were 2 patients with an abnormal response to metyrapone after prednisone treatment, while one was borderline. With chlorimipramine administration no patient showed an abnormal reaction: in all it was normal or at the lower limit of normal. Thus, chlorimipramine seems to improve also the responsiveness of the HPA system to metyrapone. This was confirmed further, by the finding of a significantly more pronounced decrement of desoxycortisol levels after treatment with prednisone, when compared with the decrement found after treatment with both drugs. So, the responsiveness of the whole HPA-axis appears to improve.

On the other hand we could not demonstrate a significant elevation of plasma cortisol levels either during the period of chlorimipramine administration or during operation.

The results obtained from ACTH and metyrapone stimulation tests seem to confirm to some extent our hypothesis regarding the importance of a serotonin enhancing effect of chlorimipramine on HPA function. The recovery of the hypothalamo-pituitary part of the axis seems to be more pronounced than the adrenal part of the axis. It was not possible to prevent completely a suppression of the HPA-axis with this dose of chlorimipramine. The dose which was chosen is a moderate one; in psychiatric patients with severe depression higher doses are used (1). Moreover, chlorimipramine has to be given in a rising dose in order to diminish unwanted side-effects (2). Finally, a plateau in the concentration of the active metabolite of this drug is reached only after 2-3 days

of administration (1,2,3). So the duration of administration was relatively short and the maximum dose of chlorimipramine was limited.

Thirty mg of prednisone (equivalent to 120 mg cortisol) constitute a large glucocorticoid potency, and represent at least four times the normal daily production of cortisol by the adrenal glands. Moreover, the fact that this dose is divided over 3 doses a day causes a constant, 24 hour suppression of the HPA-axis (4). The extent of suppression of the HPA-axis correlates well with the amount of glucocorticoids given (5). Chlorimipramine was able to improve the maximal responses of the HPA-axis after stimulation either with ACTH or metyrapone (after all, it might be stressed that in those patients who had received chlorimipramine together with prednisone no abnormal responses to ACTH stimulation and no abnormal responses after metyrapone were observed). Thus it seems reasonable to attribute these results to the presumed serotonin enhancing actions of chlorimipramine.

As we discussed in chapter I there is convincing evidence that the recovery of the suppression of the HPA-axis originates in the enhanced secretion of CRF by the hypothalamus (Graber et al. (6); Buckingham (7)). Therefore we tried to prevent suppression of the HPA-axis by stimulating this process of recovery *in man*, in a similar way as we demonstrated in rats (see chapter III).

It is unclear why basal plasma cortisol levels during operation in chlorimipramine treated patients were similar as those observed after prednisone alone. When considering the cortisol levels during operation in chlorimipramine treated patients, we observe a pattern similar to that observed in prednisone treated patients. After moderately high initial levels the plasma cortisol levels fell to much lower values, and again (as found before) a nadir was reached at 09.30 hour. However, cortisol levels during operation in chlorimipramine treated patients were not significantly different from those in prednisone treated patients. In this case we could not demonstrate an enhancing effect of chlorimipramine on the HPA-system. When the cortisol levels at 08.00 hour during the administration of chlorimipramine and prednisone are compared with the plasma cortisol levels in patients treated with prednisone only, no significant increase could be found. Again the profound suppressing effect of this dose of prednisone on HPA function seems to prevent an elevation of cortisol levels in a basal state.

V.E. CONCLUSIONS

The results of this study demonstrate that chlorimipramine seems to exert some positive effects on the HPA function. Chlorimipramine enhances the responsiveness of the HPA system as measured by ACTH- and metyrapone stimulation tests, but cannot prevent all the suppressive effects of prednisone.

In basal states and during a period of stress in anesthesia and surgery, however, there was no significant rise in cortisol levels in chlorimipramine treated patients. We suggest that either lower doses of prednisone or higher doses of chlorimipramine over a more prolonged period might show such elevations. These results seem to indicate that administration of drugs, which induce a serotonin enhancing effect on the hypothalamus might result in the (partial) prevention of suppression of the HPA-axis by glucocorticoids.

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Chapter VI

General discussion and summary

The suppressive effects of prolonged administration of pharmacologic doses of glucocorticoids on the HPA function are wellknown and often represent a major problem in clinical practice: it is unknown after what duration of administration, and for what dosages of glucocorticoids precisely suppression of HPA function can be expected.

