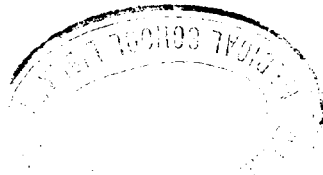


The effect of certain drugs and hormones on cortisol and  
cortisol binding globulin levels in human plasma

by

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A thesis submitted for the degree of Doctor of Philosophy in  
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ABSTRACT

The effect of certain drugs and hormones on plasma cortisol and cortisol-binding globulin (CBG) has been investigated. Total plasma cortisol was measured fluorimetrically and by RIA. CBG, as the cortisol-binding capacity (CBC) was measured by competitive adsorption and by a new two-tier column method. Free plasma cortisol was measured using a specially developed dialysis method.

Patients receiving hormone treatment for rheumatoid arthritis were studied, and because all were receiving salicylates in addition to hormones, a preliminary trial of the effect of salicylates alone on cortisol and CBG was carried out. This demonstrated no consistent effect of salicylates. Hormone treatment was either with ACTH gel, Zn-tetracosactrin or prednisolone. These had the expected effect on plasma cortisol, the long-acting form of ACTH causing a rise and prednisolone a fall. No significant effect on CBG was seen during therapy with either type of ACTH, but prednisolone significantly reduced the levels.

Plasma total and free cortisol and CBG levels were measured hourly for twenty-four hours in two volunteers. Total and free cortisol varied as expected. CBG levels also showed some variation during this period. Fluctuations in total and free cortisol levels were shown to occur within 15 minutes during saline and hydrocortisone infusions in two volunteers. Cortisol levels increased in response to hydrocortisone infusion and had returned to normal 30 minutes after stopping the hydrocortisone.

Cortisol and CBG levels were measured in the plasma of women throughout the menstrual cycle, and the effect of "low oestrogen" oral contraceptives and oestrogen-free oral contraceptives was studied. No regular pattern of either total cortisol or CBG was seen during the menstrual cycle and no effect on the levels of either was caused by the oestrogen-free contraceptives, the mean ratio of cortisol/CBC being below unity. Low oestrogen contraceptives produced a marked rise in both total cortisol and CBG throughout the cycle, and the cortisol/CBC ratio rose to above unity. This suggested that the drug was causing a rise in free cortisol and not in the protein-bound fraction alone.

In order to confirm this, a method for measuring free cortisol, using dialysis, was developed. It was shown that women taking low oestrogen contraceptives had a free cortisol about twice that of control women. The possibility that the free cortisol was not consistently raised was investigated by sampling through the day and at various times in the menstrual cycle. Free cortisol together with total cortisol and CBG were consistently raised during these times. There was no difference however in urinary cortisol between the pill and control groups.

CBG was measured in normal men and women and in women having severe premenstrual syndrome using the two-tier column method. CBG levels were low in the women having premenstrual syndrome.

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## ABBREVIATIONS

|      |                                     |
|------|-------------------------------------|
| ACTH | Adrenocorticotrophic hormone        |
| CPB  | Competitive protein binding         |
| CBC  | Cortisol binding capacity           |
| CBG  | Cortisol binding globulin           |
| CRH  | Corticotrophin-releasing hormone    |
| FSH  | Follicle stimulating hormone        |
| HPA  | Hypothalamic-pituitary-adrenal axis |
| LH   | Luteinising hormone                 |
| PMS  | Premenstrual syndrome               |
| RIA  | Radioimmunoassay                    |
| RA   | Rheumatoid arthritis                |
| SD   | Standard deviation                  |
| SHBG | Sex-hormone binding globulin        |
| TBG  | Thyroxine binding globulin          |
| TLG  | Thin-layer gel                      |
| UFC  | Urinary free cortisol               |

## INTRODUCTION

## Chapter 1: Cortisol

### Introduction

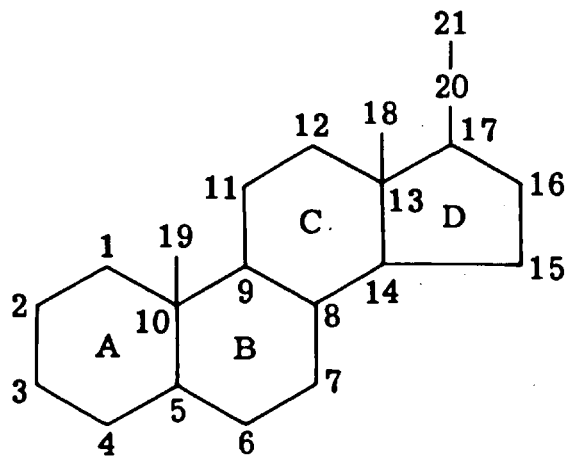
Metabolism in man is controlled through the action of hormones, most of which are secreted by endocrine glands and which influence the rate of many reactions and systems of reactions in the body. Hormones act on tissues distant from their site of synthesis, and the rates of synthesis, degradation, secretion and transport of the hormones are themselves under metabolic control, to ensure proper integration of the various aspects of metabolism. An important group of hormones secreted by glands derived from the embryological mesoderm are steroidal in structure and of these several are secreted by the adrenal cortex. In fact, over forty steroids have now been isolated from the human adrenal cortex: not all are active hormones, many are intermediates or precursors, and some are only detectable in minute quantities. Cortisol and aldosterone are the most important of the corticosteroids in man, and are essential to maintain good health.

The corticosteroids may be divided into the glucocorticoids primarily cortisol, which have an important regulatory effect on intermediary metabolism and also have anti-inflammatory activity, and the mineralocorticoids such as aldosterone, noted for their sodium-retaining activity.

### 1. Structure

Cortisol was first isolated and described as "Substance M" by Reichstein (1937) and by Mason et al (1938) who called it "Compound F". The constitution and configuration were established by Reichstein (1937), Mason et al (1938) and further by Von Euw and Reichstein in 1942. It was identified as the adrenal cortical hormone by Reich et al (1950). Cortisol was first isolated in human blood together with corticosterone by Bush and Sandberg in 1953 and isolated in crystalline form by Romanoff et al (1953) from human adrenal vein blood.

Cortisol belongs to the pregnane series of steroids in which there is a two carbon side chain attached to carbon 17 of the steroid nucleus thus giving a 21 carbon molecule. The structure and numbering of the carbon atoms of cortisol are shown in Figure 1. An 11-oxygen function



CORTISOL

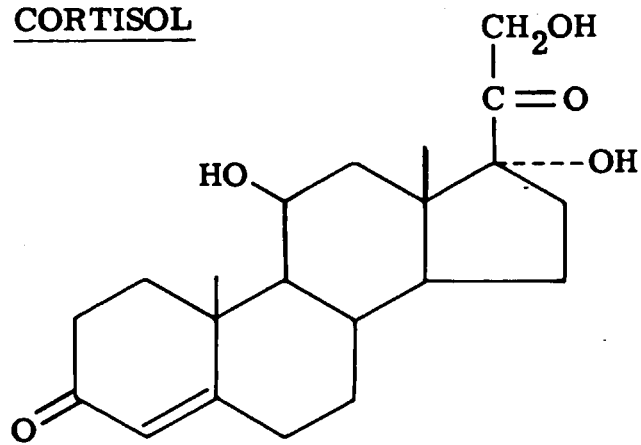


Fig. 1 : The structure and numbering of the carbon skeleton of cortisol.

at C<sub>11</sub> in the  $\beta$  position is required for glucocorticoid activity. The hydroxyl group at C<sub>21</sub> and the carbonyl group at C<sub>20</sub> are also necessary for full glucocorticoid activity (Rosner, 1969).

## 2. Synthesis

Cortisol is synthesised in the zona reticularis and the zona fasciculata of the adrenal cortex. Cholesterol, which is abundantly available, is the major precursor of steroid biosynthesis in the adrenal. The main outlines of the synthetic pathways are shown in Figure 2 (review: Cope, 1972).  $\Delta^5$ -pregnenolone is formed from cholesterol, which then acts as a precursor of all types of adrenal corticosteroids. The formation of pregnenolone is thought to be under the control of adrenocorticotrophic hormone, ACTH (Stone and Hechter, 1954). It has recently been suggested that ACTH may also affect late stages of cortisol synthesis (McKenna et al, 1979). Numerous studies have established that pregnenolone undergoes a series of enzymatically controlled hydroxylations at the 17 $\alpha$ , 21 and 11 $\beta$  positions to give respectively 17 $\alpha$ -hydroxypregnenolone then, utilising 3 $\beta$  hydroxysteroid dehydrogenase, 17 $\alpha$ -hydroxyprogesterone, 11-desoxycortisol and cortisol itself.

There is some evidence that there may be alternative pathways using for example 17 $\alpha$ -hydroxypregnenolone as a precursor of cortisol (Lipsett and Hockfelt, 1961; Mulrow et al, 1962; Welicky and Engel, 1962).

## 3. Metabolism

The biological half-life of cortisol in plasma is between 60 and 120 minutes, the main site of metabolic inactivation being the liver. The major process of inactivation is the enzymatic reduction of ring A to form tetrahydrocortisol, and oxidation of the 11-hydroxyl to the 11-ketone to yield tetrahydrocortisone. Further reduction of these compounds at C<sub>20</sub> gives respectively cortol and cortolone (see Fig. 3). These cortisol metabolites are conjugated mostly with glucuronic acid to produce water soluble products which are readily excreted by the kidneys. The amount of unmetabolised cortisol excreted in the urine is dependent on the concentration of plasma cortisol, the degree of protein binding, the glomerular filtration rate and the rate of tubular reabsorption (review: Cope, 1972).

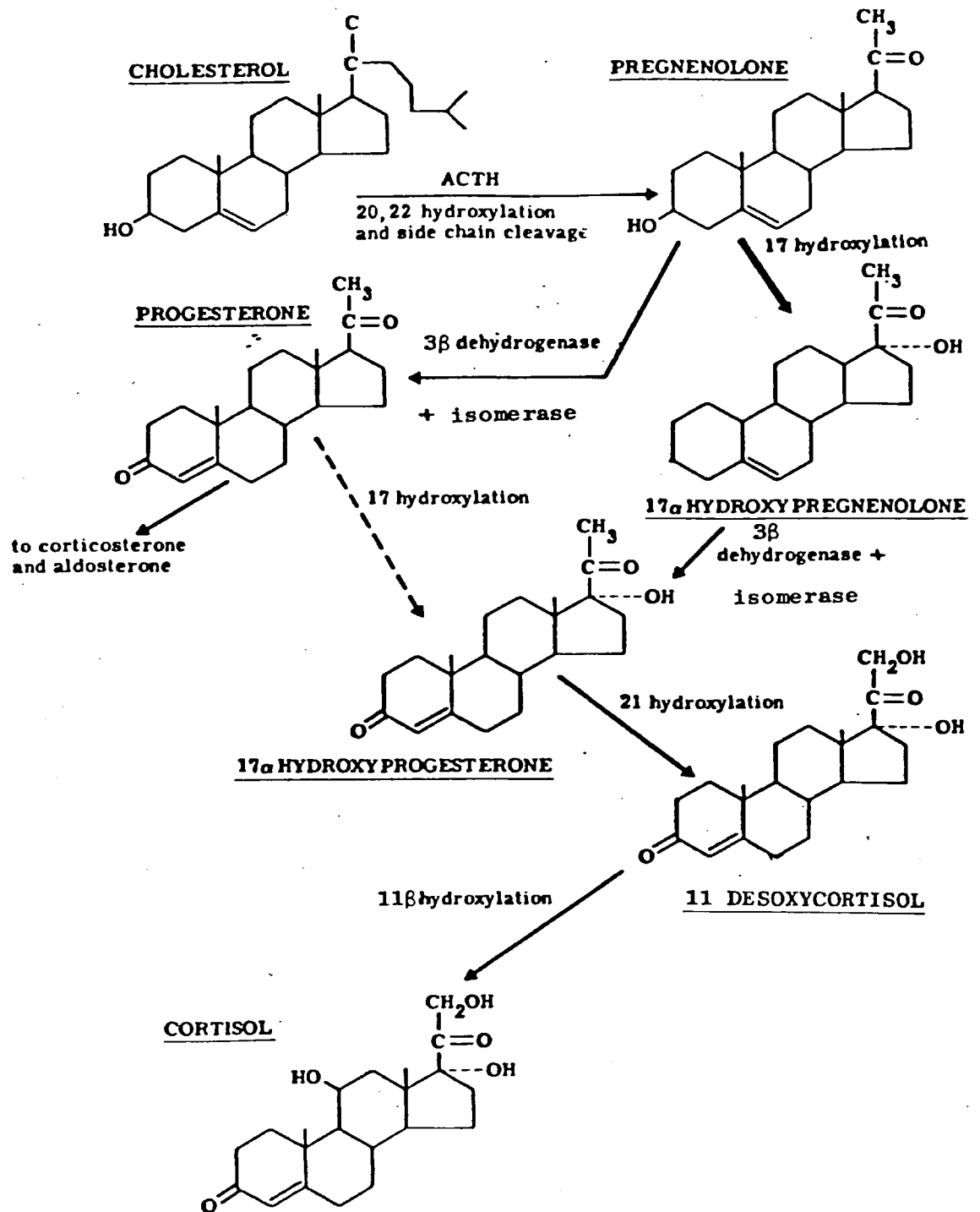


Fig. 2 : Biosynthesis of cortisol.

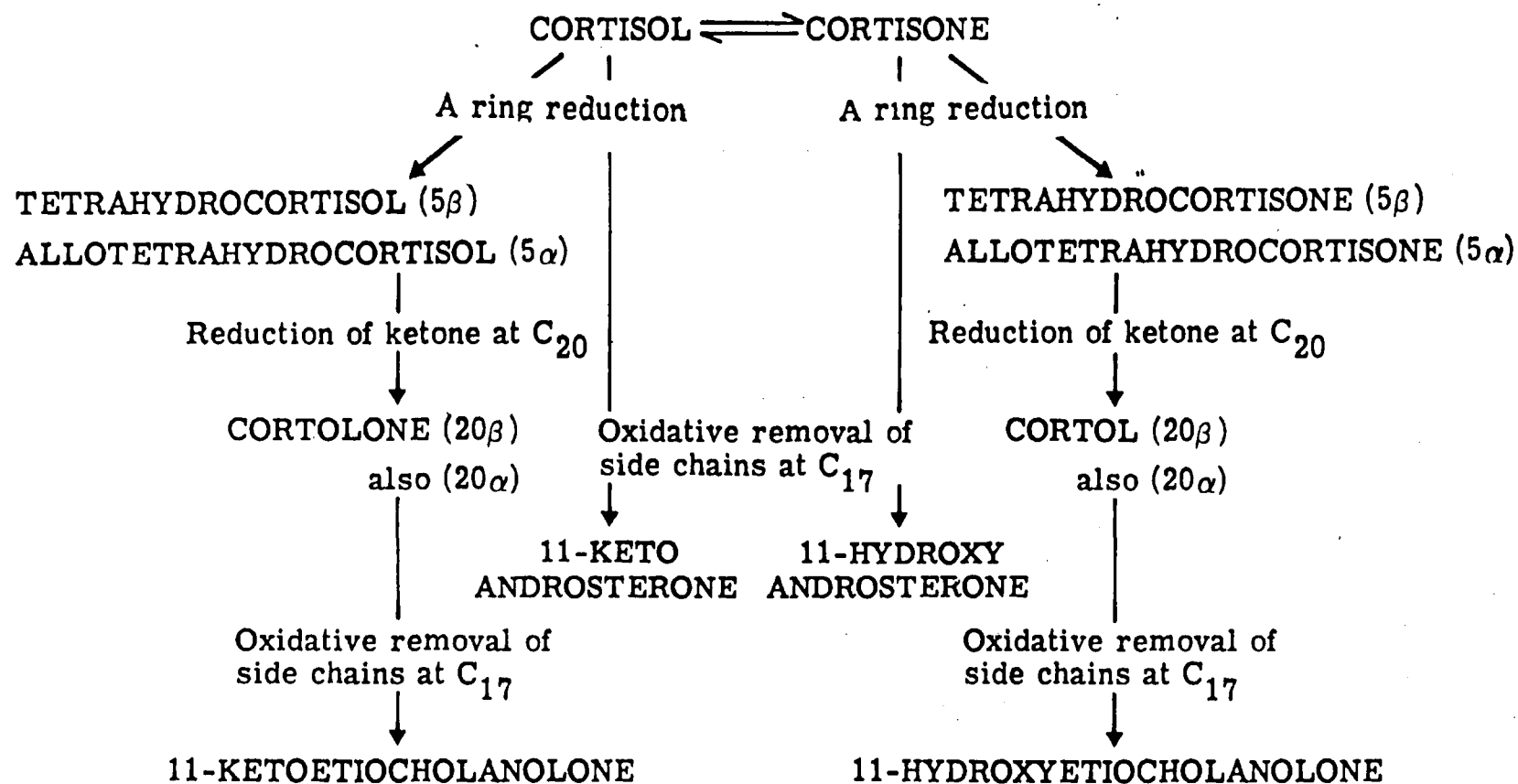


Fig. 3 : Primary excretion products of cortisol.

#### 4. Action at the cellular level

Current ideas on the action of cortisol in target cells are based on the hormone-gene hypothesis first put forward by Karlson (1963). Evidence supporting this view for the action of cortisol has been reviewed by, for example, King and Mainwaring (1974) and Baulieu (1975).

Cortisol enters the target cells, possibly by a process of simple diffusion. Once in the cell a cytoplasmic complex is formed by the high affinity binding of cortisol to receptor protein. Binding to the specific hormone receptors is dependent on the presence of the  $11\beta$  - hydroxyl group, characteristic of most biologically active glucocorticoids. There is a firm correlation between the extent of binding of the glucocorticoids to their receptors and their biochemical response (Munck and Brinck-Johnson (1968). The formation of this initial hormone-receptor complex is followed by the transfer of the entire complex to the nuclear chromatin, ultimately to DNA. The stimulation of genetic transcription and of nuclear RNA synthesis gives rise to an increased rate of protein synthesis, the end product being the formation of enzymes or other specific proteins. The response is reversible on withdrawal of the steroid from the intact cell, and it may be repressed by the addition of steroids with anti-glucocorticoid activity, for example 11-deoxy- $17\alpha$  -hydroxycorticosterone (Kattwinkel and Munck, 1966).

There is no obligatory involvement of RNA synthesis however - the deposition of glycogen (Ray et al , 1964) and the induction of tyrosine aminotransferase in liver (Leviton and Webb, 1969) can proceed to a limited extent in the absence of RNA synthesis.

#### 5. Physiological function

Cortisol provides the major glucocorticoid activity and also contributes significantly to mineralocorticoid activity in man. In contrast, the very small amount of aldosterone secreted by the adrenal has negligible glucocorticoid activity, but is the most active

mineralocorticoid. Corticosterone has both glucocorticoid and mineralocorticoid effects, but is present in less than one tenth the concentration of cortisol.

One of the earliest indications of biological activity of the glucocorticoids was the stimulation of gluconeogenesis from amino acid and carbohydrate precursors in liver slices after administration of the steroid in vivo (Long et al , 1940; Koepf et al , 1941). In addition to the stimulation of gluconeogenesis, cortisol has been found to have many varied effects on metabolism. (These have been reviewed by, for example, Hall et al , 1974). Cortisol is involved in the regulation of protein, lipid, carbohydrate and nucleic acid metabolism. There is a decrease in the peripheral uptake of glucose and an increase in the deposition of glycogen in the liver. Cortisol also controls the distribution of water between the intracellular and extracellular fluid and maintains normal glomerular filtration. Together with aldosterone, cortisol promotes the retention of sodium and loss of potassium from cells.

The relationship between these physiological actions and the biochemical properties of cortisol is uncertain. The overall effect is catabolic, but it is unknown whether the various responses of the different tissues to cortisol are based on a common mechanism or on a different mechanism for each tissue. However, studies from a wide range of cell types indicate that protein synthesis seems to be the major cellular event (review: King and Mainwaring, 1974). Cortisol is known to enhance the activity of many enzymes by a process of enzyme induction, particularly in the liver (Lin and Knox, 1957). The ability of cortisol to stabilise lysosomal membranes has been suggested to account for the anti-inflammatory effect of cortisol (Pollock and Brown, 1971).

## 6. Regulation of secretion

The production of cortisol by the adrenal is controlled by ACTH. ACTH release is controlled by corticotrophin-releasing factor (CRF)

which is synthesised in the hypothalamus and transported to the anterior pituitary via the portal vascular system (De Groot and Harris, 1950). This chain of regulatory hormones is termed the hypothalamic-pituitary-adrenal (HPA) axis. ACTH is discussed further in Chapter 2. There are three main physiological mechanisms that control the secretion of CRF and thus ACTH and cortisol: the nyctohemeral rhythm, negative feedback mechanism and the response to stress.

a. Nyctohemeral rhythm

The level of cortisol is not constant through the day, but has a regular periodicity with peak levels in the early morning (0600-0800 hr), falling to the lowest levels at around midnight. The variations in level are due to alterations in secretion rate by the adrenal, produced by corresponding variations in ACTH and CRF secretion. These changes are related to the sleep-wake cycle and are not acutely altered by sleeping habits although they will adapt after approximately one week (Orth et al., 1967). This mechanism is under hypothalamic control. The secretion of ACTH is not continuous through the day, but is episodic. Variation in the amplitude and frequency of these episodes gives rise to the nyctohemeral rhythm. (Hellman et al., 1970; Krieger et al., 1971; Weitzman et al., 1971).

Loss of this rhythm may occur in various pathological states, notably in Cushing's syndrome and may be modified or abolished with both natural and synthetic glucocorticoids (Ceresa et al., 1970).

b. Negative feedback mechanism

i. Long feedback control. There is a reciprocal as well as a direct relationship between ACTH secretion and corticosteroid levels, such that ACTH production is increased when cortisol levels are inappropriately low, and conversely, decreased when cortisol levels are inappropriately high. The non-protein bound fraction of cortisol is thought to operate this system, the receptors apparently lying within the hypothalamus, although the anterior pituitary is now thought to be the major feedback site (Kendall et al., 1972;

Jones et al, 1972, 1974; Dallman and Yates, 1969). Recently, this mechanism has been described in the rat as consisting of two components: immediate feedback inhibition, "fast feedback", followed by a silent period that precedes the "slow feedback" component (Jones, 1976).

ii. Short feedback control. A similar mechanism may also occur between ACTH and CRF - the short-loop feedback (Motta et al, 1969).

c. Response to stress

This is the most powerful of the three control mechanisms, causing a sharp rise in ACTH and cortisol levels irrespective of the time of day or existing level of hormones. The stress response occurs with a wide variety of stimuli: emotional, metabolic or external; and although its purpose is little understood it is vital - lack of this response, due, for example, to adrenocortical insufficiency, may result in collapse in circulatory failure on exposure to severe stress.

7. Protein binding of cortisol in the blood

Introduction

Cortisol is secreted in the free form, but normally in the plasma up to 90% of cortisol is associated with protein, this portion being in equilibrium with the free or non-protein bound fraction.

Daughaday (1958), Slaunwhite and Sandberg (1959) and Bush (1957) demonstrated the existence of at least two types of cortisol binding proteins present in plasma: a high affinity, low capacity protein called 'transcortin' by Sandberg & Slaunwhite, and 'cortisol binding globulin' by Daughaday - due to its association with the globulin fraction on electrophoresis, and a low affinity, high capacity protein, albumin.

a. Albumin

Albumin binds all steroid hormones, but has the greatest affinity for the oestrogens, less for progesterone and testosterone, and least for cortisol, and appears to have little importance in binding cortisol in physiological conditions.

The studies of Eik-Nes et al (1954) led to the formation of the polarity rule to explain the gradation in binding of structurally related steroids to albumin, i.e. the binding affinity between a protein and a steroid decreases with an increasing number of hydrophilic or polar groups. There is relatively little interference between the binding of different steroids to albumin and there may be different binding sites for each steroid. The  $\Delta^4$ -3-keto configuration in ring A is important in the binding of the adrenal hormones to albumin and the introduction of a hydroxyl group at C<sub>11</sub> will interfere with binding (Cope, 1972). Albumin binds cortisol with low affinity, i.e. approximately four times lower than high affinity sites such as those on cortisol binding globulin. There is a change in association constant with varying ratios of steroid to albumin (Brunkhorst and Hess, 1965) from one to up to 20 sites per molecule. The approximate free energy change is low - 21-25kJM<sup>-1</sup>, an indication of the formation of relatively weak protein-steroid bonds (Sandberg et al, 1957; Westphal et al, 1958).

#### b. Cortisol binding globulin

Cortisol binding globulin (CBG) is the most important glucocorticoid binding protein, and has been found in all vertebrates studied (Seal and Doe, 1966). The concentration ranges between 433-883 nmol cortisol bd./l. as shown in Table 1. This is sufficient to bind cortisol completely in peripheral blood in normal subjects. The chemistry of CBG has been reviewed by, for example, Westphal (1971). It is an  $\alpha_1$  glycoprotein with a molecular weight of around 52,000. It has been isolated, purified, and the amino acid composition determined for several species (Muldoon and Westphal, 1967). Carbohydrates account for between 26 and 29% of the molecule and the amino acid composition varies somewhat particularly in regard to the sulphur-containing amino acids cysteine and methionine. Sialic acid is present as in most glycoproteins, although its removal is not accompanied by any loss of binding capacity. Portions of the amino acid sequence in CBG responsible for its antigenic determinants must contain species differences however, because antibodies raised against purified proteins do not cross-react. Westphal (1970) has reported reversible polymerisation of the rat CBG molecule. On removal of corticosteroids from the CBG complex, the protein aggregated and diverse, tetrameric and octameric forms were recognised. This pattern reversed on addition of corticosteroid.

TABLE 1

Concentration of cortisol binding globulin in normal plasma

| Method                   | Temperature (°C) | n   | CBG (nmol cortisol/l) | Reference                    |
|--------------------------|------------------|-----|-----------------------|------------------------------|
| Gel exchange             | 22               | 30  | 646 $\pm$ 124         | Frajira <u>et al.</u> , 1976 |
| Equilibrium dialysis     | 9                | 12M | 883 $\pm$ 152         | Murphy <u>et al.</u> 1963    |
|                          | 9                | 12F | 856 $\pm$ 124         | "                            |
| Electrophoresis          | 22               | 26  | 442 $\pm$ 110         | Osorio & Schats, 1964        |
|                          | 4                | 9   | 629 $\pm$ 99          | "                            |
| Gel filtration           | 4                | 19M | 662 $\pm$ 68          | Doe <u>et al.</u> 1964       |
|                          | 4                | 16F | 624 $\pm$ 79          | "                            |
| Gel filtration           | 22               | 83M | 713 $\pm$ 28          | DeMoor, 1962                 |
|                          | 22               | 32F | 720 $\pm$ 33          | "                            |
| Gel filtration           | 4                | 35  | 662 $\pm$ 221         | Burger <u>et al.</u> 1970    |
| Fluorimetry              | 22               | 22  | 433 $\pm$ 36          | Smith & Muehlbacher, 1969    |
| Adsorption               | 4                | 36  | 547                   | Burger <u>et al.</u> 1970    |
| Adsorption               | 4                | 15M | 513 $\pm$ 52          | Trapp & West, 1969           |
| Adsorption               | 4                | 52F | 442 $\pm$ 52          | Moore et al, 1978            |
|                          |                  | 46M | 428 $\pm$ 83          |                              |
| Filtration using biogels | 22               | -   | 580 $\pm$ 58          | Golikov, 1969                |

CBG binds cortisol and corticosterone with high affinity as well as 17-hydroxyprogesterone, progesterone and the synthetic glucocorticoid, prednisolone (Daughaday, 1958). For this steroid binding to occur the  $\Delta^4$ -3-ketone complex in ring A is essential and affinity for CBG is increased by the presence of hydroxyl groups in the 11 $\beta$ , 17 $\alpha$  and 21 positions (Rosner, 1969).

The association constant of CBG for cortisol decreases with increasing temperature for example, at 37 $^{\circ}$ ,  $K_a = 0.3 \times 10^8 \text{ M}^{-1}$  and at 4 $^{\circ}$ ,  $K_a = 6 \times 10^8 \text{ M}^{-1}$  (Westphal, 1967). CBG binding is destroyed by heating to 60 $^{\circ}$ C for 20 mins (Daughaday et al, 1962) or by boiling for 5 minutes (Murphy et al, 1963) although under these conditions binding to albumin still occurs. This strong temperature dependence of association of cortisol with CBG is in marked contrast to albumin binding which shows only a slight temperature influence (Klotz and Urquhart, 1949). Association of the cortisol - CBG complex is a rapid process at 4 $^{\circ}$ C, being complete in minutes, and virtually instantaneous at 37 $^{\circ}$  (Rosner et al, 1973). Dissociation is however slow; the half dissociation times were found by Rosner to be 5 minutes at 4 $^{\circ}$ C, and about 5 seconds at 37 $^{\circ}$ C. The binding affinity between corticosteroid hormones and CBG is dependent on the hydrogen ion concentration. A maximal value has been observed at approximately pH 8 (De Moor et al, 1963); at pH 5 and below, irreversible denaturation appears to take place with loss of binding affinity. CBG is produced by the liver and its biosynthesis may be stimulated by thyroxine (Labrie et al, 1965, 1968).

Normal concentrations of CBG have been found in adrenal insufficiency, Cushing's syndrome, hypo- and hyperthyroidism, hypopituitarism and acromegaly. A low CBG level may be found in Cushing's syndrome and in those conditions where albumin concentration is decreased, such as cirrhosis and nephrosis (Rosner, 1969). Pregnancy and oestrogen treatment result in marked elevation of CBG (Sandberg and Slaunwhite, 1959; Seal and Doe, 1967); this is discussed further in Chapter 2.

c. The distribution of cortisol in plasma

The reversible nature of the binding of cortisol to albumin and CBG is regulated by a binding equilibrium according to the Law of Mass Action, and the relative amounts of cortisol bound to albumin, CBG and the free cortisol may be calculated:

$$C = \frac{CP_c + CP_a}{K_c (M_c - CP_c) + K_a (M_a - CP_a)}$$

where C = concentration of unbound cortisol

CP<sub>c</sub> = concentration of CBG-bound cortisol

CP<sub>a</sub> = concentration of albumin-bound cortisol

K<sub>c</sub> = apparent association constant for CBG

K<sub>a</sub> = apparent association constant for albumin

M<sub>c</sub> = total concentration of CBG binding sites

M<sub>a</sub> = total concentration of albumin binding sites

This equation is based on the assumption that free cortisol is in reversible equilibrium with each of the two binding proteins and that equilibrium can be described by a single apparent association constant (Sandberg et al, 1966).

Under normal circumstances most of the binding sites on CBG are occupied and only a small fraction of cortisol is unbound. As the cortisol concentration rises the increase in binding is largely due to albumin, and as a consequence of the lower affinity binding a relatively larger proportion of the total cortisol is not protein-bound, i.e. it is physiologically active (Mills, 1961).

d. The physiological role of the binding proteins

The physiological role of the binding proteins is uncertain. The ubiquitous occurrence of CBG in all species studied (Seal and Doe, 1966) would seem to suggest some essential function in the control of vertebrate metabolism in the regulation of corticosteroid activity.

At physiological concentrations the presence of protein is not necessary to solubilise plasma steroids. Beisel (1964) has shown that the rate of disappearance of free cortisol from plasma is twice

as rapid as that of the disappearance of bound cortisol – CBG having a seemingly protective role against reduction of cortisol by red cell and liver metabolising enzymes. Cortisol bound to protein will not be readily filtered by the glomerulus into the urine, unlike free cortisol.

It is accepted that binding proteins act as a reservoir of physiologically inactive cortisol (Labrie et al, 1965) and/or act as a buffer system for unbound steroid, allowing cortisol levels to be closely and rapidly adjusted. Keller et al (1969) suggested that CBG may increase the sensitivity of the adrenocortical system by having a different effect on different target tissues.

## 8. Disorders of Cortisol Production

### Introduction

Adrenocortical dysfunction can be seen as Cushing's syndrome, Addison's disease, adrenogenital syndrome, Conn's syndrome (primary aldosteronism) and feminising and virilising syndrome. The first three syndromes are concerned with alterations in circulating levels of cortisol.

#### a. Cushing's syndrome

Cushing's syndrome, first described by Harvey Cushing in 1932, results from prolonged elevation of the circulating cortisol level.

Cushing's syndrome affects women four times more commonly than men, and most often occurs between the ages of 35 and 50 years. The major clinical signs are proximal obesity, purple abdominal striae, spontaneous bruising, hypertension, osteoporosis and spontaneous fractures with amenorrhoea and hirsutism in women. The syndrome may be divided into two main groups, depending on whether or not the condition derives from exposure to excess ACTH. The most common cause, about 80% of cases, is pituitary-dependent Cushing's disease, with another approximately 10% of cases due to adrenocortical tumours, adenomas and carcinomas, which may secrete very large amounts of cortisol, often with various androgens (review: Burke, 1978). Secretion of ACTH by malignant or benign tumours of

non-endocrine origin, the ectopic ACTH syndrome, will also cause Cushing's syndrome.

Cushing's disease describes the condition where the adrenal is hyperactive due to an ACTH-secreting pituitary tumour. Although it is thought that the hypothalamus is the primary site of disturbance in most cases, pituitary tumours are nearly always found (O'Neal, 1964). The excessive ACTH production by the pituitary which is not inhibited by normal cortisol levels, stimulates the adrenal to over-produce cortisol. In autonomous adrenal tumours, cortisol is synthesised by the tumour independently of the pituitary (ACTH). ACTH production is suppressed by the high circulating levels of cortisol and this can lead to atrophy of the ACTH dependent cells of the adrenal cortex. Differential diagnosis is therefore essential when considering treatment of Cushing's syndrome.

Diagnosis requires the confirmation of over-production of cortisol, one of the earliest signs of which may be the loss of the circadian rhythm (Vetter et al, 1977), followed by the determination of the cause of the excess cortisol production, preferably by the measurement of ACTH. High levels of ACTH in the plasma indicate Cushing's disease (pituitary-dependent adrenocortical hyperplasia) or ectopic ACTH syndrome; very low plasma ACTH levels indicate adrenal tumour (adenoma or carcinoma).

Dynamic tests with either dexamethasone (9  $\alpha$ fluoro-16  $\alpha$ -methyl-prednisolone) or metyrapone (2-methyl-1,2-bis-(3-pyridyl)-1-propanone) are, therefore, also used to help distinguish the various causes of the syndrome (Liddle et al, 1959 ; Metcalf and Beaver, 1968).

Dexamethasone, a synthetic corticosteroid, acts by inhibiting ACTH output via the feedback mechanism, so blocking adrenocortical production. On administration of dexamethasone, patients with adrenal tumours or ectopic ACTH syndrome fail to suppress cortisol production.

Metyrapone specifically inhibits 11 $\beta$ -hydroxylase, the final step of cortisol synthesis. This leads to decreased cortisol production and via the feedback system, to an increase in ACTH output. Patients with bilateral adrenal hyperplasia (Cushing's disease) show an excessive

response to metyrapone; patients with adrenal tumours or ectopic ACTH syndrome however show impaired or no response.

The best treatment of Cushing's disease is to remove the source of ACTH, whether pituitary or ectopic as in, for example, carcinoma of the bronchus. This is not always possible however, and a combination of pituitary irradiation and bilateral adrenalectomy followed by replacement therapy is commonly used in these circumstances. Pituitary irradiation is necessary to inhibit pituitary enlargement following adrenalectomy (Nelson's syndrome). Metyrapone is also used for a short term pre-operatively and for the long-term control of Cushing's disease. (Besser and Jeffcoate, 1976). It has the disadvantage of long-term use in women because of the virilising effect of the cortisol precursors.

#### b. Adrenogenital syndrome

Adrenogenital syndrome is a congenital absence or limitation of one of the enzymes involved in cortisol biosynthesis. The enzyme deficiencies result in inadequate cortisol production, giving rise to excessive ACTH output from the pituitary via the feedback mechanism resulting in adrenal hyperplasia and an increased production of androgens. This syndrome is associated therefore, with excessive circulating levels of androgens which result in masculinisation in the female and precocious puberty in the male - in those who survive neonatal electrolyte imbalances. The most common form of the syndrome involves a deficiency of adrenal 21-hydroxylase. More rarely, a block at 11 $\beta$ -hydroxylase can occur, while even more rarely, blocks have been described at the 3 $\beta$ -hydroxydehydrogenase and 17 $\alpha$ -hydroxylase step (see Fig. 3 ) (review: Mason, 1972). Treatment is by replacement therapy to replace circulating cortisol and to suppress androgen production. This is instituted as soon as possible to avoid growth stunting due to *early* epiphyseal fusion.

#### c. Addison's disease

(i) Primary adrenal insufficiency or Addison's disease was first described by Addison in 1855. The dominant features of the disease, as

described by Addison are "general languor and debility, remarkable feebleness of the heart's action, irritability of the stomach and a peculiar change of the colour of the skin." It is a relatively rare condition occurring at any age and in both sexes with equal frequency. This disorder results from partial or complete destruction of all three zones of the cortex. The clinical features of the disease reflect therefore the lack of glucocorticoids and mineralocorticoids. The most usual cause of the disease is idiopathic atrophy showing lymphocytic infiltration. Autoimmune adrenal atrophy (review Irvine and Barnes, 1975) or tuberculosis are the commonest causes of Addison's disease, more rarely, secondary tumours and adrenal haemorrhage are implicated.

Definitive diagnosis depends on the demonstration of low corticosteroid production in the presence of high plasma ACTH concentrations. In Addison's disease, the adrenal is maximally stimulated by high levels of circulating ACTH. Injection of ACTH or synacthen (synacthen test) will therefore produce no change in cortisol levels. Treatment is by replacement therapy.

#### (ii) Secondary adrenal insufficiency

Deficient pituitary ACTH secretion will produce a secondary adrenal insufficiency. The adrenal cortex atrophies and the production of corticosteroids diminishes. Aldosterone production is not affected however, as it is not under the control of ACTH. Clinical presentation is similar to Addison's disease, except for the lack of pigmentation due to the low ACTH levels. Impaired ACTH secretion may be either hypothalamic or pituitary in origin. Pituitary tumours or pituitary failure associated with long-term corticosteroid treatment (see Chapter 2) can cause a drop in ACTH production.