A review of the literature discloses only a few definite answers to these questions as summarized in chapter I. However, it is certain that the HPA-axis is defined by a closed loop negative feedback system. The existence of corticotrophin releasing factors (CRF's) has been proved by a large amount of "circumstantial" evidence, but the exact nature of the main compound remained uncertain, until the recent isolation and characterization of an ovine CRF, which was a polypeptide of 41 aminoacids. It seems very probably however that other compounds will be detected which also exert CRF-like activity, and that not one "universal" CRF can be found.

The evidence for the existence of a "short" loop negative feedback effect of ACTH on CRF release has been reviewed also in chapter I. Most studies appeared in the late sixties and early seventies and almost all data were obtained from rats under very special circumstances, and the physiological implications of this phenomenon remained uncertain.

The importance of neurotransmitters in the synthesis and release of CRF has been demonstrated convincingly by many authors. The existence of cholinergic and adrenergic pathways were known for a considerable time. The importance of serotonin as a stimulatory neurotransmitter gained increased attention in the last ten years.

In this study our main aim was to determine whether stimulation of neurotransmitter activity with drugs might be able to enhance the HPA function to such extent that suppression by high circulating glucocorticoid levels could be prevented.

In chapter II the model of the transplantable ACTH/PRL secreting tumor 7315a is described. This model enabled us to study the importance of a postulated "short" loop negative feedback mechanism of ACTH and CRF. We demonstrated that the high circulating corticosterone levels were the main factor in inducing a negative feedback mechanism on pituitary ACTH synthesis and

release. We could not find conclusive evidence for the existence of a "short" loop negative feedback mechanism of ACTH on CRF in this model.

In chapter III we described our efforts to influence the hypothalamo-pituitary axis in rats with different drugs with postulated serotonergic or cholinergic actions. The manipulation of the hypothalamo-pituitary axis with serotonergic drugs was much more successful than cholinergic stimulation. Especially chlorimipramine was able to prevent suppression of the pituitary ACTH content and ACTH release in the simultaneous presence of high circulating corticosterone concentrations. The effects of fenfluramine were less pronounced.

In chapter IV we investigated the effects of relatively high doses of prednisone on the HPA function in man in a prospective protocol. It was made clear that, even when prednisone was administered for such a short period as 10 days, there was an extensive suppression of the HPA-axis in hypercalcemic patients. In several patients there was biochemical evidence of adrenocortical insufficiency. During surgery the HPA response in those patients was significantly less then in a comparable control group of patients. This indicates that stressful procedures may be hazardous even after glucocorticoid administration for a short time. On the other hand the recovery of the HPA function was fast. One day after operation the adrenal response to ACTH stimulation was completely normal again.

Finally, in chapter V we tried to influence the HPA function in a manner similar to the experiments in rats, using chlorimipramine simultaneously with an identical dose of prednisone as used in the hypercalcemic patients studied in the first group. As far as we know, there are no reports in the literature in which the HPA-axis *in man* was manipulated with drugs with presumed neurotransmitter (c.q. serotonin) enhancing activity in order to prevent the suppression of HPA function by glucocorticoids. Although the results in rats were very promising, those obtained in our patients treated with both drugs were less clear. However, no cases of evident biochemical adrenocortical insufficiency were noted after chlorimipramine, as were seen in the patients treated with prednisone only. There was a significant improvement in the responsiveness of the whole HPA-axis after treatment with chlorimipramine, when evaluated with ACTH stimulation and metyrapone testing.

On the other hand the plasma cortisol levels, neither during the 10 days period of the combined drug regimen nor during surgery showed differences in comparison with those patients treated with prednisone alone. So we were not able to prevent with chlorimipramine all suppression of the HPA function caused by glucocorticoids. The most probable explanation is that the dose of glucocorticoids used, was too high to prevent suppression with this moderate dose of chlorimipramine. Lower doses of glucocorticoids in combination with higher doses of serotonin enhancing drugs as chlorimipramine and may be

fenfluramine might yield better and more promising results. This, however, has yet to be investigated. Nevertheless, the results of our experiments in rats and some of the results in man suggest that the HPA-axis can be influenced by modulating neurotransmitter activity with drugs with putative stimulating actions on the CRF synthesis and release.

Samenvatting

Het supprimerend effect op de hypothalamus-hypofyse-bijnieras van farmacologische doses glucocorticoïden is algemeen bekend, en vormt regelmatig een reëel probleem in de kliniek; in feite is niet goed bekend boven welke doseringen en na welke tijdsduur van toediening deze suppressie optreedt.

In hoofdstuk I wordt een overzicht gegeven van de literatuur die op deze vragen betrekking heeft. Het blijkt dat ook hieruit slechts weinig duidelijke antwoorden gedistilleerd kunnen worden. Zeker is wel dat de hypothalamus-hypofyse-bijnieras bepaald en gereguleerd wordt door een gesloten negatief terugkoppelingssysteem. Er is reeds lang een grote hoeveelheid bewijsmateriaal - zij het voor een belangrijk deel indirect - voorhanden, waaruit blijkt dat er stoffen bestaan die de afgifte van ACTH bevorderen. Tot voor kort was de exacte samenstelling hiervan onduidelijk maar recent werd een 'corticotropin releasing factor' (CRF) geïsoleerd uit de hypothalami van schapen. Dit bleek een polypeptide met 41 aminozuren te zijn. Het lijkt echter waarschijnlijk dat bij andere diersoorten dergelijke stoffen, met een andere aminozuur samenstelling, ontdekt zullen worden en dat er geen sprake zal blijken te zijn van één 'universeel' CRF.

In hetzelfde hoofdstuk wordt ook een overzicht gegeven van de beschikbare literatuur betreffende het veronderstelde supprimerende effect van ACTH zelf op de afgifte van CRF: het zogenaamde 'short' loop negative feedback effect. Deze studies verschenen vooral in de jaren zestig en in het begin van de jaren zeventig, en bijna alle gegevens werden verkregen uit experimenten met ratten onder zeer bijzondere omstandigheden. Het blijft de vraag in hoeverre dit verschijnsel ook in fysiologische omstandigheden van belang is.

Het belang van neurotransmitters voor de synthese en afgifte van CRF is door vele auteurs overtuigend aangegeven. Dat cholinergische en adrenergische neuronen hierbij een rol spelen is reeds vrij lang bekend.

Serotonine heeft de laatste jaren in toenemende mate de aandacht getrokken als een neurotransmitter die een stimulerend effect op CRF kon hebben.

In dit onderzoek hebben wij ons als belangrijke vraag gesteld of het mogelijk zou zijn, met behulp van bestaande geneesmiddelen, die neurotransmitter activiteit zodanig te stimuleren, dat het supprimerend effect van hoge doses glucocorticoïden op de functie van de hypothalamus-hypofyse-bijnieras geheel zou worden voorkomen.

In hoofdstuk II wordt het model van de transplanteerbare, ACTH en prolactine producerende, tumor 7315a beschreven. Met gebruik van dit model bij ratten was het mogelijk, het belang van het reeds eerder genoemde 'short' loop negative feedback mechanisme na te gaan. Wij hebben aangetoond dat in dit model de hoge glucocorticoïd concentratie in de circulatie de belangrijkste factor was die de negatieve terugkoppeling op de synthese en afgifte van ACTH bewerkstelligde. In dit model althans, hebben wij geen duidelijk bewijs kunnen vinden voor het bestaan van een terugkoppelingssysteem, waarin ACTH zelf een negatief effect heeft op de synthese en afgifte van CRF.

In hoofdstuk III worden de experimenten bij ratten beschreven waarin gepoogd werd de hypothalamus-hypofyseas te stimuleren met behulp van stoffen met veronderstelde serotonergische en cholinergische effecten. Het manipuleren van de hypothalamus-hypofyseas slaagde met serotonergische stoffen aanzienlijk beter dan met cholinergische. Met name chlorimipramine (Anafranil®) was in staat de suppressie van zowel de hoeveelheid ACTH in de hypofyse voorkwab, als de afgifte van ACTH aan de circulatie te voorkomen, in aanwezigheid van een hoge concentratie corticosteron. Het positief effect van fenfluramine (Ponderal®) was minder uitgesproken.