## Chapter 2: Steroid Therapy in Clinical Medicine

### 1. Corticosteroids and adrenocorticotrophic hormone

#### Introduction

Corticosteroids and ACTH are among the most widely used drugs in clinical medicine, and yet their mode of action is obscure. Corticosteroids are powerful anti-inflammatory agents with many clinical applications and yet they can have serious side effects such as the development of some of the features of Cushing's syndrome and the activation of peptic ulceration and of diabetes mellitus.

They were first proved to be useful in the treatment of rheumatoid arthritis – a systemic disorder of unknown cause, in which symptoms and inflammatory changes predominate in joints. Most commonly, ACTH, either porcine or synthetic, or a synthetic corticosteroid such as prednisolone, are used in the treatment of RA.

The aim of both corticosteroid and ACTH therapy is the same – to increase the level of circulating glucocorticoids, which have a beneficial, pain-relieving and anti-inflammatory effect. There is believed to be no other therapeutic effect of ACTH than its action on the adrenal cortex. ACTH causes a general enhancement of adrenal secretion and will produce the systemic effects of this increase. Corticosteroid treatment, on the other hand, will inhibit all adrenal secretion via the negative feedback mechanism, except that of aldosterone which is not under the control of ACTH. The degree and duration of inhibition will depend on the type of drug used, the dosage and the time of administration. If the dosage is high enough there will be complete inhibition of the production of cortisol, and this will lead to suppression of the HPA axis, that is to chronic inhibition, such that its function will not immediately recover after cessation of therapy or in response to stress. There is variation in individual responsiveness and in the readiness with which suppression is produced, but it broadly relates to the daily dose, type and duration of therapy. The adrenal cortex atrophies during prolonged corticosteroid therapy and is hypertrophied during prolonged ACTH therapy. The changes reported in the pituitary and hypothalamus

are similar following both forms of therapy (review: Myles and Daly, 1974). ACTH therapy appears to cause less suppression of HPA responsiveness to stress, possibly because the increased responsiveness of the patients' adrenals partly compensates for the lessened pituitary ACTH output.

a) Prednisolone

Prednisolone (11 $\beta$ , 17 $\alpha$ , 21-trihydroxy-1, 4-pregnadiene-3, 20-dione) was one of the earliest analogues of the glucocorticoids to be synthesised. It was first shown to be an active anti-rheumatic agent by Bunim et al (1955) and Hart et al (1955). Prednisolone is given by mouth but may also be given as either an intravenous (prednisolone phosphate) or an intramuscular (prednisolone acetate) injection.

Prednisolone differs from cortisol in having a double bond in ring A between C1 and C2 (Fig. 4). This modification results in approximately four times the glucocorticoid activity of cortisol on a weight for weight basis (Bunim et al, 1955). The mechanism of disappearance from plasma and degradation by the liver are similar to those of cortisol (Slaunwhite and Sandberg, 1956). The modification of ring A makes prednisolone less accessible to the enzymes which normally inactivate cortisol by the reduction of the 3-ketone and of the 4-5 double bond.

There is no appreciable conversion of prednisolone to cortisol and cortisone in the body. It competes with cortisol for the binding site on CBG but has slightly less affinity (Vermeulen, 1959). The free, unconjugated steroid persists much longer in the plasma; this may account for its greater anti-inflammatory activity (Peterson, 1959).

The incidence of side effects is related to the dose and duration of treatment. Side effects are produced on the gastro-intestinal system e.g. increased tendency to peptic ulceration. Musculo-skeletal complications, for example myopathy and osteoporosis

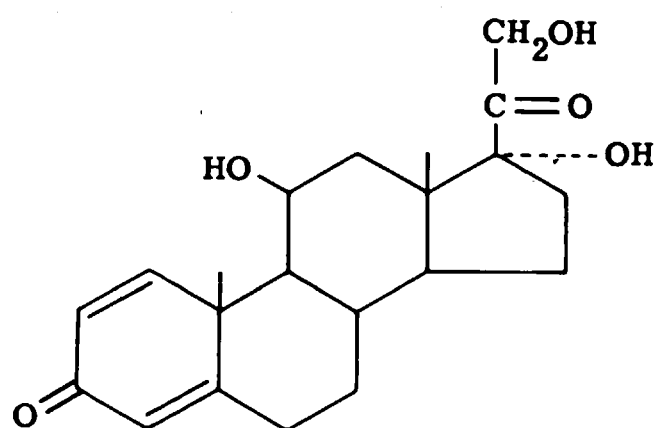


Fig. 4 : Structure of prednisolone.

also occur. Cataract development, an increased susceptibility to infection and the retardation of growth in children are also found with prednisolone treatment. (Treadwell et al, 1964; Blodgett et al, 1956).

b) Adrenocorticotrophic hormone

ACTH is a straight chain polypeptide of 39 amino acids, with a molecular weight of around 4500. The N-terminal sequence of amino acids, approximately 1 - 24, are identical in all mammalian species so far studied and are essential for the full biological activity of ACTH on the adrenal cortex. The remaining C-terminal amino acids contain the most antigenic portion of the polypeptide and are species specific (Riniker et al, 1972).

The mode of action of ACTH on the adrenal is unclear, but the initial event appears to be the binding of the hormone to a stereospecific receptor on the plasma membrane of the adrenal cell. This combination with the receptor site results in the activation of adenylate cyclase which is also associated with the cell membrane and catalyses the reaction of adenosine triphosphate (ATP) to adenosine cyclic 3-5 monophosphate (cyclic AMP). An increase in cyclic AMP and in protein synthesis is necessary for ACTH to produce its steroidogenic effect, but the mechanism is not understood. It seems clear, however, that ACTH exerts a controlling effect on the steroid biosynthetic pathway between cholesterol and pregnenolone. The structure, regulation and action of ACTH have been recently reviewed by Krieger and Ganong (1977).

On injection of ACTH, plasma corticosteroid levels increase in about 3-6 minutes. There is a log-dose response which is linear over a tenfold range; a maximal response may be reached after which an increase in the ACTH level causes no further increase in corticosteroid output (Lipscomb & Nelson, 1962). ACTH is rapidly destroyed after intramuscular injection - about six hours - unless modified in some way to prolong its action, for example combined with hydrolysed gelatin (Acthar gel, Armour) which will prolong the half life in plasma to about 12-18 hours. Tetracosactrin (Synacthen, CIBA; Cortrosyn, Organon) is a synthetic polypeptide

$\alpha$  1-24 ACTH, with a short duration of action, approximately two hours. In order to prolong its physiological activity further, tetrasactrin is adsorbed on to a zinc phosphate complex (Depot synacthen, CIBA). This acts for up to 48 hours (Treadwell and Dennis, 1969).

Prolonged therapy with ACTH will produce chronic over-stimulation of the adrenal cortex and may produce pituitary inhibition via the negative feedback system, although there is apparently less suppression than with oral steroids (Daly and Glass, 1971; Irvine et al, 1973).

The side effects of ACTH therapy are predominantly produced by the increase in circulating adrenal steroids, except for aldosterone which has only a transient rise as it is not predominantly under the control of ACTH. There is a sustained increase in the production of corticosterone and 11-desoxycorticosterone however, and these, together with cortisol, may cause side effects as discussed in the previous section. Dehydro-epiandrosterone is also increased by the action of ACTH and although itself a weak androgen this may be converted to the strongly androgenic testosterone in the peripheral tissues. This increase in androgen levels may be troublesome in women producing hirsuties and acne. Sodium retention hypertension and pigmentation together with acne are characteristic side effects of ACTH (Treadwell et al, 1964).

## 2. Oestrogens

### Introduction

Oestrogens are responsible for initiating and maintaining the female genitalia and secondary sexual characteristics, and together with progesterone are responsible for the maintenance and control of normal menstruation. Oestrogens also affect the metabolism of other body tissues e.g. they tend to antagonise the effects of androgens on the skin, cause deposition of subcutaneous adipose tissue and are responsible for the induction of the synthesis of certain specific proteins in the liver. Oestrogens inhibit the secretion of FSH and in large doses cause sodium and water retention (review: Hall et al. 1974)

17 $\beta$  oestradiol and oestrone are the primary oestrogens produced by the ovary, the former being the most potent. The structures of

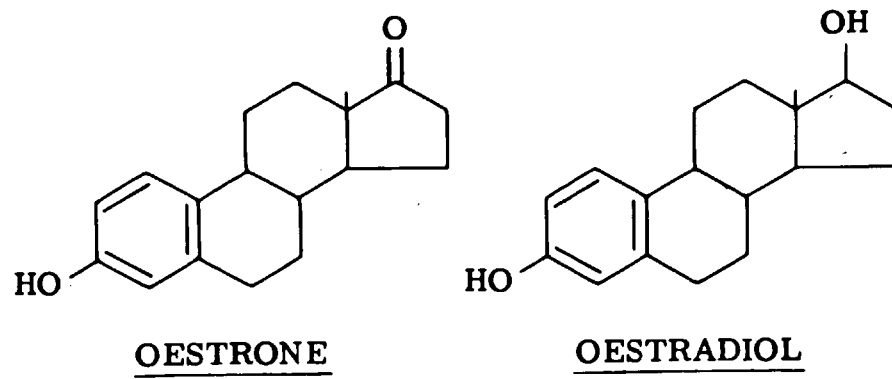


Fig. 5 : The structure of oestrone and oestradiol.

oestrone and oestradiol are shown in Fig. 5. The ovaries also synthesise progesterone, which is also produced by the adrenal cortex as a precursor in corticosteroid biosynthesis. The ovarian hormones circulate in the blood bound to protein; the oestrogens mainly bound to sex hormone binding globulin and progesterone to albumin and CBG. The biosynthesis of these steroids is dependent on the pituitary gonadotrophins luteinising hormone and follicular stimulating hormone. Their production is normally cyclical unless fertilisation of an ovum occurs, when ovarian secretion becomes continuous (review: London and Shaw, 1978).

a) The menstrual cycle and pregnancy

During the first half of the menstrual cycle the Graafian follicle develops under the influence of the secretion of small amounts of LH and FSH. (The developing follicle secretes oestrogens and  $17\alpha$ -hydroxyprogesterone). There is a gradual increase in the secretion of  $17\beta$ -oestradiol which stimulates the production of LH/FSH-releasing hormone by hypothalamus, causing a 'surge' of LH and FSH secretion from the anterior pituitary. The activity of the follicle declines just before ovulation. The surge of LH causes the follicle to rupture and release the ovum (Baird and Fraser, 1974; Smith et al, 1971). The surrounding theca interna cells invade the corpus luteum and are probably responsible for the rise in  $17\beta$ -oestradiol and  $17\alpha$ -hydroxyprogesterone in the second half of the cycle. After about 14 days the corpus luteum regresses and the levels of oestrogens and progesterone fall and menstruation occurs (general review: Hall et al, 1974).

If fertilisation of the ovum occurs in the oviduct, there is a high probability of implantation and development of the zygote in the oestrogen-progesterone primed endometrium. The initiation and continuation of the pregnancy depends on corpus luteum function until the placenta begins to elaborate large quantities of steroids, becoming the major source of hormones necessary for the maintenance of the pregnancy. Oestrogens and progesterone rise steadily throughout the pregnancy, progesterone levelling off a few weeks from term.

b. Effects of Oestrogens on Cortisol and CBG

The rise in plasma cortisol levels during human pregnancy was reported in 1953 by Gemzell and has been repeatedly verified since (eg. Doe et al, 1969). Robertson et al, (1959) later showed that oestrogen administration in man was associated with low urinary 17-hydroxysteroid excretion. Sandberg and Slaunwhite in the same year demonstrated that there was an increase in the cortisol binding in both sexes induced by the administration of oestrogens. The increase in protein binding was found to occur within 5-10 days of administration of oestrogen (Mills et al, 1960; Sandberg et al, 1960) and disappears within about the same time after stopping oestrogen. This effect is not shown by progesterone nor by androgen therapy where the levels of CBG are significantly decreased (Dickinson et al, 1969). Barbosa et al (1971) have shown that for changes in plasma proteins to occur, a 17  $\alpha$ -alkyl group and to a lesser degree a C-3 ketone group are necessary. Additional unsaturation in ring A may result in changes in CBG and is consistent with the effect of oestrogen on this protein.

Although the cortisol level rises considerably during pregnancy, no overt clinical signs of hypercortisolism have been found. It was postulated therefore, that the rise in CBG during pregnancy keeps the physiologically available cortisol level low enough so as not to cause Cushing's syndrome (Sandberg et al, 1966).

Oestrogens have been found to decrease the ability of the liver to metabolise cortisol and increase hepatic synthesis of proteins (Peterson, 1959; Sandberg et al, 1969). There is, however, a high urinary free cortisol excretion in pregnancy often reaching levels seen in Cushing's syndrome (Murphy, 1968).

The concentration of unbound cortisol in pregnancy has been measured but the results are contradictory. Booth et al (1961) and Murray (1967) did not find a consistent increase of the free cortisol level. Doe et al (1960), Layne et al (1962), Rosenthal et al (1969), O'Connell and Welsh (1969), Burke and Poulet (1970) and Brien and Dalrymple (1976), however, have all found an increase in free cortisol level during pregnancy. Westphal (1966) has calculated that the increase in progesterone during

pregnancy and the subsequent displacement of cortisol from CBG could account, at least in part, for the increase in free cortisol.

Burke and Poulet (1970) and Nolten et al (1979) have shown that there is a normal diurnal rhythm of cortisol although the levels are above normal through twenty-four hours. Additional evidence in favour of an increase in metabolically active cortisol in pregnancy is given by Doe et al (1969) who showed that a cortisol-like aminoaciduria was present in pregnancy and that pregnant women not infrequently had purplish striae of the abdomen, buttocks and axillae, diabetic glucose tolerance curves, a tendency to bruising, hypertension and fluid retention, all of which are consistent with a mild Cushingoid state. In addition, patients with rheumatoid arthritis frequently have a remission during pregnancy.

Mukherjee and Swyer (1972) measured ACTH and cortisol in pregnancy and found that in normal pregnancy the ACTH was lowered so that the cortisol/ACTH ratio was very much increased. Similarly, Doe et al (1969) and Nolten et al (1978) have suggested that during pregnancy, a resetting of the set point in the negative feedback mechanism between free cortisol and ACTH may take place. However, Genazzani et al (1975) showed that human chorionic corticotrophin (HCC), a hormone produced by the placenta, has measurable ACTH-like activity by bioassay and immunoassay. They found in contrast with Mukherjee and Swyer (1972) and Rees et al (1975) an increase in immunoreactive ACTH in pregnant women.

### 3. Oral Contraceptives

#### (i) Types of oral contraceptives

The most commonly prescribed oral contraceptives are those in which a fixed oestrogen-progestin combination is given orally for 21 days, followed by a withdrawal period of 7 days. These are referred to as "the combined type". In the sequential oral contraceptives, oestrogen is given for 11 or 16 days followed by an oestrogen-progestogen combination for 10 days and 5 days respectively. In the progestogen-only type of oral contraceptive small doses of compounds such as chlormadinone acetate or norethisterone are administered

without interruption through the cycle (Elstein, 1970).

(ii) Contraceptive oestrogens

The contraceptive oestrogens used are derivatives of ethinyl oestradiol, a synthetic steroid, for example mestranol (the 3-methyl ether of ethinyl oestradiol) (Fig. 6). The metabolism of these compounds differs from that of the natural oestrogen, 17- $\beta$ -oestradiol (e.g. Williams, 1969). Ethinyl oestradiol is more avidly bound to receptors in the uterus than natural oestrogens (Korenman et al, 1970) and storage and slow release from fat depots prolong considerably the activity of the steroids (Williams, 1967).

(iii) Contraceptive progestins

All presently used synthetic oral progestins can be derived from two synthetic prototypes; norethisterone (17 $\alpha$ -ethinyl-19-nortestosterone) and its derivatives, the most potent being norgestrel (Fig. 6). The other synthetic prototype is 17 $\alpha$ -acetoxy-progesterone and its derivatives, for example chlormadinone acetate.

(iv) Biochemical effects of oral contraceptives

These have been recently reviewed by Briggs (1976).

Combined oral contraceptives eliminate the mid-cycle "surge" of FSH and LH (Achtari et al, 1971), the luteal phase rise in progesterone and the mid- and late-cycle rise in plasma oestradiol (Briggs and Briggs, 1972).

Both major hormones from the adrenal cortex, aldosterone and cortisol, are increased (Beckerhoff et al, 1973; Daly and Elstein, 1972), as are growth hormone and testosterone (August et al, 1969; Spellacy et al, 1970; Briggs and Briggs, 1972) while changes are seen in the Renin-Angiotensin system (Beckerhoff et al, 1973).

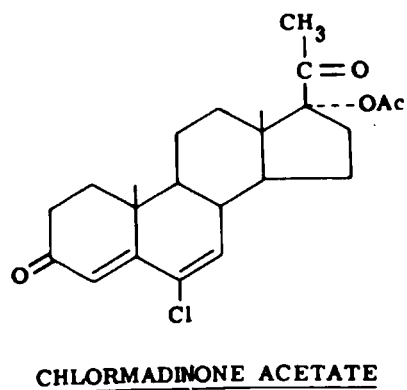
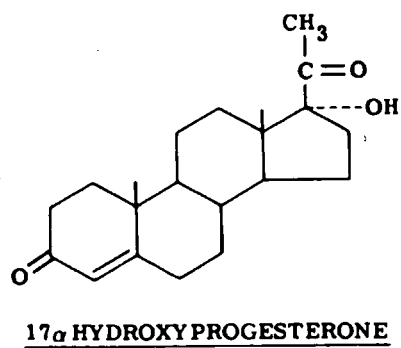
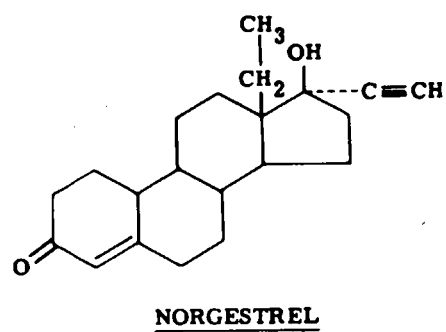
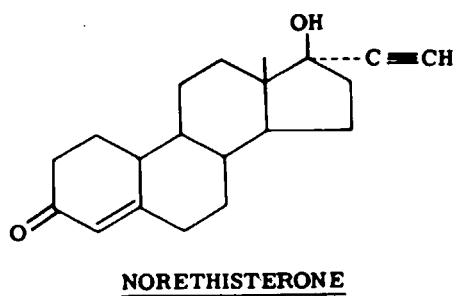
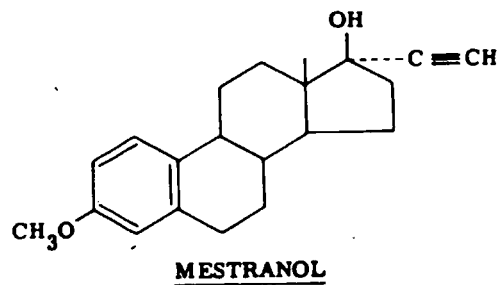
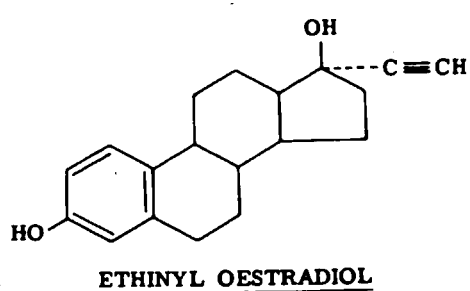


Fig. 6 : The structure of certain oestrogens and progestogens used in oral contraceptive preparations.

Oral contraceptives induce significant changes in the pattern of a wide range of serum proteins. Certain metalloproteins such as transferrin and caeruloplasmin are increased, as well as the specific hormone-binding proteins, CBG, SHBG and TBG .

A number of proteins show significant decreases, for example, albumin (Briggs and Briggs, 1972). Significant changes have also been found in minerals, vitamins, enzymes and lipids (Beck, 1973; Anderson et al, 1976; Briggs, 1976).

(v) Mode of action

The transport and metabolism of the oral contraceptives at cell level has been little investigated. Knowledge is rudimentary on the disposal and metabolism of the contraceptive steroids (Fotherby and James, 1972) and also in the relationship between chemical structure and pharmacological activity in the human species (review: Briggs, 1976).

The balance of evidence at present indicates that the combined oral contraceptives inhibit ovulation in the majority of cases and in addition may affect the ovaries, fallopian tubes, endometrium, endocervix and spermatozoa (reviews - Diczfalusy, 1971; Elstein, 1970; Briggs and Diczfalusy, 1974).

Reeves et al (1970) found that the binding of some contraceptive steroids to CBG is considerably less than that of progesterone. It has been suggested that SHBG is the carrier protein for d-norgestrel (Victor et al, 1976).

### Chapter 3: Methods for the Measurement of Cortisol and CBG

#### 1. Measurement of total plasma cortisol

Total plasma cortisol can be measured using either sulphuric acid fluorescence, or by competitive protein binding methods.

##### a. Sulphuric acid fluorescence techniques

Cortisol and corticosterone develop an intense fluorescence to light of appropriate wavelength when in a strong sulphuric acid solution. This is a sensitive reaction, allowing the use of small volumes of plasma and is specific for the  $\Delta^4$ -3 keto-11-hydroxysteroids. The fluorescence is excited maximally at 470-475 nm and emits maximally at about 520-530 nm. The mechanism of this fluorescence has been studied by Sadee et al, 1971. Specificity has been intensively studied by various investigators e.g. Sweat (1954), DeMoor et al (1960) and Mattingly (1962). Corticosterone which fluoresces at about two and one half times the intensity of cortisol, and oestradiol which fluoresces at about one fifth the intensity of cortisol (with 70% sulphuric acid) are the main interfering steroids. However, human plasma contains very little oestradiol and the corticosterone level is usually less than 10% of the cortisol concentration (Udenfriend, 1969).

Accuracy, as assessed by the recovery of added cortisol to plasma is satisfactory and has been found for example to range from 90-105% by Silber et al, 1958.

Fluorescence is eliminated by the introduction of a  $\Delta^1$  double bond or a 9  $\alpha$ -fluoro function. Thus many of the currently used anti-inflammatory steroids are not detected e.g. prednisolone, dexamethasone. Cortisol levels can therefore be monitored during therapy or during dynamic tests for HPA function. Spironolactone (3-(3-oxo-7  $\alpha$ -acetylthio-17  $\beta$ -hydroxy-4-androsten-17  $\alpha$ -yl)-propionic acid- $\gamma$ -lactone) an aldosterone antagonist has been found to fluoresce in sulphuric acid

reagent and this has been used to monitor the drug plasma levels (Gochman and Gantt, 1962).

Fluorescence was first used to measure cortisol by Sweat and Farrell (1952) and was investigated in human plasma by De Moor et al (1960) and Mattingly (1962). In Mattingly's method, unconjugated cortisol and corticosterone are extracted into dichloromethane. The extract is back-extracted with a sulphuric acid-ethanol reagent and the resulting fluorescence read. Cortisol standards and a water blank are treated similarly.

Fluorescence methods based on the Mattingly method are widely used and agreement on normal levels of cortisol is good (Udenfriend, 1969). However, there is some disagreement and this may be due to interference from non-specific fluorescence (Daly and Spencer-Peet, 1964).

Investigation of this background fluorescence indicates that development is rapid in the first two to three minutes (Stahl and Dorner, 1966), followed by a slow increase over several hours (Daly and Spencer-Peet, 1964). Cortisol and corticosterone develop their own fluorescence which is proportional to the amount of steroid and which reaches a plateau in 8-20 minutes.

In order to reduce this non-specific fluorescence, which can account for up to 41% of the total (James et al, 1967), several methods have been used. Preliminary purification of the plasma extract made by partitioning between benzene and water has been used to reduce spurious fluorescence (e.g. Braunsberg and James, 1962; Rudd, 1963), and this method has also been used to separate cortisol and corticosterone (Van der Vies, 1961). Washing plasma with petroleum ether or iso-octane before extraction (e.g. Silber et al, 1958) and washing the plasma extract with dilute sodium hydroxide (e.g. Braunsberg and James, 1962) have also been used. A different approach was taken by Spencer-Peet et al (1965). They used the linear increase in spurious fluorescence with time, and subtracted from this the constant fluorescence due to cortisol and corticosterone. This method was found by Spencer-Peet et al to be as specific as the other methods in reducing blank values and without the danger of loss of steroid incurred with the more elaborate extraction procedures and

was, therefore, the method used for the assay of cortisol in this study.

b. Competitive protein binding

Assays employing these methods have come into widespread use since their original publication by Yalow and Berson (1960) for the measurement of serum insulin, and by Ekins (1960) for the measurement of serum thyroxine. Currently they are the most common method of assay for hormones.

The principle behind these assays depends on the competition between radio-actively labelled hormone and standards or samples for combination with a specific binding protein or an antibody prepared against the hormone. The reaction is governed by the Law of Mass Action and so, provided a specific reagent concentration (usually the binding protein) used in the system is saturable and held constant, the distribution of hormone following reaction is dependent on the amount of hormone initially present.

For example: when cortisol is added to a mixture containing binding protein and  $^3\text{H}$ -cortisol (F) there is a decrease in bound  $^3\text{H}$ -cortisol (B), as some of the isotope-labelled hormone is displaced from the antibody complex by the native (added) hormone i.e. the ratio of B to F decreases as an increasing amount of unlabelled cortisol is added to the mixture. By comparing the B to F ratio produced by unknown samples containing cortisol with that produced by a series of known cortisol standards, a quantitative estimate of the amount of cortisol in the unknown sample can be determined. Such assays can be used to measure either the binding protein or the hormone which it binds.

Two types of binding protein are used in the measurement of cortisol. Specifically raised antibodies as by for example Ruder et al (1972) and the naturally occurring binding protein, cortisol binding globulin pioneered by Murphy et al (1963, 1964).

This type of assay allows a large number of samples to be measured and is very sensitive, the binding proteins being used in high dilution. Tracer amounts of high-activity labelled hormone are used and the binding proteins are highly specific and of known cross-reactivity with other hormones.

## 2. Measurement of plasma cortisol binding globulin levels

### Introduction

Available methods for the measurement of CBG assess the concentration of the protein indirectly by measuring the cortisol binding capacity. This can be expressed in the weight of steroid bound per weight of protein or, more usually, volume of plasma. The molar concentrations of the binding protein, P, may be expressed in terms of the molar concentration of its binding protein sites, N, where N is the product of n, the number of binding sites and P.

$$N = nP$$

More information on binding can be obtained by determining binding when the protein concentration is kept constant and the steroid concentration is varied. The results can then be presented in graphical form as in the Scatchard plot or a plot of the ratio of the degree of binding and the free ligand concentration versus the degree of binding (Scatchard, 1949) i.e. B/U is plotted against B; B being the bound steroid, U being the unbound steroid. If there is only one set of independent binding sites then a straight line is obtained. If more than one set of binding sites are present, then a curve will be produced. The equation governing this plot is:-

$$B/U = K_A (P_T - B)$$

where

$K_A$  = association constant

$P_T$  = total protein concentration

From these plots the association constant can be determined and the binding capacity estimated directly from the curve. Problems in the interpretation and representation in these plots have been discussed by, for example, Weder et al (1974) and Chamness and McGuire (1975).

When saturation analysis is used however this obviates the need for Scatchard analysis. However, the non-specific binding due to albumin has to be eliminated. The sample is then incubated with saturating amounts of steroid. This is a useful method when many samples have to be assayed.

CBG can be measured by two different types of assay, one where the equilibrium is maintained throughout the experiment and one where it is not maintained, that is the free cortisol is separated from the bound cortisol. The first group includes equilibrium dialysis, gel equilibration and ultrafiltration and gives results which are thermodynamically valid for the conditions of the equilibrium. The second group includes gel filtration and competitive protein binding and although they do not give thermodynamically correct results, under strictly controlled conditions they can give useful results. They also have the advantage of being more practical to perform and requiring relatively small amounts of sample.

### 3. Techniques where equilibrium is maintained

#### (a) Equilibrium dialysis

For the measurement of total binding, equilibrium should be maintained and equilibrium dialysis is the reference method. Westphal et al (1961), Slaunwhite and Sandberg (1959), Bush (1957), and Daughaday (1958) have all used this technique to measure both the bound and the free steroid concentrations.

In the dialysis technique, a trace amount of radioactively-labelled hormone is dialysed to equilibrium against a diluted serum sample in a dialysis sac of cellulose membrane. Since the membrane is freely permeable to the small molecule (ligand) and not at all to the larger protein molecules, the concentration of the ligand in the absence of binding is the same on both sides of the membrane at equilibrium. If binding occurs, the concentration of ligand will be greater inside the bag, since only the unbound ligand is in equilibrium across the semi-permeable membrane. If the solutions on both sides of the membrane are sampled at equilibrium, the amount of ligand bound can easily be calculated. The outside solution thus represents the concentration of unbound steroid; the bound steroid concentration is obtained by subtracting the unbound from the total steroid concentration inside the dialysis bag. Complete equilibration must have occurred between any labelled hormone and endogenous hormone as well as across the membrane.

Steroids do not usually carry charges which could interfere with the dialysis; although adsorption to the membrane and to the wall surfaces may be a problem. This need not be taken into account, however, as although this loss will be reflected in the absolute amounts of ligand, the proportion across the membrane will remain the same.

The principle advantage of this method is its thermodynamic validity. Since the unbound steroid can move freely through the semi-permeable membrane, its equilibrium concentration is equal on both sides of the membrane. The main disadvantage of equilibrium dialysis is the slow rate at which the steroid diffuses across the membrane, 12-72 hours. There may also be handling problems, especially where, in order to try and reduce diffusion time, a dialysis sac with a large surface area is used with small volumes of fluid.

#### (b) Gel equilibration

Pearlman and Crepy (1967) have developed a technique for measuring bound and free steroid using small pore Sephadex, in principle similar to equilibrium dialysis methods. Since only the free steroid enters the Sephadex molecules the free and bound fractions can be calculated on the assumption that the free steroid is distributed between the Sephadex molecules and the buffer, and the bound steroid restricted to the buffer, taking into account the partition coefficient of the steroid between the buffer and the Sephadex. As the bound and free steroid remain in contact throughout the experiment, this method can be considered as maintaining the equilibrium.

The main advantage is its rapidity owing to the high surface area of the gel in relation to the sample volume. Reversible adsorption of steroids to the gel occurs however, the degree of affinity for the gel is dependent on the steroid involved (Burke, 1969). This provides a reversible binding system which competes for the binding sites on the protein for the ligand. Pearlman and Crepy compensate for this in their calculations, although the validity of this remains unproven.

(c) Ultrafiltration

Ultrafiltration has been used to measure CBG, for example, by Keller et al (1966), Ryan and Hanna (1971) and Chen et al (1961). It has a small effect on the equilibrium if less than about 1/10th of the total plasma volume is filtered, Keller et al (1966). In Ryan and Hanna's method of equilibrium ultrafiltration, a plasma solution of total unknown steroid concentration is ultrafiltered through a cell bounded by a membrane permeable to solvent and steroid, and impermeable to protein. The concentration of the unbound steroid can be varied while in equilibrium with bound steroid by altering the centrifugation time and a binding curve may be constructed. The technique involves a fairly complex (as compared to equilibrium dialysis) apparatus and considerable expertise and is not suitable for measurement of many plasma samples. This is a very rapid method, although it has the disadvantage that large volumes of plasma are necessary. A new type of membrane has been used by Ryan and Hanna which has a higher flow rate than Visking tubing, but this gives 25% more binding than that estimated by equilibrium dialysis (Golikov, 1969).

(d) Steady-state gel filtration

In the original method of Burke (1969), plasma is injected onto a gel column. As the plasma moves down the column the unbound cortisol in it moves more slowly than the bound complex which is excluded from the gel and therefore the bound complex continuously dissociates during its passage. As the protein becomes progressively stripped of cortisol, the amount of cortisol dissociating diminishes. Providing the column is not long enough to remove all the cortisol from the protein, some cortisol will emerge still bound to protein, followed by cortisol freed from the complex low in the column and progressively higher concentrations of cortisol freed higher up the column. Cortisol freed at the top of the column emerges with the original unbound cortisol. The sample size is very important. If serum is continuously passed down a column, bound complex at its leading edge will be extensively

dissociated and stripped of cortisol, but if the sample is large enough there will come a point where the cortisol concentration in the gel is equal to that of the unbound concentration in the original sample. This cortisol is retarded by the gel, and any more of the sample passed into the column after this point has been reached will pass through it at equilibrium. The necessary sample size was found by Burke to be approximately 1.6 times the void volume of the column; equilibrium being shown by the concentration plateau of both constituents i.e. total bound and free cortisol eluate.

The method is thermodynamically stable but time consuming and tedious to perform. It also requires relatively large volumes of plasma - approximately 8 ml.

#### 4. Techniques where the equilibrium is not maintained

##### (a) Gel filtration

Doe et al (1964) have used small pore (G25-G50) Sephadex gel filtration to measure CBG. Physical separation of the bound and unbound portion of the ligand is accomplished by chromatography based on the principle of molecular sieving in which unbound cortisol penetrates the "pores" of a gel suspension of a cross-linked dextran, Sephadex. Labelled or unlabelled cortisol is added to plasma in excess of its binding capacity, equilibrated and then the plasma applied to the column. Free cortisol is retained by the Sephadex and therefore is slowed down, as compared to the protein and protein-bound cortisol which are excluded from the gel and are eluted immediately after the void volume of the column.

Some dissociation of the CBG-cortisol does occur, but it is difficult to estimate the extent to which it does so, several factors being involved; the rate of dissociation of the complexes, the volume and protein concentration of the sample, height of the column, the rate of elution and the temperature.

As the plasma passes down the column, the cortisol-albumin complex dissociates due to its low association constant, leaving only CBG-cortisol complex in the eluate.

This is not an equilibrium procedure, as there is some dissociation of the CBG-cortisol complex down the column. The actual dissociation is small at 4°C due to the relatively high association constant and a slow rate of equilibration. At higher temperatures the binding affinity decreases and the equilibration rate becomes greater (Doe et al , 1964).

This is a simple and rapid method and gives reproducible results. It does, however, have a limitation on the number of samples processed, and conditions such as elution rate and temperature must be strictly controlled (Westphal, 1969).

#### (b) Electrophoretic methods

Electrophoresis is the name given to the movement of small charged particles through an electrolyte subjected to an electric field. The rate of migration of particles of like charge will depend on the number of charges each carries. As a result of those different rates of migration, plasma proteins can be separated into a number of fractions of like mobility. The albumin moves most rapidly and travels furthest followed by the  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$  and  $\gamma$  globulins.

CBG has been measured by utilising its electrophoretic characteristics i.e. alpha globulin mobility in continuous flow electrophoresis (Daughaday, 1958) by, for example, Osorio and Schatz (1964).

The samples are incubated with saturating amounts of labelled cortisol followed by paper electrophoresis carried out at 4°C for 2 hours. The paper strips are then cut in half and one half stained to locate the protein bands and the other half cut into parallel bands and the radioactivity counted. A quantitative measure of the CBG levels can then be calculated.

It is specific for CBG, and technically easy to perform. However, there is no binding to albumin shown by this method and background readings are high.

#### (c) Adsorption methods

DeMoor and Heyns (1968) have used adsorbents to obtain a competitive equilibrium between binding proteins and adsorbent, and knowing the affinity of the protein for the adsorbent, the binding protein concentration can be calculated. (The principles of binding assays were discussed earlier). This method has the main advantage that it is rapid but there

must be no adsorption to the walls of the vessel.

The removal of the free steroid by the use of various adsorbents, for example charcoal, florisil, and Fullers earth, have been compared by Murphy (1967). She added adsorbent for a short time only, in order to prevent the dissociation of the steroid-protein complex while still removing the free ligand completely. With very strictly controlled conditions this is a useful way to obtain values of binding as in, for example, the method of Trapp and West (1969).

All these techniques depend on the instantaneous removal of the free ligand, but this is not possible in practice as the removal takes time, and dissociation of the complexes may occur. They are, however, more practical than equilibrium dialysis methods for the study of large numbers of samples.

## 5. Measurement of free plasma cortisol

### (a) Direct measurement

Equilibrium dialysis (eg Slaunwhite and Sandberg, 1959; Doe et al, (1969), steady-state gel filtration (Burke, 1969) and ultrafiltration (eg O'Connell and Welsh, 1969; Robin et al, 1978) have all been used to measure free cortisol levels in plasma. These techniques were discussed earlier.

### (b) Indirect measurement

#### (i) Free cortisol index

These methods are based on the uptake of free cortisol by either charcoal (Baumann et al, 1975; Nolten et al, 1979) or an anion exchange resin (Brien and Hingerty, 1975). The principles of binding assays were discussed earlier. Although not a direct measure of free cortisol, these methods have the advantages of requiring only small amounts of plasma and being technically easy to perform and they do not require expensive equipment.

(ii) Urinary free cortisol

Only the unbound cortisol is filtered by the renal glomeruli and so measurement of the urinary cortisol can be used to provide a measure of the plasma free cortisol. (eg. Burke and Beardwell, 1972).

Urinary free cortisol can be measured both by fluorimetry (eg Mattingly, 1962) and by competitive protein binding (eg Beardwell et al, 1968).

(iii) Salivary Cortisol

As with urinary cortisol, salivary cortisol is non-protein bound and should therefore reflect the free cortisol concentration.

Walker et al (1978) have published a specific and sensitive radio-immunoassay for the direct measurement of cortisol in saliva. They have shown that levels of cortisol found in saliva, closely reflect the plasma cortisol concentration.

The ease and lack of stress involved in the collection of saliva give this method of assessing adrenal function potentially distinct advantages over the measurement of cortisol in plasma or urine.

## METHODS

## Chapter 4: Measurement of Total Plasma Cortisol

Plasma cortisol levels may be estimated in a variety of ways including colorimetric methods (e.g. Nelson and Samuels, 1952; Peterson et al, 1957), fluorimetric methods (e.g. Mattingly, 1962) and binding assays (e.g. Murphy and Patee, 1964; Beardwell et al, 1968; Ruder et al, 1972).

### 1. Cortisol assay by sulphuric acid fluorescence

Plasma cortisol measurement using fluorescence is widely used. These methods are simple to operate and sensitive, allowing the use of small volumes of plasma (James et al, 1971). They have the advantage from the clinical viewpoint that they are unaffected by the synthetic steroids commonly used for therapy and for tests of adrenocortical function, for example, prednisolone and dexamethasone. They have the disadvantage that corticosterone is measured as well as cortisol although the main disadvantages relate to the introduction of non-specific fluorescence from the plasma, for example drugs such as spironolactone. Glassware and reagents used in the assay may also contribute to the non-specific fluorescence (Usui and Kawamoto, 1970).