In hoofdstuk IV hebben wij in een prospectieve studie bij de mens het effect nagegaan van relatief hoge doses prednison op het functioneren van de hypothalamus-hypofyse-bijnieras. Het is duidelijk geworden dat prednison, zelfs wanneer dit slechts gedurende 10 dagen gegeven wordt, een duidelijke suppressie van de functie van deze as bewerkstelligt. Bij enkele patienten kon het bestaan van een bijnierinsufficiëntie op biochemisch niveau worden aangetoond. De met prednison behandelde patienten hadden tijdens een halsexploratie significant lagere cortisolwaarden dan een vergelijkbare groep controle patienten.

Dit kan er op wijzen dat procedures die gepaard gaan met stress een potentiëel gevaar opleveren, ook wanneer glucocorticoïden slechts gedurende een korte tijd toegediend worden. Anderzijds is gebleken dat het herstel van de functie van de hypothalamus-hypofyse-bijnieras ook weer snel intreedt: één dag na de operatie was de respons van de bijnier op stimulatie met ACTH weer geheel normaal.

In hoofdstuk V tenslotte, worden de pogingen beschreven de functie van de hypothalamus-hypofyse-bijnieras te stimuleren door gebruik te maken van gelijktijdige toediening van prednison en chlorimipramine, analoog aan de desbetreffende experimenten in de rat. Voor zover ons bekend zijn er in de literatuur geen studies verschenen waarin getracht is de hypothalamus-hypofyse-bijnieras te stimuleren door gebruik te maken van stoffen met neurotransmitter activiteit (met name serotonergische activiteit), teneinde suppressie van de as door glucocorticoïden te voorkomen.

De veelbelovende resultaten, verkregen bij de dierexperimenten, zijn bij de

patienten die beide middelen toegediend kregen niet geheel geëvenaard. Enerzijds werden er na toediening van chlorimipramine en prednison op biochemisch niveau geen tekenen meer gevonden van bijnierinsufficiëntie, zoals eerder bij de patienten die alleen met prednison behandeld werden. Bovendien was er een significant beter reactievermogen van de hypothalamus-hypofyse-bijnieras na toediening van de chlorimipramine, wanneer dat geëvalueerd werd met behulp van ACTH- en metopiron stimulatie testen. Anderzijds waren de plasma cortisolwaarden, noch tijdens de periode van 10 dagen waarin beide farmaca gelijktijdig werden toegediend, noch tijdens de operaties verschillend van de cortisolwaarden bij patienten die alleen prednison toegediend kregen. Wij zijn dus niet in staat geweest alle supprimerende effecten van glucocorticoïden te voorkomen.

De meest voor de hand liggende verklaring is dat de dosis glucocorticoïden toch te hoog is om elke vorm van suppressie met behulp van deze dosis chlorimipramine te voorkomen.

Het is goed mogelijk dat het toedienen van glucocorticoïden in lagere doseringen, in combinatie met hogere doseringen van stoffen met serotonergische eigenschappen zoals chlorimipramine en wellicht ook fenfluramine, betere resultaten oplevert. Niettemin suggereren onze studies bij de rat en de mens dat het mogelijk is de hypothalamus-hypofyse-bijnieras te beïnvloeden door gebruik te maken van stoffen die door hun neurotransmitter activiteit de synthese en afgifte van CRF kunnen stimuleren.

Verantwoording

Het schrijven van een proefschrift doet de aandacht te eenzijdig uitgaan naar de schrijver. Pas in de verantwoording, achterin opgenomen, blijkt hoezeer de auteur dank verschuldigd is aan al diegenen die het onderzoek, het beschikken over de talloze laboratoriumfaciliteiten en het verrichten van de vele laboratoriumbepalingen mogelijk gemaakt hebben.

Zo ook in mijn geval. Dank uiteraard, in de eerste plaats aan mijn promotor, Steven Lamberts die de stuwende kracht achter de totstandkoming van dit werk is geweest, en met zijn ongebreidelde energie, aanstekelijk enthousiasme en 'positief' denken mij door de dieptepunten, zoals die in elk onderzoek voorkomen, heeft geleid. De vele uren die wij samen doorbrachten discussiërend over meer dan alleen dit proefschrift zijn voor mij van grote betekenis geweest. Mijn tweede promotor, Prof. Dr J.C. Birkenhäger, die steeds met grote belangstelling en zijn vermaarde kritische instelling de totstandkoming van dit boekje begeleid heeft, ben ik bovenal dank verschuldigd voor de voortreffelijke en gevarieerde wijze waarop hij als mijn opleider en leermeester de vorming van een assistent ter hand genomen heeft. Zijn encyclopedische belangstelling maakt hem tot een - onbereikbaar - voorbeeld voor anderen.