### Cortisol Assay

Glass distilled water was used throughout the assay. Glassware was acid washed and finally rinsed three times in glass distilled water. All reagents are of the highest purity available. Materials, see Appendix I.

#### a) Standards

A stock solution of cortisol in ethanol was made to give 2500  $\mu\text{mol/l}$ . Dilutions of this were made in ethanol to give 250, 500 and 1000 nmol working standards for each assay.

#### b) Preparation of extracts

0.5 ml of plasma, standards, or water (as blank) are diluted to 1 ml with water in glass tubes, 5 ml of dichloromethane is added from a glass dispenser and the tubes stoppered. They are then mixed for 5 minutes on a gramophone-type mixer. 3 ml of the extract are then removed into glass assay tubes and the tubes stoppered. These extracts may be kept for up to 24 hours at 4°C.

c) Fluorescence reagent

75 ml of cold ( $4^{\circ}\text{C}$ ) sulphuric acid are added from a burette to 25 ml of ethanol in a flask, which is in ice. The ethanol is shaken by hand or stirred with a magnetic stirrer and a glass stirring bar during the addition of the acid. The reagent may be kept for up to 24 hours at  $4^{\circ}\text{C}$ .

d) Assay

1.5 ml of cold fluorescence reagent is added from a glass dispenser to the dichloromethane extracts. At 30 second intervals, the tubes are stoppered, inverted and shaken vigorously for 20 seconds. At precisely 10 minutes and 20 minutes following the addition of the reagent, the fluorescence is read in a spectrophotofluorimeter, activating wavelength is 470 nm, emission wavelength is 530 nm.

e) Calculation

The 10 minute galvanometer reading is subtracted from that at 20 minutes, and the difference subtracted from the 10 minute reading. The level of cortisol in each sample may then be calculated from the linear calibration curve.

f) Characterisation of the method

Using this assay, levels of cortisol have been measured in plasma samples. The assay was found to be reproducible – interassay variation at various concentrations over a period of four months was  $784 \pm 39$  (SD),  $472 \pm 25$ , and  $246 \pm 11$ ; a coefficient of variation of approximately 5%.

The assay is sensitive, levels of cortisol down to 60 nmol/l can be measured.

Addition of 270 and 540 nmoles of cortisol to several plasmas was used to assess the accuracy of the method. The mean recovery was  $97\% \pm 6\%$  ( $n = 7$ ) for 270 nmoles added, and  $99\% \pm 8\%$  ( $n = 6$ ) for 540 nmoles.

## 2. Radioimmunoassay of Cortisol

The principles of radioimmunoassay were discussed in Chapter 3. The first radioligand assay of cortisol was described in 1963 by Murphy et al using CBG as the binding protein. Since then, radioimmunoassays have been developed by for example Ruder et al (1972) and Farmer and Pierce (1974) using antibodies raised against cortisol 21-hemisuccinate conjugated to bovine serum albumin and by, for example, Fahmy et al (1975) against cortisol - 3-BSA.

These assays are more sensitive than those using CBG as binding protein and are more specific although requiring prior extraction of the plasma and having the disadvantages of using a tritium-label requiring the use of liquid scintillation counting.

### a. Materials

see Appendix 2.

Glass disposable tubes 10 x 75 mm were used throughout the assay. Dichloromethane (nanograde) was used without further purification. Phosphate-buffered saline was the buffer used in the assay. Dextran-coated charcoal was used for the separation of bound and free hormone; this was freshly made for each assay. <sup>3</sup>H-cortisol was measured in scintillation fluid in a liquid scintillation counter.

### b. Extraction of cortisol from plasma

25 µl of plasma are pipetted into a glass tube and 500 µl of dichloromethane added. This is then vortex mixed for 20 seconds and the tubes placed in an ice bath. Two aliquots of 25 µl of the dichloromethane layer are removed for assay into clean glass tubes. The dichloromethane is allowed to evaporate and then 100 µl of buffer added.

### c. Extraction of cortisol from urine

As for plasma except that usually 50 µl of urine is extracted.

d. Preparation of standard curve

A stock cortisol standard is stored, 250  $\mu\text{mol/l}$  in ethanol. This is freshly diluted for each assay by 1 in 1000 with buffer (250 nmol/l); then the first standard is a 1 in 6.4 dilution of this (40 nmol/l). The other standards are made by serially diluting  $S_1$  to give a range of 40–0.32 nmol/l ( $S_1$ – $S_8$ ).

100  $\mu\text{l}$  of each standard are used in the assay.

e. Cortisol antibody

The antibody was obtained from Guildhay Antisera, University of Surrey. It was raised in sheep using cortisol-3-CMO-BSA as an immunogen. This antibody was chosen for its specificity.

The antibody is stored undiluted at 4°C. For each assay, an initial dilution is made with buffer of 1 in 4000. This produces a 1 in 12000 final dilution in the assay.

f.  $^3\text{H}$ -cortisol

Cortisol-1,2,6,7- $^3\text{H}$ , 1 mCi/ml ampoule is made up to 25 ml with acetone. This is then stored at 4°C. For each assay it is diluted in buffer to give approximately 6000 cpm/tube. The label is chromatographed to check its purity (see Chapter 6).

g. Assay

1. In glass tubes: 100  $\mu\text{l}$  of buffer for total counts, zero binding and blank, or 100  $\mu\text{l}$  standards and samples.
2. 100  $\mu\text{l}$  of the antibody is added and the tubes are left at room temperature for 30 minutes.
3. 100  $\mu\text{l}$  of  $^3\text{H}$ -cortisol label is added and the tubes vortex-mixed and left to equilibrate overnight at 4°C.
4. 500  $\mu\text{l}$  of dextran-coated charcoal at 4°C is added and the tubes vortex-mixed.
5. The tubes are then centrifuged at 2500 r.p.m. for 15 minutes at 4°C.
6. The supernatant is decanted into 8 ml of scintillation fluid and the radioactivity counted in the scintillation counter.

h. Calculation

All results are calculated as a percentage of total binding (zero tube). The standard curve is then plotted on 3-cycle log graph paper and the sample values read from the curve.

Then:

Plasma cortisol (nmol/l) = value from graph x 84

Urine cortisol (nmol/TV) = value from graph x 44 x TV.

3. Assessment of cortisol radioimmunoassay

a. Standard curve

The antibody was used in a final dilution of 1 in 12000. A typical standard curve is drawn in Fig. 7. Approximately 45% of the counts are bound initially (zero binding). The non-specific counts (blank) averaged less than 2% of the total counts added and were therefore not subtracted prior to calculation.

b. Cross Reactivity of the antibody

Shown below are the experimental values for the cross-reactivity of various steroids with the antibody, measured as 50% displacement of the <sup>3</sup>H-cortisol label at a final dilution of the antibody of 1 in 12000.

Antibody cross-reactivity of various steroids

| <u>Steroid</u>                   | <u>%Cross-reactivity</u><br><u>(50% displacement)</u> |
|----------------------------------|-------------------------------------------------------|
| Cortisol                         | 100                                                   |
| Corticosterone                   | 100                                                   |
| 11-Deoxycortisol                 | 9                                                     |
| Prednisolone                     | 43                                                    |
| 17 $\alpha$ -Hydroxyprogesterone | 1                                                     |
| Progesterone                     | 4                                                     |

c. Recovery experiments

(1) Extraction from dichloromethane

A trace amount of  $^3\text{H}$ -cortisol was added to a plasma pool and a urine sample. The plasma and urine sample were extracted as described and the  $^3\text{H}$ -cortisol measured before and after extraction. 97-101 % of the label was recovered in both cases (n = 10).

(2) "Cold" recovery

Known amounts of cortisol were added to a plasma and a urine sample. These together with untreated aliquots of the urine and plasma were measured in the assay. The results are shown below:

| <u>Sample</u>                    | <u>Cortisol (nmol/l)</u> | <u>Recovery (%)</u> |
|----------------------------------|--------------------------|---------------------|
| Plasma (1)                       | $580 \pm 80$             | 104                 |
| Plasma (1) + 125 nmol/l cortisol | $710 \pm 60$             |                     |
| Urine                            | $105 \pm 25$             | 108                 |
| Urine + 125 nmol/l cortisol      | $240 \pm 40$             |                     |
| Plasma (2)                       | $400 \pm 50$             | 94                  |
| Plasma (2) + 63 nmol/l cortisol  | $459 \pm 60$             |                     |

d. Precision

The within-batch precision of the assay was found by estimation of cortisol in two plasma pools. This gave a mean value ( $\pm$  SD) of  $740 \pm 60$  and  $580 \pm 60$  nmol/l (n = 10). These give a coefficient of variation of 8 and 10% respectively.

Between batch precision was measured using three control plasmas, measured in duplicate in each assay. These gave mean values ( $\pm$  SD) of  $540 \pm 42$ ,  $346 \pm 29$  and  $487 \pm 28$  (n = 6).

e. Comparison with other methods

Plasma samples were measured by both the fluorimetric method and by radioimmunoassay. The results are shown below:

Cortisol levels in plasma (nmol/l)

| <u>Fluorimetric Method</u> | <u>RIA</u> |
|----------------------------|------------|
| 660                        | 690        |
| 675                        | 644        |
| 340                        | 340        |
| 460                        | 432        |
| 515                        | 478        |
| 220                        | 202        |
| 530                        | 515        |
| 350                        | 352        |
| 220                        | 184        |
| 490                        | 486        |
| 210                        | 178        |
| 386                        | 324        |

Regression analysis (shown in Fig. 8) gives  $r = 0.99$ . The slope of the line is 0.97 and has a positive intercept of 38. The results obtained using the RIA are, on average, 6 per cent lower than those obtained using the fluorimetric assay.

Urines were similarly compared with a competitive protein binding method (Beardwell et al, 1968) and found to give similar results as shown below. ( $r = 0.96$ ). The slope of the line is 0.99 and has a negative intercept of 4.

Urine Cortisol levels (nmol/TV)

| <u>Competitive protein binding</u> | <u>RIA</u> |
|------------------------------------|------------|
| 98                                 | 128        |
| 154                                | 133        |
| 88                                 | 55         |
| 98                                 | 92         |
| 141                                | 176        |
| 118                                | 131        |
| 40                                 | 37         |
| 298                                | 310        |
| 70                                 | 65         |
| 125                                | 130        |

Fig 7: Cortisol RIA standard curve - 1 in 12,000 f.d. 48% binding

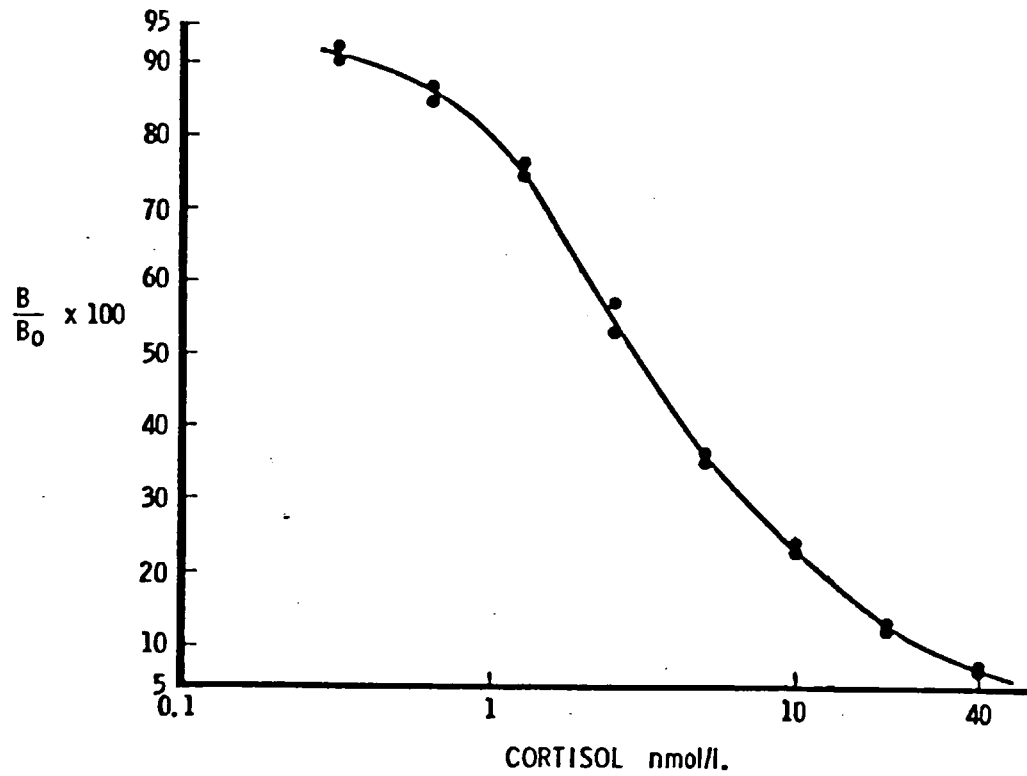
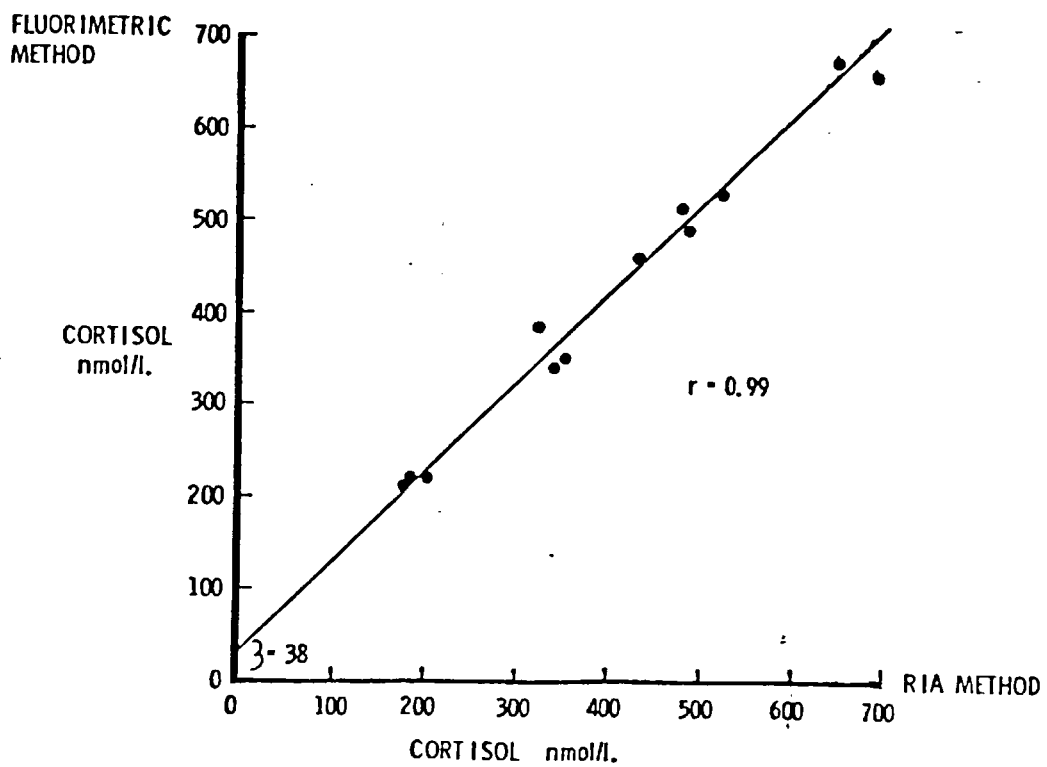


Fig 8: Regression line for plasma cortisol measured by fluorimetry and RIA



## Chapter 5: The measurement of cortisol binding globulin

### I . An adsorption method

#### Introduction

The method chosen is based on that of Trapp and West (1969) where the free and bound steroid are separated by dextran-coated florisil (DCF) and the latter fraction measured. This method has the advantages that it is simple and many samples may be assayed fairly quickly but has the disadvantages of protein-binding assays in that it is not an equilibrium method and the adsorbent will probably displace the binding equilibrium. However, florisil has a relatively low affinity for cortisol (Murphy, 1967) and CBG has a high association constant,  $5-6 \times 10^8 \text{ M}^{-1}$  (Muldoon and Westphal, 1967), thus the displacement effect is probably small.

The estimation of the cortisol binding capacity by Trapp and West in normal adults gave  $513 \pm 52$  nmol cortisol/l plasma, and is comparable with other published values, for example, that obtained by Daughaday (1961) by equilibrium dialysis:  $555 \pm 144$  (Table 1).

Trapp and West vary the amount of excess cortisol added, depending on the expected amount of endogenous CBG in the plasma. This was impossible in a "blind" trial and it seemed therefore that it would be easier if the plasma cortisol level was measured fluorimetrically (see Chapter 4) to add a constant amount of cortisol to the assay.

#### a) Assay of cortisol binding globulin

Materials are given in Appendix 1.

##### 1) Preparation of Samples

Four tubes containing 0.1 ml of plasma are set up for each sample except for the 'total' and 'blank' which contain 0.1 ml buffer. Two of these tubes are stoppered and heated to  $60^\circ$  in a waterbath for 25 minutes in order to denature the CBG.

## 2) Assay

0.5 ml of phosphate buffer (0.1 M, pH = 7.4) containing 0.1 M KCl, an excess of cortisol (276 nmol/l) and a tracer dose of tritiated cortisol (0.15  $\mu$ Cl or 2.8 nmol/l) is added to each tube. The tubes are then allowed to equilibrate overnight at 4°C.

The free steroid is removed by adding 100 mg DCF with a scoop, followed by shaking for one hour in an ice-box.

The tubes are then centrifuged at 2500 rpm at 4°C for 10 minutes. 0.1 ml of the supernatant is removed for counting in 10 ml of scintillation fluid.

## 3) Calculation

The 'total' will give the total amount of tritiated cortisol added and the 'blank' gives a measure of the efficiency of the DCF in removing cortisol.

Subtraction of the value of the heated sample from that of the unheated sample for each plasma will give the binding due to CBG:

$$\text{Cortisol binding capacity, CBC, (nmol cortisol/l).} = \frac{(\text{cpm unheated sample} - \text{cpm heated sample}) (\text{cortisol in sample} + \text{cortisol added})}{\text{Total cpm added.}}$$

As there is only one binding site on CBG, the CBC is equivalent to the concentration of CBG.

## b) Assessment of cortisol binding globulin assay

### 1) Precision

The precision of the assay was found by estimation of the CBC of a plasma pool. On one occasion this gave a mean value of 558 nmol cortisol/l with standard deviation of 24 (n = 10). On a repeat assay of the same plasma pool this gave a mean value of 558 nmol/l and an S.D. of 17 (n = 10). This gives a within-batch coefficient of variation of approximately 5%. Between-batch precision was calculated at different plasma levels, and the variation was found to be  $701 \pm 25$ ;  $513 \pm 17$  (mean  $\pm$  s.d. nmol cortisol/l, n = 8) for each assay over a period of three months.

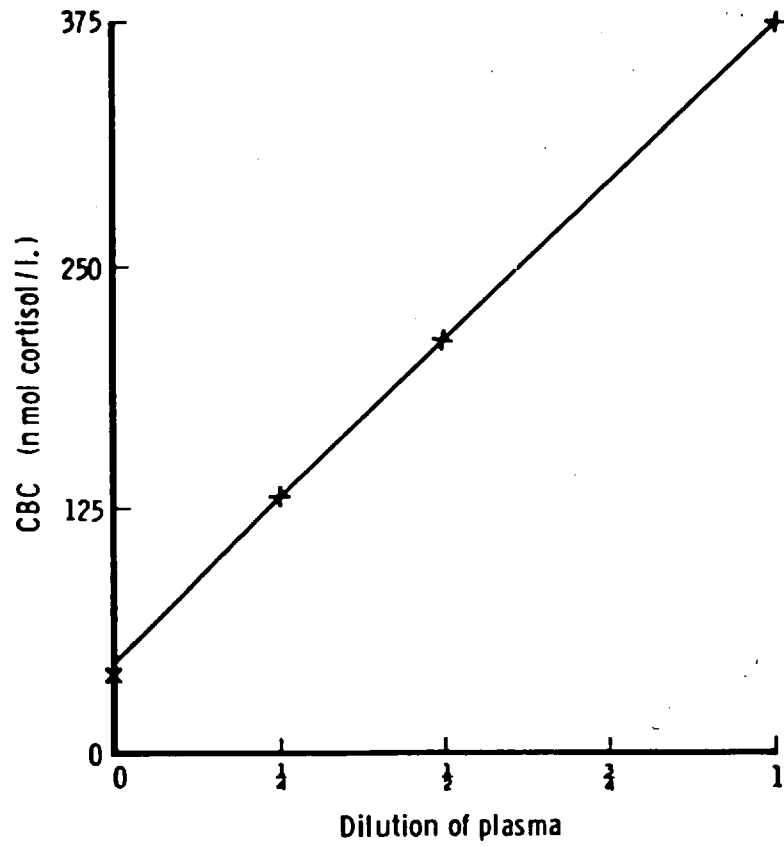


Fig. 9: The effect of dilution of plasma on the cortisol binding globulin

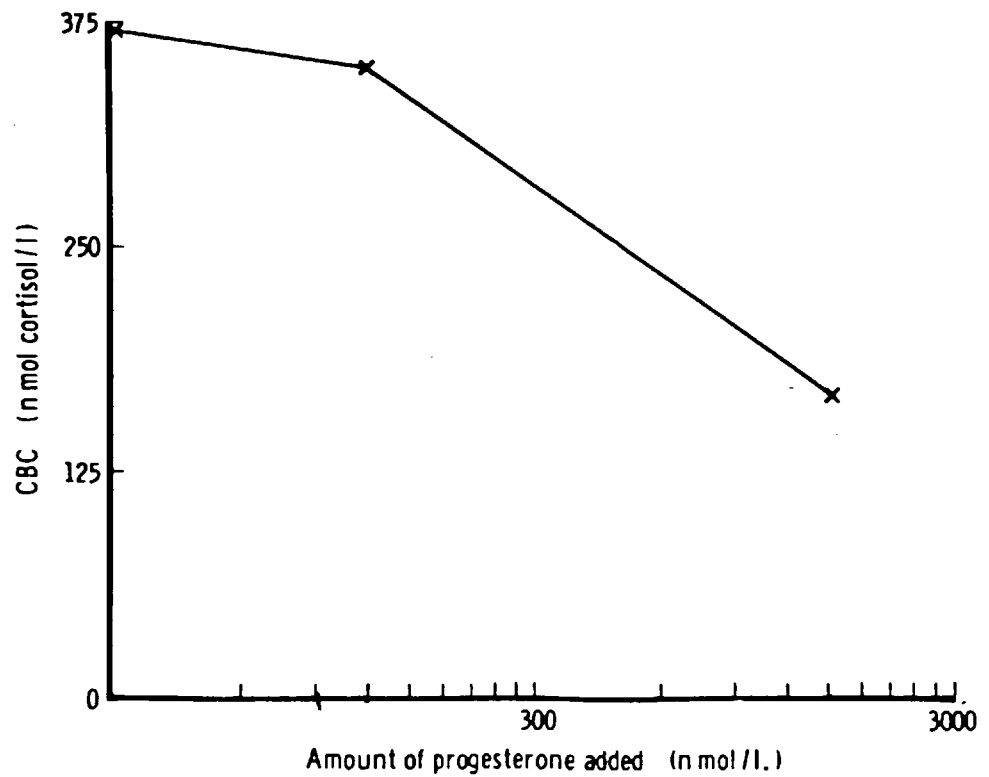


Fig. 10: The effect of progesterone on the cortisol binding globulin

## 2) Parallelism

Parallelism was tested by diluting plasma and measuring the CBG of the various dilutions. One such experiment is shown in Fig. 9.

Plasma was diluted with buffer and the CBG measured. The assay is shown to be linear over the range studied.

## 3) Effect of progesterone

As the assay was to be used for the measurement of CBG through the menstrual cycle, the effect of progesterone - which binds with high affinity to CBG - in the assay was investigated.

Increasing amounts of progesterone were added to plasma in the assay buffer, the results are shown in Fig. 10. As can be seen, physiological levels of progesterone such as those reached in luteal phase of up to 40 nmol/l appear to have little effect on the measurable CBG.

## c) Normal range of cortisol binding globulin

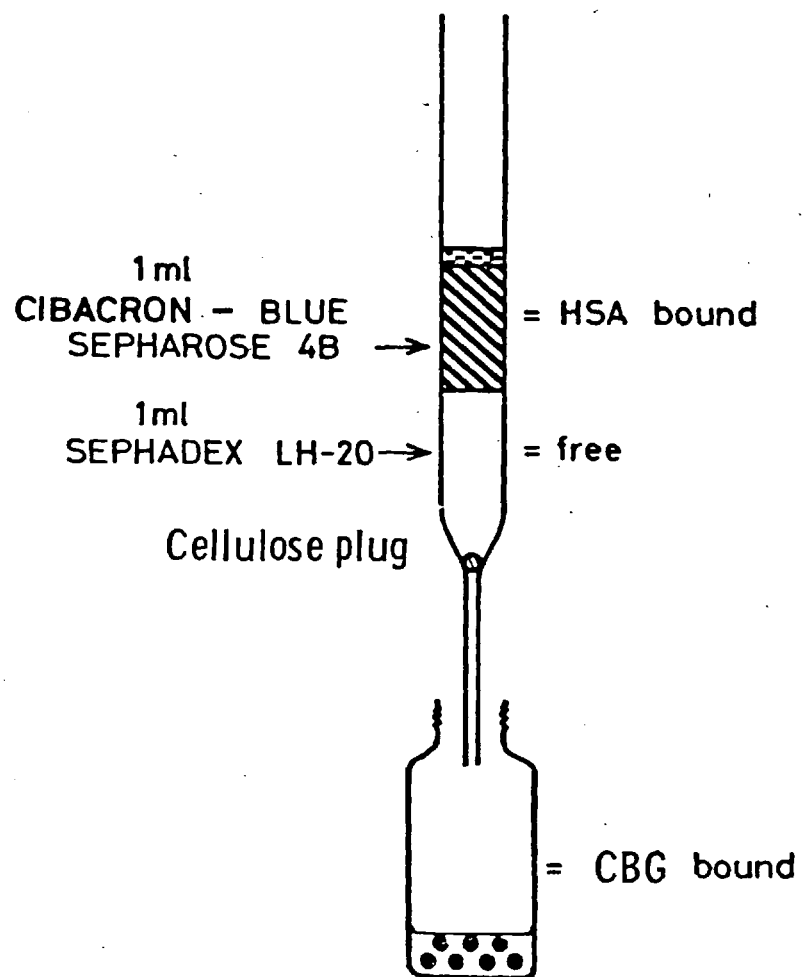
Cortisol binding globulin was measured as the cortisol binding capacity in forty-nine healthy men and women between the ages of 20 and 50 years old. The mean CBG was  $449 \pm 60$  nmols cortisol/l.

## 2. Measurement of cortisol binding globulin by a two-tier column method

An alternative method for the measurement of CBG was devised, based on the two-tier column method of Iqbal and Johnson (1977) to measure SHBG.

Albumin is removed from diluted plasma by an affinity matrix-Cibacron Blue F3G-A-Sepharose gel (Travis et al, 1976). The treated plasma is then passed through Sephadex LH20 gel, where the free cortisol is retarded by the gel. The initial eluate from the column is therefore, CBG-bound cortisol.

Fig. 11: Two-tier column



The principles of gel filtration have been discussed earlier (Chapter 3 ) while the separation of glucocorticoids by Sephadex LH20 chromatography has been shown by, for example, Murphy et al, 1971 .

Retention of the albumin fraction of plasma by the Sepharose, means that small columns of LH20 can be used. This has the advantage of minimising dissociation in the column, as discussed previously . The CBG can therefore be measured rapidly on a single column (see Fig. 11).

a) Column preparation - investigation of gels LH20 and G50, and column size

Dye-linked Sepharose was prepared as described by Iqbal and Johnson (1977) and stored at 4°C in tris buffer (pH = 7.4 0.05 M, Tris 0.005M Ca Cl<sub>2</sub>) to give a 50:50 solution. Sephadex G50 and LH20 were prepared and stored similarly.

Various amounts of gel were poured, between 0.5 and 5 ml, in pasteur pipette columns. Using tritium-labelled cortisol in tris buffer at 4°C, the void volume  $V_o$ , was found for each column and the <sup>3</sup>H-cortisol-labelled plasma was applied to each column. Both G50 and LH20 columns retained the cortisol, i.e. the labelled cortisol was eluted in a volume greater than  $V_o$ .

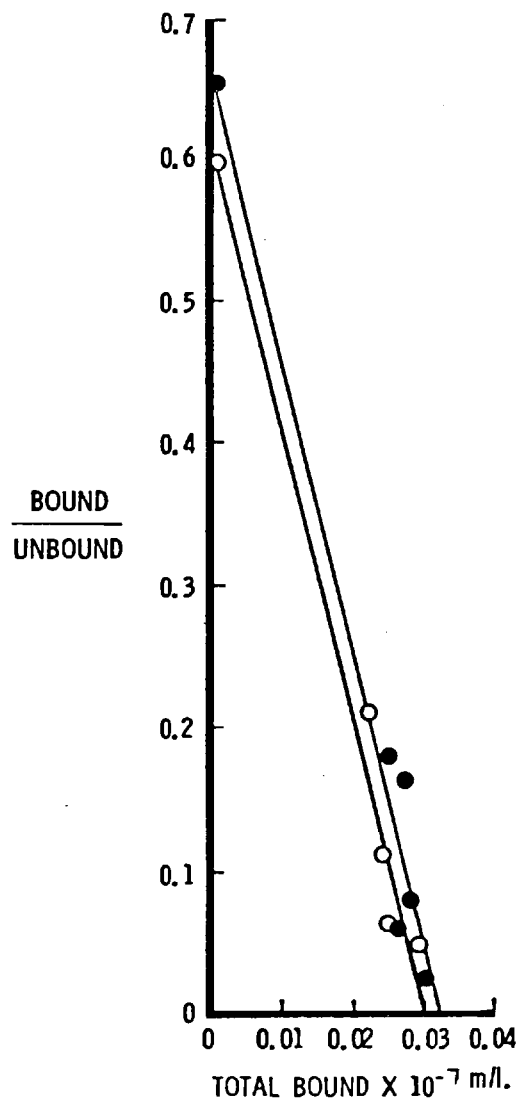
The 2 ml columns were chosen as being the smallest amount of gel giving sufficient retention of the cortisol -  $V_o$  was 1.5 ml and <sup>3</sup>H-cortisol was eluted from 2.2 ml buffer added. G50 and LH20 gave similar results. The LH20 columns were chosen however, as they were easier to pour.

On addition of 1 ml of dye-linked Sepharose to the 2 ml LH20 columns  $V_o$  was 1.7 ml and <sup>3</sup>H-cortisol was eluted from 2.4 ml buffer added.

b) Scatchard Plot - investigation of binding in the system

Plasma or 5% human albumin as blank was diluted 1 in 5 in tris buffer (0.05 M tris, 0.005 M CaCl<sub>2</sub> HCl, pH = 7.4) in small glass tubes. A trace amount of <sup>3</sup>H-cortisol was added together with various amounts of cortisol - between 0 and 0.75 nmoles. They were then incubated for one hour at 4°C. Duplicate 100 µl aliquots were taken from each

Fig.12: Scatchard analysis of 'two-tier' column method



tube and applied to the columns (previously equilibrated at 4°C with tris buffer). Tris buffer(2.3ml) was gradually added to each column and the eluate collected. Scintillation fluid (12 ml) was added to the eluate, which was then counted. 100 µl aliquots were removed from the remaining diluted equilibrated plasma as totals. Results are shown in Fig. 12. The plots show the presence of only one binding system present.

Calculation of the CBC from these plots, gives a value of 300 nmol cortisol/l for the female plasma and 310 nmol cortisol/l for male plasma. A value of 970 nmol cortisol/l was found for a late pregnancy plasma. The association constant as calculated from the male and female plasma is  $2.1 \times 10^{-8}$  M/L. This is of the same order as other published values for  $K_a$  at 4°C, for example,  $0.6 \times 10^9 \text{ M}^{-1}$  found by Westphal (1967).

#### c) Precision

Intra-assay variation was found by repeat estimations of a plasma pool. This gave a mean CBC of  $322 \pm 19$  nmol cortisol/l ( $n = 9$ ). The same pool was measured in duplicate in six consecutive assays; giving an interassay variation for CBC of  $324 \pm 25$  nmol cortisol/l.

#### d) Comparison with the adsorption method

Plasma was measured by the two different techniques. The results are shown below.

CBC (nmol cortisol/l)

|                  | n  | Adsorption Method | Column Method |
|------------------|----|-------------------|---------------|
| Male Plasma      | 7  | $362 \pm 25$      | $324 \pm 25$  |
| Female Plasma    | 12 | $380 \pm 22$      | $355 \pm 23$  |
| Pregnancy Plasma | 5  | $726 \pm 29$      | $683 \pm 34$  |

The regression line between the adsorption method and the column method has a slope of 0.96 and a negative intercept of -20.

## Chapter 6: The measurement of free cortisol

### Introduction

Available methods for the measurement of free cortisol at the start of this study required large volumes of plasma, greater than 10 ml and were often technically difficult to perform. In order to have some idea of the free cortisol levels in plasma, the ratio of cortisol and CBC was used. This was not, however, satisfactory although it showed that the measurement of the free cortisol levels in plasma would be very useful. Methods for the measurement of free cortisol in plasma were therefore investigated.

### 1. Column gel filtration methods

Of the available methods to measure free cortisol Burke's gel filtration method (Burke, 1969), which does not disturb the equilibrium or alter the protein concentration, and can be carried out at 37<sup>o</sup> with strict pH control, seemed to be the method of choice. However, it has the major drawback for this purpose, that 10 ml of plasma are required – too large a volume for experiments involving the rapid and frequent sampling of blood. It was proposed therefore to try to miniaturise this method and thus enable small volumes of plasma to be measured.

#### a) Gel filtration method of Burke

In the original method of Burke (1969), plasma is injected onto a gel column. As the plasma moves down the column the unbound cortisol in it moves more slowly than the bound complex which is excluded from the gel and therefore the bound complex continuously dissociates during its passage. As the protein becomes progressively stripped of cortisol, the amount of cortisol dissociating diminishes. Providing the column is not long enough to remove all the cortisol from the protein, some cortisol will emerge still bound to protein, followed by cortisol freed from the complex low in the column and progressively higher concentrations of cortisol freed higher up the column. Cortisol freed at the top of the column emerges with the

original unbound cortisol. The sample size is very important. If serum is continuously passed down a column bound complex at its leading edge it will be extensively dissociated and stripped of cortisol, but if the sample is large enough there will come a point where sufficient cortisol has dissociated into the column to create within it a cortisol concentration equal to that of the unbound concentration in the original sample. This cortisol is retarded by the gel, and any more of the sample passed into the column after this point has been reached will pass through it at equilibrium. The necessary sample size was found by Burke to be approximately 1.6 times the void volume of the column; equilibrium being shown by the concentration plateau of both constituents in the eluate.

b) Experiments using a 30 x 1.4 cm jacketed column

Materials - see Appendix 1.

(i) Preparation of columns: Jacketed columns 1.4 x 30 cm are packed with Sephadex G 50 (fine) which has been previously swollen in phosphate buffer (pH 7.4, 0.15 M) and degassed. The packing of the columns are checked using Blue Dextran and then equilibrated at 37° overnight before use.

(ii) Preparation of sample: Serum was equilibrated with a tracer dose of tritiated cortisol (10  $\mu$ Ci/ml) at 45° for 10 minutes, then at 37° for 30 minutes.

(iii) Assay: 10 ml samples were then injected onto the column, the flow rate being approximately 1 ml/2 minutes. 0.5 ml fractions were collected using a fraction collector and 0.1 ml from each fraction was taken for counting into 10 ml of scintillation fluid. Protein was easily visible in the fractions as a yellow colour. This was checked by measuring the optical density at 280 nm on a UV spectrophotometer and found to be accurate.

A typical experiment is shown in Fig. 13.

(iv) Assay precision: Repeated estimations on aliquots of the same plasma sample gave a percentage free cortisol of: 5.2, 5.4, and 5.3 giving a mean free cortisol of 52 nmol/l (total cortisol was estimated fluorimetrically - see Chapter 4).

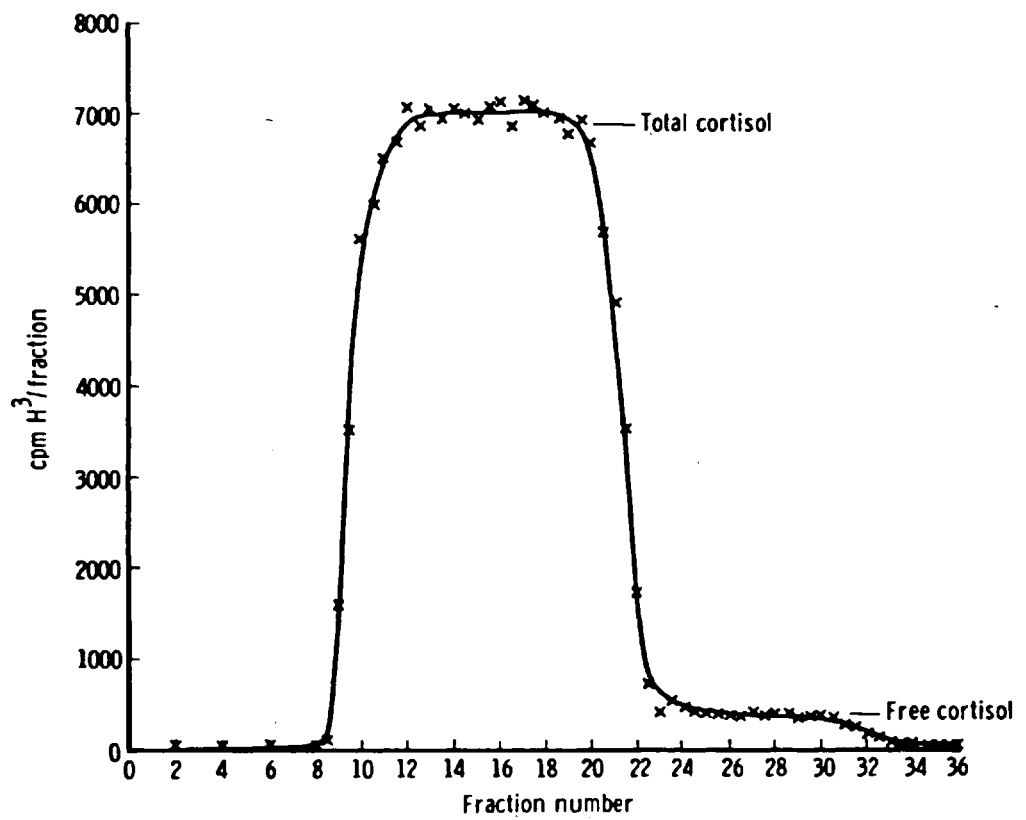


Fig. 13: One experiment using a Sephadex gel column to measure free cortisol levels.