Mijn eerste wankele stappen op het pad van de ACTH-bepaling werden met altijd hulpvaardige, en soms kritische belangstelling gadegeslagen door de medewerkers van het laboratorium en de 4 Zuid zijvleugel: Ellen Bons, die mij alle laboratoriumwerk met veel noodzakelijk geduld geleerd heeft, Piet Uitterlinden, Rob Oosterom, Theo Verleun, Joke Zuiderwijk en Ronald Lammers die altijd bereid waren tot hulp bij de vele dierexperimenten. Aan hen allen zeer veel dank. Uiteraard ook aan Dr G.H. Mulder die zo goed was ons de door hem ontwikkelde ACTH-bioassay ter beschikking te stellen. De zeer bewerkelijke procedures nodig voor de vele metopiron- en ACTH-stimulatietesten werden op voortreffelijke wijze verricht op de afdeling endocrinologie, 4 Noord, door Zr Paula Schuyff en de haren, waarbij met name Zr Jean Heuff en Zr Cis Baarschens genoemd moeten worden. De vermaarde organisatie van Zr Schuyff stond garant voor een perfecte afwikkeling van het gecompliceerde protocol.

Dr Frank de Jong en zijn laboratoriumwerkers waren telkens bereid de honderden cortisol- en desoxycortisolmonsters met voorrang te bepalen. Ik

ben Dr de Jong bovendien dank verschuldigd voor zijn gewaardeerd commentaar. Dr H.A. Bruining trad steeds als 'vaste' chirurg op en omzeilde enthousiast alle bestaande wachtlijsten om aan de regels van het protocol voor wat betreft de operaties te kunnen voldoen. Ook zijn spontane medewerking en belangstelling heb ik op zijn grote waarde geschat.

Bas van Ouwerkerk en Rini Baggen waren in 1983 tijdens de operaties aanwezig om de cortisolmonsters af te nemen. Laatstgenoemde was mij bovendien frequent op zaterdag en zondagochtend behulpzaam bij de injecties in de ratten, zij het in eerste instantie met gemengde gevoelens ten aanzien van de ratten.

Mijn collegae in de maatschap interne van het Diakonessenhuis Refaja vingen mijn afwezigheid waar nodig op en droegen bovendien in 10 maanden tijd liefst 3 van de 6 patienten met primaire hyperparathyreoidie aan, die in het onderzoeksprotocol konden worden opgenomen.

Anke de Graaff verrichtte op zeer nauwgezette en voortreffelijke wijze al het vele typewerk voor dit proefschrift; een zwaar en tijdrovend werk waarvoor mijn dank niet groot genoeg kan zijn.

Tot slot, maar niet als minste, dankbaarheid ten aanzien van mijn moeder, die met mijn vader mogelijk gemaakt heeft, wat heden bereikt wordt.

Aan mijn vader is niettemin dit proefschrift opgedragen.

Curriculum vitae

De schrijver dezes werd in 1951 in Rotterdam geboren. Aan het Gymnasium Erasmianum te Rotterdam werd in 1969 het eindexamen gymnasium β afgelegd. De studie medicijnen werd in 1969 aangevangen aan de Rijksuniversiteit te Utrecht. In juni 1972 volgde het candidaatsexamen, terwijl in februari 1975 het doctoraal examen werd afgelegd. Het artsexamen werd op 24 maart 1976 behaald. Daarop volgde van maart 1976 tot oktober 1977 de dienstplicht bij de Koninklijke Marine. Op 1 januari 1978 werd de opleiding interne geneeskunde aangevangen in het Majella Ziekenhuis te Bussum (opleider Dr D. Maingay). Vanaf januari 1979 tot en met december 1982 werd de opleiding op de afdeling interne geneeskunde III van het Academisch Ziekenhuis Dijkzigt te Rotterdam (opleider Prof. Dr J.C. Birkenhäger) voortgezet en afgerond. Dit proefschrift werd op dezelfde afdeling bewerkt.

Sinds 1 januari 1983 is de schrijver als internist verbonden aan het Diakonessenhuis Refaja te Dordrecht.