Another experiment on a plasma pool gave respectively 10.4, 8.6, 10.4, 9.1 and 9.5 percent free cortisol : a mean free cortisol of 35 nmol/l.

c) Experiments using 20 x 1 cm jacketed columns

Using a smaller column 20 x 1 cm, and a sample volume of 8 ml, three experiments gave 11.8, 10.9 and 10.4% free cortisol for the plasma pool. A 15 x 1 cm column gave similar results for the plasma pool but still required a sample size of 4 ml.

d) Conclusion

It seemed that it would be difficult to reduce the volume of the sample used in this column method much below 2 ml and this, together with the wish to be able to analyse large numbers of samples at the same time, made this method appear unsuitable for the measurement of free cortisol in this study.

2. Thin layer gel filtration

A thin layer gel filtration technique seemed to offer several advantages - reduced running time, small sample size and several samples may be run at the same time. By using this technique and the theory of Burke's column method, it was hoped to be able to use this method to measure free cortisol in small volumes of plasma.

The method used was a composite one, taken from the general instructions, as given by Pharmacia, together with those of Burke (1969) for the column method.

a) Preparation of thin layer plates

In this method, using the Pharmacia thin layer gel (TLG) apparatus (Fig. 14 ), a layer of Sephadex G50 (superfine) gel is poured onto a glass plate (20 x 20 cm) and spread with a simple rod-type spreader to form a thin layer (approximately 0.6 mm thick), and the plate placed in the apparatus. Filter paper wicks (Whatman 3 mm) are attached to either end of the plate and dipped into troughs containing phosphate buffer (pH = 7.4, 0.15M). The lid is replaced and the plate allowed to equilibrate for 5 to 6 hours.

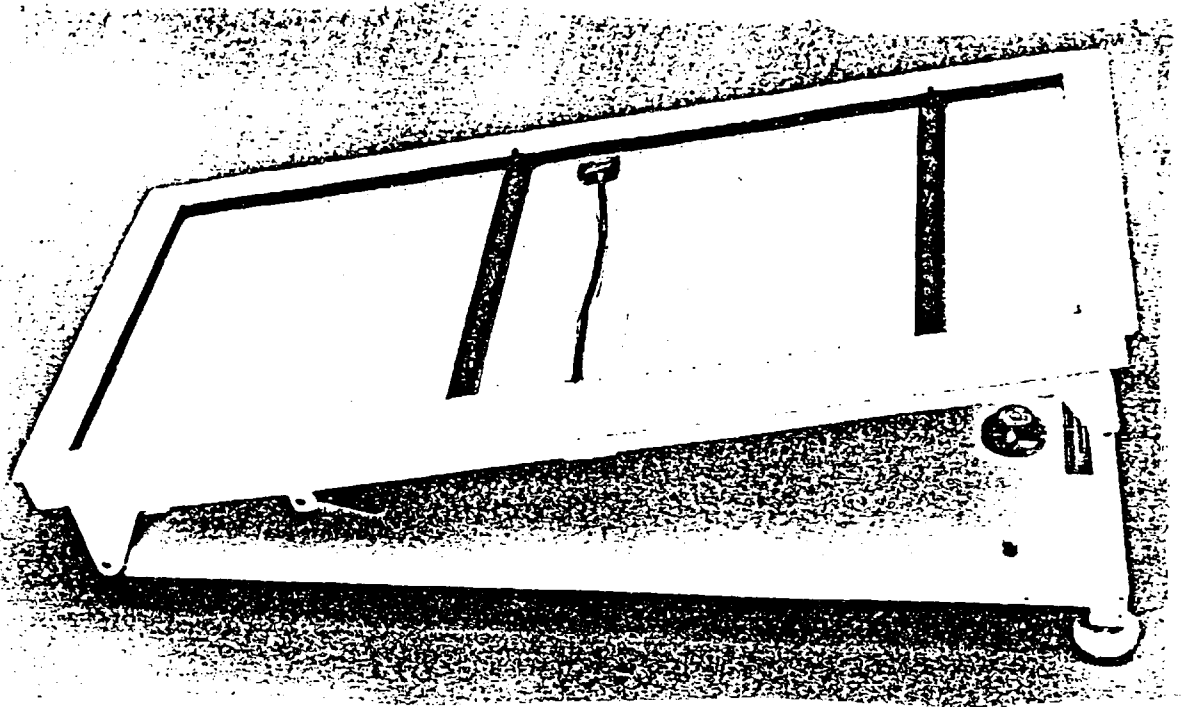


Fig. 14 : Thin layer gel apparatus

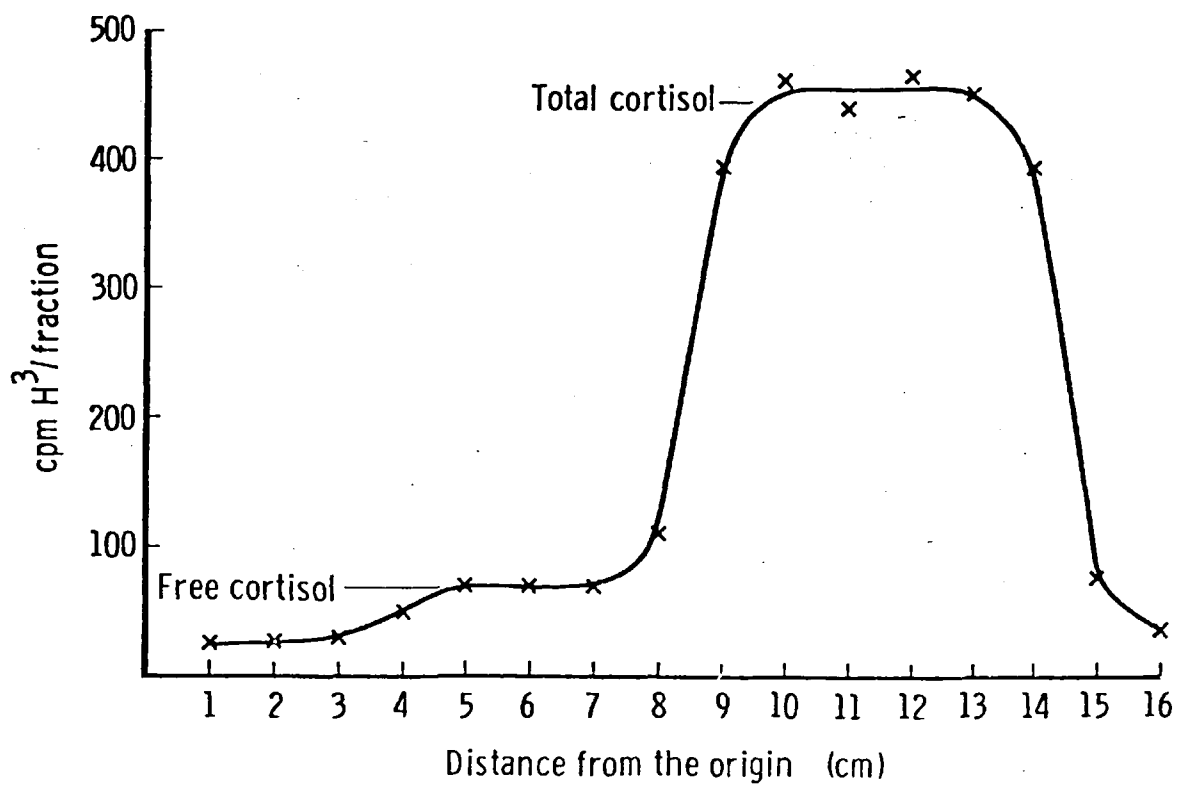


Fig. 15 : One experiment to measure plasma free cortisol using the thin layer gel apparatus

b) Preparation of sample

The sample is equilibrated with a tracer dose of tritiated cortisol in exactly the same way as for the column method.

c) Assay

The sample is applied to the gel in a long narrow band (6 x 2 cm), the lid replaced and the apparatus tilted to an angle of  $10^{\circ}$  to allow flow through the gel. After approximately 4 hours, the apparatus is brought back to the horizontal position to stop the development of the chromatogram. Protein can be seen clearly as a translucent zone.

A 1 cm metal grid is pressed onto the gel and then excess gel is scraped off in 1 cm portions, into small plastic tubes. 0.5 ml buffer is added to elute off the cortisol, and the radioactivity of the supernatant counted in 10 ml of scintillation fluid.

d) Development of the assay

The apparatus is fairly simple to use and the plates are, with practice, easy to pour.

(i) Sample application: Applying the sample proved difficult.

Following the criteria given by Burke for the amount of sample required -  $V_0 \times 1.6$  ml for a 20 x 20 cm plate with 35 ml gel spread in a layer 0.6 cm thick, a sample path 2.0 cm wide travelling 10 cm would travel through 35/20 ml gel. Assuming  $V_0$  is about 1/3 total gel volume of the sample path, the sample volume required is approximately 0.7 ml. Various methods for applying this amount of serum were tried. Using a microcap syringe, it proved very difficult to avoid damaging the gel and to control the area of application. A cover slip was tried - the sample was dropped with a microcap onto a cover slip and then this was tilted and touched against the gel - again it was difficult to apply an even streak and avoid damaging the gel. The most successful method proved to be the sideways application (for the running of the plate) with a syringe, of bands of sample 2 x 6 cm applied through an application slot.

(ii) Sample identification: The protein band was easily visible as a yellow-translucent area. A marker of amido black labelled serum was run as a check.

(iii) Measurement of sample: At first a standard technique given by Pharmacia was used to assess the gel; that of taking a paper replica. At the end of chromatogram development, a strip of Whatman 3 MM chromatography paper was rolled onto the gel and left for 5-10 minutes. It was then carefully removed and dried at 100°C. After drying, the strip was cut into half lengthwise and half stained for protein with e.g. Lissamine green or ninhydrin and the other half cut into 1 cm strips, placed into vials and 10 ml scintillation fluid added and counted in scintillation counter.

The results obtained were low, and scraping off the gel in sections was tried. This was more successful. In order to speed this process, a wire mesh of 1 cm square was made. After development this was placed over the gel, and the excess gel - that not in the 2 cm path - scraped away. The remaining gel was scraped off into small vials and the cortisol eluted out with buffer, which was then counted. The results of one such assay is shown in Fig. 15.

#### e) Conclusion

Results obtained indicated that if the resolution could be improved this method could be used to estimate free cortisol. The main problem, however, was sample application. It is very difficult to apply the right amount of sample, which is very large by thin layer standards (0.7 ml), to the plate without damaging the gel irrevocably.

### 3. Equilibrium dialysis method

While working on the thin layer method as previously described, a small scale equilibrium dialysis method for measuring tri-iodothyroxine and thyroxine was published by Ellis and Ekins (1973).

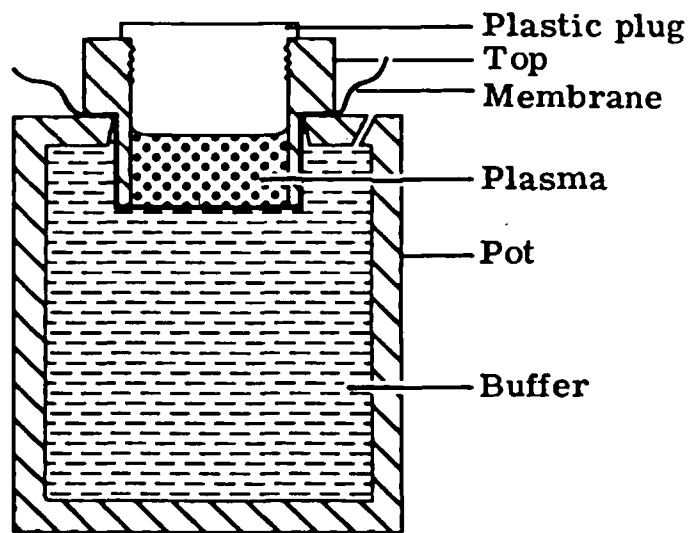


Fig.16: The equilibrium dialysis cell.

The simplicity of this method seemed excellently suited to the measurement of free cortisol in small volumes of plasma.

In this method, serum is placed on a Visking membrane in a small dialysis cell - as shown in Fig. 16. The dialysis cells were made from PTFE rod, containing Hepes buffer. The system is sealed and allowed to equilibrate in a 37°C shaking water bath for 18 hours. The hormone levels on both sides of the membrane are then directly measured using an optimised radioimmunoassay.

For the measurement of free cortisol, a tracer label can be used rather than directly measuring cortisol. Tritium-labelled cortisol is available at a high specific activity and at a minimum 97% purity which permits the use of a tracer. This simplifies the method and enables results to be obtained more rapidly.

a) To find the time taken for equilibration to occur

0.15 ug/ml of <sup>3</sup>H-cortisol was added to Hepes buffer (0.08 m, pH = 7.4). The dialysis cells were filled with 5 ml of unlabelled buffer; a square of Visking membrane placed on top of the cell and the top pressed firmly in place - thus trapping the membrane and holding it taut.

0.5 ml of the labelled buffer was then gently pipetted onto the membrane and the top sealed with a plastic plug-type stopper. The cells were then left at 37°C in a shaking water bath to equilibrate.

At intervals, cells were removed from the shaker, and two 0.1 ml samples taken from above (top) and below (bottom) the membrane for scintillation counting - as described earlier.

The results of one experiment are shown in Fig. 17. Equilibration is seen to have occurred within 20 hours.

b) Measurement of the total amount of cortisol in the dialysate and dialysand

Aliquots of a pooled plasma were dialysed as previously described for 24 hours. Six of the cells were sampled, as described; the total dialysate and dialysand of the remaining cells were removed and extracted with

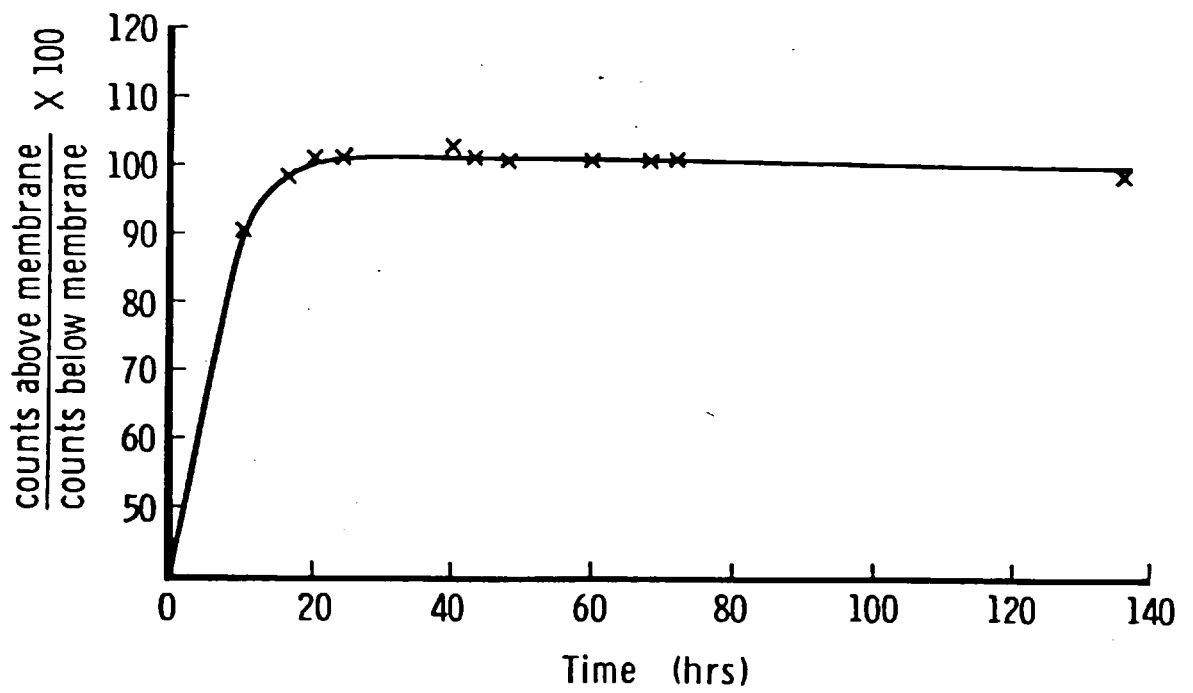


Fig. 17: Time taken for equilibration of cortisol to occur in the dialysis cells.

dichloromethane. The cortisol content of the extracts was then measured using the radioimmunoassay described in Chapter 4. The results are shown below:

| <u>Method</u>                                                  | <u>Mean Percentage</u><br><u>free cortisol</u> |
|----------------------------------------------------------------|------------------------------------------------|
| Measurement total cortisol                                     | $5.7 \pm 0.4$                                  |
| Measurement of cortisol in sample<br>using $^3\text{H}$ tracer | $5.7 \pm 0.2$                                  |

c) Checking purity of Tritium-labelled cortisol

(1,2,6,7,- $^3\text{H}$ ) cortisol, specific activity 80-105 Ci/mmol was obtained from the Radiochemical Centre at minimum 97% purity. The purity of the label was checked using a thin-layer silica gel plate activated for 30 minutes at  $110^\circ\text{C}$ , and then chromatographed in ethyl acetate. 98-99% of the  $^3\text{H}$  was found to lie with the cortisol (located by its fluorescence). Each batch of  $^3\text{H}$ -cortisol was checked in this way.

d) Method of plasma labelling with tritiated cortisol

In order to avoid labelling each sample individually, a time consuming procedure, the effect of labelling the buffer with  $^3\text{H}$ -cortisol was investigated. Buffer and plasma pool were labelled as previously described. The percentage free cortisol was then measured using:

- (i) labelled plasma with unlabelled buffer, or
- (ii) labelled buffer and unlabelled plasma.

Labelling the plasma gave a mean of 9.7% ( $n = 6$ ) free cortisol, whilst the labelled buffer gave a mean of 9.3% ( $n = 7$ ).

e) Effect of quenching in the measurement of free cortisol

Correction of the scintillation counter results for quenching was investigated. The mean percentage free cortisol was  $7.93 \pm 0.25$  before correction,  $8.06 \pm 0.25$  after correction ( $n = 8$ ). It was decided therefore to ignore the slight variations in quenching in future experiments.

f) Effect of evaporation in the measurement of free cortisol in the serum

Cells were prepared as previously described – 0.5 ml of a labelled serum and 0.2 ml of buffer were dialysed against 4.8 ml buffer for two different times – 27 hours and 94 hours. The mean and standard deviation are shown below.

| <u>Time Dialysis</u><br>(hours) | <u>n</u> | <u>Mean Percentage</u><br><u>free cortisol</u> | <u>S.D.</u> |
|---------------------------------|----------|------------------------------------------------|-------------|
| 27                              | 6        | 4.5                                            | 0.26        |
| 94                              | 4        | 4.9                                            | 0.30        |

There seems therefore to be little evaporation loss during prolonged dialysis.

g) Effect of various buffers on dialysis

Phosphate and Hepes buffer were compared using two different molarities in each case. Hepes is a Zwitterionic buffer of the type first described by Good et al (1966) and widely used in cell culture for their superior buffering capacity over long periods of time as compared to the common ionic buffers.

Cells were prepared as previously described. A plasma pool was incubated with  $^3\text{H}$ -cortisol (40 nCi/ml) for 30 minutes at  $37^\circ\text{C}$ . 0.5 ml aliquots of this pool were then dialysed against the different buffers.

| <u>Buffer used</u>              | <u>Mean</u><br><u>Percentage free cortisol</u> |
|---------------------------------|------------------------------------------------|
| Phosphate buffer 0.05M pH = 7.4 | $5.5 \pm 0.2$                                  |
| 0.15M pH = 7.4                  | $5.7 \pm 0.2$                                  |
| Hepes buffer 0.01 M pH = 7.4    | $5.6 \pm 0.2$                                  |
| 0.08M pH = 7.4                  | $5.7 \pm 0.2$                                  |

As there were problems caused by growth of micro-organisms with the phosphate buffers, despite the addition of anti-bacterial agents, it was decided to use Hepes buffer for the assay.

The osmolality of the 0.01 M Hepes buffer was 154 mOsm/kg, the 0.08M which was isomolar with plasma gave an osmolality of 288 mOsm/kg. This was therefore used in the assay.

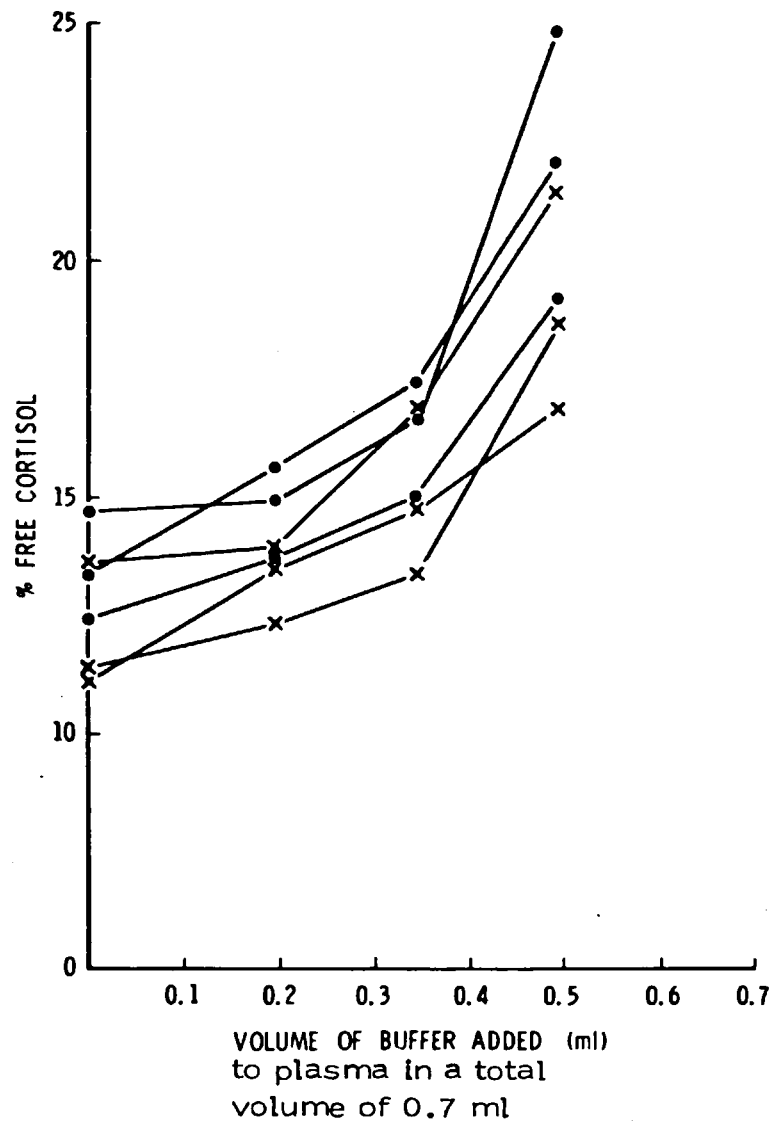


Fig.18: The effect of dilution of plasma on dialysis.

●—● = 5 ml cells

x—x = 2 ml cells

h) Effect of dilution of plasma on dialysis

Cells were made similar to those made previously, except that the volume below the membrane contained 2 ml and not 5 ml. These cells were used to measure the effect of dilution of plasma on free cortisol.

Ellis and Ekins (1973) used 0.2 ml - 0.5 ml of serum and, to aid dialysis and reduce the evaporation effects, added to this a small amount of buffer from the pot.

Varying amounts of buffer were added to the plasma in a total volume of 0.7 ml, in 2 ml or 5 ml cells; the results are shown in Fig. 18.

The results obtained with the smaller cells were on average 10% lower than those obtained from the 5 ml cells. Dilution of the plasma by the addition of 0.2 ml of buffer has little effect on the results obtained by dialysis. This is in agreement with the results obtained by Demey-Ponsart et al (1977).

i) Comparison of equilibrium dialysis method and Burkes' column method

Aliquots of a serum pool were measured using the two different techniques. The results are shown below:

| <u>Method</u>  | <u>No.</u> | <u>Mean percentage</u><br><u>free cortisol</u> |
|----------------|------------|------------------------------------------------|
| Burkes' Method | 3          | $5.3 \pm 0.1$                                  |
| Dialysis       | 6          | $4.5 \pm 0.3$                                  |

j) Albumin binding

Aliquots of a pooled serum were used, half of which were heated to 60°C for 30 minutes in order to destroy the CBG. The plasma was then dialysed as previously described. The results are shown below:

|               | <u>Mean percentage</u><br><u>free cortisol</u> |
|---------------|------------------------------------------------|
| Plasma        | 5.1                                            |
| Heated plasma | 46.2                                           |

k) Conclusions

The dialysis cells were simple to use and the method is reproducible and can be used to measure free cortisol in small volumes of plasma. Intra-assay variation is 6%; interassay variation on a plasma pool was 9% over a period of two months.

The <sup>3</sup>H-cortisol was checked for purity and the <sup>3</sup>H was found to be 98-99% associated with cortisol. Further purification was thought unnecessary. The validity of this assumption and of the use of a tracer label was further investigated by comparing results obtained on a plasma pool using the trace label with those obtained using the same pool but directly measuring the total and free cortisol after dialysis. The results were almost identical, and the use of a tracer label was therefore deemed satisfactory.

The effect of dilution on plasma cortisol measurement by equilibrium dialysis were found to be of the order of 10% under the chosen experimental conditions. The theory of this effect has been discussed by Tait (1964). Provided the same set of experimental conditions are used however, comparative studies can be made. In fact, measurements made by the dialysis method and by Burkes' column method, discussed earlier, gave very similar results.

Destroying the CBG in plasma by heating to 60°C, increased the free cortisol almost ten fold.

## Chapter 7: The Measurement of Salicylates by the Double Iodide Method of Trinder

Aspirin or acetyl-salicylic acid (Fig. 19) is rapidly hydrolysed to salicylic acid on ingestion and so the acetylated compound is rarely found later than three hours after ingestion (Varley et al, 1976).

Salicylates may be determined by simple methods which are based on reactions of the phenol group as in the method devised by Trinder (1954).

In this method serum is diluted with a ferric chloride-mercuric chloride mixture in acid solution. The precipitated protein is centrifuged off, and the colour of the supernatant is read at 540 nm in a spectrophotometer.

### 1. Reagents

Materials are given in Appendix 1.

#### a) Colour reagent

40 g of mercuric chloride is dissolved in 850 ml of distilled water, 120 ml of molar hydrochloric acid, and 40 g of ferric nitrate are added. This is then stirred until dissolved and the solution made up to one litre with distilled water.

#### b) Stock standard

580 mg of sodium salicylate is dissolved in 500 ml distilled water, with a few drops of chloroform added. This gives a solution of 1 g/l.

### 2. Assay

To 1 ml of serum, standard or water (as blank), 5 ml of colour reagent are added with shaking. The tubes are then vortex-mixed and centrifuged. The supernatant of the unknowns and the standards are read against the blank at 540 nm.

The salicylate values of the sera are found by:

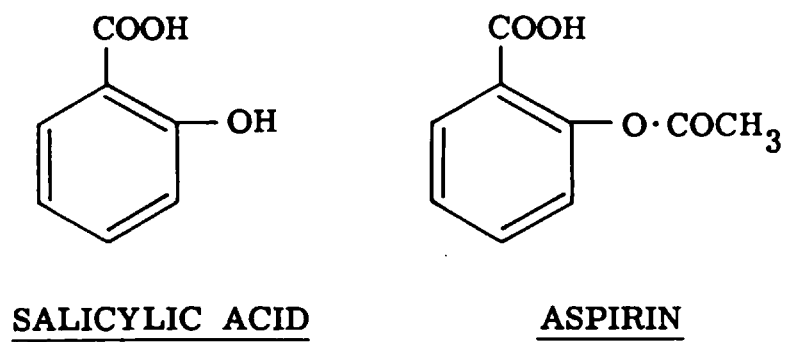


Fig. 19: The structure of salicylic acid and of aspirin.

$$\text{Serum salicylate (mg/l)} = \frac{\text{Readings of unknown}}{\text{Reading of standard}} \times \text{standard value}$$

## CLINICAL STUDIES

## Chapter 8: Variation in Total and Free Cortisol and CBG Levels through the Day and in response to Hydrocortisone infusion

Fluctuations in cortisol levels have been extensively studied by, for example, Hellman et al (1970) and Krieger et al (1971). These studies have characterised the normal pattern of cortisol secretion as occurring in episodic bursts of activity. A major proportion of these episodes occur during the night and early morning, (0300 - 0900 hrs). A study of the reproducibility of secretion from day to day and person to person showed that the pattern of activity was remarkably similar (James et al, 1978). James et al inferred from these results that cortisol secretion occurs "in response to programmed central nervous system control rather than to changes in peripheral steroid patterns."

As free cortisol is the physiologically active steroid, it was decided to investigate the free cortisol levels and their relationship to the binding protein CBG, through twenty-four hours. Free and total cortisol were also more intensively studied over a four-hour period during saline infusions. The response of cortisol to hydrocortisone infusion was similarly studied.

### 1. Plasma total and free cortisol and CBG levels through twenty-four hours in normal subjects

Hourly blood samples were measured in one man (RF) and one woman (JP) in order to investigate any changes in levels of cortisol and CBG occurring during this period. The subjects were in good health and taking no medications. JP was in the luteal phase of her menstrual cycle.

Samples were taken by intravenous catheterisation within the home environment by Dr. M. Dalton of the Royal Free Hospital, London. The normal activity pattern was not hindered. The samples were centrifuged, frozen and stored at  $-20^{\circ}\text{C}$  until assayed.

Total and free cortisol and CBG levels were measured on each sample, as previously described ( Methods). The results are shown in Fig. 20 a and b and in Appendix 2.

Fig 20a: Total and free cortisol and CBG levels in one woman, JP.

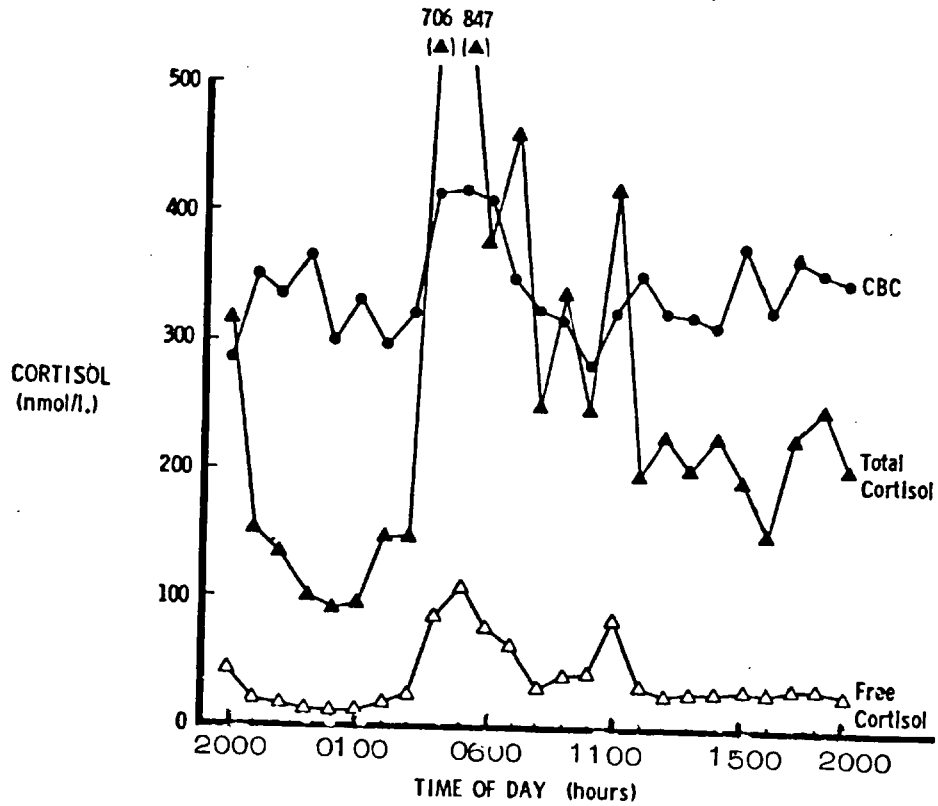
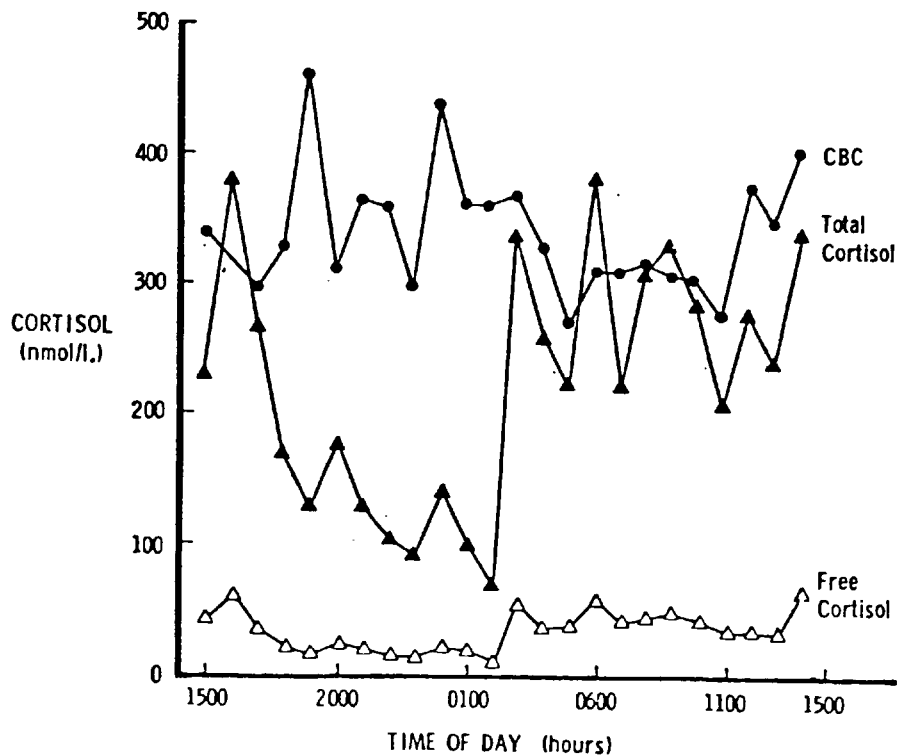


Fig 20b: Total and free cortisol and CBG levels in one man, RF.



## 2. Free cortisol levels during saline and hydrocortisone infusions

Normal saline or hydrocortisone were continuously infused into two healthy male volunteers using a Sage Pump connected to an indwelling, intravenous catheter. The infusions were carried out by Dr J Alaghband-Zadeh at Charing Cross Hospital. The experiments were performed separately but under the same conditions and started at the same time.

Each patient had one continuous four hour saline infusion and one infusion in which hydrocortisone, at a rate of 2 mg/hour or 3 mg/hour (i.e. 5.6 or 8.3  $\mu\text{m}$ /hour) was introduced with the saline for the first two hours. Samples were taken every fifteen minutes, immediately centrifuged and the serum stored at  $-20^{\circ}\text{C}$  until assayed. Total and free cortisol were measured as described in Methods.

The results are shown in Figs 21 a and b and Appendix 2.

## 3. Conclusions

As expected, the results clearly show that total cortisol levels vary throughout the day. The free cortisol levels follow the total cortisol levels closely. Peaks are seen during sleep in both subjects and are thought to be associated with REM sleep (Krieger et al, 1971).

The CBC levels show some variation, tending to be higher at night when compared with those obtained during the day. However, there is no significant difference between these times.

In both subjects, total and free cortisol are raised on hydrocortisone infusion and the increase is proportional to the dose of hydrocortisone given. In both experiments, an increase in cortisol is seen after 30 minutes, during the hydrocortisone infusion.

Fig 21a: Saline Infusions In two male subjects JD and PH.

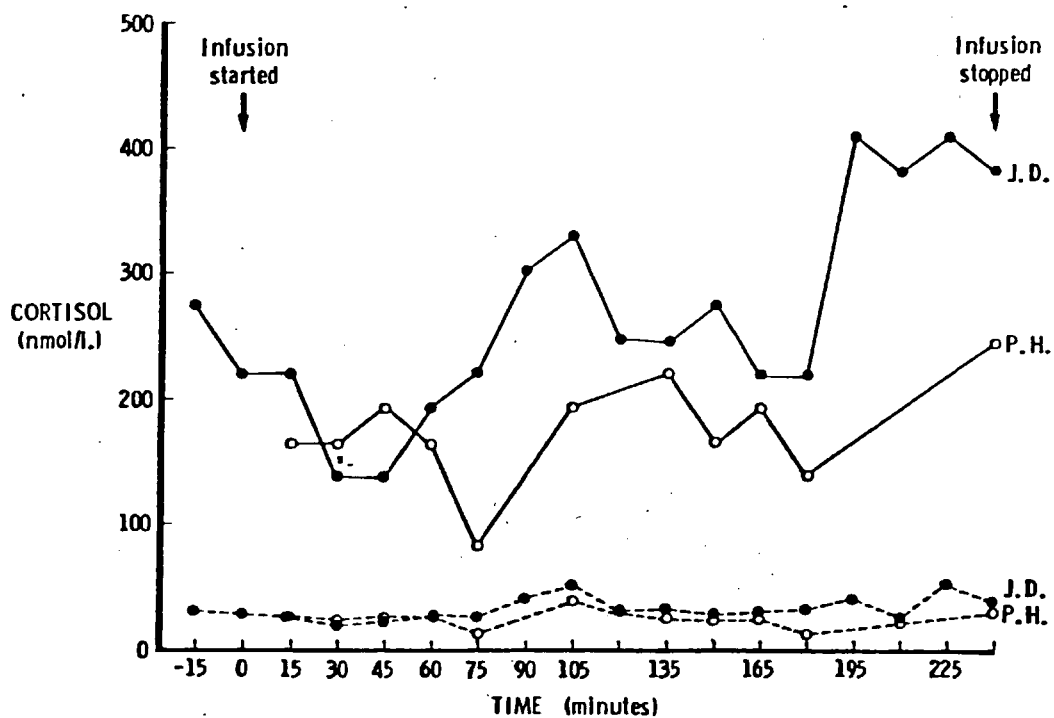
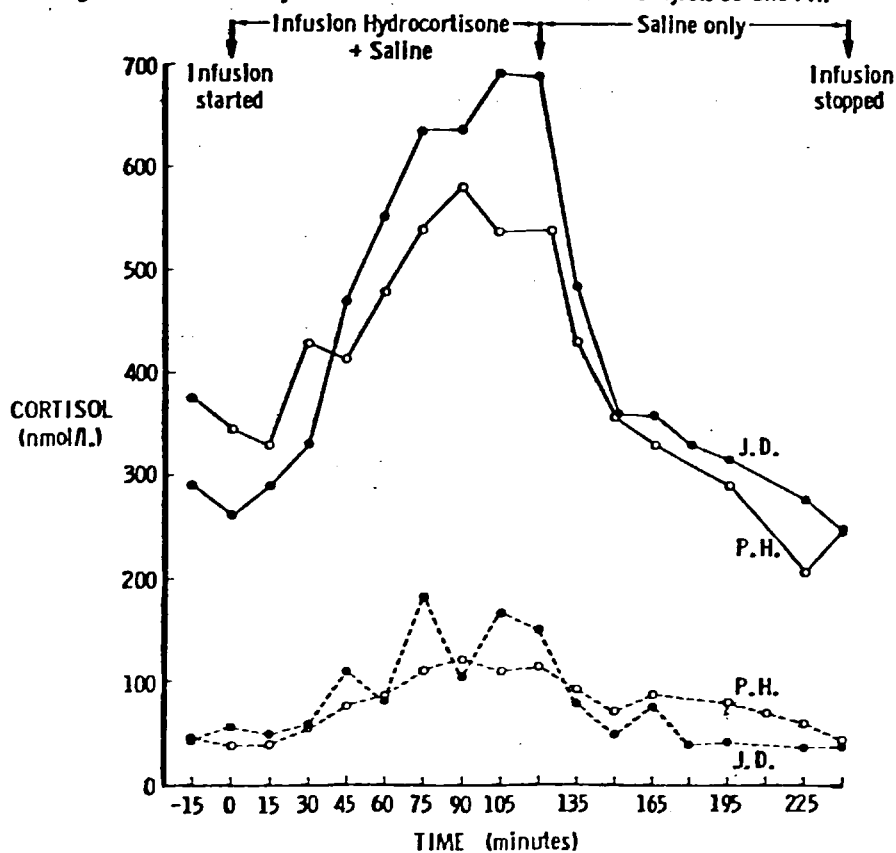


Fig 21b: Saline and hydrocortisone infusions in two male subjects JD and PH.



## Chapter 9: The effect of steroid therapy on the levels of cortisol and cortisol binding globulin levels in rheumatoid arthritis.

### Introduction

The treatment of rheumatoid arthritis is commonly rest, physical therapy, salicylates to relieve pain, and often adrenocorticoids for their non-specific anti-inflammatory action.

As already discussed, prolonged administration of corticosteroids and ACTH results in varying degrees of suppression of the HPA axis and may lead to failure of the normal response to stress.

These drug-induced effects on the HPA axis may be reflected in the levels of cortisol and CBG. Indeed, variations in cortisol and CBG levels could be one of the factors contributing to the severity of and susceptibility to side effects produced by treatment.

Stenlake et al (1969) reported an increase in free cortisol after injection with Synacthen Depot and treatment with acetyl salicylic acid. This increase was due to displacement of cortisol mainly from the albumin-binding sites. As salicylates are used in the treatment of rheumatoid arthritis, the effect of salicylate on the cortisol and CBG levels in normal adults was investigated and salicylate levels in rheumatoid patients were monitored.

Sandberg and Slaunwhite (1959) suggested that the CBG content of plasma might be abnormal in rheumatoid arthritis but more recently Winter et al (1966) found that the plasma CBG content was the same in RA patients as in controls.

Lewis et al (1971) showed that the mean daily dose of prednisolone, the frequency of side effects and the serum albumin levels could be correlated. They found that when the serum albumin is less than 25 g/l the frequency of steroid side effects was doubled. They suggested, however, that the albumin levels are important only insofar as they reflect liver function. Albumin levels had been measured

for all patients and those few (3) who had low, i.e. less than 25 g/l levels, or any other evidence of liver damage were excluded.

1. The effect of salicylates on cortisol and cortisol binding globulin levels in normal subjects

A small pilot study was undertaken to discover if salicylates had any effect on the cortisol and cortisol binding capacity.

Seven volunteers were given a gradually increasing dose of aspirin over four days. Four 300 mg tablets were given on the first day (1.2 g), 8 tablets on the second day (2.4 g), 12 tablets on the third day (3.6 g), increasing to 16 tablets on the fourth day (4.8 g). Then the tablets were stopped. The dose was divided through the day, the tablets being taken at approximately the same time each day - 0800 hr, 1300 hr, 1800 hr and 2200 hr.

Two other volunteers took no aspirin and acted as controls.

No participant had taken salicylates for at least two weeks before taking part in the study.

Blood was taken between 0915 and 0945 hr on the day before starting aspirin (day 1) and for the next three days (days 2 to 4).

After withdrawal, the blood was centrifuged and stored frozen at  $-20^{\circ}\text{C}$  until assayed, and salicylate, cortisol and CBG levels were measured. The results are given in Appendix 2 and Fig. 22. Two subjects failed to take all their aspirin, as indicated by the salicylate levels and were excluded (R.E. and R.M.).

2. The effects of various steroid therapies on cortisol and cortisol binding globulin in rheumatoid patients

Cortisol and CBG were measured in patients attending the rheumatology out-patient clinics at the West London Hospital and at the Edinburgh Royal Infirmary.

Rheumatoid patients who had not been prescribed any steroid drugs were used as controls. Otherwise patients were grouped according to the therapy they were receiving - long-acting

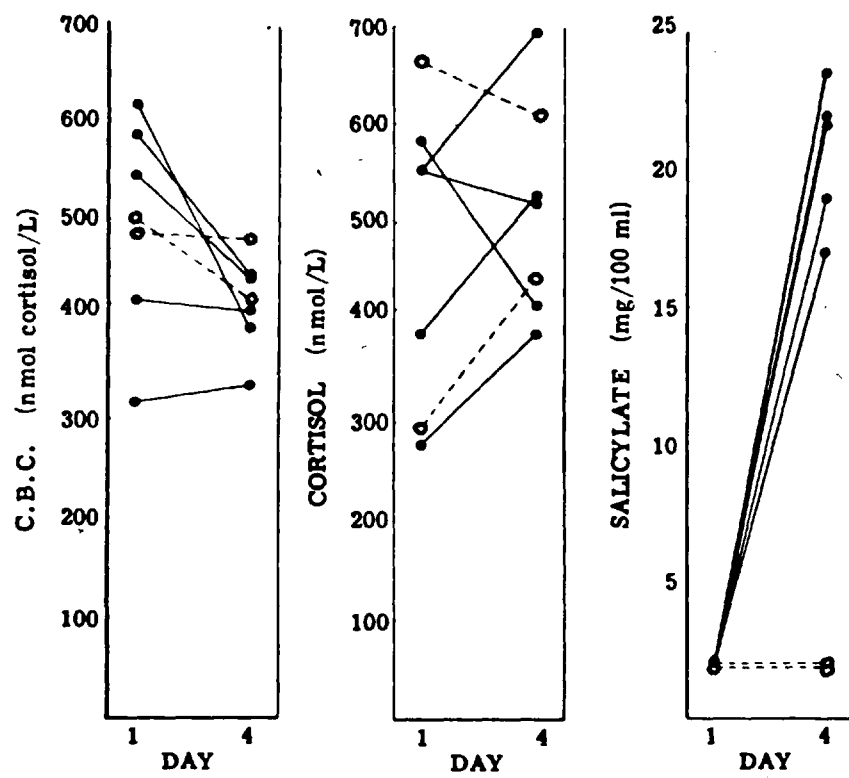


Fig. 22 : The effect of salicylates on cortisol and cortisol binding globulin

synthetic ACTH (Synacthen Depot, CIBA); ACTH (Acthar Gel, Armour) and Prednisolone (Prednesol, Glaxo; Precortisyl, Roussel). The various groups were approximately matched as to sex and age but not for degree of joint involvement.

Blood was taken between 0830 and 1000 hr , before any treatment was given that day. Plasma was stored at  $-20^{\circ}\text{C}$  until assayed.

Albumin levels had been routinely measured on the rheumatoid patients and salicylate levels were measured as described in Chapter 7. Those few patients who had a low serum albumin, that is less than 25 g/l, were excluded from the study, as described earlier.

The results obtained for cortisol and CBG levels are shown in Appendix 2 and the mean and standard deviation in Table 2. The ratio of cortisol to the CBG was calculated for each sample and the results shown in Fig. 23 and the mean in Table 2.

Results were log-transformed to allow for the skewed distribution of cortisol indicated by the increasing standard deviation with higher levels of cortisol. The results are shown in Table 3 and Fig. 24.

### 3. Conclusions

The results from the aspirin studies in normal adults taking "step-up" doses of aspirin show no consistent acute effect of aspirin. There is no significant correlation between salicylate levels and either CBG or cortisol.

ACTH had no significant effect on CBG ( $p > 0.95$ ) and at the time of sampling, no longer showed any effect on plasma cortisol levels. Under treatment with the Synacthen Depot, however, cortisol levels were significantly raised ( $p < 0.01$ ). In prednisolone treated patients, cortisol levels were significantly lowered when compared to the non-treated group ( $p < 0.01$ ).

These changes are reflected in the cortisol/CBG ratio - an indication of the free cortisol levels. During prednisolone treatment,

TABLE 2      The effect of three drugs on the levels of  
cortisol and cortisol binding globulin in rheumatoid arthritis

| Drug treatment  | n  | Mean cortisol<br>(nmol/l) | S.D. | Mean CBG<br>(nmol cortisol/l) | S.D. | Cortisol/CBG | S.E.M. |
|-----------------|----|---------------------------|------|-------------------------------|------|--------------|--------|
| None            | 36 | 373                       | 25   | 547                           | 17   | 0.7          | 0.07   |
| Prednisolone    | 39 | 157                       | 22   | 461                           | 19   | 0.3          | 0.05   |
| Acthar Gel      | 26 | 444                       | 83   | 549                           | 19   | 0.8          | 0.12   |
| Synacthen Depot | 34 | 588                       | 102  | 489                           | 22   | 1.2          | 0.18   |

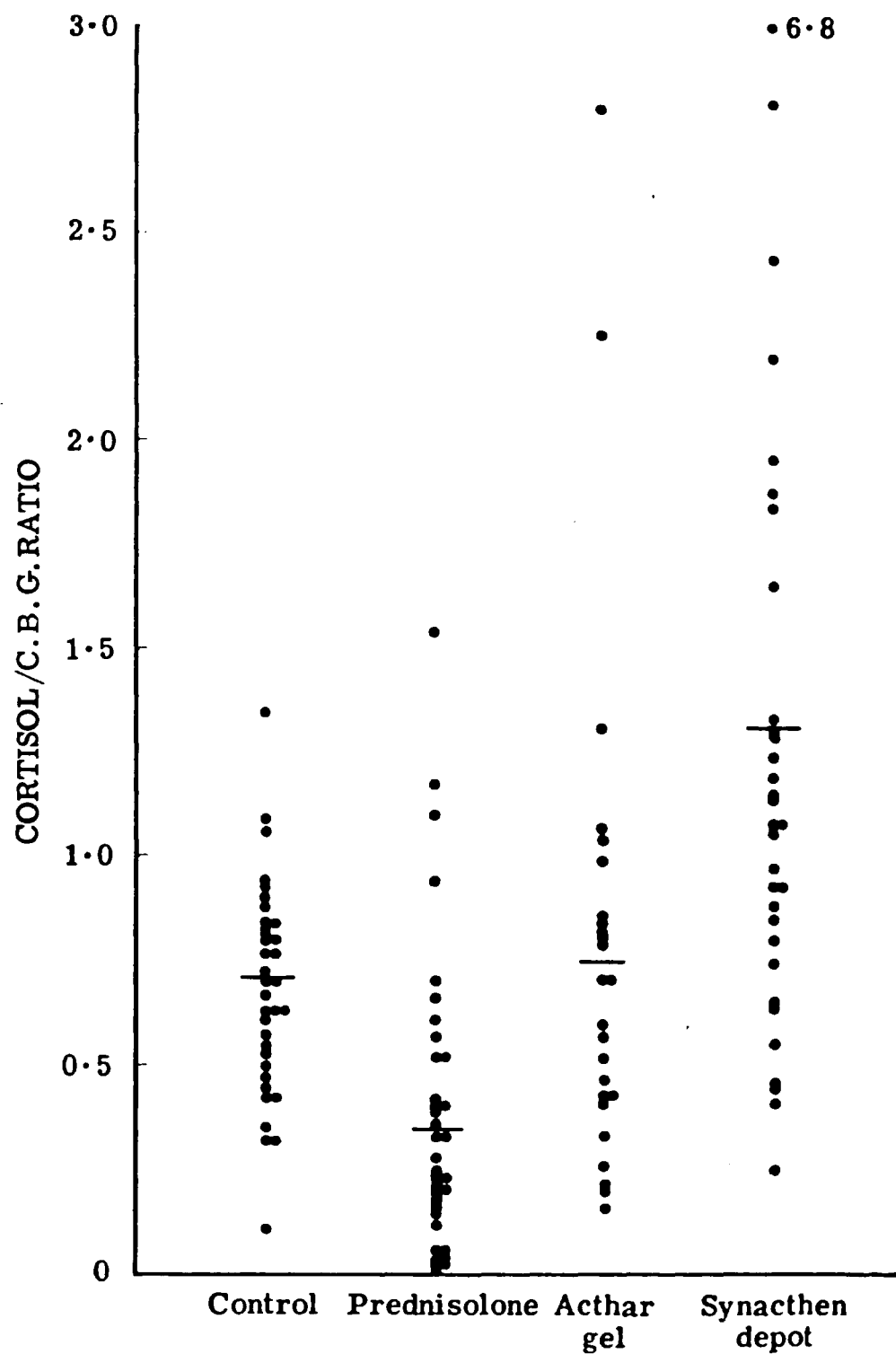


Fig. 23 : The effect of three drugs on the ratio of plasma cortisol to cortisol binding globulin

TABLE 3      The effect of three drugs on the levels of  
cortisol and cortisol binding globulin in  
rheumatoid arthritis - backtransformed data

| Drug treatment  | n  | backtransformed<br>mean-cortisol<br>(nmol/l) | SD | Backtransformed<br>mean- CBG<br>(nmol cortisol/l) | SD |
|-----------------|----|----------------------------------------------|----|---------------------------------------------------|----|
| None            | 36 | 338                                          | 44 | 539                                               | 33 |
| Prednisolone    | 39 | 113                                          | 67 | 438                                               | 35 |
| Acthar Gel      | 26 | 319                                          | 67 | 540                                               | 33 |
| Synacthen Depot | 34 | 512                                          | 48 | 505                                               | 41 |

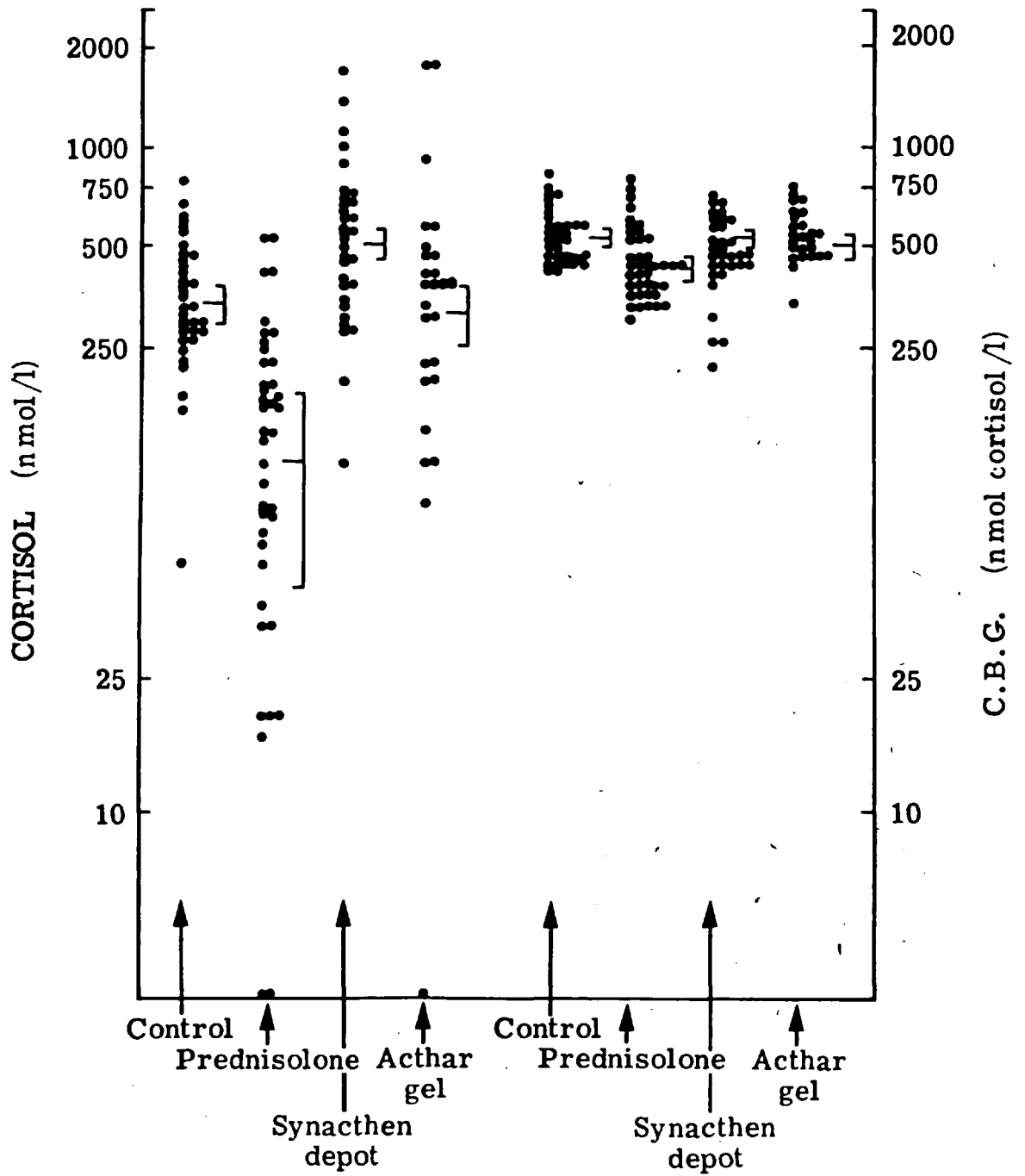


Fig. 24 : The effect of three drugs on plasma cortisol and cortisol binding globulin levels

the ratio is reduced to approximately half that of the controls, while under long-acting ACTH treatment, the ratio is increased by approximately one and a half times. ACTH gel treatment appears to have little significant effect.

## Chapter 10: The effect of oral contraceptives on the total cortisol and cortisol binding globulin in women through the menstrual cycle

### Introduction

Although cortisol and CBG levels have been measured in women, little or no account has been taken of the variation of their hormonal status during the menstrual cycle, i.e. of the stage of the menstrual cycle at which blood was taken. Oestrogens are known to affect cortisol and CBG levels as previously discussed. Either increased secretion or oral administration of progesterone could lead to the displacement of cortisol from the common binding protein CBG. Although probably transient in effect, the rise in the concentration of unbound cortisol, by the negative feedback system, would cause a decrease in secretion of cortisol.

However, although progesterone readily displaces cortisol from CBG in vitro, in the plasma in vivo, at least four to five times more progesterone than cortisol is required to displace cortisol. This is due to the fact that the association constants of albumin and other proteins for progesterone are relatively high and the binding systems have a high capacity for the steroid. Synthetic steroids may, however, have a high affinity for CBG and less for albumin, and so by competition contribute to the level of unbound cortisol.

A trial was set up to investigate levels of cortisol and CBG throughout the menstrual cycle in normal women, and in women taking 'low dose' (i.e. 50 ug oestrogen) combined-type oral contraceptives, or progestogen-only contraceptives.

Levels of cortisol and CBG in women were measured throughout the menstrual cycle.

Ten of the women studied were taking various combined-type oral contraceptives and had been doing so for at least six months prior to sampling. Fourteen women took no steroids and acted as controls. Two women received a progestin-only contraceptive, norethisterone 0.35 mg.

The combined oral contraceptives were:

Norinyl-1 (Syntex): mestranol 50 ug, norethisterone 1 mg.  
(6 subjects)

Ovulen 50 (Searle): ethinyl oestradiol 50 ug, ethynodiol diacetate 1 mg.  
(2 subjects)

Gynovlar (Schering): ethinyl oestradiol 50 ug, norethisterone acetate 3 mg. (1 subject)

Minovlar (Schering): ethinyl oestradiol 50 ug, norethisterone acetate 1 mg. (1 subject).

Blood was taken at intervals throughout the menstrual cycle in the morning between 0900 and 1000 hr, and the samples stored at  $-20^{\circ}\text{C}$  until assayed.

Several women were studied throughout more than one cycle; the data are therefore derived from 15 cycles from women on combined preparations, 2 cycles from women on the progestin-only preparation and 18 cycles from controls.

Measurements have been grouped into three-day periods for clarity and to allow for sufficient data at each point.

Day one of the cycle is taken, as is clinically conventional, as the first day of menstrual bleeding.

TABLE 4

Plasma cortisol and cortisol binding globulin through the menstrual cycle

All cycles

| Cycle Day |    | CONTROL CYCLES (18)  |     |                          |     | PILL CYCLES ( 15) |                      |     |                           |     |
|-----------|----|----------------------|-----|--------------------------|-----|-------------------|----------------------|-----|---------------------------|-----|
|           | n  | Cortisol<br>(nmol/l) |     | CBG<br>(nmol cortisol/l) |     | n                 | Cortisol<br>(nmol/l) |     | CBG<br>(n mol cortisol/l) |     |
|           |    | mean                 | sem | mean                     | sem |                   | mean                 | sem | mean                      | sem |
| 1 - 3     | 25 | 458                  | 27  | 475                      | 30  | 21                | 845                  | 81  | 974                       | 57  |
| 4 - 6     | 24 | 492                  | 62  | 525                      | 51  | 18                | 759                  | 56  | 676                       | 32  |
| 7 - 9     | 21 | 427                  | 38  | 507                      | 37  | 17                | 777                  | 60  | 734                       | 33  |
| 10 - 12   | 26 | 345                  | 28  | 472                      | 23  | 19                | 774                  | 42  | 810                       | 40  |
| 13 - 15   | 22 | 401                  | 28  | 479                      | 30  | 22                | 808                  | 40  | 833                       | 39  |
| 16 - 18   | 22 | 356                  | 31  | 439                      | 22  | 16                | 888                  | 85  | 873                       | 57  |
| 19 - 21   | 25 | 396                  | 31  | 495                      | 22  | 20                | 849                  | 70  | 783                       | 45  |
| 22 - 24   | 24 | 350                  | 35  | 500                      | 20  | 21                | 815                  | 63  | 815                       | 40  |
| 25 - 27   | 18 | 363                  | 35  | 518                      | 40  | 17                | 987                  | 78  | 904                       | 34  |
| 28 - 30   | 11 | 368                  | 56  | 522                      | 42  | 7                 | 858                  | 106 | 841                       | 77  |

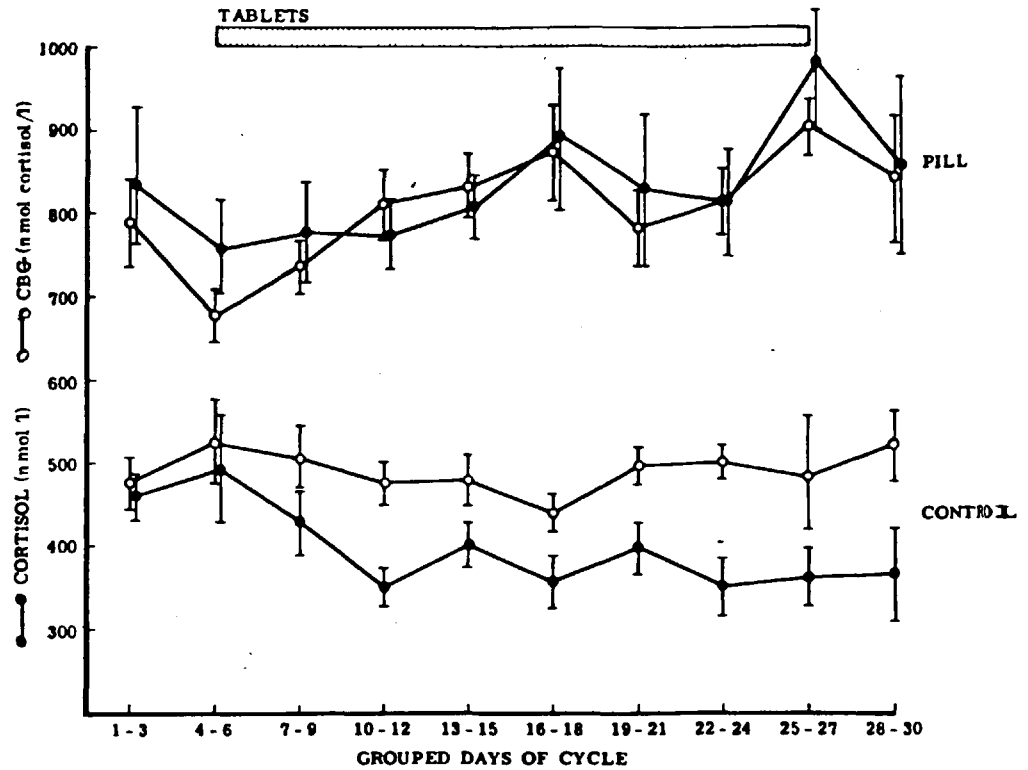


Fig. 25 : Plasma cortisol and cortisol binding globulin levels through the menstrual cycle:  
1. All cycles.

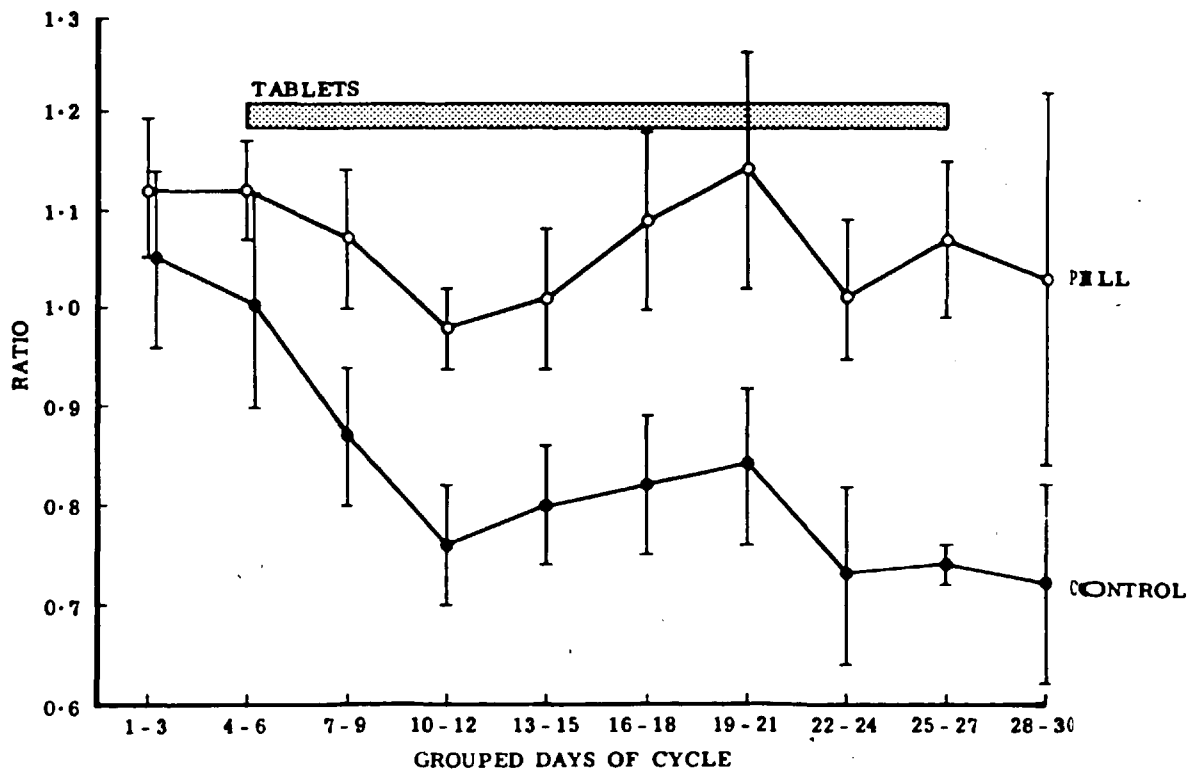


Fig. 26 : Ratio of cortisol to cortisol binding globulin levels through the menstrual cycle.  
1. All cycles.

1. Cortisol and cortisol binding globulin in pill and control cycles

(i) The mean  $\pm$  SEM for cortisol and CBG levels for each three-day period were calculated for the pill and control groups. The results are shown in Table 4 and Fig. 25. Individual results are shown in Appendix 2.

The ratio of cortisol to the CBG was calculated for each sample, and the mean  $\pm$  SEM for each three-day period calculated. These are shown in Table 5 and Fig. 26.

The plasma cortisol and CBG in the pill group are higher throughout the cycle than in the control group.

Analysis of variance (see Appendix 3 (2)) showed significant differences in the pattern of cortisol and CBG levels through the cycle between the pill and control groups. Cortisol levels in the pill group show a trough from day 4 to day 15 and a peak at day 25 through to day 3 of the next cycle. CBG levels showed a trough on days 4-9 and a peak on days 16-18. The control group showed a peak in cortisol on days 4-9 followed by a significant trough on days 10-12. The CBG however showed no significant variation throughout the cycle.

(ii) Using only the first cycle from each woman, i.e. 14 control cycles and 10 pill cycles, the mean cortisol and CBG levels, together with the ratio of cortisol to CBG were calculated. These are shown in Table 5 and 6 and Figures 27 and 28.

Student's 't' test (see Appendix 3 (1)) showed no significant difference between the two sets of data, ( $p > 0.5$ ).

(iii) Where more than one cycle was measured (4 'control' and 4 'pill' women) Student's 't' test showed no significant differences between the two groups of cycles ( $p > 0.4$ ).

2. Cortisol and cortisol binding globulin in women taking different oestrogen-containing preparations - ethinyl oestradiol and mestranol

The three-day means of cortisol and CBG in 6 women taking mestranol (Norinyl-1) were compared with those from the four women

TABLE 5      Ratio of cortisol to cortisol binding globulin in control women  
and in women taking oral contraceptives through the menstrual  
cycle

| Cycle days | All Cycles |      |      |      | One Cycle per woman |      |      |      |
|------------|------------|------|------|------|---------------------|------|------|------|
|            | Control    |      | Pill |      | Control             |      | Pill |      |
|            | mean       | sem  | mean | sem  | mean                | sem  | mean | sem  |
| 1 - 3      | 1.05       | 0.09 | 1.12 | 0.07 | 1.12                | 0.10 | 1.10 | 0.12 |
| 4 - 6      | 1.01       | 0.11 | 1.12 | 0.05 | 0.95                | 0.10 | 1.14 | 0.07 |
| 7 - 9      | 0.87       | 0.07 | 1.07 | 0.07 | 0.90                | 0.08 | 1.10 | 0.10 |
| 10 - 12    | 0.76       | 0.06 | 0.98 | 0.04 | 0.79                | 0.07 | 0.92 | 0.06 |
| 13 - 15    | 0.80       | 0.06 | 1.01 | 0.07 | 0.83                | 0.08 | 0.89 | 0.05 |
| 16 - 18    | 0.82       | 0.07 | 1.09 | 0.09 | 0.83                | 0.09 | 1.05 | 0.10 |
| 19 - 21    | 0.84       | 0.08 | 1.14 | 0.12 | 0.88                | 0.12 | 0.98 | 0.12 |
| 22 - 24    | 0.73       | 0.09 | 1.02 | 0.07 | 0.75                | 0.10 | 0.90 | 0.09 |
| 25 - 27    | 0.74       | 0.02 | 1.07 | 0.08 | 0.72                | 0.09 | 1.05 | 0.06 |
| 28 - 30    | 0.72       | 0.10 | 1.03 | 0.19 | 0.73                | 0.11 | 1.01 | 0.06 |

TABLE 6

Plasma cortisol and cortisol binding globulin throughout the menstrual cycleOne cycle per woman

| Cycle day | CONTROL CYCLES (14) |                      |     |                             |     |    | PILL CYCLES (10)     |     |                             |     |
|-----------|---------------------|----------------------|-----|-----------------------------|-----|----|----------------------|-----|-----------------------------|-----|
|           | n                   | Cortisol<br>(nmol/l) |     | C.B.C.<br>(nmol cortisol/l) |     | n  | Cortisol<br>(nmol/l) |     | C.B.C.<br>(nmol cortisol/l) |     |
|           |                     | mean                 | sem | mean                        | sem |    | mean                 | sem | mean                        | sem |
| 1 - 3     | 20                  | 470                  | 33  | 447                         | 29  | 13 | 832                  | 114 | 779                         | 78  |
| 4 - 6     | 19                  | 449                  | 47  | 527                         | 23  | 13 | 764                  | 66  | 674                         | 48  |
| 7 - 9     | 16                  | 449                  | 46  | 579                         | 48  | 12 | 779                  | 76  | 716                         | 38  |
| 10 - 12   | 20                  | 351                  | 35  | 461                         | 29  | 13 | 739                  | 39  | 825                         | 51  |
| 13 - 15   | 17                  | 404                  | 35  | 465                         | 36  | 13 | 805                  | 52  | 901                         | 34  |
| 16 - 18   | 17                  | 353                  | 40  | 433                         | 26  | 12 | 864                  | 96  | 897                         | 59  |
| 19 - 21   | 20                  | 399                  | 35  | 479                         | 25  | 12 | 825                  | 91  | 864                         | 48  |
| 22 - 24   | 19                  | 363                  | 41  | 488                         | 22  | 13 | 766                  | 75  | 851                         | 53  |
| 25 - 27   | 14                  | 382                  | 40  | 550                         | 45  | 12 | 967                  | 64  | 927                         | 41  |
| 28 - 30   | 7                   | 378                  | 69  | 513                         | 29  | 3  | 786                  | 113 | 932                         | 156 |

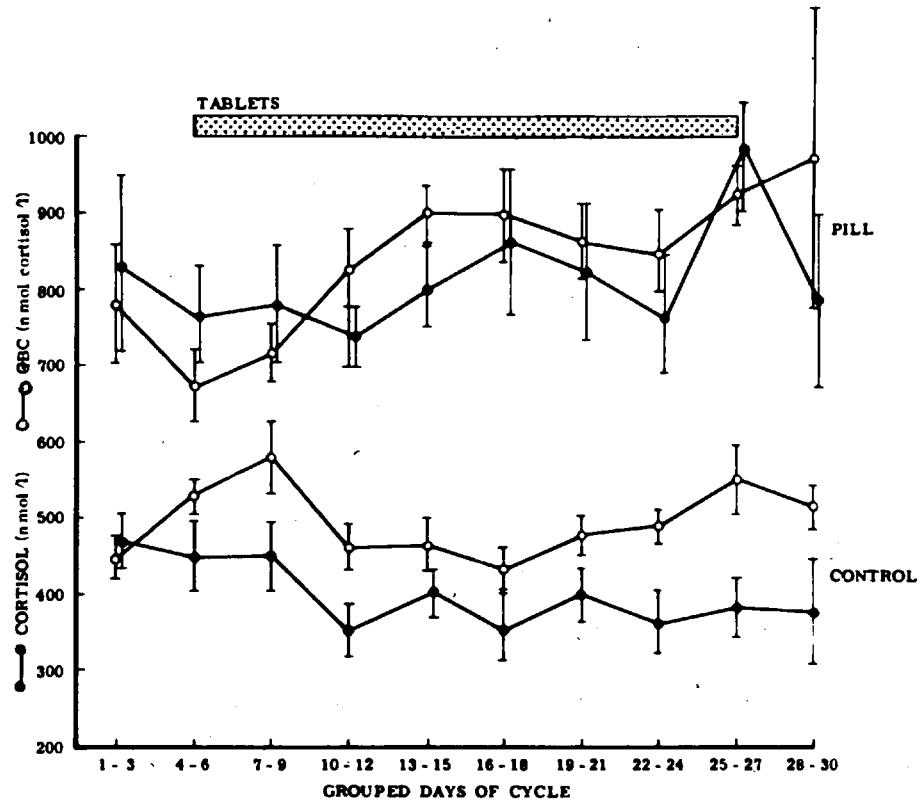


Fig. 27: Plasma cortisol and cortisol binding globulin levels through the menstrual cycle: 2. One cycle per woman.

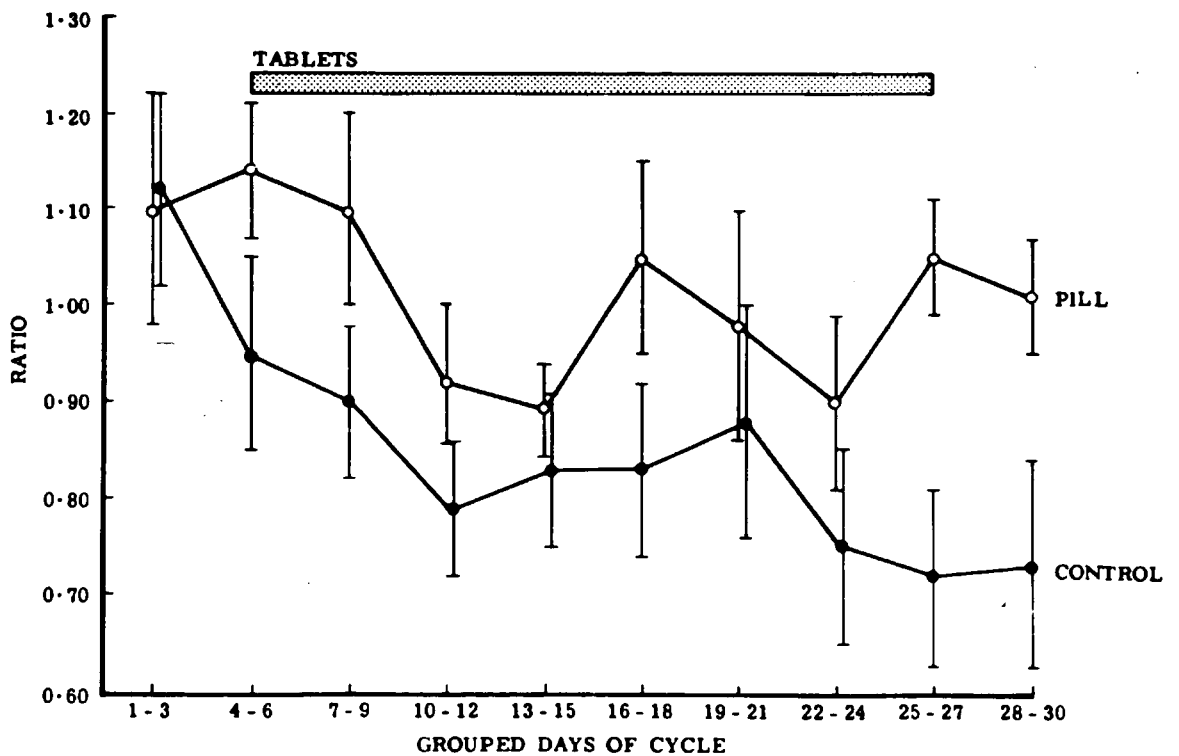


Fig. 28 : Ratio of cortisol to cortisol binding globulin through the menstrual cycle: 2. One cycle per woman.

taking ethinyl oestradiol (Ovulen 50, Gynovlar, Minovlar) (Table 7).

Student's 't' test showed no significant difference between the two groups ( $p > 0.5$ ).

### 3. Cortisol and cortisol binding globulin in women taking the norethisterone contraceptive pill

(i) The three-day mean values for cortisol and CBG from the two women taking Net 0.35 (Norethisterone acetate 0.35 mg) are shown in Table 8.

Student's 't' test shows no significant difference between the results of these cycles and those from the control cycles ( $p > 0.6$ ).

(ii) One of the women, MH, had two cycles measured while taking no steroids. Comparison of the three cycles shows no significant difference between them ( $p > 0.8$ ).

### 4. Conclusions

Levels of both cortisol and CBG are significantly raised in women taking the oral contraceptive pill at all stages of the menstrual cycle. This would appear to be due to the oestrogen component - there being no such rise following progestin-only treatment.

Although ethinyl oestradiol is approximately 1.5 times more potent in suppressing ovulation than mestranol (Goldzieher et al, 1966) no significant difference was found between the levels of cortisol and CBG in each different oestrogen group. Cortisol levels and CBG seem to be fairly consistent for each woman, there being no significant difference between two cycles in the same woman.

Cortisol and CBG levels remained fairly constant in the control group, cortisol levels peaking at the beginning of the cycle, days 4-9, and reaching their lowest on days 10-12.

In contrast, the pill group show a marked periodicity in both cortisol and CBG, both lower at the beginning of the cycle and rising to peak values at the end of the cycle. The fairly sharp increase from day 4 to day 15 is probably related to the beginning of a new course of tablets at or around day 4. The oestrogen component of

TABLE 7      Cortisol and cortisol binding globulin levels in women taking oral contraceptives:  
comparisons between pills containing mestranol and ethinyl oestradiol.

| mestranol (Norinyl-1) (11 cycles) |    |                   |     |                       |     | ethinyl oestradiol (Ovulen, Gynovlar, Minovlar (4 cycles) |                   |     |                        |     |
|-----------------------------------|----|-------------------|-----|-----------------------|-----|-----------------------------------------------------------|-------------------|-----|------------------------|-----|
| Cycle day                         | n  | Cortisol (nmol/l) |     | CBG (nmol cortisol/l) |     | n                                                         | Cortisol (nmol/l) |     | CBG (n mol cortisol/l) |     |
|                                   |    | mean              | sem | mean                  | sem |                                                           | mean              | sem | mean                   | sem |
| 1 - 3                             | 16 | 864               | 95  | 795                   | 74  | 5                                                         | 872               | 156 | 792                    | 58  |
| 4 - 6                             | 12 | 800               | 73  | 701                   | 50  | 6                                                         | 676               | 78  | 627                    | 52  |
| 7 - 9                             | 13 | 787               | 75  | 751                   | 40  | 4                                                         | 745               | 97  | 682                    | 49  |
| 10 - 12                           | 14 | 787               | 54  | 828                   | 47  | 5                                                         | 742               | 52  | 756                    | 68  |
| 13 - 15                           | 16 | 784               | 51  | 809                   | 50  | 6                                                         | 872               | 49  | 897                    | 46  |
| 16 - 18                           | 12 | 897               | 103 | 880                   | 67  | 4                                                         | 875               | 131 | 869                    | 105 |
| 19 - 21                           | 13 | 817               | 77  | 756                   | 55  | 6                                                         | 919               | 152 | 858                    | 65  |
| 22 - 24                           | 16 | 809               | 73  | 781                   | 48  | 5                                                         | 839               | 137 | 922                    | 67  |
| 25 - 27                           | 12 | 1007              | 108 | 889                   | 80  | 5                                                         | 872               | 153 | 784                    | 26  |
| 28 - 30                           | 5  | 828               | 143 | 803                   | 65  | 2                                                         | 897               | 41  | 1038                   | 201 |

TABLE 8      Plasma cortisol and cortisol binding globulin through the  
menstrual cycle: Norethisterone Acetate

| Cycle day | n | Cortisol<br>nmol/l | CBG<br>(nmol cortisol/l) | Cortisol/CBG |
|-----------|---|--------------------|--------------------------|--------------|
| 1 - 3     | 3 | 211                | 282                      | 1.28         |
| 4 - 6     | 2 | 280                | 399                      | 0.75         |
| 7 - 9     | 4 | 298                | 368                      | 0.72         |
| 10 - 12   | 2 | 226                | 353                      | 0.64         |
| 13 - 15   | 1 | 359                | 475                      | 0.76         |
| 16 - 18   | 1 | 268                | 400                      | 0.67         |
| 19 - 21   | 3 | 296                | 341                      | 0.86         |
| 22 - 24   | 2 | 276                | 294                      | 0.97         |
| 25 - 27   | 1 | 248                | 317                      | 0.78         |
| 28 - 30   | - | -                  | -                        | -            |

the oral contraceptives stimulates the rise in CBG which in turn is followed by a rise in cortisol levels.

The ratio of cortisol to CBG shows a significant increase at the end of the cycle in the pill group compared with the control group, the level of cortisol exceeding that of the CBG. The ratio is similarly, although not so greatly, higher at the beginning of the cycle, i.e. there is an increase in free cortisol in the pill group throughout the cycle.

## Chapter 11: The determination of free cortisol in men and women and in women taking oral contraceptives

### Introduction

Raised levels of both cortisol and CBG have been found in oral contraceptive therapy, although there is some disagreement as to whether the free cortisol levels are raised (Briggs and Briggs, 1972; Baumann *et al*, 1975) or not (Burke, 1970; Bulbrook *et al*, 1973; Brien, 1975). These increases appear dependent on the dose of oestrogen as they were not seen with the progestogen-only preparations (Daly and Elstein, 1972; Burke, 1970).

Available methods for the measurement of free cortisol are tedious and require fairly large (10 ml or more) volumes of plasma. A method was developed, therefore, which required small amounts of plasma (0.5 ml) and was relatively simple to perform (Chapter 6). The assay was then used to measure free cortisol in normal adults and in women taking the oral contraceptive 'low dose' pill.

The possibility that variations occurring during the day and/or during the menstrual cycle may account for the differences in published levels of free cortisol was also investigated. Plasma cortisol and CBG levels were measured in the morning and in the evening and in early and late stages of the menstrual cycle in a small group of women, some of whom were taking 'low-dose' oral contraceptives. Urine cortisol levels were also measured.

### 1. Total and free cortisol in normal adults and in women taking oral contraceptives

The levels of total and free cortisol were measured in normal healthy adults and in women taking various oral contraceptives. No attempt was made to select the type of oral contraceptive used, but all the women were taking 'low-dose', i.e. containing 50 µg oestrogen, combined-type oral contraceptives and had been doing so for at least six months.

TABLE 9 The concentration of free cortisol in plasma - comparison of some published results

| Author and reference     |   | Mean percentage free cortisol | Mean free cortisol (nmol/l) | Technique used              |
|--------------------------|---|-------------------------------|-----------------------------|-----------------------------|
| Baumann et al (1975)     |   | $10.6 \pm 2.8$                | $38 \pm 17$                 | Free cortisol index         |
| Booth et al (1961)       | m | -                             | 31                          | Equilibrium dialysis        |
|                          | f | -                             | 18                          |                             |
| Burke (1970)             | m | 6.3                           | 19(3-50)                    | Steady-state gel filtration |
|                          | f | 5.5                           | 18(3-59)                    |                             |
| Doe et al (1969)         | m | 7.7                           | $35 \pm 14$                 | Equilibrium dialysis        |
|                          | f | 7.4                           | $39 \pm 11$                 |                             |
| Kunugi et al (1973)      |   | 10.5                          | 51                          | Gel filtration              |
| Nolten et al (1979)      |   |                               | $24 \pm 3$                  | Free cortisol index         |
| O'Connell & Welsh (1969) | f | 7.6                           | 27                          | Ultrafiltration             |
| Plager et al (1964)      |   | 13.0                          | 35                          | Ultracentrifugation         |
| Robin et al (1977)       |   | $9.7 \pm 2.6$                 | $41 \pm 20$                 | Ultrafiltration             |
| Rosenthal et al (1969)   |   | $8.5 \pm 0.6$                 | 18                          | Ultracentrifugation         |
| Sandberg et al (1967)    |   | 10.0                          | 30-35                       | Equilibrium dialysis        |
| This study               | m | $10.6 \pm 1.7$                | $41 \pm 17$                 | Equilibrium dialysis        |
|                          | f | $8.6 \pm 1.0$                 | $28 \pm 8$                  |                             |

Blood was taken from 48 men and women, within the age group 20-35 years, between 0900 and 0930 hrs. After centrifugation the plasma was frozen immediately and stored at  $-20^{\circ}\text{C}$  until assayed.

Total plasma cortisol was measured fluorimetrically and the free cortisol measured by the small scale equilibrium dialysis method, the development of which has been described in Chapter 6.

The results of the assays are shown in Fig. 29 and Table 10, and in Appendix 2(4).

These results are compared with others published in the last few years in Tables 9 and 10.

Student's 't' test (see Appendix 3(i)) was performed following log transformation of the results, to give an approximately normal distribution. A significant difference was found between the pill-taking and normal women - both the total and free cortisol levels were higher in the pill group ( $p < 0.01$ ). There were also significantly higher cortisol levels in the men as compared to the control women ( $p < 0.01$ ).

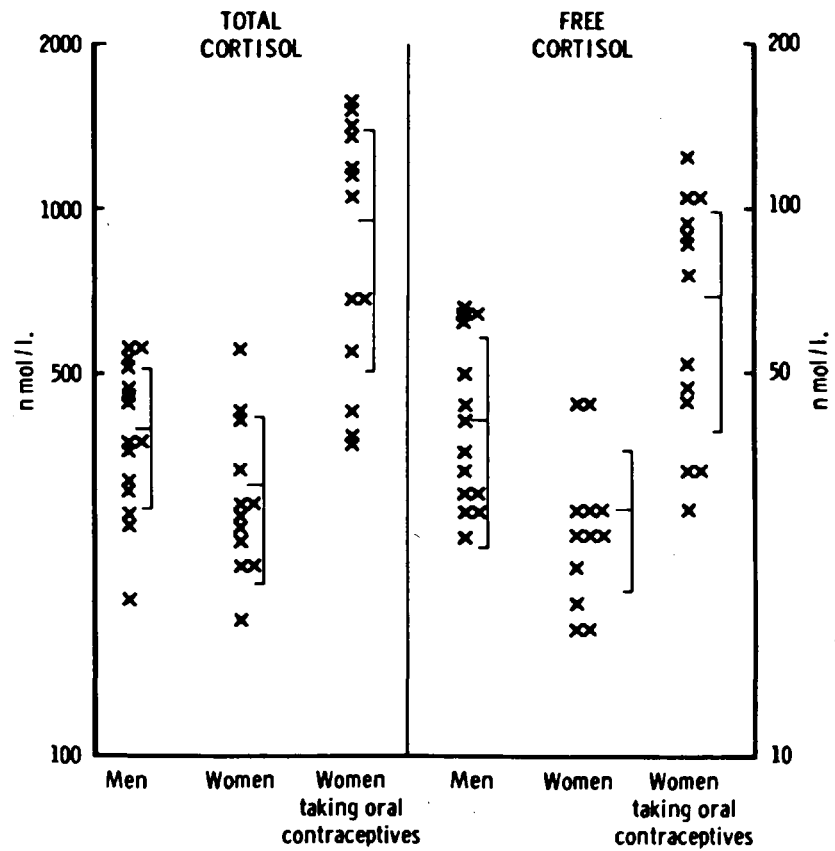


Fig. 29 : Plasma free cortisol levels in normal adults and in woman taking oral contraceptives.

TABLE 10

The concentration of free cortisol in women taking oral contraceptives - comparison of results

|                         |                           | n  | Percentage free<br>Cortisol | Total Cortisol<br>nmol/l | Free Cortisol<br>nmol/l |
|-------------------------|---------------------------|----|-----------------------------|--------------------------|-------------------------|
| This study              | Male                      | 22 | 10.6 $\pm$ 1.7              | 397 $\pm$ 113            | 41 $\pm$ 17             |
|                         | Female                    | 12 | 8.6 $\pm$ 1.0               | 312 $\pm$ 105            | 28 $\pm$ 8              |
|                         | Female on 'low-dose' pill | 14 | 7.6 $\pm$ 1.2               | 952 $\pm$ 447            | 69 $\pm$ 30             |
| Baumann et<br>al (1975) | Male and female           | 18 | 10.6 $\pm$ 2.8              | 356 $\pm$ 113            | 38 $\pm$ 17             |
|                         | Female on 'low-dose' pill | 17 | 5.7 $\pm$ 1.9               | 955 $\pm$ 362            | 56 $\pm$ 22             |
| Burke<br>(1970)         | Male                      | 36 | 6.3                         | 298(152-442)             | 19(3-50)                |
|                         | Female                    | 31 | 5.5                         | 331(110-563)             | 19(3-50)                |
|                         | Female on 'low-dose' pill | 5  | 3.2                         | 384(248-483)             | 12(8-17)                |
|                         | Female on 'standard' pill | 16 | 3.2                         | 886(442-1623)            | 28(14-50)               |

## 2. Total and free cortisol levels at various times of the day and stages of the menstrual cycle

Blood was taken between 0900 and 1000 hr and again between 1630 and 1730 hr from twelve women, some of whom were taking various 'low-dose' combined-type oral contraceptives. Samples were taken during days 6-9 and again between days 23-26 of the menstrual cycle. Plasma was stored and assayed as described in the previous chapter. Twenty-four hour urines were collected on the same days that blood was taken and the cortisol levels measured using radioimmunoassay.

The results are shown in Table 11 and in Figs. 30 and 31.

**TABLE 11** Mean total and free plasma cortisol levels and cortisol binding capacity in women taking oral contraceptives and in control women in the morning and evening at the beginning and end of the menstrual cycle together with their 24-hour urine cortisol levels

| <u>Time</u> | <u>Total Cortisol (nmol/l)</u> |                 |                            |                 |
|-------------|--------------------------------|-----------------|----------------------------|-----------------|
|             | <u>Controls</u>                |                 | <u>Oral Contraceptives</u> |                 |
|             | days<br>6 - 9                  | days<br>23 - 26 | days<br>6 - 9              | days<br>23 - 26 |
| 0900-1000   | 374 $\pm$ 123                  | 495 $\pm$ 352   | 892 $\pm$ 118              | 1196 $\pm$ 352  |
| 1630-1730   | 197 $\pm$ 99                   | 202 $\pm$ 153   | 506 $\pm$ 97               | 567 $\pm$ 192   |
|             | <u>Free Cortisol (nmol/l)</u>  |                 |                            |                 |
|             | <u>Controls</u>                |                 | <u>Oral Contraceptives</u> |                 |
|             | days<br>6 - 9                  | days<br>23 - 26 | days<br>6 - 9              | days<br>23 - 26 |
| 0900-1000   | 46 $\pm$ 19                    | 52 $\pm$ 33     | 91 $\pm$ 10                | 103 $\pm$ 35    |
| 1630-1730   | 20 $\pm$ 13                    | 22 $\pm$ 19     | 43 $\pm$ 9                 | 42 $\pm$ 18     |
|             | <u>CBG (nmol cortisol/l)</u>   |                 |                            |                 |
|             | <u>Controls</u>                |                 | <u>Oral Contraceptives</u> |                 |
|             | days<br>6 - 9                  | days<br>23 - 26 | days<br>6 - 9              | days<br>23 - 26 |

Table 11 contd...

|           |                                    |               |                            |                |
|-----------|------------------------------------|---------------|----------------------------|----------------|
| 0900-1000 | 453 $\pm$ 202                      | 398 $\pm$ 210 | 692 $\pm$ 173              | 1036 $\pm$ 228 |
| 1630-1730 | 404 $\pm$ 220                      | 424 $\pm$ 185 | 715 $\pm$ 136              | 1023 $\pm$ 194 |
|           | <u>Urinary Cortisol nmol/24 hr</u> |               | <u>Oral Contraceptives</u> |                |
|           | days                               | days          | days                       | days           |
|           | 6 - 9                              | 23 - 26       | 6 - 9                      | 23 - 26        |
|           | 98 $\pm$ 38                        | 116 $\pm$ 31  | 107 $\pm$ 30               | 88 $\pm$ 14    |

Levels of both total and free cortisol are higher in the morning than in the evening in both groups ( $p < 0.01$ ), and the levels of the pill group are higher than those of the control women ( $p < 0.01$ ) at both times of day and both phases of the cycle.

The difference between the morning and evening cortisol in the pill group appears more marked at the end than at the beginning of the cycle. These differences however do not achieve significance ( $p < 0.3$ ).

The CBC levels do not vary with the time or dates of sampling in the control group. Treatment with oral contraceptives, however, causes a marked rise in the level of CBC at both times of the day and of the cycle with an even more marked rise towards the end of the cycle ( $p < 0.01$ ).

The urinary cortisol shows no significant differences between either days of the cycle or with oral contraceptive treatment.

As cortisol varies through the day, the ratio of CBC to urinary cortisol for each patient was calculated. The results are shown below and in Fig. 32.

#### Urinary Cortisol as a Percentage Function of CBC

|                    |                  |                |
|--------------------|------------------|----------------|
|                    | <u>Day/Cycle</u> |                |
|                    | <u>6 - 9</u>     | <u>23 - 26</u> |
| Controls           | 23.0             | 31.4           |
| Oral Contraceptive | 15.2             | 8.6            |

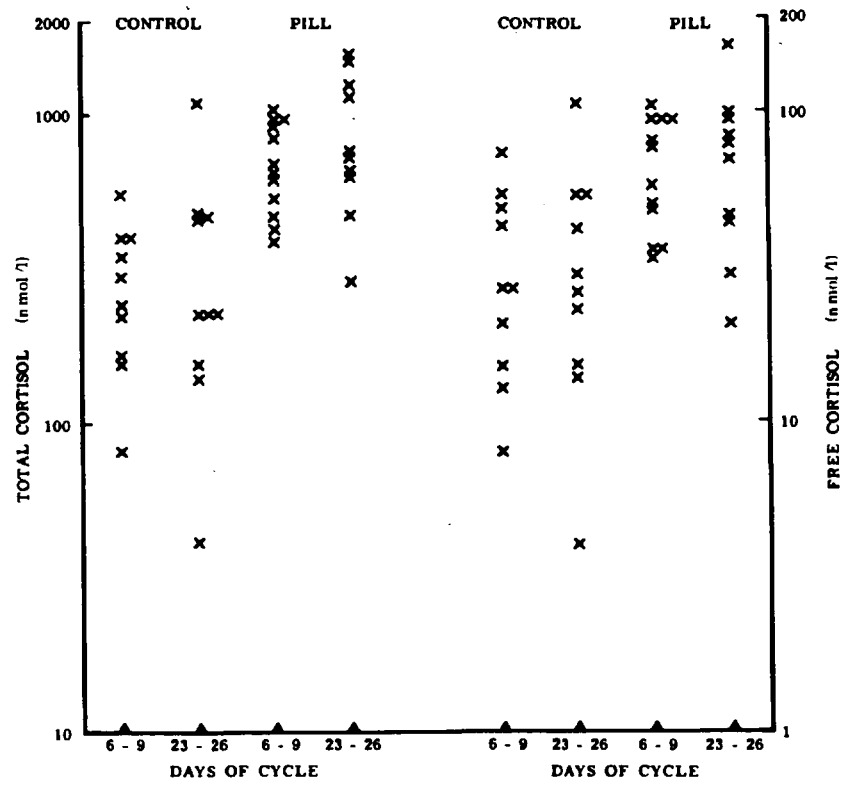


Fig. 30 : Total and free plasma cortisol levels in women in the morning and at different times during the menstrual cycle.

Fig. 31: Plasma cortisol binding globulin and urinary cortisol measured in women taking oral contraceptives (● — ●) or no medication (○ — ○) at different times of their menstrual cycle.

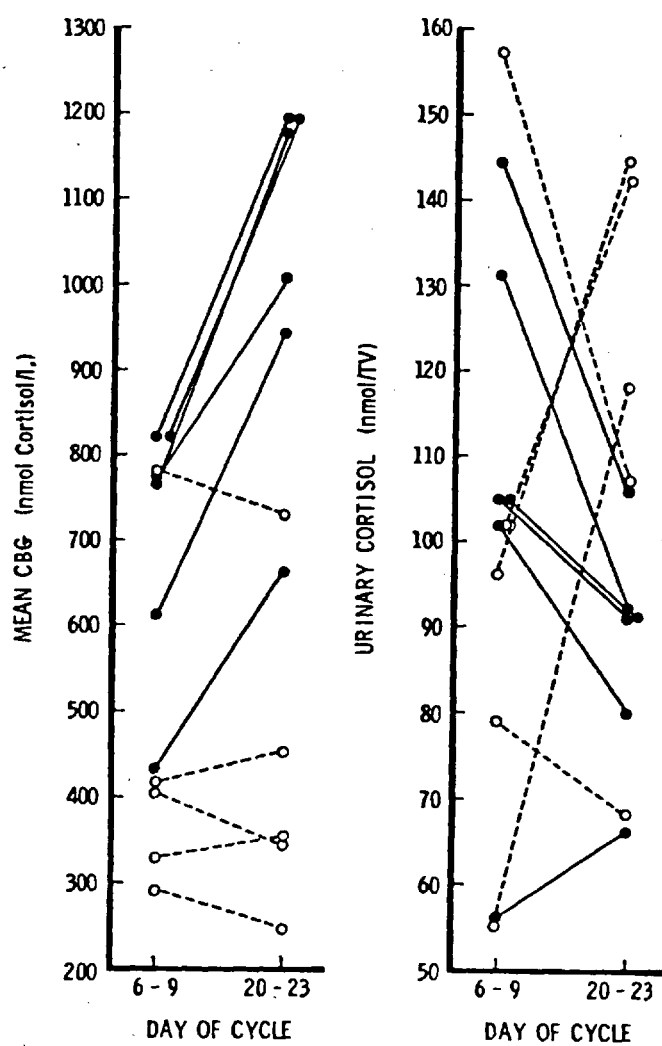
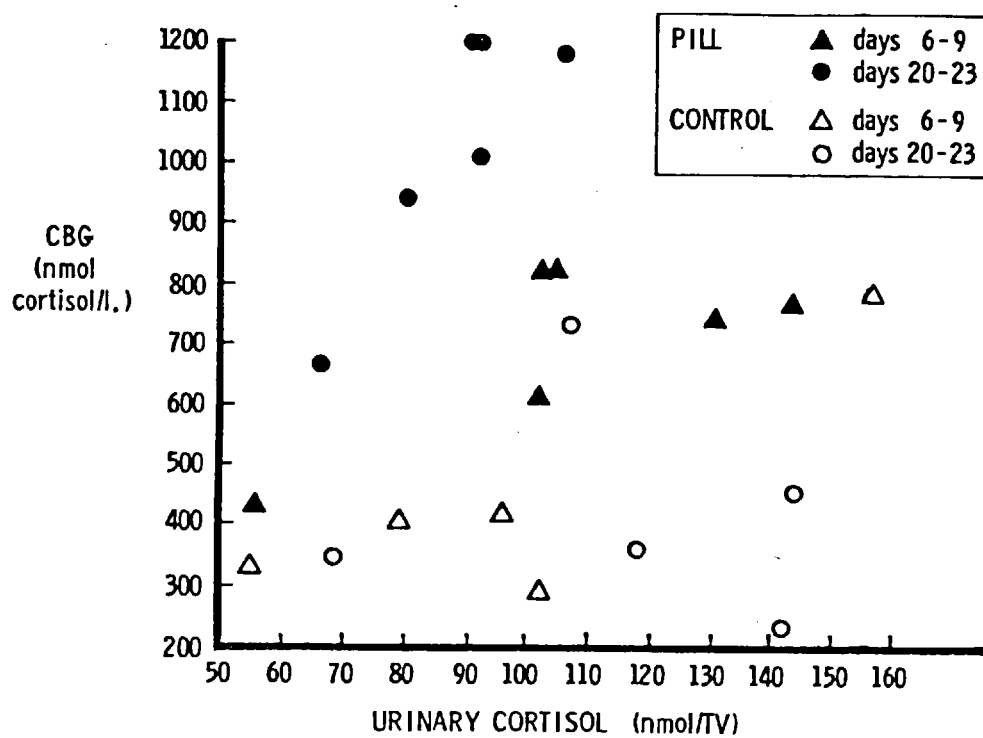


Fig. 32: Comparison of cortisol binding globulin and urinary cortisol levels in women .



### 3. Conclusions

The estimation of free cortisol in adults , as measured by the dialysis method described in the Methods section, Chapter 7, give results which are comparable to others published, as shown in Table 10.

Men tended to have a slightly higher free cortisol than women. Women taking low-dose oral contraceptives had a raised total and free cortisol when compared to the other groups in both studies and in the second study this difference was shown to occur both in the morning and in the evening and at the beginning and end of the cycle.

CBG, as measured by the CBC, is raised during oral contraceptive treatment, as previously noted and there is also a significantly higher level of CBG towards the end of the cycle in these women.

Surprisingly there was little difference shown in the urinary cortisol levels. There were only six women in this trial however and this may account for the lack of any differences.

This is further discussed in the Discussion section.

Chapter 12: Cortisol binding globulin levels in men and women and in women having premenstrual syndrome as measured by the 'two-tier' column method

Introduction: Premenstrual syndrome

Premenstrual tension or premenstrual syndrome (PMS) (Dalton, 1977) covers a wide variety of symptoms which increase in intensity for 5-10 days before menstruation. Termination of symptoms may occur abruptly with the onset of full menstrual flow. The most frequent complaints are a sense of abdominal bloating, breast tenderness, headache, irritability, mental depression and an increase in weight which may be associated with oedema of the legs. The incidence of the syndrome depends on the criteria used for diagnosis, but has been estimated to be around 40% of women (Dalton, 1977). Diagnosis of the syndrome depends on the recognition of the cyclical occurrence of symptoms rather than on the type of symptoms as currently, there is little evidence of any biochemical tests which are useful. The aetiology of PMS has been the subject of much debate. One of the earliest ideas was put forward by Frank (1931) who suggested that symptoms were produced by a rise in "female sex-hormone" levels. Gillman (1942) has suggested that progesterone was the cause of PMS and an abnormal oestrogen-progesterone ratio has also been put forward as the cause of PMS by for example Israel (1938). Water and sodium retention have been implicated by for example Greenhill and Freed (1941) but they do not correlate with the presence or severity of PMS (Russel, 1972).

It is currently thought that the wide fluctuation in female sex hormone levels in the menstrual cycle provide the critical "triggering" mechanisms for the pathophysiological changes in peripheral tissues, as well as in the central nervous system.

Continuation of these symptoms of PMS after hysterectomy and after the menopause suggest that the hypothalamic-pituitary axis is important in the aetiology of PMS and Dalton (1977) has suggested that there may be a fault in the "progesterone feed-back pathway".

### 1. Cortisol binding globulin levels in normal men and women

Blood was taken from healthy volunteers between the ages of 20 and 45 years, in order to establish a normal range of values for CBG levels in plasma using the 'two-tier' column method described in Chapter 5.

The blood was centrifuged and the plasma stored at  $-20^{\circ}\text{C}$  until assayed.

The mean CBG levels for men and women are shown in Table 12 and in Appendix 2.

TABLE 12: Levels of cortisol binding globulin in men and women and in women having pre-menstrual syndrome as measured using the "two-tier" column method.

|                | n  | CBG<br>(nmol cortisol/l) |
|----------------|----|--------------------------|
| Men            | 14 | $352 \pm 24$             |
| Women          | 16 | $358 \pm 25$             |
| Women with PMS | 30 | $213 \pm 73$             |

### 2. Cortisol binding globulin levels in women with premenstrual syndrome

In view of the lack of evidence of specific changes associated with the premenstrual syndrome it was useful to measure CBG levels in PMS.

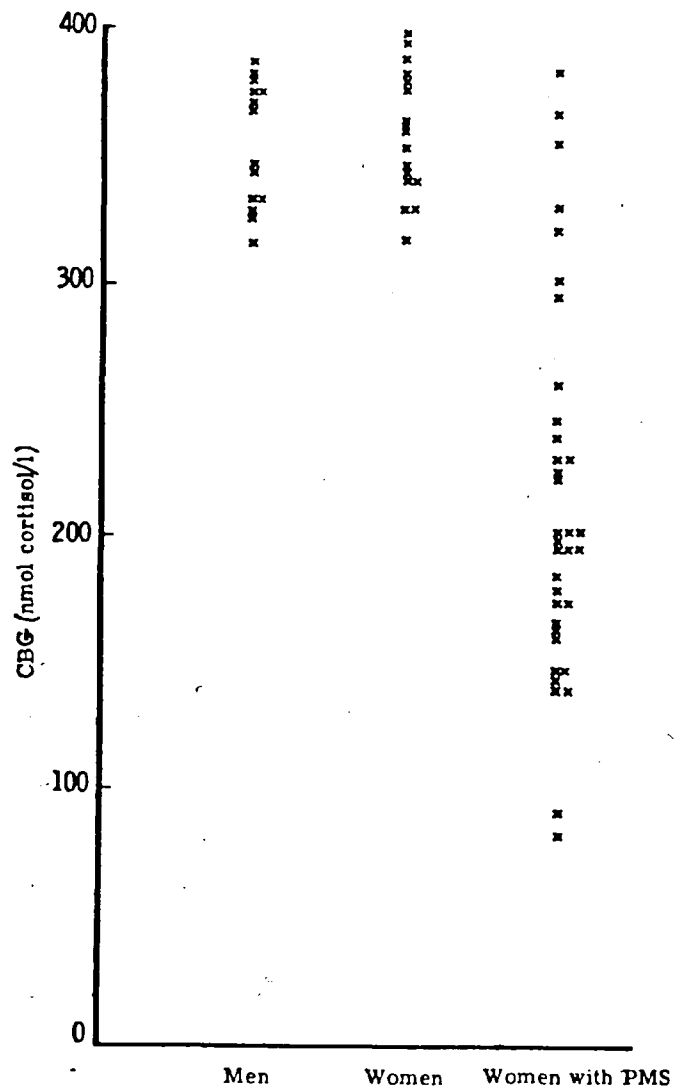
The patients, age range, 16-52 years, were all attending a private premenstrual syndrome practice and had received no medication for the two months prior to blood samples being taken. Premenstrual syndrome was diagnosed by recurrence of symptoms in the same phase of each menstrual cycle with an absence of symptoms post-menstrually. Blood was taken on day 21 of the menstrual cycle and the plasma stored at  $-20^{\circ}\text{C}$  until assayed.

Results are shown in Table 12 and Appendix 2 (I).

### 3. Conclusions

The two-tier column method gives CBG levels which are lower than others published (Table 1), for example, DeMoor (1962) found levels of 713 and 720 nmol cortisol/l. These were however only a fairly small group.

Fig. 33: Cortisol binding globulin levels measured using the two tier column method in men and women and in women having severe PMS



Women having PMS are shown to have low levels of CBG when compared to the normal controls. This is very interesting especially as low levels of SHBG were found in these women by Dr M Dalton (in preparation). This is discussed further in the Discussion section.

## DISCUSSION

## DISCUSSION

### 1. Methods

Methods used to measure plasma levels of cortisol and CBG are reviewed in Chapter 3.

#### (a) Cortisol

Cortisol was measured using two different standard techniques - fluorimetry and RIA. The fluorimetric method was found to be sensitive and precise with an interassay variation of approximately 5%. The method has the advantage that prednisolone does not interfere in the assay, which was important in the rheumatoid study.

The standard curve and recovery experiments showed that the RIA method was sensitive and precise. Interassay variation was found to be 9%. Cross-reactivity of other steroids for the antibody was investigated and corticosterone and prednisolone were found to be the main interfering steroids. This method has the advantages of allowing many samples to be measured in one assay and it can be used to measure cortisol in both plasma and urine.

Comparison of the results obtained on plasma by the two methods showed that RIA gave slightly lower results. Regression analysis (Fig. 8) gave a slope of 0.99 with a positive intercept for fluorimetry of 38 nmol/l.

#### (b) Cortisol binding globulin

In this study CBG was measured by two different methods. One was an adsorption method based on that of Trapp and West (1969). It was convenient to perform, sensitive and had an interassay variation of approximately 5%. There was found to be no interference from progesterone levels up to 100 nmol/l - higher than those found in the normal luteal phase of the menstrual cycle. CBG levels in normal men and women were found to be  $449 \pm 60$  nmols cortisol/l using this assay. These levels are similar to those published as shown in Table 1.

Another method for the measurement of CBG was developed to compare with the binding method. This was based on the two-tier column method for the measurement of SHBG of Iqbal and Johnson (1977). In this method, albumin is removed by Cibacron Blue F3G-A-Sepharose gel

followed by the separation of CBG-bound and free cortisol by the LH20 gel. Sephadex G50 and LH20 gel columns were compared in the assay and were shown to give similar results. Scatchard analysis of the columns showed the presence of only one binding system.

Calculation of the association constant for CBG from these plots gave a value of  $2.1 \times 10^8 \text{ M}^{-1}$  which is of the same order for  $K_a$  at  $4^\circ\text{C}$  as those published for example  $7.9 \times 10^8 \text{ M}^{-1}$  by Stroupe et al (1978) and  $2.1 \times 10^8 \text{ M}^{-1}$  by Chen et al (1977), both using an equilibrium dialysis technique and a value of  $3 \times 10^8 \text{ M}^{-1}$  by Trapp and West (1969) using an adsorption technique. The assay is rapid, sensitive and gives reproducible results - the interassay variation was about 8%.

Comparison of the two methods using male, female and pregnancy plasma pools showed a good correlation between the two methods.

The normal range for the adsorption method was  $449 \pm 60 \text{ nmol cortisol/l}$  for men and women ( $n = 49$ ) whereas the column method gave a mean of  $355 \pm 25 \text{ nmol cortisol/l}$  ( $n = 30$ ). The difference between the two 'normal ranges' of around 20% is larger than that found in the pooled plasmas of around 10%. This may be accounted for by the fact that different groups were used and that the adsorption method group was larger. There is a strong possibility that heat-labile residual binding may account for some of the differences between the two methods. The two-tier column method provides HSA binding and then the gel delays the free cortisol leaving bound cortisol to pass through the column. On the other hand, in the adsorption method the plasma is heated to  $60^\circ\text{C}$  for 25 minutes to destroy CBG-bound cortisol and the difference between heated and non-heated binding taken as specifically CBG-bound cortisol.

Iqbal and Dalton (1980, in preparation) have shown an inverse linear relationship between the concentration of the specific binding of HSA and SHBG and the non-specific binding or residual binding. They found that after HSA and SHBG are removed from plasma by affinity chromatography, there is approximately 11% residual binding for testosterone and 12% for  $17\beta$  oestradiol. A similar order of residual binding for cortisol could therefore account for the differences between the two methods.

(c) Free cortisol

In order to get a measure of the degree of free cortisol in small volumes of plasma, a sensitive small-scale dialysis method was developed, based on the method of Ellis and Ekins (1973) for thyroxine and tri-iodotyronine. Various small-scale gel filtration methods were investigated based on Burkes' steady-state method (1969). Results obtained using the original method of Burke gave values for free cortisol of 35-52 nmol/l (see Table 9). It was found to be very difficult however to reduce the 8 ml plasma samples much below 2 ml using conventional columns. Thin layer gel filtration seemed to offer many advantages including requiring only a small sample size and allowing several samples to be run on the same plate. However, severe difficulties were encountered with the sample application on to the gel. It proved to be very difficult to apply sufficient sample (0.7 ml) without damaging the gel layer. The dialysis method using specially designed dialysis cells published by Ellis and Ekins (1973) therefore had the advantages of simplicity. Investigation of this method showed that a tracer-label could be used instead of the direct measurement of cortisol in the dialysate and dialysand provided the purity of the label was greater than 97%. This simplified the method further. The effects of various buffers, time of equilibration and evaporation from the dialysis cells were investigated. The effects of dilution of the samples on the percentage free cortisol was investigated and found to be of the order of 10%. As the experimental conditions were kept strictly however, this was felt not to detract from the usefulness of the method. Comparison of the free cortisol in a plasma pool measured by the dialysis method with Burke's column method, in fact gave closely similar results. Destroying CBG by heating to 60°C for 25 minutes, increased the percentage free cortisol from 5.1% to 46.2%. The remaining binding was mostly due to albumin. The dialysis system was then used to measure "normal" adult levels of free cortisol, using only 500 µl of plasma. These were found to be in agreement with those published, as shown in Table 9.

## 2. Cortisol binding globulin levels during infusions and through 24 hours

In order to study the changes, occurring throughout the day in cortisol and CBG levels in normal healthy adults; hourly blood samples were taken from two volunteers over a twenty-four hour period. Total plasma cortisol levels fluctuate (Fig. 20) as described by others, for example Hellman et al (1970), Krieger et al (1971), James et al (1978). There is a series of episodic bursts of secretion closely correlated with ACTH secretion, in the later part of the sleep period.

Peaks occurring during sleep in both subjects are thought to be associated with REM sleep (Krieger et al, 1971). It is difficult to fit this pattern of activity into the negative feedback system (Yates and Urquhart, 1969), as it has been shown that episodic release of ACTH occurs even when plasma steroid levels remain low for a prolonged period as in Addison's disease (Krieger and Gewirtz, 1974). James et al (1978) have studied the reproducibility of the pattern of plasma cortisol secretion. They find that the pattern is similar and reproducible from person to person and from day to day. From this, they suggest that "secretion occurs in response to programmed central nervous system control, rather than to changes in peripheral steroid levels."

Although the cortisol circadian rhythm has been largely documented and investigated, free cortisol and CBG levels have not been so thoroughly investigated, and are not generally taken into account in models of feedback control. The free cortisol levels found in this study closely follow the total cortisol levels. The CBG levels show some variation but there are no significant peaks and troughs. Doe et al (1969) and Burke and Poulet (1970) have both reported low free cortisol levels during the night.

Baumann et al (1976) and Clerico et al (1979) have measured free cortisol at several times during the day. They also found that the free cortisol levels closely follow the total cortisol pattern. Angeli et al (1978) found clear fluctuations in CBG levels with a trough occurring during the night. Doe et al (1964) also found a slight decrease in CBG during the night. De Moor et al (1962) however found no

variation in CBG levels throughout the twenty-four hour period. Angeli et al and Doe et al have taken their conclusions from six and four observations in 24 hrs respectively. The variation shown in the levels in this study of only two subjects but taken at hourly intervals shows that this can be misleading.

Shorter sampling times of 15 minutes were used to study total and free cortisol levels over a four hour period in response to saline infusion and to hydrocortisone and saline infusions in two healthy male volunteers. The total cortisol levels in both subjects fluctuated during the saline infusion although the free cortisol levels remained fairly constant (Fig.21a). Unfortunately insufficient blood was taken to measure the CBG levels during the infusions.

During the hydrocortisone infusion, both total and free plasma cortisol rose sharply in response to the drug. The two men were given different doses of hydrocortisone and the level of the cortisol response in the two men is proportional to the dose given (Fig.21b). The rise in cortisol reaches a plateau then starts to return to normal levels when the infusion is stopped, and achieves normal levels after about 30 minutes. As the rise in free cortisol level is roughly proportional to the increase in total cortisol levels, it is unlikely that the CBG levels have altered.

### 3. Cortisol and cortisol binding globulin levels in rheumatoid arthritis

Long term treatment of rheumatoid arthritis with cortico-steroid-type drugs, including for this purpose, ACTH, is common, but although very useful these drugs can have severe side effects and may lead to adrenal or pituitary suppression via the negative feedback mechanism. The incidence of side effects is dependent on the dose, duration and type of therapy used, and these will be reflected in the cortisol and CBG levels. Commonly Acthar Gel, Synacthen Depot, or prednisolone are used in the treatment of rheumatoid arthritis, together with or instead of salicylates.

Before cortisol and CBG levels were investigated in those rheumatoid patients receiving these drugs, levels in patients receiving salicylates only were first investigated. A small trial was carried out to investigate the acute effects, if any, of therapeutic levels of aspirin on cortisol and CBG, and this showed they had no consistent effect (Fig. 22). The results are in good agreement with others published, e.g. those of Jensen et al (1971) and Stenlake et al (1968). Salicylic acid does bind to albumin, (McArthur et al, 1971) but in the method used for the determination of CBG binding due to albumin is subtracted from the total CBC to give that due to CBG alone, and hence the salicylate binding does not interfere in the method.

Measurement of cortisol and CBG in patients receiving one of the three drugs (Fig. 24) showed that Synacthen Depot and prednisolone had the most effect on the cortisol and CBG levels compared to controls; Acthar Gel having little or no effect.

Treatment with prednisolone caused the cortisol levels to fall to about one third of those found in the control group, and the CBG to fall by about one fifth of the control values. The reduced level of CBG may, however, only be apparent, because of the presence of prednisolone in the plasma. Prednisolone binds to CBG with a slightly lower affinity than cortisol, but this could interfere with the measurement of the CBG levels by CPB method. It may also be, in part, due to an actual reduction in the CBG level.

The ratio of cortisol to CBG, an indication of the free, physiologically active hormone, is reduced during prednisolone treatment to about one half that of the controls (Fig. 23.)

Prednisolone, as a synthetic glucocorticoid will activate the feedback receptors in the pituitary and/or hypothalamus resulting in the reduction of the total and free cortisol levels, via the negative feedback mechanism.

There seems to be little or no effect of Acthar Gel treatment on the cortisol and CBG levels. This is at first surprising as ACTH is known to stimulate cortisol secretion. However, as previously mentioned (Chapter 2 ) Acthar Gel, on injection, has a short half life and the blood samples were taken from these patients before the next injection was given. At this time the effects of the previous injection on the adrenal would be negligible. This would indicate therefore, from the cortisol and CBG levels found during Acthar Gel treatment that there is no constant effect on the adrenal ie the feedback mechanism for the control of cortisol levels is unaffected in these patients.

Treatment with Synacthen Depot, the action of which in the body is considerably more prolonged than that of Acthar Gel, results in cortisol levels of approximately one and a half times that of the controls; the CBG level is about the same as in the control group. The cortisol to CBG ratio is almost twice that found in the controls. It seems that the long-acting preparation has had a sustained effect on the adrenal, resulting in a prolonged rise in plasma cortisol. As there is no concomitant rise in the specific binding protein CBG, this increase in plasma cortisol results in a disproportionate increase in the free cortisol levels; that is an increase in the level of physiologically active cortisol in the plasma.

It seems therefore, that these drugs, used in the treatment of rheumatoid arthritis, are having some effect on the adrenal as reflected by the ratio of CBG to cortisol .

Their pharmacological effect is mainly in the increase in glucocorticoid levels. This also however, produces unwanted side effects and the degree of severity of these could be related to the level of cortisol and CBG.

Lewis et al (1971) have described a correlation between the incidence of side effects with prednisolone treatment and the plasma albumin levels. When plasma albumin levels fell below 25g/l, the incidence of side effects doubled. The results presented here indicate that this could also be true for ACTH treatment.

#### 4. Cortisol and cortisol binding globulin levels in the menstrual cycle

Plasma cortisol levels are known to increase during pregnancy and in oestrogen treatment as discussed in Chapter 2 (e.g. Sandberg et al , 1959). Variations in the levels of cortisol and of CBG in response to the changing hormonal environment of the menstrual cycle had not previously been investigated in detail however, and the measurement of cortisol and CBG levels in women have taken little or no account of these possible changes.

On investigation, normal adult women showed fairly constant levels of binding protein during the menstrual cycle, but the cortisol levels varied throughout the cycle, lower levels being found after day 10-13 (Fig. 25 ).

The cortisol/CBG ratio (Fig.26) falls steadily through cycle days 1-12, then remains fairly steady to the end of the cycle. The value of the ratio remaining is less than unity - with cortisol apparently being adequately bound by the available CBG.

A decrease in cortisol secretion late in the normal cycle has also been shown by Beck et al (1972). They suggested that this was a result of inhibition of the HPA axis and possibly the effect of direct adrenocortical suppression by progesterone. Genazzani et al (1975) measured the LH and cortisol levels through the menstrual cycle. This study showed a fall in cortisol levels, followed by a rise occurring just before ovulation, which is then sustained throughout the cycle. They also showed a cyclic variation in basal levels of ACTH during the menstrual cycle, with a peak of ACTH preceding ovulation.

Golstein et al (1974), Angeli et al (1978), Schöneshofer and Wagner (1977) and Carr et al (1979) have all found no significant difference in true plasma cortisol levels in the early follicular and in those found in the luteal phase of the menstrual cycle. Angeli et al (1978) did, however, find an increase in the CBG levels in three out of four of the women studied. Moore et al (1978) measured CBG through the menstrual cycle in five women. They also found little variation and also found that there was no correlation with oestradiol levels.

Progestogens have no discernable effects on either the cortisol or the CBG levels through the menstrual cycle when compared to the controls (Table 8). This is in agreement with the studies of Burke, 1970, Daly and Elstein, 1972, and Briggs and Briggs, 1972.

The levels of cortisol and CBG measured in women taking the combined oral contraceptives are, however, markedly different from the controls (Fig. 25). Both the cortisol and the CBG levels in the treated women are significantly higher throughout the cycle. There is an increase in CBG from days 4-6 as the new course of tablets is started, to about days 13-15; the level then remains steady to the end of the course of tablets (days 25-27) when the level falls. Cortisol remains steady until days 10-12, when it rises to a peak on days 25-27, then falls slightly.

The rise in the levels of cortisol and CBG follows the administration of a course of oral contraceptives; the oestrogen content of the oral contraceptives stimulating the production of CBG, which rises in response to therapy. As described in Chapter 2, pregnancy, or the administration of oestrogens or oral contraceptives, cause an increase in the plasma cortisol and CBG level in women, e.g. De Moor et al, 1966; Doe et al, 1969) and an oestrogen dose-related response in CBG levels has been shown (e.g. Swartz et al, 1974). Cortisol levels rise in response to the oestrogens and/or to the increase in CBG levels.

The ratio of cortisol to CBG is consistently higher throughout the cycle of treated women compared to the controls. It varies slightly during the cycle, falling from days 4-6 to days 10-12, after which it rises. The value of the ratio however remains above or around unity. This is suggestive of an increase in the fraction of cortisol which is bound to albumin. There is, however, some controversy over the question of a raised free cortisol in women taking 'low dose' oral contraceptives; Burke (1969) showed increases in free cortisol with oral contraceptives containing 100 µg oestrogen, but no significant rise was found with those pills containing only 50 µg oestrogen. These results were borne out by Bulbrook et al (1973).

## 5. Cortisol and cortisol binding globulin levels in women taking oral contraceptives

In order to investigate the effect of oral contraceptives further, plasma free cortisol levels together with total cortisol and CBG levels were measured in men and women and in women taking oral contraceptives. (see Fig. 29).

Characterisation of the dialysis method for free cortisol measurement showed that the results obtained in women were similar to those found by others (Table 9); The levels found in men, however, tended to be higher than the womens' level. This was also found by Schöneshofer and Wagner (1977), especially during the follicular phase of the menstrual cycle. The groups were not very large and there was a marked variation in the levels found in women as compared to those found in men. This increased variation probably accounts for the difference found between the two groups.

Levels of plasma free cortisol found in women on the 'low-dose' pill were found to differ from those previously published by, for example, Burke (1969), in that women taking 50  $\mu$ g of oestrogen daily in this study have a higher free and total cortisol than normal women. Baumann et al (1975) have more recently published results which agree closely with those presented here and these, together with Burke's, are compared in Table 10.

The standard deviation of the cortisol levels in the pill group was larger than that in either of the other two groups. This could be one reason for the discrepancy between published results. The various methods used to measure plasma free cortisol may also account for differences. For the measurement of free cortisol Baumann used both an equilibrium dialysis and a charcoal uptake technique, while Burke used his steady-state gel filtration method (Burke, 1969). For the measurement of total cortisol however both Baumann and Burke used a competitive protein binding technique, whereas the values obtained in this study were found by a fluorimetric technique (see Chapter 4).

The significance of raised plasma free cortisol levels in oestrogen treatment is uncertain. Clinical observation shows that adrenal gland function remains normal in women using oral contraceptives. The adrenal response to injected ACTH and to dexamethasone, a synthetic corticosteroid, is normal in women receiving oral contraceptives (e.g. Mestman et al, 1968; Streeter et al, 1969). A decreased response is found with metyrapone which acts by inhibiting 11 $\beta$ -hydroxylase activity (Sprunt et al, 1968) which would explain the apparent lack of response.

There is no evidence that women develop symptoms or signs of hypercortisolism during long term treatment with these oral contraceptives. Some changes do occur which seem to be similar to those of steroid diabetes (Wynn and Doar, 1970). Whether these changes are related to the raised cortisol level itself, or are due to the effect of the oestrogens is speculative and requires further investigation. Only the cortisol circulating in the free form is believed to be physiologically active, consequently significant changes in the free cortisol level may occur which are not reflected in the total plasma cortisol level and which may yet have marked effects.

One explanation of the apparent lack of effect of the raised plasma free cortisol levels may be that the free cortisol is not consistently raised throughout the day or throughout the menstrual cycle. The total amount of cortisol bathing the tissues may not therefore be higher than normal over twenty-four hours or 28 day cycle.

In order to investigate this possibility the plasma free and total cortisol and CBG was measured in the morning (0900-1000 hr) and evening (1630-1730 hr) at different times during the menstrual cycle, on days 6-9 and days 23-36, in normal adult women and in women taking oral contraceptives containing 50  $\mu$ g of oestrogen. Urinary free cortisol was measured at the same time.

Although there were only six women in each group the plasma total and free cortisol were significantly raised in the pill group throughout the day and at different times in the cycle as compared to the controls (Fig. 30). These results are similar to those found by Baumann et al (1975).

There is an apparent increase in total cortisol in the control and pill group towards the end of the cycle. The variation within the group was also markedly increased. Statistical analysis of these two groups showed that there was no significant difference between the controls but there is a difference between the two in the pill group ( $p < 0.1$ ). There is no such significance in the cortisol levels however. This may be explained by the significant increase in CBG levels found towards the end of the cycle in the pill group.

It appears, therefore that there is a definite constant increase in total and free cortisol and CBG levels throughout the cycle in women taking oral contraceptives. The urinary free cortisol (UFC) measured at the same times in these women, show no apparent differences between or within the two groups. This is in agreement with previously published results, e.g. those of Burke (1970). Bulbrook and Hayward (1969) however have shown that the excretion of 17-OH-corticosteroids decreases in women taking oral contraceptives. The type and dosage of the pills was not recorded however and this would be expected to be a dose-related phenomenon (Burke, 1970).

It seems that it is the unbound cortisol which is ultrafiltered by the kidney, the amount filtered being dependent on the degree of protein binding (Daughaday, 1961). There has been shown to be a dependence of UFC on the plasma free cortisol levels (Burke and Beardwell, 1973; Sederberg-Olsen et al , 1975), and UFC is commonly used as an indicator of changes in plasma cortisol levels as in, for example, Cushings' Syndrome (Burke and Beardwell, 1972). UFC is known to be dependent on the kidney function and that the amount reabsorbed by the kidney is a function of the amount filtered, up to 220 nmol/l (Lindholm, 1973; Sedersberg-Olsen et al , 1975). More recently, it has shown that there is a relationship between creatinine clearance and UFC (Burke and Beardwell, 1973; Gilliland and Phillips, 1978). However, in this study although the plasma free cortisol levels are twice the control levels at 9 a.m. and 5 p.m. in the pill women, the UFC is the same.

The possibility that this may be related to the CBG levels was examined by looking at the ratio of UFC and CBG. This was lower in the pill group than in the controls. This would suggest that the CBG is not simply acting as an inert carrier or that as for testosterone, there is a decrease in the metabolic clearance rate and an increase in the rate of peripheral metabolism (Bird et al , 1969). Oestrogens are known to affect the hepatic metabolism of the cortisol and cortisol binding protein (e.g. Musa et al, 1967; Peterson, 1959). The effect of the increased CBG levels on the hepatic extraction of cortisol are unknown.

The problem still remains, therefore, why do women taking oral contraceptives containing 50 µg of oestrogen and having a raised free cortisol not show clinical signs of hypercortisolism? There may be therefore a threshold level of free cortisol above which signs and symptoms occur and which is not reached as a consequence of contraceptive treatment.

The existence of the raised free cortisol itself is anomalous as the level should be regulated by the negative feedback system operated by cortisol and thought to act on the hypothalamus to control the production of ACTH (Kendall et al, 1972). The apparent failure of this regulating mechanism is puzzling. It may be that relatively gross changes in free cortisol are controlled in this way, and although the plasma level is raised the actual increase at the tissue level is minimal, or that the system reacts to a rapid change in the level, but sluggishly if at all to a slow rise, until a threshold level is reached, when the control system will react. This is analogous to the 'fast feedback' described by Dallman and Yates (1969) and Jones et al (1972; 1974). There is some evidence that this is also true in man (J. R. Daly; Personal Communication). Doe et al (1969) have suggested that there may be a re-setting of the negative feedback mechanism between cortisol and ACTH in response to the oestrogens.

## 6. Cortisol binding globulin levels in women having severe PMS

Using the two-tier column method for the measurement of CBG levels in plasma, low levels of CBG were found in women having severe premenstrual syndrome. These women comprised a tightly diagnosed group who were referred to a PMS clinic. They had received no treatment for two months while their symptoms were evaluated (K Dalton, 1977) and they all showed complete absence of symptoms postmenstrually. This finding would support one of the first theories to account for premenstrual tension (Frank, 1931) that of an imbalance between the oestradiol and progesterone, as was shown earlier in this study, oestrogens do affect the binding protein levels. SHBG levels in these women were also found to be low (M Dalton, in preparation) although Backstrom et al (1976) found no difference in levels.

17  $\beta$  -oestradiol levels in women with PMS are either normal or raised (Backstrom and Carstensen, 1974). Progesterone levels are found to be low (Munday, 1977) while prolactin levels are found to be variable (Munday, 1977, Halbreich et al, 1976). Some of these variations in hormone levels may be due to incorrect diagnosis and to different subgroups within PMS dependent on the main presenting symptoms. However, in 90% of the patients studied in this group, the CBG was low. Further investigations of the hormone binding proteins in PMS may prove their measurement to be useful in the diagnosis of PMS.

## 7. Summary

To summarise then, several methods have been used in order to investigate cortisol and cortisol binding in plasma. A small-scale dialysis method has been developed which can measure plasma free cortisol using only 500  $\mu$ l of plasma. A 'two-tier' column method for the measurement of CBG has also been developed. When compared with the adsorption method there was good correlation between the two methods, although the column method gave lower results. Both methods are suitable for routine use being simple to perform and the two-tier column method especially gives rapid results.

Investigation of the changes occurring in cortisol and CBG, through twenty-four hours, showed that the free cortisol followed the variations in the total cortisol level. CBG however remained relatively constant. The patterns of cortisol and CBG were similar to those previously reported although the importance of frequent sampling to investigate changes occurring in the binding protein are shown.

Drugs such as prednisolone and Synacthen used in the treatment of rheumatoid arthritis were shown by this study to affect cortisol levels. The effects of these drugs can also extend to the binding protein CBG. These alterations in CBG may possibly correlate with the occurrence of side effects of treatment. Unfortunately the existence and/or severity of side effects was not noted during the trial.

The study of the menstrual cycle in normal ovulating women showed the constancy of CBG levels maintained through the cycle. This was the first study carried out which provides a detailed description of changes in cortisol and CBG through the menstrual cycle. The study of women taking 50 µg oestrogen in oral contraceptive treatment showed conclusively that cortisol and CBG were raised throughout the cycle. The ratio of cortisol and CBG suggested that free cortisol may be raised in these women.

Further investigation of this showed that women taking oral contraceptives did have a raised free cortisol - approximately twice that of normal women. This was in contradiction with some published work, but in agreement with others. In order to examine this further, cortisol and CBG levels and UFC were measured at different times of the day and at different times of the cycle. Again, the women taking oral contraceptives had increased free and total cortisol and CBG both in the morning and in the evening and at different times of the cycle. The UFC levels were however the same. This can be explained by the decreased metabolic clearance rate. The effect of oestrogens on the liver would also be involved in this. However there is also the problem of why the increased free cortisol is not

controlled by the HPA feedback mechanism. It is suggested that there must be in some way, a re-setting of the feedback by the oestrogen and there may be a threshold level over which this can occur.

Measurement of CBG using the 'two-tier' column method in normal women and in women with severe premenstrual syndrome showed that the women with PMS had low CBG levels. This is an important finding, especially in the light of the confused clinical and biochemical picture in this syndrome.

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"Adrenal glucocorticoid control system."

## APPENDICES

## APPENDIX I

### Materials

#### a. Chemicals

All chemicals used were of 'Analar' grade unless otherwise specified.

|                                      |                       |
|--------------------------------------|-----------------------|
| Toluene (scintillation grade)        | Koch-Light Labs. Ltd. |
| Butyl PBD                            | "                     |
| Triton X-100                         | "                     |
| Cortisol                             | Sigma Ltd.            |
| Progesterone                         | "                     |
| Dextran 60C                          | "                     |
| Florisil                             | "                     |
| Thimerosal                           | "                     |
| Hepes buffer                         | Microbio Labs. Ltd.   |
| Sepharose 4B                         | Pharmacia Ltd.        |
| Sephadex G-50 (fine)                 | "                     |
| Sephadex LH20                        | "                     |
| Cortisol-1, 2, 6, 7 - H <sup>3</sup> | Radiochemical Centre  |
| Cortisol-1, 2, - H <sup>3</sup>      | "                     |
| Dichloromethane (nanograde)          | Camlab Ltd.           |
| Lissamine Green                      | B.D.H. Ltd.           |
| Ninhydrin spray                      | "                     |
| Cibacron Blue F3G-A Sepharose        | Dr J. Iqbal           |

#### b. Apparatus

|                                                         |                        |
|---------------------------------------------------------|------------------------|
| Chromatography columns                                  | Whatman Ltd.           |
| Thin layer gel apparatus                                | Pharmacia Ltd          |
| Visking Tubing                                          | Jencons Ltd.           |
| Pyrex glass pasteur pipettes                            | "                      |
| Glass cortisol assay tubes<br>(Brown's oestrogen tubes) | V A Howe Ltd.          |
| Plastic assay tubes                                     | Luckhams Ltd.          |
| Scintillation vials                                     | Intertechnique Ltd.    |
| Fraction collector                                      | LKB Instruments Ltd.   |
| Spectrophotofluorimeter                                 | Aminco Bowman Co. Ltd. |

Small pyrex glass columns  
Cellulose plugs

Jencons Ltd.  
Bilbate Ltd.

c. Solutions

(i) Scintillation fluid

1% Butyl PBD dissolved in scintillation grade Toluene  
(3 vols): Triton X-100 (1vol)

(ii) 0.1 m phosphate buffer, pH = 7.4

Disodium hydrogen orthophosphate ( $\text{Na}_2\text{HPO}_4$ , 14.2g/l) and sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 19.2g/l) are mixed in the approximate ratio 4:1 and the pH adjusted to 7.4.

(iii) 0.15m phosphate buffer pH = 7.4

Solutions of disodium hydrogen orthophosphate ( $\text{Na}_2\text{HPO}_4$ , 21.3g/l) and sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 28.8g/l) are mixed in the approximate ratio of 4:1 and the pH adjusted to 7.4.

(iv) Hepes buffer

0.08 M Hepes in distilled deionised water containing 0.62% sodium chloride and 1 in 10,000 FD Thimerosal. The pH was 7.4.

(v) 0.05 M Tris buffer, pH = 7.4

Tris (6.1 g/l) and calcium chloride ( $\text{CaCl}_2$ , 1.11g/l) are made up to one litre with distilled water and the pH adjusted to 7.4 with hydrochloric acid.

d. Preparation of dextran-coated Florisil (DCF)

50g of Dextran, type 60C, was dissolved in 300 ml of distilled, deionised water and 50 g of Florisil (activated magnesium silicate, 60-100 mesh) added. The slurry was shaken overnight and then decanted and filtered on a scintered glass funnel. The DCF was then dried in a vacuum dessicator.

A. Total and Free Cortisol Levels in Infusion Experiments

| <u>Time</u><br>(mins) | <u>Saline</u>     |                   |                   |                   | <u>Hydrocortisone</u> |                   |                     |                   |
|-----------------------|-------------------|-------------------|-------------------|-------------------|-----------------------|-------------------|---------------------|-------------------|
|                       | <u>JRD</u>        |                   | <u>PH</u>         |                   | <u>JRD (3 mg/hr)</u>  |                   | <u>PH (2 mg/hr)</u> |                   |
|                       | <u>T Cortisol</u> | <u>F Cortisol</u> | <u>T Cortisol</u> | <u>F Cortisol</u> | <u>T Cortisol</u>     | <u>F Cortisol</u> | <u>T Cortisol</u>   | <u>F Cortisol</u> |
| -15                   | -                 | -                 | 276               | 30                | 290                   | 38                | 373                 | 40                |
| 0                     | -                 | -                 | 221               | 28                | 262                   | 56                | 345                 | 37                |
| +15                   | 166               | 25                | 221               | 25                | 290                   | 50                | 331                 | 39                |
| 30                    | 166               | 22                | 138               | 19                | 331                   | 57                | 428                 | 55                |
| 45                    | 193               | 25                | 138               | 22                | 469                   | 111               | 414                 | 74                |
| 60                    | 166               | 28                | 193               | 28                | 552                   | 82                | 483                 | 84                |
| 75                    | 83                | 14                | 221               | 25                | 635                   | 182               | 538                 | 109               |
| 90                    | -                 | -                 | 304               | 41                | 635                   | 105               | 580                 | 121               |
| 105                   | 193               | 39                | 331               | 52                | 690                   | 168               | 538                 | 112               |
| 120                   | -                 | -                 | 248               | 33                | 690                   | 151               | 538                 | 114               |
| 135                   | 221               | 25                | 248               | 33                | 483                   | 79                | 431                 | 92                |
| 150                   | 166               | 22                | 276               | 28                | 359                   | 49                | 359                 | 70                |
| 165                   | 193               | 22                | 221               | 30                | 359                   | 77                | 331                 | 87                |
| 180                   | 138               | 14                | 221               | 33                | 331                   | 38                | -                   | -                 |
| 195                   | -                 | -                 | 414               | 41                | 317                   | 41                | 290                 | 80                |
| 210                   | -                 | -                 | 386               | 25                | -                     | -                 | 207                 | 69                |
| 225                   | -                 | -                 | 414               | 52                | 276                   | 36                | 207                 | 57                |
| 240                   | 248               | 30                | 386               | 39                | 248                   | 38                | 248                 | 39                |

B. Plasma total and free cortisol and cortisol binding globulin levels through twenty-four hours in two normal subjects

a. R F. ♂

|        | <u>Time</u><br>(hrs) | <u>Total Cortisol</u><br>(nmol/l) | <u>Free Cortisol</u><br>(nmol/l) | <u>CBG</u><br>(nmol cortisol/l) |
|--------|----------------------|-----------------------------------|----------------------------------|---------------------------------|
| Start: | 1500                 | 231                               | 40                               | 338                             |
|        | 1600                 | 378                               | 61                               | 317                             |
|        | 1700                 | 269                               | 35                               | 295                             |
|        | 1800                 | 172                               | 21                               | 328                             |
|        | 1900                 | 130                               | 15                               | 460                             |
|        | 2000                 | 179                               | 23                               | 309                             |
|        | 2100                 | 130                               | 19                               | 363                             |
|        | 2200                 | 104                               | 15                               | 356                             |
|        | 2300                 | 92                                | 13                               | 296                             |
|        | 2400                 | 139                               | 19                               | 433                             |
|        | 0100                 | 99                                | 17                               | 359                             |
|        | 0200                 | 67                                | 10                               | 359                             |
|        | 0300                 | 336                               | 55                               | 366                             |
|        | 0400                 | 258                               | 38                               | 326                             |
|        | 0500                 | 218                               | 38                               | 268                             |
|        | 0600                 | 378                               | 59                               | 308                             |
|        | 0700                 | 218                               | 38                               | 307                             |
|        | 0800                 | 307                               | 42                               | 313                             |
|        | 0900                 | 328                               | 47                               | 303                             |
|        | 1000                 | 269                               | 40                               | 300                             |
|        | 1100                 | 202                               | 33                               | 272                             |
|        | 1200                 | 273                               | 34                               | 374                             |
|        | 1300                 | 233                               | 31                               | 345                             |
|        | 1400/1500            | 336                               | 59                               | 401                             |

b. J.P. Q

|        | <u>Time</u><br>(hrs) | <u>Total Cortisol</u><br>(nmol/l) | <u>Free Cortisol</u><br>(nmol/l) | <u>CBG</u><br>(nmol cortisol/l) |
|--------|----------------------|-----------------------------------|----------------------------------|---------------------------------|
| Start: | 1945                 | 315                               | 42                               | 285                             |
|        | 2045                 | 151                               | 20                               | 350                             |
|        | 2145                 | 134                               | 15                               | 336                             |
|        | 2245                 | 101                               | 12                               | 364                             |
|        | 2345                 | 92                                | 11                               | 298                             |
|        | 0045                 | 95                                | 13                               | 331                             |
|        | 0145                 | 147                               | 20                               | 297                             |
|        | 0245                 | 147                               | 27                               | 322                             |
|        | 0345                 | 706                               | 85                               | 415                             |
|        | 0445                 | 847                               | 108                              | 416                             |
|        | 0545                 | 378                               | 79                               | 407                             |
|        | 0645                 | 462                               | 65                               | 349                             |
|        | 0745                 | 248                               | 33                               | 324                             |
|        | 0845                 | 336                               | 42                               | 318                             |
|        | 0945                 | 256                               | 44                               | 281                             |
|        | 1045                 | 420                               | 85                               | 324                             |
|        | 1145                 | 197                               | 35                               | 353                             |
|        | 1245                 | 227                               | 27                               | 324                             |
|        | 1345                 | 200                               | 29                               | 321                             |
|        | 1445                 | 227                               | 29                               | 312                             |
|        | 1545                 | 193                               | 32                               | 374                             |
|        | 1645                 | 151                               | 30                               | 323                             |
|        | 1745                 | 227                               | 32                               | 363                             |
|        | 1845                 | 252                               | 33                               | 354                             |
|        | 1945                 | 202                               | 28                               | 348                             |

C. The effect of salicylate treatment on cortisol, cortisol binding globulin and salicylate levels in normal adults

| <u>Subject</u> | <u>Day</u> | <u>Salicylate</u><br>(mg/100 ml.) | <u>Cortisol</u><br>(nmol/l) | <u>CBG</u><br>(nmol Cortisol/l) |
|----------------|------------|-----------------------------------|-----------------------------|---------------------------------|
| J.P.           | 1          | < 2                               | 386                         | 585                             |
|                | 2          | 8.8                               | 442                         | 530                             |
|                | 3          | 16.0                              | 469                         | 442                             |
|                | 4          | 22.0                              | 524                         | 444                             |
|                | 5          | 21.3                              | 304                         | 367                             |
|                | 8          | < 2                               | 524                         | 348                             |
| S.N.           | 1          | < 2                               | 580                         | 414                             |
|                | 2          | 4.0                               | 524                         | 414                             |
|                | 3          | 9.3                               | 662                         | 309                             |
|                | 4          | 17.0                              | 414                         | 334                             |
|                | 8          | < 2                               | 552                         | 348                             |
| M.C.           | 1          | < 2                               | 276                         | 544                             |
|                | 2          | 6.3                               | 386                         | 442                             |
|                | 3          | 14.3                              | 442                         | 524                             |
|                | 4          | 21.7                              | 386                         | 442                             |
|                | 5          | 23.0                              | 221                         | 392                             |
|                | 8          | < 2                               | 221                         | 213                             |
| D.G.           | 1          | < 2                               | 552                         | 616                             |
|                | 2          | 5.0                               | 582                         | 466                             |
|                | 3          | 13.6                              | 552                         | 428                             |
|                | 4          | 23.6                              | 519                         | 389                             |
|                | 5          | 18.6                              | 461                         | 417                             |
|                | 8          | < 2                               | 607                         | 516                             |
| T.R.           | 1          | < 2                               | 552                         | 420                             |
|                | 2          | 2.0                               | 552                         | 420                             |
|                | 3          | 11.3                              | 248                         | 422                             |
|                | 4          | 19.0                              | 690                         | 409                             |
|                | 5          | 4.0                               | 607                         | 422                             |

| <u>Subject</u> | <u>Day</u> | <u>Salicylate</u><br>(mg/100 ml.) | <u>Cortisol</u><br>(nmol/l) | <u>CBG</u><br>(nmol Cortisol/l) |
|----------------|------------|-----------------------------------|-----------------------------|---------------------------------|
| R.E.           | 1          | < 2                               | 497                         | 386                             |
|                | 2          | < 2                               | 497                         | 386                             |
|                | 3          | 3.2                               | 580                         | 348                             |
|                | 4          | 6.0                               | 497                         | 403                             |
|                | 5          | 10.3                              | 607                         | 397                             |
|                | 8          | < 2                               | 497                         | 331                             |
| R.M.           | 1          | < 2                               | 442                         | 414                             |
|                | 2          | < 2                               | 359                         | 420                             |
|                | 3          | 3.6                               | 386                         | 395                             |
|                | 4          | 5.0                               | 359                         | 353                             |
|                | 5          | 3.2                               | 414                         | 417                             |
|                | 8          | < 2                               | 497                         | 455                             |
| S.D.           | 1          | < 2                               | 662                         | 486                             |
|                | 2          | < 2                               | 662                         | 491                             |
|                | 3          | < 2                               | 497                         | 469                             |
|                | 4          | < 2                               | 607                         | 480                             |
|                | 5          | < 2                               | 635                         | 552                             |
|                | 8          | < 2                               | 828                         | 500                             |
| J.Z.           | 1          | < 2                               | 293                         | 500                             |
|                | 2          | < 2                               | 635                         | 530                             |
|                | 3          | < 2                               | 469                         | 417                             |
|                | 4          | < 2                               | 442                         | 420                             |
|                | 5          | < 2                               | 414                         | 461                             |
|                | 8          | < 2                               | 414                         | 328                             |

D . Cortisol and Cortisol Binding Globulin Levels in Rheumatoid Arthritis Patients

| 1. <u>No steroid treatment</u> |                      |                          |         |                      |                          |
|--------------------------------|----------------------|--------------------------|---------|----------------------|--------------------------|
| Patient                        | Cortisol<br>(nmol/l) | CBG<br>(nmol Cortisol/l) | Patient | Cortisol<br>(nmol/l) | CBG<br>(nmol Cortisol/l) |
| E.N.                           | 472                  | 524                      | H.A.    | 224                  | 469                      |
| F.O.                           | 472                  | 582                      | M.A.    | 381                  | 715                      |
| H.O.                           | 389                  | 442                      | F.D.    | 270                  | 610                      |
| C.O.                           | 293                  | 431                      | F.N.    | 328                  | 447                      |
| R.I.                           | 787                  | 740                      | K.I.    | 414                  | 588                      |
| R.E.                           | 552                  | 508                      | H.O.    | 560                  | 591                      |
| C.R.                           | 177                  | 541                      | G.A.    | 450                  | 588                      |
| W.I.                           | 580                  | 505                      | O.R.    | 607                  | 723                      |
| M.A.                           | 671                  | 836                      |         |                      |                          |
| W.O.                           | 218                  | 511                      |         |                      |                          |
| M.C.                           | 295                  | 464                      |         |                      |                          |
| A.Y.                           | 367                  | 660                      |         |                      |                          |
| A.N.                           | 160                  | 497                      |         |                      |                          |
| M.A.                           | 276                  | 480                      |         |                      |                          |
| C.O.                           | 643                  | 475                      |         |                      |                          |
| K.E.                           | 259                  | 558                      |         |                      |                          |
| F.L.                           | 259                  | 729                      |         |                      |                          |
| B.O.                           | 243                  | 574                      |         |                      |                          |
| B.A.                           | 328                  | 651                      |         |                      |                          |
| G.R.                           | 500                  | 535                      |         |                      |                          |
| L.U.                           | 397                  | 560                      |         |                      |                          |
| V.A.                           | 433                  | 541                      |         |                      |                          |
| B.U.                           | 295                  | 464                      |         |                      |                          |
| A.R.                           | 315                  | 406                      |         |                      |                          |
| G.A.                           | 295                  | 464                      |         |                      |                          |
| R.O.                           | 276                  | 447                      |         |                      |                          |
| A.N.                           | 373                  | 439                      |         |                      |                          |
| R.A.                           | 353                  | 417                      |         |                      |                          |

Cortisol and Cortisol Binding Capacity Levels in Rheumatoid Arthritis Patients

| 2. <u>Prednisolone treatment</u> |                      |                          |         |                      |                          |
|----------------------------------|----------------------|--------------------------|---------|----------------------|--------------------------|
| Patient                          | Cortisol<br>(nmol/l) | CBG<br>(nmol Cortisol/l) | Patient | Cortisol<br>(nmol/l) | CBG<br>(nmol Cortisol/l) |
| W.H.                             | 97                   | 433                      | S.M.    | 77                   | 326                      |
| C.L.                             | 163                  | 400                      | C.O.    | 221                  | 519                      |
| L.E.                             | 130                  | 389                      | R.O.    | 248                  | 351                      |
| B.A.                             | 80                   | 378                      | C.O.    | 110                  | 574                      |
| J.O.                             | 80                   | 384                      | C.A.    | 55                   | 362                      |
| H.O.                             | 530                  | 453                      | M.A.    | 276                  | 480                      |
| K.E.                             | 41                   | 326                      | W.H.    | 414                  | 375                      |
| T.H.                             | 190                  | 362                      | C.S.    | 166                  | 315                      |
| O.S.                             | 635                  | 342                      | G.I.    | 524                  | 340                      |
| B.E.                             | 19                   | 458                      | C.O.    | 83                   | 340                      |
| C.L.                             | 414                  | 439                      |         |                      |                          |
| W.O.                             | 19                   | 560                      |         |                      |                          |
| H.O.                             | 19                   | 660                      |         |                      |                          |
| F.L.                             | 254                  | 753                      |         |                      |                          |
| W.A.                             | 298                  | 447                      |         |                      |                          |
| P.H.                             | 171                  | 729                      |         |                      |                          |
| E.V.                             | 0                    | 348                      |         |                      |                          |
| C.O.                             | 17                   | 436                      |         |                      |                          |
| W.O.                             | 0                    | 431                      |         |                      |                          |
| A.K.                             | 190                  | 519                      |         |                      |                          |
| S.U.                             | 138                  | 549                      |         |                      |                          |
| S.M.                             | 224                  | 784                      |         |                      |                          |
| S.T.                             | 36                   | 516                      |         |                      |                          |
| P.R.                             | 174                  | 607                      |         |                      |                          |
| G.I.                             | 69                   | 428                      |         |                      |                          |
| W.E.                             | 36                   | 566                      |         |                      |                          |
| B.U.                             | 185                  | 466                      |         |                      |                          |
| P.A.                             | 77                   | 378                      |         |                      |                          |
| C.L.                             | 276                  | 447                      |         |                      |                          |

3. ACTH Treatment

| <u>Patient</u> | <u>Cortisol</u><br>(nmol/l) | <u>CBG</u><br>(nmol Cortisol/l) |
|----------------|-----------------------------|---------------------------------|
| W.A.           | 304                         | 547                             |
| L.I.           | 386                         | 547                             |
| P.A.           | 304                         | 643                             |
| B.U.           | 1739                        | 621                             |
| H.O.           | 497                         | 464                             |
| T.H.           | 1739                        | 767                             |
| A.K.           | 193                         | 574                             |
| E.V.           | 386                         | 467                             |
| G.O.           | 580                         | 582                             |
| E.V.           | 0                           | 348                             |
| M.O.           | 911                         | 693                             |
| L.Y.           | 386                         | 549                             |
| B.O.           | 110                         | 500                             |
| P.I.           | 220                         | 527                             |
| C.O.           | 110                         | 538                             |
| L.Y.           | 386                         | 638                             |
| G.W.           | 386                         | 447                             |
| G.A.           | 331                         | 417                             |
| M.O.           | 409                         | 555                             |
| P.I.           | 193                         | 444                             |
| P.I.           | 138                         | 513                             |
| M.O.           | 414                         | 723                             |
| D.A.           | 469                         | 447                             |
| C.O.           | 220                         | 502                             |
| C.R.           | 83                          | 500                             |
| M.O.           | 571                         | 698                             |

4. Synacthen Treatment

| <u>Patient</u> | <u>Cortisol</u><br>(nmol/l) | <u>C BG</u><br>(nmol Cortisol/l) | <u>Patient</u> | <u>Cortisol</u><br>(nmol/l) | <u>C BG</u><br>(nmol Cortisol/l) |
|----------------|-----------------------------|----------------------------------|----------------|-----------------------------|----------------------------------|
| W.A.           | 690                         | 599                              | S.A.           | 386                         | 436                              |
| E.L.           | 450                         | 480                              | R.O.           | 662                         | 533                              |
| B.A.           | 547                         | 502                              | M.C.           | 331                         | 447                              |
| C.U.           | 690                         | 516                              | M.C.           | 718                         | 389                              |
| F.R.           | 560                         | 431                              | B.A.           | 276                         | 604                              |
| M.K.           | 1002                        | 533                              | B.A.           | 497                         | 461                              |
| K.I.           | 519                         | 213                              | M.K.           | 455                         | 420                              |
| R.O.           | 345                         | 403                              |                |                             |                                  |
| M.L.           | 466                         | 580                              |                |                             |                                  |
| K.I.           | 707                         | 428                              |                |                             |                                  |
| S.Q.           | 287                         | 618                              |                |                             |                                  |
| M.C.           | 607                         | 309                              |                |                             |                                  |
| R.O.           | 276                         | 259                              |                |                             |                                  |
| M.O.           | 552                         | 461                              |                |                             |                                  |
| M.P.           | 304                         | 469                              |                |                             |                                  |
| R.O.           | 1366                        | 486                              |                |                             |                                  |
| F.R.           | 400                         | 715                              |                |                             |                                  |
| K.I.           | 1104                        | 497                              |                |                             |                                  |
| M.D.           | 883                         | 685                              |                |                             |                                  |
| G.R.           | 607                         | 621                              |                |                             |                                  |
| R.O.           | 635                         | 679                              |                |                             |                                  |
| M.C.           | 731                         | 638                              |                |                             |                                  |
| W.I.           | 196                         | 475                              |                |                             |                                  |
| M.K.           | 1684                        | 257                              |                |                             |                                  |
| C.U.           | 110                         | 425                              |                |                             |                                  |
| K.I.           | 386                         | 588                              |                |                             |                                  |

E. Cortisol and Cortisol Binding Capacity Levels in Women through the menstrual cycle

1. Cortisol Levels in "Control" Women

| Day of Cycle | E.M. | A.J. | FIRST CYCLE |      |      |      | N.V. | S.D. | A.B. | F.R. | V.R. | T.M. | L.C. | J.T. | M.H. | SECOND CYCLE |      |      |  |
|--------------|------|------|-------------|------|------|------|------|------|------|------|------|------|------|------|------|--------------|------|------|--|
|              |      |      | M.L.        | R.E. | J.W. | R.E. |      |      |      |      |      |      |      |      |      | N.V.         | S.D. | M.H. |  |
| 1            | 580  | 317  | 635         |      |      | 530  |      |      |      |      | 508  | 345  | 425  | 522  |      |              | 535  | 466  |  |
| 2            |      |      |             | 166  | 331  |      | 356  |      | 431  |      |      |      |      |      | 298  |              |      |      |  |
| 3            | 800  | 359  | 635         |      |      | 428  |      | 290  |      | 635  |      | 466  |      | 497  |      |              | 475  | 384  |  |
| 4            |      |      |             | 193  | 317  |      | 455  |      | 864  |      | 331  |      | 315  |      | 166  | 759          |      |      |  |
| 5            |      | 356  | 524         |      |      | 431  |      | 362  |      | 442  |      |      |      | 276  |      |              |      | 362  |  |
| 6            | 911  |      |             | 226  | 290  |      |      |      | 375  |      | 455  | 649  | 756  |      |      | 1537         | 461  |      |  |
| 7            |      |      |             |      |      | 442  |      | 478  |      | 580  |      |      |      | 331  | 276  |              |      |      |  |
| 8            | 635  | 276  |             |      |      |      | 671  |      |      |      | 276  | 243  | 756  |      |      | 428          | 522  | 298  |  |
| 9            |      |      |             | 177  | 193  | 409  | 566  |      | 533  |      |      |      |      |      | 248  |              |      |      |  |
| 10           | 539  | 315  | 331         |      |      |      | 530  | 204  |      | 649  |      | 284  |      | 400  |      |              | 461  | 193  |  |
| 11           |      |      |             | 202  |      | 400  | 262  |      | 276  |      | 331  |      | 756  |      | 221  | 409          |      |      |  |
| 12           |      | 276  |             |      | 138  |      | 240  | 276  |      | 359  |      |      |      | 248  |      | 331          |      | 317  |  |
| 13           | 745  |      |             |      | 491  | 326  | 276  |      |      |      | 373  | 386  |      |      |      |              | 582  |      |  |
| 14           |      |      |             | 182  |      |      |      |      |      | 331  |      |      |      | 359  | 248  |              |      |      |  |
| 15           | 662  | 295  | 469         |      |      |      | 362  | 428  |      |      | 414  | 243  | 522  |      |      | 386          | 431  | 317  |  |
| 16           |      |      |             | 168  | 353  | 386  |      |      | 276  |      |      | 304  |      |      | 359  |              |      |      |  |
| 17           | 497  | 166  | 380         |      |      |      | 190  | 455  |      | 580  |      |      |      | 193  |      |              | 397  | 340  |  |
| 18           |      |      |             | 287  | 262  | 811  |      |      | 276  |      | 414  |      |      |      | 304  | 428          |      |      |  |
| 19           |      | 331  | 414         | 304  |      |      | 384  | 290  |      | 331  |      |      | 522  | 221  |      |              |      | 276  |  |
| 20           | 745  |      |             |      | 718  | 535  |      |      | 235  |      | 386  | 204  | 489  |      |      | 428          | 613  |      |  |

contd....

| Day of<br>Cycle | E.M. | FIRST CYCLE |      |      | J.W. | N.V. | S.D. | A.B. | F.R. | V.R. | T.M. | L.C. | J.T. | M.H. | SECOND CYCLE |      |      | M.H. |
|-----------------|------|-------------|------|------|------|------|------|------|------|------|------|------|------|------|--------------|------|------|------|
|                 |      | A.J.        | M.L. | R.E. |      |      |      |      |      |      |      |      |      |      | R.E.         | N.V. | S.D. |      |
| 21              |      | 662         | 428  |      |      |      |      |      |      | 414  |      | 224  |      | 226  | 207          | 409  |      |      |
| 22              | 442  |             | 226  | 414  | 146  |      | 218  | 207  |      |      |      |      | 588  |      |              |      | 367  | 436  |
| 23              |      |             |      |      |      | 373  |      |      | 235  |      |      | 306  |      |      | 166          |      |      |      |
| 24              | 577  | 690         | 177  | 235  | 287  |      | 334  | 304  |      | 359  |      |      |      | 773  |              |      | 276  | 414  |
| 25              |      |             |      |      |      | 453  |      |      | 262  |      |      |      | 386  |      | 138          | 224  |      |      |
| 26              |      |             |      | 221  | 312  |      | 552  | 334  |      | 124  |      | 364  |      | 662  |              | 406  |      | 414  |
| 27              | 627  |             |      |      |      | 331  |      |      |      |      |      |      | 386  |      |              |      |      |      |
| 28              |      |             |      |      | 375  |      |      |      |      |      |      |      |      | 480  | 166          | 549  | 522  |      |
| 29              |      |             |      | 193  |      |      | 718  |      |      |      |      |      | 386  |      |              |      |      |      |
| 30              |      |             |      | 193  | 301  |      |      |      |      |      |      |      |      |      | 166          |      |      |      |

2. C B G Levels in "Control Women"

| Day of<br>Cycle | E.M. | FIRST CYCLE |      |      |      | J.W. | N.V. | S.D. | A.B. | F.R. | V.R. | T.M. | L.C. | J.T. | M.H. | SECOND CYCLE |      |     |     | M.H. |
|-----------------|------|-------------|------|------|------|------|------|------|------|------|------|------|------|------|------|--------------|------|-----|-----|------|
|                 |      | A.J.        | M.L. | R.E. | R.E. |      |      |      |      |      |      |      |      |      |      | N.V.         | S.D. |     |     |      |
| 1               | 455  | 257         | 433  |      |      | 304  |      |      |      |      | 530  | 367  | 621  | 715  |      |              |      | 861 | 494 |      |
| 2               |      |             |      | 367  | 359  |      | 547  |      | 375  |      |      |      |      |      |      | 574          |      |     |     |      |
| 3               | 489  | 323         | 502  |      |      | 524  |      | 543  |      | 364  |      | 621  |      | 232  |      |              |      | 538 | 453 |      |
| 4               |      |             |      | 224  | 411  |      | 453  |      | 433  |      | 375  |      | 685  |      |      | 422          | 384  |     |     |      |
| 5               |      | 320         | 367  |      |      | 428  |      | 433  |      | 497  |      |      |      | 516  |      |              |      |     | 439 |      |
| 6               | 480  |             |      | 442  | 337  |      |      |      | 1369 |      | 478  | 599  | 1076 |      |      |              | 654  | 682 |     |      |
| 7               |      |             |      |      |      | 342  |      | 541  |      | 483  |      |      |      | 472  |      | 511          |      |     |     |      |
| 8               | 431  | 331         | 505  |      |      |      | 591  |      |      |      | 386  | 712  | 1115 |      |      |              | 414  | 563 | 414 |      |
| 9               |      |             |      | 527  | 268  | 558  | 527  |      | 522  |      |      |      |      |      |      | 425          |      |     |     |      |
| 10              | 403  | 284         | 345  |      |      |      | 593  | 478  |      | 469  |      | 668  |      | 527  |      |              |      | 613 | 450 |      |
| 11              |      |             |      | 533  |      | 627  | 511  |      | 331  |      | 478  |      | 654  |      |      | 571          | 458  |     |     |      |
| 12              |      | 284         |      |      | 304  |      | 577  | 494  |      | 323  |      |      |      | 337  |      |              | 472  |     | 489 |      |
| 13              | 616  |             |      |      | 124  | 577  | 472  |      |      |      | 489  | 489  |      |      |      |              |      | 671 |     |      |
| 14              |      |             |      | 497  |      |      |      |      |      | 323  |      |      |      | 431  |      | 395          |      |     |     |      |
| 15              | 403  | 301         | 356  |      |      |      | 654  | 453  |      |      | 431  | 544  | 754  |      |      |              | 469  | 613 | 480 |      |
| 16              |      |             |      | 522  | 232  | 555  |      |      | 373  |      |      | 469  |      |      |      | 436          |      |     |     |      |
| 17              | 505  | 331         | 406  |      |      |      | 582  | 417  |      | 373  |      |      |      | 370  |      |              |      | 588 | 433 |      |
| 18              |      |             |      | 389  | 364  | 690  |      |      | 487  |      | 364  |      |      |      |      | 364          | 475  |     |     |      |
| 19              |      | 287         | 453  | 433  |      |      | 522  | 439  |      | 502  |      |      | 762  | 511  |      |              |      |     | 511 |      |
| 20              | 505  |             |      |      | 381  | 409  |      |      | 373  |      | 417  | 552  | 709  |      |      | 508          | 519  | 707 |     |      |

Contd...

Contd. . .

| Day of cycle | E.M. | FIRST CYCLE |      |      |      | J.W. | N.V. | S.D. | A.B. | F.R. | V.R. | T.M. | L.C. | J.T. | M.H. | SECOND CYCLE |      |      |  |
|--------------|------|-------------|------|------|------|------|------|------|------|------|------|------|------|------|------|--------------|------|------|--|
|              |      | A.J.        | M.L. | R.E. | R.E. |      |      |      |      |      |      |      |      |      |      | N.V.         | S.D. | M.H. |  |
| 21           |      | 334         | 508  |      |      |      |      |      |      |      | 431  |      | 535  |      | 508  |              | 535  |      |  |
| 22           | 582  |             | 662  | 593  | 411  |      | 593  | 453  |      |      |      |      | 649  |      |      |              | 624  | 486  |  |
| 23           |      |             |      |      |      | 527  |      |      | 442  |      |      |      | 478  |      | 500  |              |      |      |  |
| 24           | 489  | 326         | 353  | 392  | 395  |      | 494  | 397  |      | 486  |      |      |      | 544  |      |              | 640  | 494  |  |
| 25           |      |             |      |      |      | 582  |      |      | 442  |      |      |      | 773  |      | 466  | 351          |      |      |  |
| 26           |      |             |      | 458  | 342  |      | 574  | 359  |      | 428  |      | 552  |      | 547  |      | 491          |      | 295  |  |
| 27           | 577  |             |      |      |      | 985  |      |      | 447  |      |      |      | 660  |      |      |              |      |      |  |
| 28           |      |             |      |      | 561  |      |      |      |      |      |      |      |      | 486  | 521  | 513          | 690  |      |  |
| 29           |      |             |      | 527  |      |      | 552  |      |      |      |      |      | 585  |      |      |              |      |      |  |
| 30           |      |             |      | 356  | 527  |      |      |      |      |      |      |      |      |      | 483  |              |      |      |  |

3. Cortisol Levels in Women taking Oral Contraceptives

| Day of Cycle | M.M. | FIRST CYCLE |      |      | S.H. | J.K. | J.W. | L.B. | C.K. | R.M. | M.M. | SECOND CYCLE |      |     | L.B. | C.K. |
|--------------|------|-------------|------|------|------|------|------|------|------|------|------|--------------|------|-----|------|------|
|              |      | S.B.        | P.T. | M.M. |      |      |      |      |      |      |      | S.B.         | J.W. |     |      |      |
| 1            | 635  | 1546        |      | 1435 | 900  |      |      | 731  | 580  |      | 789  |              | 1380 | 966 | 679  |      |
| 2            |      |             | 869  |      |      |      | 1532 |      |      |      |      |              |      |     |      |      |
| 3            | 497  |             |      |      | 580  | 580  |      |      | 309  | 621  | 513  |              | 1256 | 773 | 999  |      |
| 4            |      | 1159        | 828  | 966  |      |      | 980  |      |      |      |      |              |      |     |      |      |
| 5            |      |             |      |      |      | 552  |      | 781  |      | 447  |      | 980          |      |     |      |      |
| 6            | 566  | 1049        | 662  | 604  | 442  |      | 897  |      |      |      | 513  |              | 1035 | 759 | 447  |      |
| 7            |      |             |      |      |      | 483  | 1435 |      | 265  |      |      | 938          |      |     |      |      |
| 8            | 607  | 856         |      | 828  | 734  |      |      | 751  |      | 814  | 513  |              |      | 773 | 555  |      |
| 9            |      |             | 938  |      |      |      | 773  |      | 367  |      |      |              |      |     |      |      |
| 10           | 552  |             |      |      | 580  | 704  |      | 828  |      | 657  | 709  | 649          | 1214 | 800 | 800  |      |
| 11           |      | 994         | 883  | 718  |      |      | 751  |      | 538  |      |      |              |      |     |      |      |
| 12           |      |             |      |      |      | 828  |      | 897  |      | 679  |      | 1090         |      |     |      |      |
| 13           | 552  | 1132        | 938  | 1032 | 765  |      | 927  |      |      |      | 773  |              | 1143 | 731 | 718  |      |
| 14           |      |             |      |      |      | 718  |      |      | 635  |      |      | 980          |      |     |      |      |
| 15           | 635  |             |      | 955  | 828  |      |      | 800  |      | 544  | 533  |              | 1046 | 718 | 662  |      |
| 16           |      |             | 1079 |      |      |      | 1256 |      | 469  |      |      |              | 1341 |     |      |      |
| 17           | 627  |             |      |      | 704  | 593  |      | 828  |      | 701  | 505  | 1104         |      | 925 |      |      |
| 18           |      | 1557        |      | 1121 |      |      | 911  |      | 524  |      |      |              |      |     |      |      |
| 19           |      |             | 635  |      |      | 673  |      | 800  |      | 544  |      | 842          |      |     |      |      |
| 20           | 428  | 1021        |      | 1361 | 745  |      | 980  |      |      |      | 524  |              | 1123 | 649 | 690  |      |

4. C B & Levels in Women taking Oral Contraceptives

| Day of Cycle | M.M. | FIRST CYCLE |      |      | S.H. | J.K. | J.W. | L.B. | C.K. | R.M. | M.M. | SECOND CYCLE |      |      | C.K. |
|--------------|------|-------------|------|------|------|------|------|------|------|------|------|--------------|------|------|------|
|              |      | S.B.        | P.T. | M.M. |      |      |      |      |      |      |      | S.B.         | J.W. | L.B. |      |
| 1            | 751  | 1270        |      | 922  | 908  |      |      | 439  | 820  |      | 600  |              | 1220 | 798  | 897  |
| 2            |      |             | 787  |      |      |      | 1278 |      |      |      |      |              |      |      |      |
| 3            | 781  |             |      |      | 632  | 715  |      |      | 522  | 309  | 447  |              | 1018 | 627  | 872  |
| 4            |      | 1032        | 640  | 806  |      |      | 770  |      |      |      |      |              |      |      |      |
| 5            |      |             |      |      |      | 720  |      | 784  |      | 392  |      | 823          |      |      |      |
| 6            | 632  | 582         | 511  | 621  | 455  |      | 811  |      |      |      | 519  |              | 847  | 610  | 607  |
| 7            |      |             |      |      |      | 593  | 941  |      | 798  |      |      | 792          |      |      |      |
| 8            | 651  | 914         |      | 679  | 817  |      |      | 734  |      | 489  | 547  |              | 949  | 740  | 856  |
| 9            |      |             | 682  |      |      |      | 643  |      | 696  |      |      |              |      |      |      |
| 10           | 696  |             |      |      | 911  | 958  |      | 911  |      | 745  | 599  | 582          | 958  | 814  | 773  |
| 11           |      | 1234        | 690  | 577  |      |      | 980  |      | 646  |      |      |              |      |      |      |
| 12           |      |             |      |      |      | 643  |      | 878  |      | 853  |      | 938          |      |      |      |
| 13           | 806  | 1057        | 831  | 974  | 756  |      | 1076 |      | 880  |      | 511  |              | 676  | 850  | 991  |
| 14           |      |             |      |      |      | 792  |      |      |      |      |      | 1013         |      |      |      |
| 15           | 1005 |             |      | 1049 | 972  |      |      | 759  |      | 767  | 403  |              | 616  | 673  | 869  |
| 16           |      |             | 936  |      |      |      | 839  |      | 665  |      |      |              | 759  |      |      |
| 17           | 817  |             |      |      | 927  | 563  |      | 911  |      | 872  | 491  | 1190         |      | 831  |      |
| 18           |      | 1405        |      | 1046 |      |      | 930  |      | 847  |      |      |              |      |      |      |
| 19           |      |             | 593  |      |      | 1021 |      | 1013 |      | 834  |      | 740          |      |      |      |
| 20           | 742  | 668         |      | 944  | 892  |      | 1156 |      |      |      | 502  |              | 502  | 497  | 880  |

3.Contd....

| Day of Cycle | M.M. | FIRST CYCLE |      |      | S.H. | J.K. | J.W. | L.B. | C.K. | R.M. | SECOND CYCLE |      |      | L.B. | C.K. |
|--------------|------|-------------|------|------|------|------|------|------|------|------|--------------|------|------|------|------|
|              |      | S.B.        | P.T. | M.M. |      |      |      |      |      |      | M.M.         | S.B. | J.W. |      |      |
| 21           |      |             | 1435 |      |      | 668  |      |      | 607  |      |              | 1021 | 1380 |      |      |
| 22           | 1159 |             |      | 1076 | 731  |      |      | 952  |      | 497  | 582          |      |      | 731  | 712  |
| 23           |      |             |      |      |      |      | 980  |      | 497  |      |              |      | 1499 |      |      |
| 24           | 414  |             | 1214 |      | 731  | 444  |      | 690  |      | 566  | 759          | 1187 |      | 897  | 803  |
| 25           |      | 1214        |      | 911  |      | 765  | 994  |      | 580  |      |              |      |      |      |      |
| 26           |      |             | 1021 |      |      |      |      | 925  |      |      |              | 980  | 1852 |      |      |
| 27           | 1132 | 1421        |      |      | 856  | 811  | 994  |      |      |      | 640          |      |      | 524  | 828  |
| 28           |      |             | 856  |      |      |      |      |      | 563  |      |              |      | 1380 |      |      |
| 29           |      |             |      |      |      | 938  |      |      |      |      |              |      |      | 676  | 803  |
| 30           |      |             |      |      |      |      |      |      |      |      |              |      |      | 718  |      |

4. contd....

| Day of Cycle | M.M. | FIRST CYCLE |      |      | S.H. | J.K. | J.W. | L.B. | C.K. | R.M. | M.M. | SECOND CYCLE |      |      | C.K. |
|--------------|------|-------------|------|------|------|------|------|------|------|------|------|--------------|------|------|------|
|              |      | S.B.        | P.T. | M.M. |      |      |      |      |      |      |      | S.B.         | J.W. | L.B. |      |
| 21           |      |             | 740  |      |      | 963  |      |      | 803  |      |      | 850          | 629  |      |      |
| 22           | 731  |             |      | 1052 |      |      |      | 1281 |      | 709  | 439  |              |      | 792  | 878  |
| 23           |      |             |      |      |      |      | 949  |      | 649  |      |      |              | 704  |      |      |
| 24           | 616  |             | 750  |      | 936  | 823  |      | 759  |      | 756  | 651  | 787          |      | 925  | 889  |
| 25           |      | 762         |      | 905  |      | 991  | 894  |      | 629  |      |      |              |      |      |      |
| 26           |      |             | 974  |      |      |      |      | 847  |      |      |      | 927          | 905  |      |      |
| 27           | 1063 | 1121        |      |      | 856  | 972  | 1104 |      |      |      | 613  |              |      | 869  | 936  |
| 28           |      |             | 836  |      |      |      |      |      | 723  |      |      |              | 640  |      |      |
| 29           |      |             |      |      |      | 1237 |      |      |      |      |      |              |      | 817  | 1030 |
| 30           |      |             |      |      |      |      |      |      |      |      |      |              |      | 806  |      |

| 5. <u>Norethisterone Treatment</u> |                   |     |                         |     |
|------------------------------------|-------------------|-----|-------------------------|-----|
| Day of Cycle                       | Cortisol (nmol/l) |     | C.B.G. (nmol Cortisol/l |     |
|                                    | I                 | J   | I                       | J   |
| 1                                  | 235               |     | 295                     |     |
| 2                                  |                   | 221 |                         | 83  |
| 3                                  | 177               |     | 466                     |     |
| 4                                  |                   |     |                         |     |
| 5                                  | 309               | 248 | 522                     | 270 |
| 6                                  |                   |     |                         |     |
| 7                                  | 257               | 221 | 370                     | 273 |
| 8                                  |                   |     |                         |     |
| 9                                  | 331               | 179 | 613                     | 218 |
| 10                                 |                   |     |                         |     |
| 11                                 |                   |     |                         |     |
| 12                                 | 276               | 177 | 414                     | 293 |
| 13                                 |                   |     |                         |     |
| 14                                 | 359               |     | 475                     |     |
| 15                                 |                   |     |                         |     |
| 16                                 |                   | 268 |                         | 400 |
| 17                                 |                   |     |                         |     |
| 18                                 |                   |     |                         |     |
| 19                                 |                   | 309 |                         | 298 |
| 20                                 |                   |     |                         |     |
| 21                                 | 414               | 166 | 422                     | 304 |
| 22                                 |                   |     |                         |     |
| 23                                 | 179               |     | 218                     |     |
| 24                                 |                   | 373 |                         | 370 |
| 25                                 |                   |     |                         |     |
| 26                                 |                   | 248 |                         | 317 |
| 27                                 |                   |     |                         |     |
| 28                                 |                   |     |                         |     |
| 29                                 |                   |     |                         |     |
| 30                                 |                   |     |                         |     |

F. Total and Free Cortisol Levels in Men and Women and in Women taking Oral Contraceptives

| 1. <u>Normal men</u> |                                |                               |
|----------------------|--------------------------------|-------------------------------|
| <u>Subject</u>       | <u>Total Cortisol (nmol/l)</u> | <u>Free Cortisol (nmol/l)</u> |
| M.W.                 | 552                            | 64                            |
| P.W.                 | 552                            | 60                            |
| R.L.                 | 442                            | 41                            |
| A.K.                 | 511                            | 61                            |
| J.D.                 | 469                            | 50                            |
| J.G.                 | 262                            | 28                            |
| C.L.                 | 276                            | 25                            |
| P.W.                 | 304                            | 30                            |
| J.D.                 | 193                            | 14                            |
| T.M.                 | 373                            | 33                            |
| G.H.                 | 524                            | 64                            |
| D.R.                 | 455                            | 44                            |
| J.Z.                 | 317                            | 28                            |
| R.M.                 | 373                            | 30                            |
| J.D.                 | 359                            | 36                            |

2. Normal women

| <u>Subject</u> | <u>Total Cortisol (nmol/l)</u> | <u>Free Cortisol (nmol/l)</u> |
|----------------|--------------------------------|-------------------------------|
| M.M.           | 331                            | 28                            |
| A.C.           | 276                            | 25                            |
| J.S.           | 414                            | 28                            |
| M.B.           | 552                            | 44                            |
| J.D.           | 262                            | 25                            |
| A.F.           | 428                            | 44                            |
| R.H.           | 290                            | 29                            |
| P.F.           | 221                            | 17                            |
| A.W.           | 179                            | 17                            |
| Y.F.           | 248                            | 19                            |
| L.R.           | 248                            | 22                            |
| E.T.           | 290                            | 23                            |

3. Women taking Oral Contraceptives (containing 50 ug oestrogen)

| <u>Subject</u> | <u>Total Cortisol (nmol/l)</u> | <u>Free Cortisol (nmol/l)</u> |
|----------------|--------------------------------|-------------------------------|
| E.V.           | 1559                           | 105                           |
| J.P.           | 1076                           | 105                           |
| R.I.           | 1352                           | 88                            |
| L.C.           | 1518                           | 124                           |
| N.F.           | 386                            | 33                            |
| A.P.           | 690                            | 47                            |
| M.B.           | 1173                           | 86                            |
| A.B.           | 428                            | 52                            |
| L.W.           | 552                            | 33                            |
| C.C.           | 690                            | 44                            |
| S.D.           | 373                            | 28                            |
| J.P.           | 1187                           | 75                            |
| E.F.           | 1408                           | 94                            |

G. Plasma Cortisol and CBG and Urinary Cortisol levels in Control and Oral Contraceptive treated women

1. Controls

| Free Cortisol<br>(nmol/l)     |     |            |     | Total Cortisol<br>(nmol/l) |      |            |     | CBG<br>(nmol cortisol/l) |     |            |      | Urinary Cortisol<br>(nmol/TV |            | Patient |
|-------------------------------|-----|------------|-----|----------------------------|------|------------|-----|--------------------------|-----|------------|------|------------------------------|------------|---------|
| Days 6-9                      |     | Days 23-26 |     | Days 6-9                   |      | Days 23-26 |     | Days 6-9                 |     | Days 23-26 |      | Days 6-9                     | Days 23-26 |         |
| 9am                           | 5pm | 9am        | 5pm | 9am                        | 5pm  | 9am        | 5pm | 9am                      | 5pm | 9am        | 5pm  |                              |            |         |
| 80                            | 50  | 94         | 48  | 825                        | 464  | 1475       | 750 | 830                      | 810 | 1221       | 1166 | 105                          | 91         | LG      |
| 95                            | 36  | 79         | 21  | 686                        | 282  | 645        | 282 | 395                      | 467 | 647        | 677  | 56                           | 66         | SW      |
| 96                            | 58  | 165        | 44  | 927                        | 605  | 1525       | 625 | 576                      | 654 | 967        | 918  | 102                          | 80         | WM      |
| 77                            | 49  | 99         | 31  | 948                        | 630  | 1220       | 468 | 765                      | 783 | 956        | 1058 | 131                          | 92         | FC      |
| 95                            | 34  | 82         | 70  | 1016                       | 529  | 1118       | 711 | 755                      | 777 | 1205       | 1150 | 144                          | 106        | MMc     |
| 106                           | 36  | 73         | 31  | 950                        | 425  | 750        | 350 | 829                      | 814 | 710        | 808  | 107                          | 103        | MM      |
| 2. <u>Oral Contraceptives</u> |     |            |     |                            |      |            |     |                          |     |            |      |                              |            |         |
| 27                            | 8   | 26         | 4   | 224                        | 81   | 224        | 41  | 305                      | 272 | ---        | 247  | 102                          | 142        | PB      |
| 77                            | 80  | 108        | 55  | 800                        | 1075 | 1088       | 450 | 802                      | 758 | ---        | 730  | 157                          | 98         | AS      |
| 55                            | 23  | 42         | 23  | 403                        | 200  | 463        | 225 | 426                      | 405 | 458        | 446  | 96                           | 144        | GV      |
| 49                            | 15  | 55         | 15  | 400                        | 163  | 475        | 138 | 328                      | 179 | ---        | 364  | 55                           | 118        | AD      |
| 27                            | 13  | 30         | 14  | 295                        | 157  | 227        | 157 | 402                      | 404 | 200        | 344  | 79                           | 68         | JH      |

H. Urinary free Cortisol/CBG ratio in controls and women taking oral contraceptives, sampled at two different days in the cycle

| Controls |         |           | Oral Contraceptives |         |           |
|----------|---------|-----------|---------------------|---------|-----------|
| Patient  | Day 6-9 | Day 23-26 | Patient             | Day 6-9 | Day 23-26 |
| PB       | 35.2    | 57.5      | LG                  | 12.8    | 7.6       |
| AS       | 20.1    | 14.7      | SW                  | 13.0    | 10.0      |
| GV       | 23.1    | 31.9      | WM                  | 16.7    | 8.5       |
| AD       | 16.8    | 33.3      | FC                  | 16.9    | 9.1       |
| JH       | 19.6    | 19.8      | MMc                 | 12.8    | 7.6       |
|          |         |           | MM                  | 18.8    | 9.0       |

1. CBG levels in men and women and in women with severe premenstrual syndrome as measured using the two-tier column method

a. Normal men and women

| <u>Men</u>     |                                 | <u>Women</u>   |                                 |
|----------------|---------------------------------|----------------|---------------------------------|
| <u>Subject</u> | <u>CBG</u><br>(nmol cortisol/l) | <u>Subject</u> | <u>CBG</u><br>(nmol cortisol/l) |
| DE             | 382                             | VC             | 344                             |
| JC             | 380                             | AS             | 398                             |
| CB             | 344                             | AF             | 388                             |
| MJ             | 316                             | GL             | 329                             |
| EM             | 369                             | AR             | 330                             |
| SB             | 326                             | MB             | 379                             |
| CN             | 375                             | AP             | 345                             |
| CA             | 332                             | CD             | 382                             |
| DB             | 335                             | SD             | 317                             |
| BK             | 387                             | AD             | 376                             |
| SC             | 327                             | CJ             | 360                             |
| ML             | 376                             | ME             | 353                             |
| GC             | 371                             | JW             | 361                             |
| JE             | 338                             | RE             | 340                             |
|                |                                 | RH             | 394                             |
|                |                                 | GF             | 340                             |

b. Women with PMS

| <u>Subject</u> | <u>CBG</u><br>(nmol cortisol/l) | <u>Subject</u> | <u>CBG</u><br>(nmol cortisol/l) |
|----------------|---------------------------------|----------------|---------------------------------|
| HO             | 173                             | RE             | 188                             |
| BR             | 355                             | IS             | 224                             |
| BA             | 182                             | CA             | 91                              |
| RE             | 82                              | GR             | 383                             |
| WI             | 201                             | CR             | 161                             |
| CU             | 321                             | KO             | 330                             |
| WI             | 259                             | BR             | 367                             |
| FR             | 245                             | CO             | 230                             |
| KI             | 199                             | WH             | 225                             |
| SO             | 197                             |                |                                 |
| WO             | 146                             |                |                                 |
| SK             | 159                             |                |                                 |
| BE             | 145                             |                |                                 |
| AK             | 201                             |                |                                 |
| BA             | 301                             |                |                                 |
| SK             | 222                             |                |                                 |
| RE             | 142                             |                |                                 |
| ED             | 195                             |                |                                 |
| LE             | 196                             |                |                                 |
| BE             | 139                             |                |                                 |
| HO             | 174                             |                |                                 |
| DY             | 139                             |                |                                 |
| CU             | 184                             |                |                                 |
| KO             | 239                             |                |                                 |
| FI             | 201                             |                |                                 |
| RO             | 296                             |                |                                 |
| AB             | 165                             |                |                                 |

### APPENDIX 3

#### Statistical Tests

a. Students "t" test for the comparison of the means of two small samples from normal populations. (Bailey, 1959).

(i) Assuming that the populative distributions are approximately normal and that the variances of these populations are the same; then:-

$$t = \frac{\bar{X}_1 - \bar{X}_2}{s \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

provides a test of the hypothesis that the two population means are the same.

where "s" is the estimate of the assumed common population standard deviation based on both samples as:-

for each sample:- mean,  $\bar{X} = \frac{\sum X}{N}$

$$\text{standard deviation, } s = \sqrt{\frac{\sum X^2 - \frac{1}{N} (\sum X)^2}{N - 1}}$$

the assumed common standard deviation of two populations  $X$  and  $y$  is therefore estimated by:-

$$s = \sqrt{\frac{\left[ \sum X^2 - \frac{1}{N_x} (\sum X)^2 \right] + \left[ \sum Y^2 - \frac{1}{N_y} (\sum Y)^2 \right]}{N_x + N_y - 2}}$$

(ii) In order to test that the variances of the two populations are the same, the variance ratio,  $F$ , is used:

$$F = \frac{S_x^2}{S_y^2} \quad \text{where } S_x \text{ is the larger of the two sample deviations.}$$

Significance limits are found by referring to tables such as those of Fisher and Yates (1963).

## 2. Analysis of variance (randomised block design)

The data is arranged as:-

| <u>Treatments</u> | <u>Blocks</u>                | <u>Treatment totals</u> | <u>Treatment means</u> |
|-------------------|------------------------------|-------------------------|------------------------|
|                   | 1 2 ..... j                  |                         |                        |
| 1                 | $x_{11} x_{12} \dots x_{1j}$ | $T_1$                   | $\bar{x}_1$            |
| 2                 | $x_{21} x_{22} \dots x_{2j}$ | $T_2$                   | $\bar{x}_2$            |
| .                 | .                            |                         |                        |
| .                 | .                            |                         |                        |
| .                 | .                            |                         |                        |
| .                 | .                            |                         |                        |
| i                 | $x_{i1} x_{i2} \dots x_{ij}$ | $T_i$                   | $\bar{x}_i$            |
| Block Totals      | B1 B2 ..... Bj               | $G = \sum T_i$          | $\bar{x} = G/ij$       |

Then calculate  $\sum_i T_i^2/j - C$   
 where  $C = G^2/ij$

Analysis of variance table is then:-

| Source of variation | Sums of Squares      | Degrees of freedom | Mean Square |
|---------------------|----------------------|--------------------|-------------|
| Treatments          | $\sum_i T_i^2/j - C$ | $i - 1$            | $M_T$       |
| Blocks              | $\sum_j B_j^2/i - C$ | $j - 1$            | $M_B$       |
| Residual            | By subtraction       | $(i - 1)(j - 1)$   | $S^2$       |
| Total               | $\sum x_{ij}^2 - C$  | $ij - 1$           |             |

Effects of blocks and treatments are tested by examining the variance ratios  $M_{B/s}^2$  and  $M_{T/s}^2$ .

The mean of the  $i^{\text{th}}$  treatment with its standard error is:

$$\bar{x}_i \pm s/\sqrt{b}$$

and the difference between any two treatments, for example  $i^{\text{th}}$  and  $j^{\text{th}}$  is:

$$(\bar{x}_i - \bar{x}_j) \pm s\sqrt{\frac{2}{b}}$$

Significance tests are performed and confidence limits are set by reference to the normal or  $t$ -distributions.

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